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# Biotechnological Innovations for Environmental Bioremediation

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*Professor Swami Dayal Tewari*
***(14th Nov. 1918–26th Jan 1992)***
***Professor, The Legendary***
***The legendary—teacher of botany***
***and nature lover***

We dedicate this book to Professor Swami Dayal Tewari, Ashwani Kumar's (AK) father. He was one of the prominent senior Botanist of India. He completed MSc Botany in 1943 at Allahabad University, Allahabad, India, at the time when professors and department heads were either from, or trained in Great Britain like Shri Ranjan (MSc Cantab; DSc from France). During the time Swami Dayal Tewari was at Allahabad University, he was endowed with teaching from prominent figures in Botany like Julian Mitter (PhD from UK), Ram Kumar Saxena (DSc from France), Birbal Sahni, the great paleobotonist and Divya Darshan Pant (a respected authority in the field). Allahabad

University also boasts of other prominent graduates like Dr. Govindjee (who is nicknamed Mr. Photosynthesis of the twenty-first Century: https://www.life.illinois.edu/govindjee/), and Prof. H.N. Bharghav (Head of Department, Gorukhpur University, Gorakhpur). Prof. Ranjan and Prof. Pant were Swami Dayal's favourite teachers who in turn had high praise for their star student. The Allahabad University was, and remains, one of the most prestigious universities in India. After graduating from Allahabad University Prof. Swami Dayal Tewari worked with Dr. K.C. Mehta of wheat rust fame, at Agra College, Agra, and subsequently joined Champa Agarwal College, Mathura, U.P., India. There he quickly rose through the ranks and retired as the head of the biology department after 29 years of illustratious career. Many of the prominent graduates of that era migrated to the USA such as Govindjee, but Prof. S.D. Tewari preferred to remain in India and devoted his entire career to teaching of Botany. He was loved and adorned by his students and lovingly called as "Guruji". A large number of his students attained prestigious positions including Dr. U.C. Lavania, D.Sc., FNA. He has expressed his memoirs in the next page. He had tremendous influence on his children. Prof. Ashwani Kumar, his elder son, took his teaching to heart and decided to became a botanist, dedicating his career to teaching and advancing the field. Upon recommendation of his father, he decided not to join Indian Administrative Service after being selected in 1972 but pursed a prominent career in botany, becoming an Alexander von Humboldt (Germany) fellow, later serving as the President of the Indian Botany Society and winning the coveted V. Puri medal for researches in Botany.

***My Revered Teacher***
***Professor Swami Dayal Tewari 'Guruji'***

It gives me immense pleasure to write about the grand persona of my revered Guru ji Shri Swami Dayal Tewari ji at the Champa Agrawal Inter College, Mathura, where I completed my Intermediate in 1969. An epitome of strict discipline with a stick in hand (posing the same as 'Samaj Sudharak') sporting towering personality, he was a 'Guru ji' for the entire fraternity of the College including teachers and students alike. He always took pride in telling the success of his past students in order to encourage the incumbent students to prosper in their academic career. He is the only teacher who used to impart Botany teaching in English medium in an otherwise Hindi medium college; he had commanding teaching skills and influencing personality to excel his students to shape their career either in medical or academic profession. I owe him the initial encouragement to have chosen Botany to pursue as my professional career. My salutes to such an ardent teacher who is adored among his students for his simplicity and love towards teaching profession.

***Umesh Chandra Lavania, PhD & DSc (Univ. of Calcutta), FNA, FNASc***

***INSA Senior Scientist, University of***
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***(Scientist in Director's Grade) & Head of the***
***Division of Genetics & Plant Breeding,***
***CSIR-Central Institute of Medicinal &***
***Aromatic Plants, Lucknow 226015)***

# Foreword

> ‘We still do not know one thousandth of one percent of what nature has revealed to us.’
> —Albert Einstein

> ‘If one way be better than another, that you may be sure is nature's way.’
> —Aristotle

We are all so lucky living in a century with so many advanced technologies and fast improving economy. The ever-increasing living standards for the majority of the global population induce a heavy burden to the natural environment. The mother nature that provides for us is suffering from severe degradation, becoming eventually unliveable with the current socioeconomic development pattern. Fortunately, we realized the problems and have been working on mitigating the damages by implementing a range of strategies including pollutant treatment, environmental remediation, circular economy, clean production, carbon neutralization, energy saving, sustainable development, etc. Among them, biotechnology is playing an important role due to the utilization of the natural healing power, its demonstrated effectiveness and endless potential to be exploited.

Industrialization not only paves the path for the modern economies but also discharges many known and emerging environmental pollutants, including heavy metals, persistent trace organic pollutants, and greenhouse gases. Also, our everyday life generates astonishing amount of wastewater and solid waste. The disposal of untreated waste and pollutants can damage the ecological system and pollute water bodies and soils. To remedy the polluted water or soils, many biologically inspired techniques and processes have been developed to extract pollutants from the spilled environment, to degrade the pollutants into benign compounds, and to convert waste into energy or fuels like biogas.

Traditionally, environmental bioremediation and phytoremediation were developed for industrial polluted sites. The applications have been extended to coastal saline environment and urban stormwater management. Anaerobic digestion of wasted sludge or organic-rich solid waste is one of the most significant biotechnologies being developed to harvest energy and reduce the final discharge of waste to the environment. Enzymatic processes were utilized to convert biomass that is abundantly available naturally into fuels and high value chemicals, which can reduce our reliance on fossil fuels. The natural plants and microorganisms can also be genetically modified to enhance their productivity and functions towards environmental remediation.

Urbanization and population growth also mean that the interaction of human beings with the natural environment becomes closer and more frequent. This has a great implication to population health due to the incidences of bacterial or viral pathogens which cross the animal-human barrier and become human pathogens. Some recent pandemics including COVID-19 have shown us the devastating damages that could be caused by a single such pathogen and the paramount importance of environmental safety and health risks. Such knowledge will ensure public health and safety while we are harnessing nature for ourselves and our future generations.

I would like to strongly recommend this book as it provides extensive information, profound knowledge, and diverse applications about environmental remediation, phytoremediation, and environment safety and health/risk assessments. The valuable resource and contents will be very useful for professionals, specialists, engineers, researchers, and students interested in environmental engineering in colleges, universities, and industries. You will always find something interesting and relevant among nearly 40 chapters in this book. The up-to-date data and information gathered from more than 100 top scholars worldwide is a great contribution to address the global environmental issues. I believe the enduring benefits of the book will be manifested when readers use it to foster their biotechnology skills and achieve their academic growth.

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Guangming Jiang

# Preface

Technology is progressing rapidly worldwide. In the race for development, it is vital that we do not ignore the environment. Accountability to the environment is vital, and environmental ignorance is not a viable option. Environmental degradation is **a process through which the natural environment is compromised in some way**, reducing biological diversity and general health of the environment. The process can be entirely natural in origin, or it can be accelerated or caused by human activities. This book has been written to promote environmental literacy. In the past two years, the world had the advantage of biotechnology to ensure its survival during the COVID-19 pandemic. At times, it is easy to forget environmental protection keeps our existence safe, as it may not bring direct economic benefits. The world is currently facing many environmental problems including global warming, climate change, groundwater depletion, water pollution, air pollution, and other challenges. To prevent unnecessary consumption of resources and keep earth habitable, sustainability is a necessity. Environmental Biotechnology works towards achieving the Sustainable Development Goals (SDGs). The science of environmental biotechnology makes us aware of green pathways and implementation in society.

Environmental biotechnology can make a significant contribution to finding solutions to many environmental problems. Biotechnology is uniquely positioned to replace polluting materials and chemical processes with more sustainable, biological alternatives. This scientific field draws from millions of years of evolution in which living beings have specialized in producing and recycling all types of compounds and materials. These biological processes can be used to efficiently break down waste and produce materials using less water, land, and energy than traditional methods. Biotechnology applications capable of improving sustainability are virtually unlimited.

Environmental biotechnology uses integrated engineering approaches to solve environmental problems by applying microbiology principles and biotechnological techniques. The twenty-first century holds significant environmental challenges. These include a projected increase in the global population to over 8.5 billion people with limited land and water resources, slow increase in crop yields, demands for cheaper and safer food, and the need to sustain and improve the surrounding environment. Biotechnology has a widely described role in pollution control. Modern molecular biotechnology applies genome knowledge to engineer organisms with beneficial traits and enables novel solutions to environmental challenges. The Fourth Industrial Revolution has added to the toolbox of humanity the tools of molecular biotechnology. These tools promise similar positive improvements in well-being like the three previous technological revolutions. Utilizing available technology is crucial as humanity strives as a species to support growing populations with fewer resources. Public fear of biotechnology, despite the tremendous advances it has already provided, may prevent these innovations from having the impact they promise. Biotechnological industries must increase efforts to educate and engage the public to ensure biotechnology truly lives up to its potential.

Many xenobiotic chemicals can be removed from solid, gaseous, and aqueous wastes using bio-treatment technologies including bioremediation and phytoremediation. Heavy metal biosorption by living or dead organisms has been found to be more effective than physical and chemical methods. SCP production and mushroom culture on organic-rich effluents from dairy, beer, starch, and fruit pulp processing plants reduce pollution while also providing valuable products. Biofertilizers and biopesticides are increasingly used to replace chemical fertilizers and pesticides, which reduces the environmental risk associated with these chemicals. Existing non-degradable plastics will be replaced by bioplastics. Biotech technologies replace traditional polluting procedures in noxious waste gas removal, paper producing units, tanning sectors, and removal of sulphur from coal, just to name a few. This is only the beginning. Certain laboratory and field successes have yet to be commercialized, so the promise of biotechnology has yet to be fulfilled. This has the potential to give new dimensionality to environmental protection. Taking care of the environment today is a long-term investment for future generations. Environmental remediation reduces radiation exposure, for example, from contaminated soil, groundwater, or surface water, and involves more than simply removing radiation sources. It is also about safeguarding people and the environment from the potentially hazardous effects of ionizing radiation exposure.

The use of fossil fuels results in rising $CO_2$ and other greenhouse gas (GHG) emissions and causes global temperature rise and climate change negatively impacting human health, food supply, and future well-being for the species. In our previous two books by Springer-Nature, *Biofuels: Greenhouse Gas Mitigation and Global Warming: Next Generation Biofuels and Role of Biotechnology* (Kumar, Ogita and Yau (Eds) 2018 https://www.springer.com/in/book/9788132237617) and subsequently *Climate Change, Photosynthesis and Advanced Biofuels: The Role of Biotechnology in the Production of Value-Added Plants Bio-Projects* (Kumar, Yau, Ogita, Scheibe (Eds) 2020 https://www.springer.com/in/book/9789811552274), we have discussed these topics in depth. In our another book by Springer-Nature, *Innovations in Environmental Biotechnology: Emerging Trends and Challenges* (Arora, Kumar, Ogita and Yau 2022) https://link.springer.com/book/10.1007/978-981-16-4445-0, we have discussed green technology, sustainability, and emerging technologies in biotechnology.

This book comprises three parts:

## Part I: Environmental Remediation

This part describes biotechnological techniques for environmental bioremediation and applications of environmental biotechnology for nourishing and healing nature. In addition, this part provides recent innovations in bioremediation and pollutant mitigation.

## Part II: Phytoremediation

Phytoremediation is a plant-based approach to reduce hazardous contaminations present in soil, water, and air. This part reveals emerging innovations for sustainable pollutant clean-up.

## Part III: Environmental Safety, Health, and Risk Assessment

Global climate change, which has become one of the most visible environmental concerns of the 21st Century, can impact human health both directly and indirectly. This section deals with health and safety issue. In this part, technologies and methods are discussed for well-managed and efficient bioremediation processes.

We are thankful to **Professor Dr. Guangming Jiang**, Senior Lecturer, School of Civil, Mining and Environmental Engineering, Faculty of Engineering and Information Sciences, University of Wollongong, Australia, for writing the Foreword for our book.

We heartily thank all of our co-authors and colleagues who have contributed to this book. We dedicate our book to renowned Botanist Professor Swami Dayal Tewari on his 103rd birth anniversary.

Jaipur, Rajasthan, India Sudipti Arora
Jaipur, Rajasthan, India Ashwani Kumar
Hiroshima, Japan Shinjiro Ogita
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# Acknowledgements

We acknowledge the constant encouragement and support received from Prof. A. B. Gupta, Prof. A. A. Kazmi (IIT, Roorkee), Prof. Guangming Jiang (University of Wollongong, Australia), Prof. Vijay R. Kumar (University of Rajasthan, Jaipur), Dr. B. Lal Gupta (Director, Dr. B. Lal Institute of Biotechnology), Dr. Aparna Datta, and Mr. Saksham Gupta (Dr. B. Lal Institute of Biotechnology, Jaipur).

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We acknowledge the authors of all the articles and their publishers who have very kindly granted licenses to reproduce or provided open access to selected figures, tables, etc. for use in this book.

We acknowledge the assistance by internal editorial board of Dr. B. Lal Institute of Biotechnology headed by Dr. Sonika Saxena for support during editing of this book. We would also like to thank Ms. Devanshi Sutaria and Mr. Saurabh Dhakad for their constant technical assistance and support.

We are forever thankful to our families for their frequent support. We would also like to thank Springer-Nature for publishing the book and the support from Ms. Priya Shankar, Ms Harshini Punithan and Dr. Akansha Tyagi of Springer-Nature for guiding us through the process and bringing out this book so nicely.

# Introduction

*Plans to protect air and water, wilderness and wildlife are in fact plans to protect man.*
***—Stewart Udall***

Technology has the paradoxical ability to be both the source of environmental damage and our best chance for healing it today and preventing it in the future. Indeed, enormous scientific and technological efforts have been undertaken over the last three decades to better understand the nature of environmental degradation and to develop methods to mitigate the impacts of past pollution.

Biotechnology is the use of biology to the solution of problems and the creation of valuable goods. To date, the development of methods to handle aqueous, solid, and gaseous wastes has been the primary emphasis of environmental biotechnology. As it becomes evident that waste streams must be addressed holistically, new environmental concerns continue to emerge. Emerging contaminants such as heavy metals and toxic pollutants in wastewater can cause contamination for our foods/drinks and animal feeds or possibly reduced yield of our crops. The main pollutants include bacteria, viruses, parasites, fertilizers, pesticides, pharmaceutical products, nitrates, phosphates, plastics, faecal waste, and even radioactive substances. These pollutants can be a potential cause of ill-health. In recent years, the demand for the use of sustainable and eco-friendly environmental processes is rapidly growing subjected to economic, public, and legislation pressure. Biotechnology provides a plethora of opportunities for effectively addressing issues pertaining to the monitoring, assessment, modelling, and treatment of contaminated water, air, and solid waste streams. In this context, source tracking of environmental pollutants and process modelling using biological based methods are becoming increasingly important, mainly owing to the accuracy and robustness of such techniques.

New technologies are currently being developed to achieve this goal. Biomembrane reactor technology and newly discovered nitrogen removal procedures using anaerobic ammonium oxidation are examples of novel aspects in the sphere of water treatment. Also, the constant pursuit of reuse of treated wastewater is the most difficult task. Indeed, water scarcity is affecting an increasing number of countries around the world, necessitating short water cycles. Finally, biotechnology plays an important role in the development of novel wastewater treatment methods based on decentralized sanitation and reuse. Solid waste is a big issue all around the world. The use of anaerobic digestion to handle biowaste has matured into a mature technique. New biotechnological approaches that are linked to physical processes, such as plasma technology, will undoubtedly receive a lot of attention in the future decades. The use of biostimulation, remediation, and augmentation to clean up soil and sediment is now well established.

Today's challenge for scientists and industrialists alike is to address the problem of environmental deterioration in a safe, environmentally sound, and cost-effective manner. **Bioremediation** is a technology that has been extensively researched. Despite the fact that the technology has gained a lot of attention, there is still a need to look into the trends that have emerged in studying bioremediation over the last decade; some of the aspects that need to be looked into are the comparability of available data and the cost of bioremediation. The applicability of available technology, availability or unavailability of technology for laboratory investigations, geographical diversity, lack of expertise in the field, regulatory bottlenecks associated with extensive trials, and a general acceptance of the technology's effectiveness may have hampered future research.

Bioremediation refers to the clean-up of pollution in soil, groundwater, surface water, and air using typically microbiological processes. It uses naturally occurring bacteria and fungi or plants to degrade, transform, or detoxify hazardous substances to human health or the environment. Microorganisms are used in bioremediation to decrease, eliminate, contain, or alter harmful pollutants in soils, sediments, water, and air. The use of microorganisms to eliminate or immobilize waste compounds by mineralization, transformation, alteration, or detoxification process which targets the toxic compounds, is gaining importance. Natural bioremediation has been employed in waste management treatment for decades. Bioremediation comprises several techniques; most importantly, Biostimulation and Bioaugmentation are the two main kinds generally in use. Bioremediation represents a field of great expansion due to the important development of new technologies. Among them, several innovations have currently been made: such as the use of (a) earthworms by ecosystem engineers as a sustainable catalyst for environmental remediation, (b) plants in bioremediation for heavy metal removal, (c) nitrite to mitigate microbial influenced corrosion of concrete sewers, (d) 'vertical cultivation' to move towards a more sustainable and eco-friendly farming, and (e) CRISPR-Cas technology, a genome-editing powerhouse, for molecular plant breeding to generate abiotic stress tolerant crop varieties, to name a few.

In this book, we cover innovative technologies, recent developments, and specific new researches applied in the field of bioremediation, with special attention to 'microorganisms'. The book includes 40 chapters, related to environmental remediation, phytoremediation, environmental health, and risk assessments as discussed below.

## Part I: Environmental Remediation

Cleaning up the environment is an important focus of the green economy. Sites that are polluted because of industrial activity, the use of pesticides and fertilizer, or the release of other pollutants must be cleaned up in order to redevelop them or return them to their natural state.

Prior to the enactment of modern environmental regulation, many companies simply released hazardous materials into the environment. They would dump chemicals and other pollutants onto unused land or into lakes, rivers, and streams. Sites also would become polluted through accidents or improperly functioning equipment. Polluted sites that can be cleaned and redeveloped are known as brownfield sites. Environmental professionals use a number of technologies to clean up polluted sites, a process known as environmental remediation.

This part comprises 10 chapters which emphasize on bioremediation approaches. They include microbial nano-biotechnology, the role of bacteria and fungus in the bio-sustainable rehabilitation of polluted sites, anaerobic digestion, mitigation of microbially influenced corrosion of concrete sewers using nitrite, and role of metabolic engineering for sustainable development.

## Part II: Phytoremediation

The use of green plants to remove, contain, or render harmless environmental toxins is known as phytoremediation. It is worth noting that this covers the utilization of plants for in situ water, sediment, soil, and air remediation. Specially selected or modified plants can be employed in this procedure to extract harmful metals from soil or water, as well as remove radioactive elements, toxic organic compounds, and, if possible, mineralize them.

This part comprises 14 chapters which emphasize on phytoremediation. Chapters includes a sustainable solution to combat pollution, phytoremediation of coastal saline vertisol through biosaline agriculture, emerging biotechnologies in agriculture for efficient farming and global food production, role of beneficial microbes in alleviating stresses in plants, genetically modified crops to combat climate change and environment protection—current status and future perspectives, to name a few.

## Part III: Environmental Safety, Health, and Risk Assessments

With rising industrialization, achieving environmental sustainability is a major global goal today. Industries are important economic generators, but they are also major polluters because their untreated/partially treated effluents are typically discharged into the aquatic environment or disposed. Industrial effluents frequently contain highly toxic and harmful contaminants, which harm the environment and pose serious health risks to humans and livestock. As a result, there is a pressing need to create sustainable methodologies for treating/detoxifying pollutant materials before they are released into the environment, as well as to identify eco-friendly strategies to manage industrial waste. It is important to assess the health impacts and risks while using the innovative technology.

This part covers 16 chapters which includes microorganism: an eco-friendly tool for waste management and environmental safety, biochemical effect of nanoparticles-treated plants extract on waterborne pathogen: a way towards future technique for water purification, modern waste management, monitoring of paralytic shellfish toxins using biological assays, role of environmental factors in increased cancer incidences and health impacts, CRISPR-Cas technology: a genome-editing powerhouse for molecular plant breeding, Bxb1-*att* site-specific recombination technology, COVID-19 mRNA vaccines, and fundamentals of SARS-CoV-2 detection in wastewater for early epidemic prediction and key learnings on treatment processes for removal of viral fragments.

This book provides state-of-the-art description of various approaches, techniques, and some basic fundamentals of bioremediation to manage a variety of organic and inorganic wastes and pollutants present in our environment. A comprehensive overview of recent advances and new development in the field of bioremediation research is provided within relevant theoretical framework to improve our understanding of the cleaning up of polluted water and contaminated land. New technologies are also included in environmental health and health risk assessments like surveillance tool for the detection of COVID-19. The book is easy to read, and language can be readily comprehended by aspiring newcomers, students, researchers, and anyone else interested in this field. Contributors of the book chapters are experienced researchers and renowned scientists around the world. In this edited book volume, we have addressed the scope of the inexpensive and energy neutral bioremediation technologies. The scope of the book extends to environmental/agricultural scientists, students, consultants, research scholars (a better word?) owners, industrial stakeholders, regulators, and policy makers.

# Contents

## Part I Environmental Remediation

**Part II Phytoremediation**

**Part III Environmental Safety, Health and Risk Assessments**

# Editors and Contributors

## About the Editors

**Sudipti Arora** is an Environmental Research Scientist at Dr. B. Lal Institute of Biotechnology, Jaipur, with a specialization in wastewater treatment by Vermifiltration technology and other nature-based sanitation solutions. She is also the founder of "Prakrit: a centre of excellence in Environmental Biotechnology". Prakrit aims to provide transformative education and innovative solutions to drive the planet towards sustainability.

Prakrit is dedicated to solving the problems pertaining to the environment, particularly water, wastewater, sanitation, health and hygiene, solid waste management, energy and climate change, soil and land use, good health and well-being fulfilling sustainable development goals.

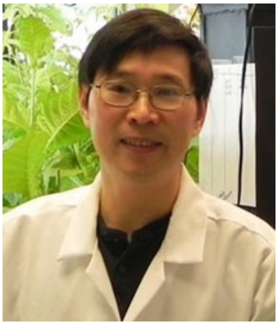
She has obtained her Ph.D. from the Indian Institute of Technology (IIT), Roorkee, and Master's in Environmental Engineering from Malaviya National Institute of Technology, Jaipur. She is the Assistant Director at Dr. B. Lal Institute of Biotechnology, Jaipur, with teaching and research experience of more than 10 years with various research publications of international repute. She has also been the guest faculty at Malaviya National Institute of Technology, Jaipur. She has been working on indigenous wastewater and faecal sludge treatment through Vermifiltration technology for 8 years and has expertise in integrated solid waste management through circular economy and wastewater-based epidemiology, particularly pathogens in wastewater and AMR. She is a member of the International Water Association and has been involved in WASH projects since 2010. She has been involved in SARS-CoV-2 wastewater-based epidemiology research from India with other Indian and UK International partners through global challenge research funds (GCRF 2020). She is also involved in an Indo-European project titled “Identifying best available technologies for decentralized wastewater treatment and resource recovery for India (Saraswati 2.0)” funded by DST, GOI, and “Optimization of the Disinfection Process for Wastewater Treatment by Hybrid Disinfection” funded by DST, in collaboration with MNIT, Jaipur. She is also involved in an integrated technology intervention project, funded by DST, GOI, on “transforming Aandhi village in Rajasthan towards a zero waste model through green technology interventions”. She is a guest editor and reviewer of various international journals, to name a few, She has authored and co-authored important discoveries in Environmental Biotechnology in more than 20 Science Citation Index Expanded (SCIE) journals. She has guided the research work of 50 UG and PG students. She has been honoured by various awards including “Research Excellence Award 2020 by INSc for outstanding research work on vermifiltration technology, Dainik Bhaskar “Green Parrot Award” in 2018 for her contribution to Indigenous water treatment, Best Poster Award at IWA conference on Global Challenges: Sustainable Wastewater Treatment and Resource Recovery in Kathmandu in 2014, and Young Scientist Award at 8th Uttarakhand State Science and Technology Congress in Dehradun in 2013. She is also been invited to give Expert Lectures at a number of national and international venues, conferences, and workshops..

**Ashwani Kumar** was born (1946) to Mr. Swami Dayal Tewari and Mrs. Shanti Devi Tewari in Bandikui, Rajasthan, India. He received his B.Sc. at Agra University and his M.Sc. (Botany) at the University of Rajasthan. He was awarded a gold medal for standing first in the order of merit. His Ph.D. (1971) was under the supervision of Professor H.C. Arya and postdoctoral with Professor Dr. K-H. Neumann, and later on with Professor Dr. Sven Schubert at Justus Liebig Universitat, Giessen, Germany, as Alexander von

Humboldt Fellow. His botanist father Professor Swami Dayal Tewari (M.Sc. in Botany) was his first teacher. Professor Ashwani Kumar was also selected for Indian Administrative Services (IAS: IPS) (1972) but he opted a career in botany, which being his family subject. His wife Mrs Vijay R. Kumar has also been a Professor of Botany at the University of Rajasthan in her own stead. He was appointed as Asst. Professor in 1969, Associate Professor in 1985 and Full Professor from 1996 to 2007. Then he was an Adjunct Professor until 2016. He along with members of COC introduced Integrated Biotechnology, a 5-year course in Rajasthan. In recognition of his research contributions, Dr. Kumar was awarded the Alexander von Humboldt Fellowship for 1977–1979, with several resumption of fellowships until 2017, British Council Visitorship, UK (1986); Visiting Professorship at Toyama Medical and Pharmaceutical University in Japan (1999–2000); Toyama Prefectural University, Japan (2011); and INSA-DFG visiting Professorship in Germany (1997). He holds a diploma in German language and has a certificate in French language. His area of research includes photosynthesis in vitro and in vivo, biotic and abiotic resistance, ethno botany, bioenergy and presently understanding salinity resistance in maize. He has also carried out research projects granted by UGC, USDA-ICAR, MNES, CSIR, DST, DBT and FACT. He attended a large number of national and international conferences as an invited speaker and served as chair or co-chair of the International Botanical Conference, Berlin, and EU Biomass conferences. He has published 220 research papers in national and international journals and 23 books, of which 10 books are authored and 13 are edited from reputed publishers such as Springer and IK. He is a member of the editorial board of *Current Trends in Biotechnology and Pharmacy*. He has guided 39 research students to Ph.D. at the University of Rajasthan, Jaipur, India. He is an elected Fellow of Botanical Society, Fellow of Phytopathological Society, Fellow of the Indian Society of Mycology and Plant Pathology, Fellow of Mendelian Association, Fellow of Association of Biotechnology and Pharmacology and Fellow of Indian Fern Society. He received V. Puri Medal as Botanist and Teacher's Excellence award of CEE in 2015. He has been a consultant in a World Bank Project sanctioned to SPRI-HPPI, President of Commonwealth Human Ecology Council (India chapter) and presently President of Indian Botanical Society and President of the Society for Promotion of Plant Science 2021–2022..

**Shinjiro Ogita** has over two decades of experience in the field of plant biotechnology. In 1992, he started his research career as a master's student at the United Graduate School of Agriculture, Tokyo University of Agriculture and Technology (TUAT), Japan, and in 1997, he received his Ph.D. in agriculture (subject: sciences of resources and environment). He is an expert in the field of cell and tissue culture and transformation technologies for higher plants. He has worked at the following institutes based on projects worked: embryogenic capacity of elite coniferous trees at the Laboratory of Cell Manipulation, Division of Bio-resources Technology, Forestry and Forest Products Research Institute, Ministry of Agriculture, Forestry and Fisheries, Japan (October 1997–September 2000).

- To establish genetically modified decaffeinated coffee plants at the Laboratory of Plant Molecular Breeding, Research and Education Centre for Genetic Information, Nara Institute of Science and Technology (NAIST), Japan (October 2000–March 2003).
- To teach plant biotechnology, microbiology and molecular biology as assistant professor (2003–2006), lecturer (2006–2010) and associate professor (2010–2015) at the Laboratory of Plant and Cell Engineering, Biotechnology Research Centre and Department of Biotechnology, Toyama Prefectural University (TPU), Japan (April 2003–March 2015). He presently works as a full professor at the Faculty of Life and Environmental Sciences, Department of Life Sciences, Prefectural University of Hiroshima (PUH), Japan (April 2015)..



**Yuan-Yeu Yau** obtained his master's and Ph.D. from the University of Wisconsin-Madison, USA. He worked as a postdoc and a specialist at the University of California-Berkeley and Plant Gene Expression Centre (USDA-ARS) in Albany, California, working in the areas of plant biotechnology, plant breeding, plant biochemistry and plant physiology. Dr. Yau worked on projects with grants supported by the NFS (National Science Foundation), NIH (National Institutes of Health), USDA, Cotton Incorporated, California Fresh Carrot Advisory Board and Northeastern State University.

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# Part I

# Environmental Remediation

# Ecosystem Engineers: A Sustainable Catalyst for Environmental Remediation

# 1

Devanshi Sutaria, Sonika Saxena, Kamlesh R. Shah,
and Sudipti Arora

## Abstract

Enzymes are robust catalysts with an increasing variety of biotechnological uses. Their characteristics make them appealing for waste/pollutant treatment procedures, and their usage may be preferable to traditional treatments. Implementing techniques for decontamination and restoration of contaminated areas has now become a priority, with bioremediation using biological agents being a potential alternative. The fundamental role of earthworm and microorganism interaction, as well as their biocatalyst, plays a critical role as ecosystem engineers in pollution mitigation. Enzyme-based approaches provide numerous benefits over microbial cells, assuming that the biocatalyst satisfies specified characteristics, such as effectiveness in removing the target pollutants, independence from costly coenzymes or cofactors, enzyme stability and a low-cost manufacturing method. The relevance of enzyme technology research directions for future progress in environmental biotechnology is discussed. The use of enzymes has been described as a long-term method to reduce pollution. Aside from biological and biochemical techniques, such as enzyme exploration and enzyme design, the chapter also examines work in adjacent research disciplines such as enzyme insolubilisation, reactor design and the use of additives. The efficacy of enzymatic procedures is obvious, especially when coupled with proven technology. The cost and stability of biocatalysts must be addressed, and collaboration and interchange between academics and industry must be strengthened further to accomplish the aim of sustainability.

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Bioremediation · Enzymatic degradation · Ecosystem engineers · Sustainability · Vermiremediation

## 1.1 Introduction

Environmental contamination has grown in recent decades as a result of increased human actions such as population growth, hazardous farming methods, unplanned urbanisation, deforestation, fast industrialisation and injudicious use of energy reserves, among other things. Chemical fertilisers, heavy metals, nuclear wastes, pesticides, herbicides, insecticides and hydrocarbons are among the pollutants that cause environmental and public health issues owing to their toxicity. Thousands of contaminated sites have been identified, with more expected to be discovered in the future decades. Pollutants are released into the environment as a result of unlawful dumping by chemical firms and enterprises. Many of the previous site clean-up procedures, such as excavating polluted soil and transporting it away to be land filled or burned, were prohibitively expensive and did not provide a lasting solution. Contaminated areas are typically the result of historical industrial operations when the health and environmental consequences of hazardous material manufacturing, usage and disposal were less well understood than they are now. The problem is widespread, and the number of polluted locations is expected to be substantial. Contaminated land is now widely recognised as a potential threat to human health, and the continued discovery of contaminated land in recent years has prompted international efforts to remediate many of these sites, either as a response to the risk of adverse health or environmental effects caused by contamination or to allow the site to be redeveloped for use.

Despite their impacts on contaminants and influence on the resource availability for other species, such as microbes and plants, earthworms and microorganisms are regarded as ecosystem engineers. However, little attention has been paid to the linkages between their environmental consequences and the consequent change of natural selection pressures on engineers as well as other species. Earthworms are considered to have a positive impact on the soil fabric as well as the putrefaction and mineralisation of litter by breaking down organic matter and producing large amounts of faeces, thereby mixing litter with mineral soil, and microorganisms are commonly used as bioremediation agents for degrading pollutant from the site (Tejada and Masciandaro 2011). As a result, they are predicted to be strong bioindicators of site integrity, which will be beneficial when planning for environmental restoration and improvement. In this chapter, we summarised existing information on vermiremediation, as well as the function of microbial enzymes as ecosystem engineers.

Bioremediation is the process of degrading environmental pollutants into less harmful forms using living creatures, mainly bacteria. It degrades or detoxifies chemicals that are detrimental to health and/or the environment using naturally occurring bacteria, fungus or plants. For bioremediation to be effective, microorganisms must enzymatically attack the pollutants and convert them to harmless products. Microbes are highly useful in remediating the polluted environment; bioremediation and natural reduction are also viewed as solutions for emerging contamination concerns (Tekere 2019). There are a lot of microorganisms, including aerobic and anaerobic bacteria. In the bioremediation process, fungus is involved. Hence, this remediation is known as micro-bioremediation. Bioremediation plays a significant role in various chemical wastes being degraded, eradicated, immobilised or detoxified. Bioremediation is a long and drawn-out procedure. Only a few bacteria and fungus species have been shown to be effective pollution degraders. The pH, temperature, oxygen, soil structure, moisture and adequate amount of nutrients, as well as low bioavailability of pollutants and the presence of other hazardous chemicals, all impact bacterial development (Azubuike et al. 2016). Although microorganisms may survive under harsh conditions, the majority of them prefer ideal conditions, which are difficult to establish outside of the laboratory.

To fulfil the needs of an expanding human population, increased industrialisation, the use of chemical pesticides to improve crop output and other anthropogenic activities have contaminated the soil. All of these activities have raised the quantity of heavy metals, crude oil, gasoline, diesel and other aromatic hydrocarbons in the soil to dangerous levels. ‘Vermiremediation’ is a non-toxic and environmentally acceptable restorative technique. Earthworms are employed in vermiremediation techniques. They consume the polluted soil, grind and digest it, and excrete the dark-coloured, porous, non-hazardous substance known as vermicast. The physicochemical characteristics and biological activity of this vermicast have been enhanced. Furthermore, these earthworms repair hazardous substances such as heavy metals and petroleum pollutants in a matter of weeks to months. Vermiremediation is now one of the most effective methods for removing hazardous chemicals from soil.

Earthworms can be used effectively in bioremediation techniques to increase organic pollutant biodegradation due to its biological and physiological activities. Earthworms have been proven to aerate and bioturbate soils, as well as enhance nutritional quality and fertility, all of which are known to restrict bioremediation. Earthworms have also been found to slow the binding of organic pollutants to soils, to liberate previously soil-bound toxins for further degradation and to encourage and spread contaminant-degrading microbes.

The biodegradation of hazardous organic pollutants is thought to involve a vast variety of enzymes from bacteria, fungus and plants. Earthworm assisted microbial enzymes drive bioremediation, which is a cost-effective and environmentally beneficial biotechnology. The study in this field would aid in the development of sophisticated bioprocess technology to minimise pollutant toxicity while also obtaining innovative valuable chemicals. The regulatory mechanisms of earthworm assisted bioremediation-related enzymes like oxidoreductases, ligninase, amylase and hydrolases have been thoroughly investigated.

## 1.2 Green Technologies for the Sustainable Development

Green technologies are currently playing an important role in shifting the course of the country's economic growth towards sustainability and providing an alternative socio-economic status that will allow present and future generations to live in a clean and healthy environment that is in harmony with nature. Green technology, also known as clean technology, contributes to the creation and extension of processes, practices and applications which improve or replace existing technologies, allowing society to meet its own needs while significantly reducing human impact on the planet and decreasing environmental risks and ecological scarcity. Green technologies are focused on the goals of sustainable development which are becoming increasingly important for ensuring sustainability.

In the last 2 years, green technologies in environmental remediation have attracted intense research interest, with an emphasis on approaches that are sustainable and ecologically benign. These methods include treatments that employ green amendments/reagents derived from various sources and/or that may be naturally decomposed or reused with minimal impact on the treated ecosystem's physico-chemical and biological characteristics. These approaches also include cost, carbon discharge, environmental health and capacity following interventions for various land use objectives. This chapter provides different perspectives of green technology in decontaminating pollutants with the help of earthworm assisted bioremediation driven by enzymatic technology for the recent advancements made towards sustainable development in the field of environmental remediation. Hence, it will focus on the eco-sustainable method for ecosystem rehabilitation.

## 1.3 Bioremediation: An Effective Tool to Manage Pollution

In today's global setting, environmental pollution is a primary cause of severe environmental harm. Industries make a significant contribution by releasing a variety of harmful chemicals into the environment via wastewater. Because of their stubborn and delayed degradation capabilities, phenolic and chlorinated pollutants appear to be the most hazardous and complex (Azubuike et al. 2016). The bioremediation process is based on the metabolic capacity of microorganisms to detoxify or convert the contaminant, which is also dependent on permeability and bioavailability. The existence of different enzymes and catabolic genes in microorganisms is responsible for their varied catabolic activity. Microbes also have additional adaptation strategies, such as using efflux pumps to lower the concentration of harmful chemicals within the cell, producing biosurfactants and modifying the cell membrane to maintain required biological activities. All of these processes and metabolic capacities combine to make microorganisms a potential cleaning tool for bioremediation of waste-contaminated areas. The addition of different microorganisms (called seeding or inoculation) to a contaminated environment to encourage higher rate of biodegradation might speed up the remediation process. The inoculations can be a combination of non-indigenous microorganisms from diverse contaminated habitats (specially chosen and grown for their varied pollutant-degrading properties) or a mixture of microbes picked from the site to be rectified or mass-cultured in the research lab. The primary benefits of bioremediation over conventional treatment are as follows: cheap cost, high efficiency and reduction of chemical and biological sludge, selectivity to specific metals, no extra fertiliser demand, biosorbent regeneration and metal recovery. Bioremediation can occur naturally (natural attenuation or intrinsic bioremediation) or can be aided by the addition of fertilisers to increase accessibility within the medium (biostimulation). A wide range of microbial enzymes that participate in the transformation of both natural and manufactured organic pollutants into intermediate combinations that may be comparable or less harmful to their parent chemicals are used in the biodegradation method. Because of their huge catabolic capacity and variety, a significant number of fungus, bacteria and Actinomycetes genera have been created to biodegrade the hazardous pollutants (Dvořák et al. 2017).

Enzyme-mediated bioremediation is a potential method since enzymes, by definition, may selectively catalyse the conversion of numerous hazardous chemicals, even when the compounds are present in the environment at low quantities for ‘chemical reaction’. As previously stated, modifying enzymes are promising biocatalysts for the long-term mitigation of various pollutants because they have the ability to degrade a wide range of lignin, methoxylated compounds, phenol, polyphenol, EDCs and nonphenolic compounds in an eco-friendly manner. Immobilisation of enzymes can result in increased catalytic potential and the generation of lignin-modifying enzymes (Zdarta et al. 2019) and via engineered microbes (Dvořák et al. 2017).

### 1.3.1 Ecosystem Engineers

Biodegradation is the process of employing various biological agents to clean up an environment that has been contaminated by organic molecules that can be alive or non-living, naturally or genetically engineered. Earthworms are sometimes referred to as ‘ecosystem engineers’ since they drastically modify the physical, chemical and biological properties of the soil profile. These changes have the potential to affect the habitat and activity of other species in the soil ecosystem. The symbiotic interactions between earthworms and microorganisms gradually break down into fragmented organic components and incorporating them into water-stable aggregates. The primary nutrients in earthworm castings and earthworm burrow linings may be used by plants. There is evidence that interactions between earthworms and microorganisms not only provide these readily available nutrients but also indirectly promote plant growth.

### 1.3.2 Conventional Bioremediation Approaches for Pollutant Mitigation: Micro-Remediation

Bioremediation is a promising method that uses natural resources such as bacteria and plants in an 'eco-friendly' manner to remove harmful organic contaminants (Tekere 2019). The present bioremediation techniques are mostly focused on biodegradation, which entails the full removal of harmless organic harmful compounds from a severely polluted medium or location. Many biological degradation mechanisms and pathways have been shown to function in the presence or absence of oxygen (Ghattas et al. 2017; Ronen and Abeliovich 2000; Wang et al. 2018). Bioremediation research focuses on strengthening the remediation process by providing optimal concentrations of biocatalysts, chemicals and nutrients, generally including oxygen, required for the breakdown and detoxification of hazardous components via microbial metabolism and/or enzymatic conversion (Adetutu et al. 2015). Microorganisms are utilised to remediate polluted areas that include a wide range of contaminants. Micro-remediation is described as the employment of microorganisms to remove, contain or transform pollutants in the environment to non-hazardous or less-hazardous forms via microbe metabolism. The discovery of the many interactions between microorganisms and various hazardous metal species led to the development of toxic metal micro-remediation. Toxic metals, on the other hand, cannot be degraded or eliminated; nevertheless, there are a variety of methods for microbes to interact with them and render them harmless. Microorganisms, for example, can start changing their chemical composition by changing their oxidation states; they can also expedite their removal by increasing the solubility of toxic metal species and allowing them to be easily washed or flushed away from the site, or, conversely, immobilise them by precipitating them out of the soil solution (Cheung 2013).

Microorganisms are used in microbial aided pollution remediation to either totally break down hazardous chemicals into water and carbon dioxide (organic pollutants) or to accelerate their conversion into less toxic forms (Malla et al. 2018). Because of its cheap cost and biology-based approach, this technique is a viable alternative to traditional chemical treatment procedures (Kang and Cao 2014). In comparison to physical and chemical techniques to treating diverse environmental pollutants, bioremediation is thus seen as a more cost-effective, flexible, effective and ecologically friendly option (Jeon and Madsen 2013). Several remediation techniques are based on a bacterial-derived enzymatic system, while others are based on bioreactors and a few others incorporate plant-based treatments. The in situ or ex situ mitigation of pollutants is accomplished using a bacterial-mediated remediation technique to clean up a contaminated site (Baric et al. 2014). In situ implies that bioremediation takes place immediately at/within the polluted site. Simultaneously, ex situ indicates that the microbiological clean-up can be performed away from the site of contamination. In the natural environment, remediation is considered sluggish and sometimes difficult to regulate. It is advantageous in this context and for ex situ remediation to employ specially built bioreactors to accelerate remediation. Bioreactors have been created for use in bioremediation procedures to obtain the best conditions for aeration, microbial growth and biodegradation in order to fulfil the various bioremediation aims. Packaged, stirred tanks, airlift, slurry phase and partitioning phase reactors have been reported to be utilised in the bioremediation of various organic contaminants.

Over the last few decades, countless bacterial species producing enzymes have been defined, and these enzymes have now been studied to remove lignin, chlorinated lignin, organic phenol and chemicals that induce endocrine disruption in humans (Bilal et al. 2019; Falade et al. 2018). As a dye decolorising peroxidase, a newer peroxidase enzyme has been identified. This enzyme has the potential to be utilised as a biocatalyst for the removal of a variety of hazardous contaminants from the environment (Brissos et al. 2017) as shown in Fig. 1.1.

### 1.3.3 Mechanism behind Degradation

Microbes interact physically and chemically with contaminants, leading to structural changes or total destruction of the target pollutants. The breakdown of organic contaminants is centred on two processes: growth and metabolism. Microbes use organic pollutants to proliferate during bioremediation. Other important nutrients, such as N and P, as well as minor nutrients such as sulphur (S) and trace elements, are also required for their growth. As a result, it offers electrons that these bacteria may employ to generate energy. The organic components are completely disintegrated as a result of this process. Furthermore, bacteria get energy by catalysing energy-generating chemical processes that entail chemical bond breakdown and electron transfer away from the contaminant. These types of chemical reactions are known as oxidation-reduction reactions because the organic contaminant is oxidised by losing electrons (electron donor), while the molecule that receives electrons is reduced (electron acceptor). The energy gained by these electron exchanges is subsequently 'spent', along with some of the pollutant's electrons and carbon, to produce additional new cells. These two components (electron donor and acceptor) are critical for cell proliferation and primary substrates. Microbes can accelerate their metabolism by combining electron donor and electron acceptor processes. In addition, they have rebuilt a slew of additional techniques for cleaning up the environment. Microbes' metabolism has been divided into two types: aerobic and anaerobic.

The major enzymatic processes that occur during aerobic biodegradation are oxidations, which are mediated by oxygenases and peroxidases and incorporate oxygen into the substrate. Biodegradable organisms' respiration is at two points in their metabolism: (Abdel-Hamid et al. 2013) during the first attack on the substrate and (Adetutu et al. 2015) at the end of the respiratory chain. Under regulated anaerobic conditions, soluble carbon molecules are disintegrated stepwise, into $CO_2$, methane ($CH_4$), ammonia ($NH^{4+}$) and hydrogen sulphide ($H_2S$) with the help of fermentative and acetogenic microbes, methanogens or sulphate reducers. Most information related to biodegradation of organic contaminants involves oxidative degradation (Tekere 2019). In the aerobic process, $O_2$ availability increases the

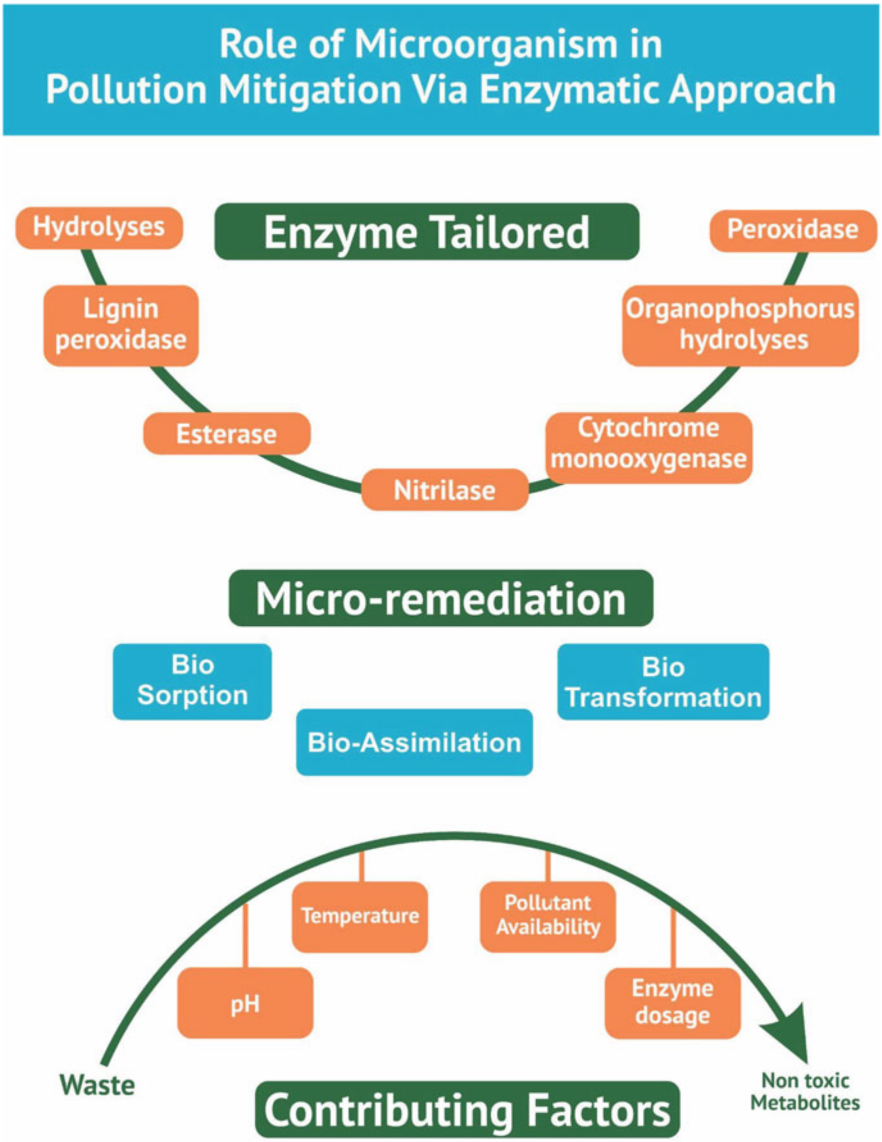

**Fig. 1.1** Role of microorganism in mitigating pollution

proliferation rate and yield of aerobic microbes. Aerobes produce monooxygenases and dioxygenases, which are helpful in the oxidation of hydrocarbons. By inhibiting reductive dehalogenation, the presence of oxygen also reduces anaerobic activities such as the breakdown of halogenated pollutants. At severely polluted locations, $O_2$ is rapidly depleted during hydrocarbon breakdown, resulting in anaerobic conditions. Anaerobic activity is common and has been documented in nitrate-, iron-, manganese- and sulphate-reducing circumstances, as well as methanogenic conditions. In normal settings, these degradation activities are frequently accompanied by changes of other molecules and other xenobiotics. Co-metabolism, co-oxidation, gratuitous metabolism and free or accidental metabolism are all names used to describe this phenomenon. Co-metabolism refers to the metabolism of an organic molecule with no nutritional benefit in the presence of a growth substrate that is consumed as both the main carbon and an energy source. It is a common occurrence of microbial activity. Bacteria release metabolic enzymes that break down the complex organic stuff that surrounds them, allowing for simpler digestion. Because these enzymes are typically nonspecific, they may work on a wide range of substrate materials, even ones that are not helpful for energy production in bacteria. Enzymes that may oxidise a wide range of substrates include methane monooxygenase and ammonia monooxygenase (Hazen et al. 2009). Co-metabolic treatment may be able to detect contaminants at trace levels as long as the substrate available for bacterial growth is maintained at appropriate quantities, as these bacteria do not rely on this pollutant for energy. Co-metabolism was once recommended for the treatment of TCE, but it is currently seldom utilised since the intermediate epoxide produced obstructs biological action much more. By inhibiting the enzymes, TCE oxidation by-products such as TCE epoxide result in the deactivation of oxygenase activity. Natural substrates can be used to overcome inhibition and inactivation. Co-metabolism may be used to treat other hazardous pollutants such as N-nitrosodimethylamine (NDMA) and 1,4-dioxane (http://www.cet-journal.de).

### 1.3.4 Sustainable Enzyme Technology for Environmental Remediation

Emerging contaminants in nature are explicit and related to numerous chronic detriments in biotic components, which eventually degrade the ecosystem as exposure occurs even at low concentrations and over long periods of time. Specific pollutant substrate is recognised by specific microbial enzymes under optimal conditions (temperature/pH/contact time/concentration) and efficiently transformed into various innocuous products enzymatically via various enzymatic reaction mechanisms such as oxidation, reduction, elimination and ring opening. Similarly, incorporating bioengineered microbes into today's study field improves the breakdown of contaminants from the recalcinated environment more effectively and efficiently.

Enzymes are the most effective bioremediation agents because they accelerate all chemical reactions on contaminants. The specificity of enzymes is generally broad enough to allow them to operate on various compounds with similar structures. Furthermore, enzymes can be engineered to improve their stability and efficiency under certain circumstances or with specific substrates (Leerachat et al. 2012; Festa et al. 2008).

Omics technologies play an important part in these advancements (Ufarte et al. 2015). Enzymes can be utilised in bioremediation in two ways: individually as isolated enzymes that are introduced to the polluted region or as a complete cell, such as bacteria, fungus or algae. Second, constant aeration, inoculation and feeding are required. Besides, environmental conditions should be prepared for microorganisms living, even though there might still be some toxic compounds in the environment that inhibit microorganisms' activity (Eibes et al. 2015; Rayu et al. 2012). Individual enzymes have some benefits over microbial whole cells, including sufficient precision, easier handling and storage, standardisable activity, greater mobility due to smaller size, activity in the presence of high concentrations of toxic compounds and biodegradability, which inhibits persistence and recalcitrance (Rao et al. 2010; Gianfreda and Rao 2004). Enzyme synthesis is minimal in the natural environment; however, it is feasible to boost enzyme production under controlled circumstances. Recombinant DNA technology and gene engineering, on the other hand, offer several potential to generate more efficient and diverse enzymes. Furthermore, nanotechnology provides various techniques for increasing enzyme stability by lowering susceptibility to mechanical stress, maintaining enzyme third structure and shielding enzymes from proteases (Rayu et al. 2012).

Enzymes can act intracellularly, that is, in the presence of or inside their originating cells; extracellularly, that is, both in the presence and absence of their originating cells; free, that is, soluble in solution with homogeneous catalysis; or immobilised, that is, linked through different links to a solid matrix with heterogeneous catalysis. The most important enzyme classes in contaminated environment remediation are hydrolases, dehalogenases, transferases and oxidoreductases. Bacteria; fungus, primarily white-rot fungi; plants; and microbe-plant relationships are the primary producers. Many of these enzymes have been shown to be capable of converting various xenobiotic compounds (Whiteley and Lee 2006). Phosphotriesterases, amidases, proteases, carbohydrases (cellulases and amylases) and depolymerase are examples of hydrolases. The primary types of oxidoreductases include mono- or dioxygenases, reductases, dehalogenases, cytochrome P450 monooxygenases, phenoloxidases (laccases, tyrosinases) and peroxidases (lignin and manganese peroxidases). Esterases, amidases and proteases may break down esteric, amidic and peptidic bonds, producing compounds with little or no toxicity. Bacterial hydrolases like carbamate or parathion hydrolases from *Achromobacter*, *Pseudomonas*, *Flavobacterium*, *Nocardia* and *Bacillus cereus*, for example, have been effectively employed in the conversion of contaminants such as carbofuran, carbaryl, diazinon and coumaphos. Likewise, carbohydrases, depolymerases, proteases and phosphatases, which are generated by a variety of bacteria and fungi, may be used to convert insoluble materials such as carbohydrates, plastics and proteins (Singh and Sharma 2002).

### 1.3.4.1 Hydrolases (EC3)

Hydrolase enzymes utilised in the bioremediation of various chemicals such as herbicides, pesticides, organophosphorus compounds, nitrile compounds and polymers include esterases, nitrilases, aminohydrolases, lipase, cutinase and organophosphorus hydrolase (Eibes et al. 2015; Ufarte et al. 2015)].

#### 1.3.4.2 Esterases (EC 3.1)

Esterases are enzymes that catalyse the breakage of ester bonds in a variety of compounds, including organophosphorus herbicides and insecticides, diethyl glycol adipate, polyurethanes and aromatic and aliphatic polyesters. Two bacteria that produce and colonise thermostable enzymes are *Escherichia coli* and *Pichia pastoris*. Furthermore, a subset of esterases identified in *E. coli* is cold-active and may act on phthalate esters. It is worth mentioning that the result of the esterase interaction with organophosphorus compounds, 3,5,6-trichloro-2-pyridinol (TCP), is converted subsequently by aminohydrolase (EC 3.5) to less hazardous molecules (Ufarte et al. 2015).

#### 1.3.4.3 Nitrilases (EC 3.5.5.1)

Nitrilases hydrolyse triple bonds between carbon and nitrogen (nitrile group) in herbicides, polymers and plastics to carboxylic acid and ammonia in a stereo-, regio- or chemoselective manner. Many species, including *Streptomyces* sp., *Fusarium solani*, *Rhodococcus rhodochrous*, *Aspergillus niger*, *Bacillus pallidus* and *Pseudomonas fluorescens*, can express these enzymes. Furthermore, an evolution strategy on *Alcaligenes faecalis* leads to identify a nitrilase that was active across a wider pH range. Furthermore, the *P. fluorescens* nitrilase gene produced in *E. coli* is likely the most promising nitrilase (Nigam et al. 2017; Martínková et al. 2017). Cyanide dihydratase (EC 3.5.5) is a nitrilase that converts cyanide to formate and ammonia. This enzyme is expressed by *Pseudomonas stutzeri* and *Bacillus pumilus*. Furthermore, fungal cyanide hydratase (EC 4.2.1.66) is a cyanide-degrading enzyme identified from *Fusarium lateritium*, *Neurospora crassa*, *Gloeocercospora sorghi* and other species that metabolise it to formamide. These enzymes show promise for bioremediation of effluent from coal coking and metal plating baths (Martínková et al. 2017).

#### 1.3.4.4 Peroxidases (EC1) Ligninolytic Peroxidases

Ligninolytic enzymes are a kind of enzyme that has a wide range of uses in bioremediation. This set of enzymes generated by white-rot fungus (WRF) is recognised as ‘ligninolytic’ in the context of nutritional restriction. In addition, lignocellulosic materials can stimulate the synthesis of these enzymes. These enzymes may digest a wide spectrum of refractory substances due to their strong nonspecificity and stereoselectivity (Kaur et al. 2016). They break down compounds through a free-radical-based chain reaction involving $H_2O_2$ and molecular oxygen (Kaur et al. 2016; Reddy and Mathew 2001). Laccase (LAC), lignin peroxidase (LiP), manganese peroxidase (MnP) and versatile peroxidase are the four major ligninolytic enzymes (VP). A good enzyme for oxidising phenolic chemicals, PAHs, dyes and pesticides is benzenediol/oxygen oxidoreductase, also known as laccase. Laccase substrates undergo one of three oxidation routes as an oxidase: (Abdel-Hamid et al. 2013) breakage of aromatic rings, (Adetutu et al. 2015) polymerisation and (Ahmad et al. 2020) breakdown of covalent links between monomers. The process is primarily composed of four copper atoms, with oxygen serving as the last electron receptor (Chauhan et al. 2017). Laccase was originally found in fungus such as *Panus conchatus* and *Polyporus* sp. Laccase was later discovered in *Azospirillum lipoferum*, the first bacterium species. Laccase is made by Gram-positive bacteria such as *Bacillus*, *Geobacillus*, *Aquisalibacillus*, *Lysinibacillus*, *Staphylococcus* and *Streptomyces*. Many bacteria manufacture laccase extracellularly, whereas others are unable to do so. Bacterial laccase is more resistant to changes in temperature and pH (Chauhan et al. 2017; Eibes et al. 2015). Laccase comes in two varieties: white and blue. The primary distinction between them is that blue laccase requires a 'mediator' to degrade nonphenolic substrates. The word 'intermediator' refers to an intermediator that laccase oxidises and converts into oxidised radicals that react with high redox potential or bulky substrates. As efficient mediators, ABTS (2,20-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) and N-heterocycles containing N-OH such as violuric acid, N-hydroxybenzotriazole and N-hydroxy-N-phenylacetamide have been employed. Every year, around 71,041,107 tonnes of dyes enter the environment (Chauhan et al. 2017). Laccase is a dye remediation enzyme. In the presence of acetosyringone as a mediator, a *Bacillus licheniformis* LS40-derived laccase can decolorise azo, indigo, and anthraquinone dyes by 80% in 1 h (Lu et al. 2012). Because of their poor solubility and rate of degradation, PAHs are classified as xenobiotic pollutants. Laccase is capable of converting PAHs to the less harmful quinine form and $CO_2$.

#### 1.3.4.5 Lignin Peroxidase

Lignin peroxidases (LiPs) are a class of monomeric haem-containing enzymes. With iron in the ferric form, their weight ranges between 38 and 43 kDa (Akbar et al. 2013; Falade et al. 2017). Because of their high redox potential (Wang et al. 2018), LiPs may break alpha and beta carbon bonds, catalyse the degradation of phenolic and nonphenolic chemicals and demethylate and liberate the aromatic ring of dyes (Christian et al. 2005). For oxidising nonphenolic materials, LiPs have a high redox potential (Falade et al. 2017).

In the presence of hydrogen peroxide as an electron acceptor, LiP activity rises. High $H_2O_2$ concentrations, on the other hand, may cause damage to the LiPs (Wang et al. 2018). $Fe^{3+}$ binds to $H_2O_2$ in the first stage of the process, forming an oxo-ferryl intermediate known as compound I, and it eventually reduces to compound II by donating one electron from the substrate; by another electron donation from the substrate, iron in haem returns to its ferric resting position, and the enzyme rejuvenates to its original form (Abdel-Hamid et al. 2013; Falade et al. 2017). Because of the slow reduction rate, compound I is accessible for interaction with $H_2O_2$ and the creation of an inert complex between LiP and superoxide (compound III).

#### 1.3.4.6 Manganese Peroxidase (EC 1.11.1.13)

Manganese peroxidases (MnPs) are haem-containing glycol proteins with a molecular weight of 32–62.5 kDa. MnP, like other ligninolytic peroxidases, works with

$H_2O_2$ and oxidises $Mn^{2+}$ to $Mn^{3+}$ by utilising $H_2O_2$. Binding an oxygen atom of $H_2O_2$ to $Mn^{3+}$ of haem is the initial step in the process. The chemical I radical is then formed by a two-electron transfer from $Fe^{3+}$ to peroxide $Fe^{4+}$oxo-porphyrin. Many methods, such as the creation of calcium alginate beads and carbon nanotubes, have been used to immobilise and improve the efficiency of MnP bioremediation (Chen et al. 2017). MnP can remediate PAHs and nitroaromatic compounds (Chen et al. 2017; Qin et al. 2014) azo dyes (Mielgo et al. 2003) and endocrine-disrupting chemicals such as bisphenol A and alkylphenols (Hirano et al. 2000; Moon and Song 2012); moreover, with the contribution of mediators such as lipid and thiyl radicals, MnP is capable of oxidising nonphenolic structures.

#### 1.3.4.7 Cytochrome p450 Monooxygenase (EC 1.14.14.1)

Cytochrome p450 monooxygenases (CYP) are a class of haem-containing enzymes that catalyse processes such as N-hydroxylation, N-dealkylation, O-dealkylation, oxidative dehalogenation and hydroxylation of C-H bonds. NADPH cytochrome p450 reductase provides necessary electrons for CYP, whereas the latter enzyme provides electrons from air oxygen. As a result, the presence of a reducing agent, such as NAD (P) H or FAD, is required. CYPs are multifunctional enzymes found in bacteria, fungi, plants and mammals. Approximately 7000 distinct CYPs have been identified so far. Among the taxa whose CYPs are employed for bioremediation include *Saccharomyces*, *Streptomyces*, *Basidiomycetes*, *Dehalococcoides*, *Rhodococcus*, *Bacillus*, *Escherichia* and *Salmonella* (Lamb et al. 2000; Kumar 2010). While bacterial CYPs are appealing due to their solubility, ease and low cost of production and self-efficiency (their electron transfer reductases, e.g. FMN, FAD and p450 monooxygenase, are on a single peptide), mammalian CYPs are membrane-bounded, are redox partner-dependent (e.g. NADPH) and have broad applications (Kumar 2010).

## 1.4 Entomo-remediation

Earthworms can be used directly in bioremediation techniques to increase organic pollutant biodegradation due to their biological, chemical and physical activities. Earthworms have been proven to aerate and bioturbate soils, as well as enhance nutritional quality and fertility, all of which are known to restrict bioremediation. Earthworms have also been found to slow the binding of organic pollutants to soils, to release previously soil-bound toxins for further degradation and to encourage and spread organic contaminant-degrading microbes. Earthworms enhance plant development and production by interacting with soil abiotic and biotic components in complicated mechanical and biochemical ways (Parvaresh et al. 2004). Earthworms absorb soil as they burrow, resulting in mechanical disintegration of soil particles and larger surface areas for biotic activity. Earthworm burrows serve as channels for water, particle movement, nutrient flow and aeration. Millions of enzymes and microbes live in the intestines of earthworms, facilitating the fast biochemical transformation and mineralisation of soil organic materials and so enriching soil.

It is well acknowledged that earthworms have the capacity to ameliorate a wide range of environmental issues. Vermitechnology is the study of employing earthworms to boost food production and address environmental and other human concerns (Mathur et al. 2006). Vermiremediation, the process by which earthworms clean up soil pollutants, is an essential component of vermitechnology that has gotten comparatively little attention (Tejada and Masciandaro 2011). Vermiremediation is the process of transforming, degrading or removing pollutants from the soil environment by utilising earthworms' biotic and abiotic interactions, life cycle, burrowing and feeding activity.

Anthropogenic pollution and contamination of soil occur globally as a result of industrial, farming and other activities. Chemicals, organic wastes, inorganic compounds or elements, particularly metals, are common soil pollutants. Due to the high costs and ecological and environmental instability associated with classic physicochemical remediation approaches, focus has shifted to biological in situ alternatives. There is some research and review studies on vermiremediation accessibility, although most of them are focused on organic pollutants (Suthar and Singh 2008). Furthermore, little attention has been devoted to the mechanisms of action of earthworms in the vermiremediation of inorganic pollutants, particularly metals. Some basic concepts used in environmental health have also not been adequately defined.

### 1.4.1 The Role of Earthworms in Pollutant Degradation

Depending on the type of the contamination, the use of earthworms for the restoration or remediation of contaminated soil might be based on a variety of techniques. Earthworms might be put into soil to promote the microbial population and improve organic pollution biodegradation. Organic pollutants may be directly mineralised as a result of ingested soil metabolism (Brulle 2010; Nahmani et al. 2007; Novais et al. 2013). The use of the earthworm *Eisenia fetida* to transform non-recyclable post-consumer paper waste modified with cow manure into a value-added product ('vermicompost') has been investigated (Gupta and Garg 2009). Vermitechnology in organic waste management would result in zero waste techno farms where organic waste would not be wasted or burnt. As a substitute for the digestion process, the efficiency of vermicomposting in stabilising municipal wastewater was investigated (Parvaresh et al. 2004). Metal contamination has the potential to disrupt soil ecosystems by altering the structure of soil invertebrate populations. Earthworms might be grown in metal solutions to study the impact of metals on their accumulation and death. Cu toxicity in the earthworm *Aporrectodea caliginosa* was studied, and it was shown that $Na^+$ and $H^+$ reduced Cu toxicity, while $Mg^{2+}$ and $Ca^{2+}$ had inconsistent effects (Arnold 2007). Arsenic promotes the production of metallothionein in earthworms, which sequesters arsenic in certain cells and tissues (Button et al. 2011; Sinha et al. 2008). Earthworm mucus may increase the concentration of dissolved organic carbon in the soil solution, resulting in more competition between arsenic and organic carbon for binding surfaces on positively charged soil constituents such as iron and manganese oxides, resulting in an increase in arsenic mobility (Sizmur et al. 2011).

Metals can be collected in various worm tissues to lower metal levels in the vermicomposting process and soil (Suthar and Singh 2008). Earthworms appear to have a variety of processes for absorbing, immobilising and excreting heavy metals and other substances (Sinha et al. 2008). One of the primary pathways in this process is metal ion diffusion through the body wall of earthworms, either cutaneous absorption or gut uptake (Oste et al. 2001). They either 'biotransform' or 'biodegrade' the chemical pollutants, rendering them harmless in their bodies. Most biotransformations and biodegradation occur in the stomach before pollutants enter the worm tissues. Metal bioavailability to terrestrial invertebrates is affected by soil physicochemical properties and pollution levels. The simulated earthworm gut (SEG) was created to assess the bioavailability of metals in soil to earthworms by simulating the composition of earthworm gastrointestinal fluid (Ma et al. 2019). Cd concentrations in soil can be reduced by earthworm treatment, and Cd causes a change in the bacterial populations in the guts of *M. posthuma* (Liang et al. 2011; Stürzenbaum and Kille 2001). The ability of earthworms to methylate inorganic Hg due to anaerobic and nutrient-rich environments in their digestive systems, as well as the activity of bacteria, was also studied. Bioaugmentation with PCB-degrading microorganisms and earthworms resulted in partial bioremediation of polychlorinated biphenyl (PCB)-contaminated soils. Earthworms aid PCB bioremediation by promoting the development and activity of indigenous PCB-degrading bacteria and hastening the dispersion of PCB-degrading bacteria in bio-augmented columns (Luepromchai et al. 2002). In recent times, the influence of earthworms on the mineralisation of the herbicide atrazine (2-chloro-ethylamino-6-isopropylamino-s-triazine) has been studied in representative soil microsites of earthworm activities (gut contents, castings and burrow linings) (Kersanté et al. 2006). In general, earthworms increase the breakdown of organic substances by around 30%; however, the mechanism behind this is unclear (Blouin et al. 2013). Organic matter is crucial in PAH biodegradation because it contributes to soil nutrients as a result of mineralisation and stimulates microbial activity. As a result, co-application of organic wastes and *E. fetida* for bioremediation of benzo(*a*)pyrene-polluted soil is sought (Tejada and Masciandaro 2011). Markman et al. (2007) discovered that earthworms may bioaccumulate considerable amounts of endocrine-disrupting chemicals (EDCs) (dibutyl phthalate, dioctyl phthalate, bisphenol A and 17β-estradiol) in their tissues (Markman et al. 2007). Earthworms can collect or break down 'organochlorine pesticide' and 'polycyclic aromatic hydrocarbons' (PAHs) residues in their feeding medium. Some fungi, including as *Penicillium*, *Mucor* and *Aspergillus*, have been discovered in the intestines of earthworms and have been shown to break down hydrocarbons. PCBs and *Mucor dieldrin* can also be degraded by *Alcaligenes*. Dieldrin is one of the most lethal toxicants (Sinha et al. 2008). The impact of increasing the concentration of earthworm (*Eudrilus eugeniae*) on the acceleration of bioremediation of used motor oil-polluted soil supplemented with poultry manure was studied (Meh et al. 2012). The efficacy of a standard biofilter and a vermifilter containing the earthworm, *Eisenia fetida*, for the treatment of residential wastewater sludge was compared, as well as the earthworm-microorganism interaction processes involved in sludge stabilisation. The addition of earthworms resulted in a considerable stabilisation of the sludge by increasing the decrease in volatile suspended solids (Arora and Kazmi 2015). Hospital waste is dangerous and must be disinfected before disposal. The use of earthworms (*Eisenia fetida* and *Eudrilus eugeniae*) to compost biodegradable parts of contaminated hospital trash had no negative effects on the environment or human health (Arora et al. 2021; Mathur et al. 2006). Endosulfan bioremediation was investigated using a bacterial strain isolate *Rhodococcus* MTCC 6716 from the stomach of an Indian earthworm (*Metaphire posthuma)* (Verma et al. 2011).

### 1.4.2 The Significance of Gut Produced Enzymes in Degradation Processes

The intestines of herbivorous animals, invertebrates and insects have piqued the interest of many researchers as a source of different aerobic and anaerobic bacteria that generate new fibrolytic enzymes with distinct molecular structure, catalytic activity and/or target specificity (Kim et al. 2017). It is still debated whether the mineralisation is caused by earthworms, microorganisms triggered in their stomach or a combined activity of both species (Blouin et al. 2013). The enzyme activity and nutrient and organic matter concentrations of the surrounding soil were compared to the equivalent characteristics of earthworm castings (Kızılkaya and Hepşen 2004). Amylases, endoglucanase, cellulase, sucrase and protease were among the enzymes tested in the stomach. *Lactobacillus viridescens*, *Lactobacillus minor*, *Bacillus pumilus*, *Bacillus licheniformis* and *Flavobacterium* were also detected (Sumathi and Thaddeus 2013). Contaminants may be destroyed in earthworms via an enzyme activity known as the 'cytochrome P450' system (Sinha et al. 2008). Carboxylesterases (CbEs) are important pesticide-detoxifying enzymes that play a role in the biochemical process of pesticide resistance in particular insect species. The inherent resistance of earthworms to pesticide toxicity was investigated. Earthworms can release pesticide-detoxifying enzymes, which have a variety of (eco)toxicological consequences and environmental uses (Sanchez-Hernandez et al. 2009). Another research looked at the tissue-specific response of ChE and carboxylesterase (CE) activities in *Lumbricus terrestris* subjected to chlorpyrifos-spiked field soils. The test of hydrolase (cellulase, phosphatase) and dehydrogenase activities was used to examine the biochemical activity in different areas of earthworm stomach and vermireactors. The dehydrogenase, hydrolytic activities and microflora are thought to offer a clear indicator of the dynamics of organic matter breakdown (Kumar et al. 2014). Cellulolysis happens as a result of the interaction of fungus and bacteria with varying needs. Earthworms have an impact on decomposition because certain species' guts contain cellulolytic activity. Because earthworms consume fungus, the action of *E. fetida* promotes fungal development during vermicomposting. The activation of microorganisms in earthworm intestines leads to more intense and effective cellulolysis during green waste vermiculture (Aira et al.

2008; Arora and Kazmi 2015). It is unclear if such cellulases are produced by earthworms or by their symbionts. In one example study, zymogram analysis revealed that one cellulase (endo-1,4-glucanase, EC3.2.1.4) digested the majority of the cellulose in *Pheretima hilgendorfi*. These findings clearly showed that the earthworm may manufacture endogenous and functional mid-foregut cellulase and utilise it with the help of the intestinal caecum. Endosymbiotic bacteria generate extracellular enzymes that digest cellulose and phenolic substances, therefore accelerating the breakdown of ingested material. Decomposition is further aided by the action of endosymbiotic bacteria found in earthworm guts. ManK, a new endo-1,4-mannanase with unusual substrate selectivity for degrading mannose-based substrates isolated from the digestive tract of *E. fetida* from *Cellulosimicrobium* sp. strain HY-13, exhibits high catalytic activity (Kim et al. 2017). In the gut of *E. eugeniae* and *E. fetida*, the activities of amylase, cellulase, xylanase, cellobiase, endoglucanase, acid phosphatase, alkaline phosphatase and nitrate reductase were measured. Earthworm enzyme activity is locally specific and regulated by physiological state and microbes. Cellulase, xylanase, acid phosphatase and alkaline phosphatase were shown to be more abundant in the stomach of *E. fetida* than *E. eugeniae*. In the gut of *E. eugeniae*, the activity of amylase, cellobiase, endoglucanase and nitrate reductase was greater than in the gut of *E. fetida*. Because of the increased abundance of microorganisms in the earthworm stomach, amylase, cellulase, acid phosphatase, alkaline phosphatase and nitrate reductase were released. Enzymes generated by earthworms and gut bacteria are thought to play an important role in the digestion and humification of soil organic matter (Thomas et al. 2020).

## 1.5 Conclusions: A Road Ahead Towards Sustainable Development

We want to shed some light on the role of enzymes in pollution bioremediation. While many physical and chemical techniques of treating polluted soil and water are inefficient, bioremediation provides a novel means of cleaning up hazardous chemicals. Enzymes, as practical instruments of living organisms, are an eco-friendly and bio-based bioremediation method. Earthworms and microorganisms play an important role in soil ecosystems because they help to maintain soil structure and fertility. They have an impact on the organic matter dynamics, structure and microbial community and are hence known as ecosystem engineers. It has also been stated how they improve soil porosity by physical and chemical characteristics altering soil organic matter by mixing leaf litter with soil, leading in the creation and stability of soil aggregates. Microorganisms exposed to polluted areas and particulate contaminants are intriguing sources of active enzymes against such pollutants. Vermiremediation is a long-term biological solution for cleaning up moderately polluted soil. It may be used as a secondary method to remove residual pollutants from soil after physicochemical remediation procedures have been applied to an openly contaminated soil environment. Overall, using enzymes for pollutant bioremediation seems to be a cost-effective, efficient and practical approach. Overall, it appears that utilising enzymes for pollution bioremediation is a cost-effective, efficient and feasible strategy. Although there is still much work to be done, further research and tests on enzyme activity and mechanism of action, as well as isolating novel enzymes, would be a potential method to minimise pollutants and create a better environment for humans and all other animals.

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# Microbial Nanobiotechnology in Environmental Pollution Management: Prospects and Challenges

## 2

M. B. Alao, C. O. Bamigboye, and E. A. Adebayo

**Abstract**

Environmental pollution is a major concern all over the world as various substances are added to nature by human activities and subsequently resulting in adverse effects on man, animals, plant and the ecosystem. In the time past, pollution has been managed conventionally by physical and chemical methods, but the pollutants persist in the environment, mostly in different forms, thus necessitating the need for a green technological approach. Environmental biotechnology is greatly accepted as it is safe and cheap and remediates all pollutants. Microbial nanobiotechnology is a viable option in pollution management in the world today as it is of high efficiency with no known toxic end products. The microorganisms producing the eco-friendly, nano-sized materials are generally regarded as safe. Diverse nanoparticles of environmental importance have been extensively synthesized intracellularly and extracellularly by bacteria, fungi, algae, yeasts, etc. Microbial nanoparticles of gold, silver and other metals have found applications in waste management, and they are of different shapes ranging from spherical, triangular, rectangular to pentagonal. Currently, in addition to remediating polluted environments, microbial nanobiotechnology is of great promise in pollution biosensory activity and prevention.

This chapter also assesses the prospects, challenges, sustainability and risks related to the application of microbial nanobiotechnology in pollution management.

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Pollution management · Biotechnology · Nanotechnology · Risk assessment · Environment

## 2.1 Environmental Pollution

Broadly, pollution can be described as the discharge or introduction of waste substances or energy into the environment through human activities in amounts which are harmful to man, crops or animals. Environmental pollution can be defined as an activity, either by individuals or corporations that can lead to the release of contaminants into the environment, thereby compromising the health status of other persons living in the area, with a clearly established causal link. Urbanization and continuous upsurge in human population has led to equal increase in industrialization and a consequential pollution of the environment. Environmental pollution is predominant in both the developing and developed countries of the world. However, extended pollution of the environment often occur in the developing countries due to poor waste management, industrial emissions, mining activities and poor sanitation.

### 2.1.1 Types of Environmental Pollution

#### 2.1.1.1 Air Pollution

This is caused by the release of toxic gases, fumes, dust, smoke, etc. Broadly, air pollution may be natural or of anthropogenic origin. Before the advent of civilization, the atmosphere was polluted to some extent from natural sources. A given sample of air may contain a host of natural contaminants such as smoke, dust, fungal spores and disease-causing bacteria. In 1909, about 1500 died in Scotland because of polluted air. It was as a result of this that the word ‘smog’ was coined as an abbreviation of smoke and fog. Atmospheric pollution progresses, most especially with the release of chlorofluorocarbon which has an effect in ozone depletion. Generally, air pollutants fall into one of four categories: particulate matter, heavy metals, persistent organic pollutants and gaseous pollutants. Particulate matters consist of varied and complex mixtures of particles suspended in the air with varied composition and size (coarse, fine and ultrafine) produced from anthropogenic and natural sources. Sources of pollution by particulate matter include activities in construction site, vehicles, industries, incinerators, dust, etc. Heavy metals are embedded in the earth’s crust, and they are not degradable and cannot be destroyed. They can be introduced into the environment via manufacturing facilities and discharge of waste water.

#### 2.1.1.2 Water Pollution

Water pollution is one of the dangerous pollution that can negatively impact the ecosystem, man, animals and plants and can also interfere with the use into which a given water is applied. Some water pollutants include pathogens (Bamigboye et al. 2020), heavy metals (Cr, Ni, Cu, Hg, Zn, Cd, Pb, etc.), petroleum products and some organic chemicals. Others include industrial waste, radioisotopes, oil spills, sewage leakages, herbicides, pesticides, animal wastes, fertilizers and contaminants from mining activities. It is notable that both groundwater and underground water can be polluted. Most freshwater resources (groundwater, lakes and rivers) are used in domestic, agricultural and industrial activities. Pollutants, especially chemicals, may be introduced into this resource during the course of these activities. For example, run-off from agricultural fields can increase the nutrient level in receiving rivers leading to the depletion of oxygen, production of toxic alga blooms and increased biomass. Agricultural activities can be a major source for introducing pesticides and other agrochemical products including persistent organic pollutants into waterbodies.

Some water pollutants are referred to as 'persistent organic pollutants' (POPs). They are described as POPs if they are toxic to animals, humans and other living organisms, bioaccumulate and resist biological, chemical and photochemical transformation processes and finally if they can be transported to far distances. They include polychlorinated dioxins, polycyclic aromatic hydrocarbons, dichlorodiphenyltrichloroethane and other emerging chemicals. These emerging chemicals might have been present in the environment but not yet detected or recognized as toxic. POPs are of concern because they can biomagnify and bioaccumulate in aquatic food webs. This dangerous trend has been demonstrated in marine mammals, fishes and human tissues and breast milk resulting from the consumption of the latter.

Also, water can be polluted substantially by leachates from landfills and hazardous waste sites. A lot of wastes are generated and discarded yearly throughout the world. They often contain infectious, radioactive and hazardous wastes, some of which end up polluting the river or underground waterbodies. Oil and chemical spills are also a cause for alarm especially in oil-producing regions leading to the pollution of drinking water in these regions. In addition, mining activities lead to the production of some heavy metals that may contaminate underground water.

#### 2.1.1.3 Soil Pollution

Soil is an important natural resource and rich in nutrients. It enhances the functioning of the ecosystem by supporting primary productivity and enhancing the flow of nutrients in the ecosystem (Milosavljevic et al. 2020). However, soil is continuously been polluted through mining, oil drilling, use of chemicals and fertilizers in agriculture, road transport, inappropriate waste disposal and industrial activities. Some pollutants introduced through these and other anthropogenic activities include heavy metals, hydrocarbons, solid waste, electronic wastes and microplastics. Previous studies have established that e-waste sites in many countries including Nigeria, Ghana, China and India are laden with organic contaminants and heavy metals, especially the vegetation, dust and soil of analysed sites. The presence of heavy metals in soil is commonly attributed to anthropogenic activities; therefore, polluted soil samples are mostly found around industrial area. Heavy metals are of concern globally since they are not biodegradable and they persist in the environment, bioaccumulate and remain toxic over a long period of time (Ma et al. 2020).

Environmental protection agency reported that Cd, Hg, Cu, As, Cr, Pb and Ni are toxic to the ecosystem; therefore, the contamination of soil by heavy metals poses a threat to environmental security and human health (Taati et al. 2020). Significant soil contamination with Cd was reported for Russia, the United States, Europe, India and Australia. Soils in Mexico and Italy were also significantly contaminated with Zn, Pb and Cu. An in-depth study of agricultural and urban soils in China also demonstrated gross pollution with heavy metals (Yuan et al. 2020).

Electronic wastes are often generated in large quantities throughout the world, with an expected increase of 3–4% per year, and about 44,700 kt of e-waste generated in 2016 worldwide (Baldé et al. 2017).

### 2.1.2 Effects of Pollution

One disturbing aspect of air pollution is that it may directly modify the intensity of sunlight, flower formation, rainfall and temperature, all of which are basic to the survival of all organisms. In addition, relatively slight rainfall or temperature change may disrupt the balance of ecosystem by eliminating plant and animal species. Moreover, air pollution may increase the earth average temperature. The atmospheric gases, including particulate matters, water vapour, carbon (IV) oxide, low level ozone and chlorofluorocarbon, are transparent to incoming solar radiation but absorb outgoing heat radiation from the earth surface. These gases reradiate the heat back to the earth. Due to the mass consumption of fuel as well as industrialization, the concentration of carbon (IV) oxide and other gases in the atmosphere increases. The increased concentration of these gases causes more heat to be radiated into the atmosphere, thereby increasing the earth temperature. This effect is beneficial because it creates extra warmth which prevent earth temperature from going below freezing point. When water is polluted by pathogens (bacteria, fungi, viruses and/or parasites), waterborne diseases are inevitable. These include diarrhoea, respiratory infections, ascariasis, typhoid and giardiasis, and damage to some body organs may also occur. Continuous exposure to heavy metals, oil spill, etc. can lead to loss of aquatic life, reduced plant diversity and cancer development.

Cardiovascular diseases have been linked with air pollution-mediated mortality and morbidity. This mostly stems from exposure to particulate matter resulting into cardiac arrhythmias and sudden death. In addition, vehicle emissions can trigger infarctions and myocardial ischaemia within hours after been exposed. Increase in the risks of hospitalizations and development of stroke has also been associated with automobile traffic emissions. Some studies have established that inhalation of some types of particles may result into increase in platelet reactivity, altered coagulation factors and increase in blood viscosity and fibrinogen. Microplastics have notable effects on soil organisms and can easily accumulate by causing alterations in the chemical contents and soil characteristics. In another study, microplastics were found to reduce the growth rate in *Lumbricus terrestris*, with no reduction in number of juvenile and final weight of adult earthworms (Chae and An 2018). Microorganisms are also affected by the presence of heavy metals in soil, and their activity, diversity, community structure and biomass are negatively influenced (Ma et al. 2020).

With regard to plants, negative effects have also been recorded. Pollutants may cause premature senescence, leaf injury, reduced yield and growth as well as photosynthetic activity. Leaves of *Albizia lebbeck* showed reduced leaf area and increase in breadth and length of leaflets. Stomata closure as a result from air pollution can inhibit fixation of carbon and may reduce the availability of $CO_2$ in leaves. Decrease in chlorophyll concentration in plants found in polluted sites has also been reported. It has been demonstrated that a significant reduction in soluble sugar is associated with most plants found at polluted sites. Other common effects on plants include depletion of cellular lipids, peroxidation of polyunsaturated fatty acids and pigment destruction. Pollution of soil with high concentrations of heavy metals can also have deleterious effects on human health. Cadmium can cause bone loss, lung cancer, adenocarcinoma and renal dysfunction. Also, lead can adversely affect the biosynthesis of haemoglobin, decrease the learning power, damage the kidney and cause behavioural disorder in children, lung cancer and central nervous system disorder. Exposure to arsenite can lead to the development of skin lesions, kidney and bladder cancer and hyperkeratosis (Taati et al. 2020).

## 2.2 Microbial Nanobiotechnology in Pollution Management

Nanotechnology, using different particles of sizes within the nanometre, is becoming an option in the technology of remediating polluted environments (Tripathi et al. 2018; Isa et al. 2019). Nanotechnology can be characterized as fabricating compounds of sizes 1–100 nm, and specific phenomena allow novel applications. It is a new environmental remediation technique and referred to as nanoremediation by many, and this technique has certainly established a new area of technology and science. When environmental clean-up includes the use of living organism-formed nanoparticles or nanomaterials, it is called nanobioremediation (Yadav et al. 2017). It has been used in the treatment of polluted water, air and soil in addition to being proven to be a modern and efficient ecological friendly technique for cleaning up residual pollution in a number of environments. The benefits of nanotechnology in environmental remediation include sensing, identification, prevention and remediation of pollution, at low cost, less time-consumption resulting in effective pollution etc. Nanoremediation is more adaptive and selective than traditional approaches and can be used in air, water and soil environments as sensors to track toxic compounds, organic contaminant and heavy metals.

Nanotechnology promises to provide the means for the design of nanomaterials, materials controlled by defined molecular structures and dynamics with tailor-made biological and physicochemical characteristics. Importance of nanotechnology in developing new products and devices has been identified, with a major effect on various areas, from sensor technology to biological medicine. Contrary to conventional physiochemical techniques, microbial nanobiotechnology, which is the biological synthesis of materials of nanosizes by microbes, has recently attracted attention as a modern, promising method to the production of greener nanoparticles. Green chemistry concepts of the discovery, making and use of chemical substance and methods to minimize or eradicate the application and production of harmful compounds combined with white biotechnology that uses plants, bacteria, moulds, enzymes and yeasts to produce compounds is greatly contributing to developing high sustainable nanomanufacturing industrial process. Microorganism-driven production of nanomaterials is an emerging biotechnology which presents an environmental friendly option to nanosynthesis of physiochemical methods (Khan et al. 2018) with microbial strains of bacteria, moulds, microalgae and yeasts. Hulkoti and Taranath (2014) reported the microbial-mediated biological synthesis of metallic (alloys), metal oxides or non-metallic nanoparticles.

Teow and Mohammad (2017) suggested complementing physical and chemical techniques of pollution control with nano-based technologies could offer new opportunities to advance the science of pollution management, especially as most presently available techniques have offered their best in ensuring adequate environment quality, particularly in the presence of emerging and persistent contaminants. Nanomaterial shows essential specific characteristics including heavy sorption, increased reactivity as well as rapid dissolution in addition to non-continuous characteristics like quantum contaminant effects, plasmon resonance of surface localization and super magnetism. Various nanomaterials applied in the treatment of environmental pollutants include nanometal oxides, nanometal and nano-adsorbents, in addition to photocatalytic and membrane techniques (Baruah et al. 2015).

### 2.2.1 Microorganisms Important in Nanobiotechnological Management of Pollution

Generally, any microorganism considered for nanobiotechnological functions must be of generally regarded as safe (GRAS) status in all fronts without any sort of controversy. Different microorganisms are known to biologically synthesize diverse nanoparticle via the NADPH-dependent reductase enzyme which reduces salts of metals to nanoparticle by electron shuttle activity of enzyme reducing metals.

#### 2.2.1.1 Bacteria

In the last decade, important inorganic nanomaterials (primarily of silver, selenium and gold) synthesized by bacteria are applied in the production of sensor devices and third-generation biosensors. Biological chemical processes that drive the bacteria biological synthesis of nanoparticles have been suggested and are presently being researched. Quite a handful of biochemical processes has been identified as part of cellular detoxification in microorganism resistance pathway that involve changes in inorganic ion solubility through reduction by enzymes and precipitating toxic soluble compounds to non-toxic nanocompounds that are insoluble. Cellular transporters as well as oxidoreductase enzymes (e.g. cysteine desulfhydrase, NADH-dependent nitrate reductase and NADPH-dependent sulphite reductase flavoprotein subunit alpha) are primarily important in intra- and extracellular biological catalytic secretion (with and probable excretion). Biomolecules-mediated physicochemical processes such as stabilization, biosorption, growth, nucleation and complexation have also been described. Some genera of bacteria have demonstrated the capability to biologically synthesize unusual organic nanostructures in addition to inorganic nanomaterials. Nanocellulolytic compounds from bacteria are mainly 3-D network of nanofibril cellulose secreted by aerobic acetic bacteria and *Gluconacetobacter*. These compounds of bacteria origin exhibit increased purity, crystallinity and mechanical balance compared to nanofibrillated and nanocrystalline cellulose (Golmohammadi et al. 2017). These celluloses of bacteria origin are therefore referred to as nanomaterials which are important in biosensor platforms like nanocomposites as well as aid in immobilizing biological elements as reported by Pourreza et al. (2015). Recently, discovery of chip-like biosensory device of bacteria origin in measuring the level of toxin was reported.

#### 2.2.1.2 Fungi

Fungi can secrete metal ions through physicochemical and biological pathways, like metabolite and polymer extracellular binding, specific polypeptide binding and accumulation depending on metabolism. In addition, even in thin solid substrate fermentation methods, fungi can easily be scaled up, and this is an important attribute of using them in nanoparticle synthesis. It is also feasible to achieve vast enzyme output as fungi are very effective secretors of extracellular enzyme. Low cost and abundant biological components are other benefits of fungi-mediated green pathway in synthesizing metal nanoparticle as diverse fungal species grow rapidly and not difficult to cultivate and store. Several studies on the importance of fungi in nanotechnology are well documented due to fungi production of important compounds in large quantities. In downstream activities, the extracellular secretion of enzymes also has an additional value. Furthermore, the intracellular metal absorption and high wall-binding are other properties of fungi that have made them preferred for nanobiotechnological functions. Fungi can secrete metal nanoparticles and nanostructure through extracellular or intracellular reduction of the enzyme and the biomimetic mineralization process. Compared to bacteria and other microorganisms, fungi are excellent protein secretors, resulting in higher nanoparticle yield. Therefore, rapid and ecological friendly biosynthesis of metal nanoparticles is possible using these dissimilar properties of fungi.

#### 2.2.1.3 Microalgae

Microalgae are one-celled photosynthetic microbes that are of interest in nanobiotechnology. In biological production of noble metal nanoparticle, microalgae like *Desmodesmus*, *Scenedesmus* and *Tetraselmis kochinensis* are well documented. For most microalgae, pathways described for nanoparticle biological secretion are nucleation phenomena, dimension control and nanoparticle structure stabilization, driven via reducing agent activities and cytoplasmic membrane enzyme in cell wall, biomolecules such as phenolic compounds, proteins, polysaccharides and polyphenols. For the future creation of new nanomaterials, the mechanisms behind biological mineralization including in vivo inorganic mineral formation have been researched to a great extent. Likely use of optical chemosensing of dissolved ammonia has been demonstrated by microalgae silver nanoparticles (Chetia et al. 2017).

### 2.2.2 Secretion and Importance of Microbial Nanoparticle in Pollution Management

In degradation of multiple pollutants, various metal-based nanomaterials have been identified, but most studies have been devoted to removing from water chlorinated organic contaminants and heavy metals. Metal oxides and metal nanomaterials exhibit benefits such as high adsorption capability and fast kinetics as highly efficient adsorbents (Santhosh et al. 2016). There are special mechanical, magnetic, catalytic and optical characteristics of metal nanoparticles, differentiating them from other metals. In this light, in the removal of radionuclides and heavy metals, nanoscale metal oxide is identified as promising alternative. Nanoscale metal oxides such as nano-$TiO_2$ and nanosilver are applied in water disinfection, biofoul prevention and organic compound remediation. Nanosilver has low human toxicity but high bactericidal properties and minimal longevity, while nano-$TiO_2$ is of increased chemical stability in addition to longer durability, but active ultraviolet is required for effectiveness. In treating groundwater pollution, particularly for arsenic removal, magnetic nanoparticles (magnetite $Fe_3O_4$) are used and retrieve with magnetic field, but stabilization is required. The fact that there is elevated reactivity, nano-zerovalent iron has a brief half-life and also needs stabilization, thus restricting its usage for certain purposes. The application of various microbial nanoparticles in environmental pollution management is presented in Table 2.1.

#### 2.2.2.1 Gold Nanoparticle

The fact that gold nanoparticles (AuNPs) are highly stable, are biologically compatible with diverse compounds and possess high oxidation resistance, they are of great interest in pollution control. More so, these special characteristics have made gold nanoparticles important in several industrial processes like in optics, electronics, sensors and coatings. The development of accurately named shape and size methods for the controlled production of gold nanoparticles is a major problem, and several chemical techniques are documented in literature, focusing on manipulating the physical characteristics of the nanoparticle. Several technologies are still at embryonic stage, and difficulties with the stability of nanoparticle preparations, crystal growth regulation and particle aggregation are often encountered. As a novel approach to the production of metal nanoparticle, roles of microorganisms in synthesizing nanosized materials have recently been well documented. Although recent efforts have been made towards nanomaterial biosynthesis, the relationship between metals and microbes has been established, and the capability of microbes to

**Table 2.1** Nanoparticles and their applications in environmental pollution management

| Nanoparticles | Applications | References |
|---|---|---|
| Immobilized nanocellulose composites of *Arthrobacter globiformis* D47 | Degradation of diuron herbicide | Liu et al. (2018) |
| Nanoscale zerovalent iron of ag, Ti, au and Mn from *Sphingomonas* sp. | Removal of decarbominated diphenyl ether, pathogens and chlorinated hydrocarbons | Kim et al. (2012) |
| Thin-film composite polyamide of *Cynomorium coccineum* L. extract | Degradation of cyanide compounds | Sebeia et al. (2019) |
| Binary mixed oxide water | Break down of methylene blue dye | Rasalingam et al. (2014) |
| Iron oxide nanopowder of soil microorganisms | Removal of azo dye direct red 23 | Kos et al. (2014) |
| Ag NPs/ag ions | Water disinfection | Xiu et al. (2012) |
| Zinc sulphide (ZnS) of enzymatic degradation by bacterially overexpressed organophosphorus hydrolase | Degradation of P-nitrophenol and acid Orange 7 | Torres-Martínez et al. (2001) |
| Titanate nanotubes | Gaseous—Nitric oxide decontamination | Chen et al. (2013) |
| Enzyme organophosphate hydrolase of paper<br>Unzipped, single-walled and multi-walled carbon nanotube | Degradation of organophosphates and heavy metals in soil and water | Mechrez et al. (2014) |
| $TiO_2$ NPs | Water and soil disinfection targeting MS-2 phage, *E. coli*, hepatitis B virus, aromatic hydrocarbons, biological nitrogen, phenanthrene | Da Silva et al. (2016) |
| Iron-based water | Heavy metal and chlorinated organic solvent degradation | Guo et al. (2017) |
| Metal-doped $TiO_2$ | Water decontamination of 2-chlorophenol, endotoxin, *E. coli*, rhodamine B, *Staphylococcus aureus* | Younas et al. (2014), Sreeja and Vidya (2016) |
| Bimetallic NPs water, soil | Brominated and chlorinated pollutant removal | Xie et al. (2014) |

accumulate or extract metal is used in industrial technological techniques like bioremediation and bioleaching.

Metallic nanoparticles and inorganic nanostructures with characteristics related to chemically secreted compounds are created by several microbes, while strict control is exercised over the particle’s composition, shape and size. Examples include the production nanoscale, semiconducting CdS crystals in *Schizosaccharomyces pombe*, secretion of magnetic nanoparticle by *magnetotactic* bacteria and the formation of palladium nanoparticle using sulphate-reducing bacteria in the presence of an exogenous electron donor. A number of organisms have been successfully demonstrated in the ability of plants, bacteria, algae, fungi, yeasts and actinomycetes to absorb gold ions from solution. For example, *Bacillus* sp., fungal species like *Fusarium* and *Verticillium*, actinomycete like *Thermomonospora* and *Rhodococcus* and lactic acid bacteria have been reported to produce gold nanoparticles. Production of nanostructures like nanowire and the development of nanoparticle by biological models like viruses, DNA, S layers and proteins are also of interest.

#### 2.2.2.2 Silver Nanoparticle

Secretion of silver nanoparticles (AgNPs) by microorganisms is owed to their protection principle (resistance pathway), and the formed nanoparticles are very useful. The bacterial cell's resistance to ions of silver in the environs is important for the synthesis of nanoparticle. In nature, ions of silver are extremely noxious to bacteria. Their cellular machinery thus aids to transform reactive ions of silver into stable silver atoms. Furthermore, pH as well as temperature plays essential part in their growth. Nanoparticle size is 50 nm at room temperature; at higher temperatures, i.e. 60 °C, the size decreases to 15 nm. This means the size decreases with temperature increase. In alkaline environment, the microorganism's secretion of nanoparticles is more similar to acidic environment. Above the pH of 10, however, death of cell is observed. *Pseudomonas stutzeri* AG259 was initially isolated from the silver mine and it is the first evidence for the secretion of silver nanoparticles. A secretion of nanoparticles by *E. coli* depends on $AgNO_3$ concentration. Silver helps to cause the organism to produce nanoparticle at lower concentration, whereas at higher concentration, it induces cell death. In the purification of microbial waste, such as water disinfection, silver nanoparticles are often used. In addition, in increasing the overall performance of the resulting nanocomposite, AgNP has been combined with many components, like metal oxides and polymers.

#### 2.2.2.3 Titanium Oxide Nanoparticles

Titanium oxides are another commonly studied metal-based substance for environmental nanotechnology bioremediation. Because of their properties ranging from photocatalytic, known cheap cost, semiconducting, gas sensing, non-toxicity, electronic and energy converting abilities, titanium oxide nanoparticles ($TiO_2$ NPs) have been widely researched in air purification, waste management, surface cleansing and photocatalysis in environmental pollution management. $TiO_2$ NPs are light-activated and are also commonly investigated for their capability to extract organic pollutants from different media. For microbes such as algae, fungi, viruses and bacteria, $TiO_2$ NPs generate highly reactive oxidants such as hydroxyl radicals acting as disinfectant. The material is usually doped with another transition metal ion to improve efficiency, since $TiO_2$ shows very restricted photocatalytic ability. In addition, magnetic metallic nano-adsorbents are specifically desirable because they are easy to preserve and isolate from treated environment. In the literature, nanoparticles of iron oxides and iron are generally researched for the remediation of various heavy metals, like $Cu^{2+}$, $Ni^{2+}$, $Co^{2+}$ and $Cd^{2+}$ and remediation of organic chlorinated solvents.

## 2.3 Principles of Nanotechnology in Pollution Management

The principles of nanotechnology in pollution management are presented in Fig. 2.1. Major nanotechnological mechanisms of pollution management including adsorption, filtration and photocatalysis are discussed in this section.

### 2.3.1 Adsorption

Adsorption is widely applied in the remediation of inorganic and organic pollutants from the environment as it is economically feasible. Adsorption includes the application of various materials such as zeolites, clay and alumina silicates to extract metals from solutions. The nanomaterial's large aspect ratio and surface area makes it one of the latest superior performance adsorbents (Yano et al. 2018). One of the common applications is in multiwall carbon nanotubes as they have greater potential for sorption of metal ions (3–4 times) than granular and powder activated carbon (Yano et al. 2018). Compared to nano-adsorbents, the conventional adsorbents available are less effective because nano-adsorbents have major advantages due to their large unique surface area and associated sorption sites, tunable pore size, short

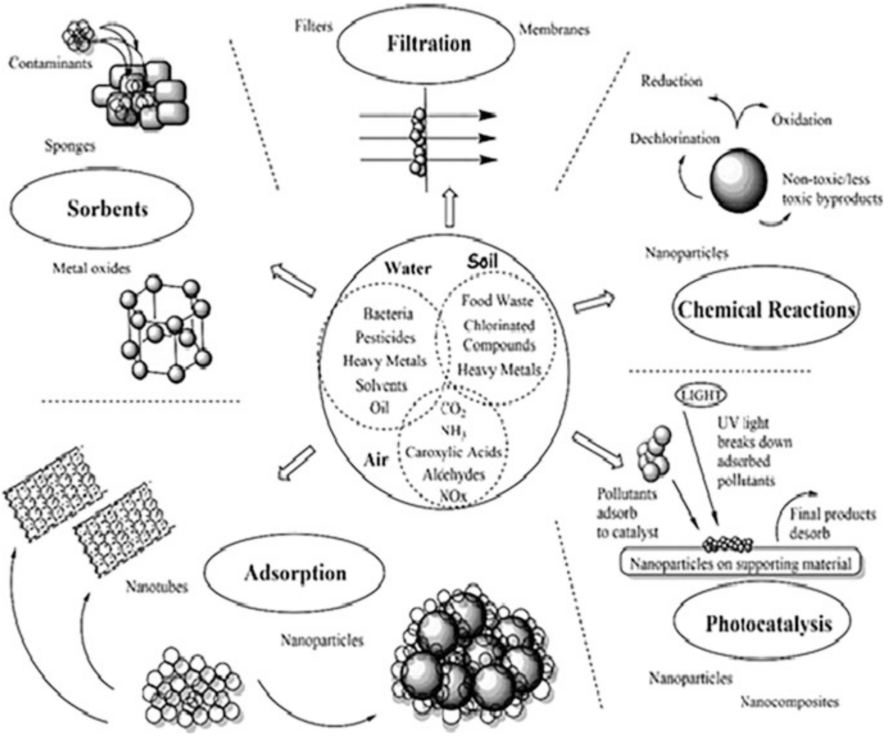

**Fig. 2.1** Principles of nanotechnology in environmental pollution management (Guerra et al. 2018)

intra-articular diffusion distance and surface chemistry, in addition to their high adsorption rates for inorganic compounds like heavy metals and micro-pollutants. Four kinds of nano-adsorbents, namely, zeolites, metal-based nano-adsorbents, polymeric nano-adsorbents and carbon-based nanomaterials, appear to be the subject of recent research.

Owing to diverse contaminant-carbon nanotube interactions and large surface area, carbon nanotubes (CNTs) are reported to be highly effective in the adsorption of organic matter and can therefore help in the breakdown of diverse organic contaminants. Compared to activated carbon, CNTs are effective in the adsorption pathway of several organic compounds, and their high adsorption is linked to large surface area and varied interactions between contaminants and CNT. Its external surfaces are the available surface area for adsorption on individual CNTs. It is easy to assess the adsorption potential of the carbon nanostructure by its textural characteristics as reported by Gupta and Saleh (2013), while photeric and other functional groups as well as oxygen can be added to carbonaceous nanomaterial surfaces, which provide new chemical adsorption sites. CNTs form loose bundles in the aqueous solution owing to the hydrophobicity of the graphitic surface, thus decreasing the adsorption effectiveness, whereas the production of aggregates requires interstitial spaces that increase the adsorption location, thereby increasing the adsorption effectiveness of different organic molecules.

Although activated carbon and CNT bundles have about the same basic surface area, there are micropores on activated carbon that are not available to many organic molecules. Because of more open sorption sites, CNTs have large pores that improve the adsorption of many molecules, so they have higher adsorption ability. Owing to diverse existing pollutants-CNT relationships, like hydrogen bonding, hydrophobic effect, covalent bonding, pep interactions and electrostatic interactions, CNTs strongly adsorb several polar organic compounds. The CNT surface rich in p electrons permits pep interactions with organic molecules. Organic compound having functional groups of –COOH, –OH and $–NH_2$ forms a hydrogen bond with the electron-donating graphitic CNT surface. Via non-covalent bonding like hydrophobic interactions, hydrogen bonding, electrostatic forces, $\pi$–$\pi$ stacking and van der Waals forces, strong interactions between organic molecules and CNT occur. Adsorption of positively charged organic compounds like certain antibiotics at sufficient pH is facilitated by electrostatic attraction. In addition, the system's stability and selectivity can also be improved, as CNT enables one or more functional groups to be added to further improve the system's efficiency.

Via functionalization or purification pathways, compound functional groups are attached to CNT surface. Owing to the condition of preparation, the carbon nanotubes may be multi-walled carbon nanotubes (MWCNT) or single-walled, while the CNT is of interest in the treatment of environmental pollution due to high stability and surface area. CNTs assist in the remediation of heavy metals ($Cd^{2+}$, $Pb^{2+}$, $Zn^{2+}$ and $Cu^{2+}$) in the environment via the adsorption process. In addition to adsorption, CNT cab aid other adsorptive materials, CNT can be used. Metal oxides (Fe, Ti and alumina) are an affordable and efficient technique in toxic metal adsorption. The dissolved metal ions in question adsorb substantially. Due to the presence of large active sites, nanoparticles clean the polluted sites with greater adsorption, and the ability of adsorption increases with size reduction.

In photocatalytic remediation, in the presence of catalyst and light, the pollutants can be quickly degraded into products of low molecular weight that are eventually transformed into $CO^2$, $H_2O$ and anions ($NO^{3-}$, $PO^{3-}$ and $Cl^-$). Different types of photocatalysts are applicable, but $TiO_2$ is the most frequently researched due to its biological stability, inexpensiveness and photostability (Guesh et al. 2016). Ultraviolet radiation is needed to separate charge due to the energy gap, and TiO2 generates reactive oxygen species (ROS) on irradiation with UV light that can fully degrade pollutants in short reaction time. In addition, $TiO_2$ NPs demonstrate no selectivity and are therefore suitable for the remediation of several forms of pollutants, like organic chlorinated compounds, aromatic polycyclic hydrocarbons, colorants, phenols, pesticide, arsenic, cyanide and heavy metals (Chen et al. 2016; Nguyen et al. 2016).

Adsorbents that have the capacity to extract both organic and heavy metals from the water are dendrimers. Their inner shells may be hydrophobic for organic compound sorption, while the outer branches may be adapted for heavy metal adsorption (e.g. hydroxyl- or amine-terminated). The adsorbent adsorption of metals and organic pollutants is based on complexation, electrostatic interactions, hydrophobic effect and bonding of hydrogen. In order to recover metal ions from aqueous solutions, the dendrimer ultrafiltration system was developed. The metal-laden dendrimers are recovered by the ultrafiltration technique and the dendrimers are regenerated by reducing the pH to 4.

### 2.3.2 Nanofiltration

Nanofiltration (NF) is a modern method of membrane filtration for waste water treatment based on the properties of charge repulsion and high permeation rate (Sharma and Sharma 2012). Nanofiltration is a method of separation with a cut-off between reverse osmosis and ultrafiltration. Centrifugal pumps are most commonly applied inside the nanomembrane for the pressure and circulation of waste water. The plant has large modules, with various membrane configurations (Sharma and Sharma 2012). The normal length of the module ranges from 0.9 to 5.5 m in nanofiltration, and the diameter varies from 100 to 300 mm. Considering the membrane pore size and the specific material to be extracted, membrane separation processes serve as an obstacle for various pollutants (Kusworo et al. 2018). Nanofiltration membranes have been effectively applied to eliminate hardness, heavy metals, colour and odour. Together with a charge-based repulsion mechanism, nanofiltration membranes have high selectivity but need considerable operating energy and are vulnerable to fouling. Alternatively, nanocomposite membranes are a fouling resistant filtration option for membrane filtration that has thermal or mechanical robustness and is widely used in reverse osmosis water treatment in removing micro-pollutants (Bhati and Rai 2017).

### 2.3.3 Photocatalysis

Photocatalysis is an oxidization mechanism in which photocatalytic oxidation degrades organic pollutants. This is done by combining a catalyst-coated filter with ultraviolet light rays. In advanced oxidation processes, nanocatalysts can efficiently be applied in chemical oxidation of inorganic and organic contaminants in the environment. These techniques are on the basis of creating highly reactive radicals, readily able to react with pollutant molecules. Owing to the expensive nature of supplying necessary energy (ozonators, UV lamps and ultrasonicators, etc.), the implementation of this method is often limited. The most substantial oxidation mechanism is photocatalysis. This is a change in the chemical reaction caused by the adsorption of a photon whose energy is greater than the energy required to resolve the interstitial force of a semiconductor's two electron shells (conductive and valentine). The electron (negatively charged particle) is moved from the valentine shell to the empty conduction shell when the photon illuminates the catalytic surface and leaves a void with a positive charge behind it. This electron–hole (e–h) pair produces highly reactive radicals that bind and thus remediate pollution molecules. There are, however, many technological problems that need to be addressed in order to allow wider practical implementation of this method, such as more effectively separating nanocatalysts after treatment and enhancement of selective properties during chemical reactions and optimization of catalysts in the utilization of available light energy.

## 2.4 Current Advances in Nanotechnological Management of Pollution

### 2.4.1 Pollution Bioremediation

A role in microbial bioremediation has been played by nanotechnology. The biggest challenge in biological degradation has been improving the solubility or bioavailability and consequently the rate of degradation. Surfactant micelles are recently applied in enhancing the solubility of the pollutants, but these appear to interfere with microorganism liposomes and are often broken down upon contact with soil, thus reducing the effectiveness of biological remediation. A promising move in this direction has been shown by recent developments in nanotechnology. An improvement in the solubilization degree of the model remediation compound phenanthrene (PHEN) using amphiphilic polyurethane (APU) nanoparticles has been reported. These particles, made of precursor chains of polyurethane acrylate anionmer (UAA) or poly(ethylene) glycol (PMUA), have hydrophobic interiors that demonstrate high PHEN affinity. By forming a nanoparticle suspension that competes with NAPLs, they improve PAH desorption and mobility. The affinity of nanoparticles to hydrophobic pollutant is improved by adjusting the hydrophobic portion of the precursor chain as opposed to surfactants, and mobility can be improved by regulating the charge density of the modified nanoparticles. Magnetite nanoparticles' form is another advantage provided by nanoparticles in biological remediation. Magnetite nanoparticles are produced in inert conditions by the co-precipitation of ferrous and ferric salts. In terms of simpler separation, reusability and cost-effectiveness, this technology imparts great industrial applicability. The use of magnetite nanoparticles by coating them with dibenzothiophene-desulfurizing bacterial strains of *P. delafieldii* and *R. erythropolis* LSSE8-1 has been documented, while biological desulfurization behaviour was observed. Cells coated with magnetite nanoparticles modified with ammonium oleate displayed repeated biological desulfurization behaviour and could be collected with an external magnet on the flask surface. In summary, magnetite nanoparticle usage has demonstrated increased desulfurization activity.

Nano-adsorbent used in powdery form in slurry reactors is highly effective as reported by Vijayageetha et al. (2018), but an extra system must be attached for the nanoparticles to be recovered. Nano-adsorbents may be applied in fixed or fluidized adsorbers by filling beads or granules with the nano-adsorbents. Fixed-bed reactors are typically associated with limits on head loss and mass transfer, but no future separation process is necessary (Miklos et al. 2018). As nano-adsorbents display good efficiency and are also inexpensive compared to other adsorbents, nano-adsorbents are commonly used in the breakdown of arsenic (Vijayageetha et al. 2018). ArsenXnp is a polymer and nanoparticles of iron oxide (Vijayageetha et al. 2018), while ADSORBSIA is a medium of nanocrystalline titanium dioxide and they are both utilized in extracting arsenic from water (Vijayageetha et al. 2018). In drinking water treatment systems, both small and medium scale, ArsenXnp and ADSORBSIA are used and proved to be cost-effective (Miklos et al. 2018; Vijayageetha et al. 2018).

Earlier studies have validated the effective breakdown of Hg and bulky dye molecule by graphite oxide-coated sand granules (Rhodamine B) with the breakdown nearly analogous to the activated carbon effectiveness. Nanoparticles of zerovalent type can easily eliminate the different metallic contaminants present in waste water and industrial effluent. In remediation of several halogenated hydrocarbons, organic compounds and radionuclides, nanoparticles have also been used. For Cr(VI) and Pb(II), the degradation degree of nanoscale zerovalent iron is 30 times higher compared to iron powder. Nano-adsorbent iron oxide is important in degrading arsenic forms (As(V) and As(III)), and the degradation rate is 5–10 times higher than that of the micron scale.

### 2.4.2 Pollution Biosensory

In many ways, nanotechnology plays a crucial role in solving pollution problems. Firstly, the ability to coat nanoparticles with a wide variety of biological and chemical ligands helps to give the sensor specificity. The capping of nanoparticles with different ligands such as proteins, DNA, enzymes, etc. has been documented by diverse scientists. The binding is extremely precise between these ligands and analytes. Secondly, through changing the shape and size of the nanoparticle, the volume to surface ratio of the nanoparticles can be managed, thereby imparting influence over the quality of contact with the analyte molecule. Thirdly, the capability to create nanoparticles of different metals aids to enhance conductivity and thus sensitivity. For sensing biological species, inorganic and organic pollutants, sensors based on nanoparticles have been utilized. The application of semiconductor nanostructures of porous silicon for organic contaminant detection has been proposed by scientists. Porous silicon shows photoluminescence phenomenon, and in the presence of inorganic or organic molecules, this luminescence is quenched. The concentration of pesticides as small as 1 ppm could be detected using this technology.

Both the transfer of energy to the triple substrate of the organic substrate and that of electron from the silicon nanocrystal conduction band to the quencher's vacant orbital are due to the reduction of photoluminescence by organic contaminant. For the sensing of different gases such as $NH_3$, $NO_2$ or $O_3$, carbon nanotube-based sensors have been applied. The electrical resistance of the nanotubes changes so dramatically when they are in contact with these gases that they are observed. The detection using nanoparticles of diverse heavy metals, like Pb, CD and Hg, is either fluorescence or calorimetric dependent. In the detection of heavy metal ions like mercury, cadmium and lead, functionalized gold nanoparticles with 11-mercaptoundecanoic acid and chitosan have been used. Mercaptoundecanoic acid and chitosan are chelating agents made of metal. The binding of these metal chelators to heavy metals makes nanoparticles being aggregated, causing a change in the absorption of wavelengths and eventually a resultant change in colour from red to blue. While it is not peculiar to a particular ion, the detection of heavy metal ions in general has been very sensitive. Different sensors such as that for Pb have been developed based on similar concepts. The gold nanoparticles were coated with lead-based DNA enzymes for this sensor.

Enzyme-induced aggregation of nanoparticles occurs in the absence of lead, while in the presence of lead, the activated enzyme cleaves the substrate and prevents aggregation. This resulted in a change of wavelength absorption. Selective mercury detection by Au nanoparticles coated with rhodamine B, which is a very fluorescent compound, has been reported. Owing to its binding with Au nanoparticles, its fluorescence is quenched. In the solution, the presence of Hg results in the release of rhodamine and thereby restores the fluorescence, with the intensity relating to the amount of Hg present in the solution. The mercury specificity was given by changing the surface of the nanoparticle with thiol ligands and adding to the solution a chelating agent. The nanosensor thus developed was highly selective and sensitive and can detect very low mercury concentrations (2 ppb) compared to the other available fluorescent chemosensors.

The detection time was also reduced to less than 10 min. Microorganisms (mainly bacteria) or their by-products that may be harmful to human health are biological pollutants. The largest bacteria contaminants belong to the family of *Enterobacteriaceae*. The application of mannose-encapsulated gold nanoparticles to detect *E. coli* was suggested, since mannose has an affinity for *E. coli* pilli type I. It was found that encapsulated mannose nanoparticles had a greater affinity for *E. coli* as opposed to free mannose. Instead of a particular sensor for a specific microbe, Yonzon et al. (2005) built a universal device for bacteria detection based on silver nanoparticles. Maltose showed affinity for carbohydrate binding protein concanavalin A, when functionalized with silver nanoparticles. Concanavalin A was also shown to show a greater tolerance for maltose compared to galactose. In addition to detection, the removal of bacteria from the media by amine functionalized magnetic nanoparticles has been suggested. The positive charge on the surface of the nanoparticles revealed that the bacterial negative charged surface had an electrostatic interaction. The efficiency was 88.5% for removing bacterial from water samples. Ho et al. (2004) coated IgG with gold nanoparticles and examined the association of IgG with protein for pathogenic microorganism sensing. This interaction was efficacious for sensing *S. aureus* and *S. saprophyticus* with related experiments carried out in order to identify other pathogenic microorganisms. The IgG antibody was coated on the surface of magnetic nanoparticles with a view to purifying water. By adding an external magnet, the trapped microorganisms have been extracted, and for the identification of microorganisms, quantum dots (QD) are often used as a fluorescent labelling method.

Zhu et al. (2004) reported that antibody-to-QD conjugation could be used for the detection of pathogens like *Giardia* and *Cryptosporidium*, while for *Salmonella typhimurium* and mutant *E. coli* strains, similar findings were also published. The simultaneous detection of four distinct toxins was proposed by Goldman et al. (2004). Using quantum dot shells and antibodies as probes, the cholera toxin, ricin, shiga-like toxin 1 and staphylococcal enterotoxin B were detected. As compared to organic dyes, quantum dots demonstrate superior photostability and multiplexing analysis. In addition to these biological pollutants, because of their potential as bioterrorism tools, the detection of *Bacillus* specifically is becoming important. The *B. anthracis* virulence is encoded by an exotoxin capsule and a tripartite. As a biomarker, the sensor for bacillus spores utilizes calcium dipicolinic acid (CaDPA). In the spores, CaDPA is strictly present and has no molecule that interferes. The deposition of spores on the surface of AgFON (silver film over nanoparticles) has been reported to have resulted in a CaDPA-unique SERS spectrum. At less than the life-threatening dose, the device was able to detect spore concentrations. A surface aided fluorescent sensing device for the detection of anthrax spores was developed by Yilmaz et al. (2010). Using ultraviolet light, the binding of DPA to the receptor was observed. The emission of blue light resulted from DPA-bonded receptors, although no bonding resulted in red light. For calculating the concentration of spores, the ratio of red to blue light might be used. The sensors thus produced were more sensitive and reliable than the current available techniques and can be reused. A nanosensor based on bacillus-generated exotoxin detection was developed by Zhang et al. (2011). Three genes, namely, Cya, Pag and Lef, are encoded by the exotoxin. The developed sensor was based on the toxin Pag gene encoding detection. This was made up of two nanoparticles, viz. magnetite and gold. A target DNA-specific first probe and tracer labelled barcode DNA were coated on gold nanoparticles. The magnetite nanoparticles were coated with the target DNA-specific second probe. A sandwich structure consisting of gold nanoparticle and magnetite was formed in the presence of the target. To separate the complex from the mixture, a magnetic field was applied, and the presence of the target DNA was detected using a nuclear tracer.

Nanoparticles are applied in the detection, concentration and identification of trace organic or inorganic contaminants. As they deliver high adsorption ability and recovery rate, with fast kinetics, CNTs are of promises in environmental analysis of organic contaminants or trace metals. With quick adsorption kinetics, the pre-concentration factors for metal ions have been demonstrated to range from 20 to 300. In order to pre-concentrate a number of organic compounds, CNTs were also extensively tested, most of which were carried out in real water samples. Adsorption to CNTs of charged species results in changes in conductance, providing the basis for the association between the concentration of analytes and current variations. Other nanomaterials have also been used, such as nano-Au and QDs. In a colorimetric assay, modified nano-Au was used to detect pesticides with high sensitivity and selectivity at ppb levels. Furthermore, modified nano-Au was shown to detect $Hg^{2+}$ and $CH_3Hg$ rapidly. The detection limits of PAHs were reduced by QD adjusted $TiO_2$ nanotubes to the level of pica-mole per litre based on fluorescence resonance energy transfer. A CoTe-based nanosensor of QDs immobilized on a glassy carbon electrode surface detect in water bisphenol A even at a concentration of 10 nM within 5 s.

### 2.4.3 Pollution Prevention

Metal oxide nanocatalysts, primarily gold nanocatalysts, have positive results in the prevention or reduction of source contamination. Pollution prevention is accomplished either by applications of pollution control, like air cleaning, or as low light-off autocatalysts and hydrogen stream purification used for fuel cells. During 2001, 27 patents were issued on gold catalysts for various activities in the field of pollution control and have since shown an upward trend. The aim of the patents for pollution prevention by gold nanocatalyst was divided by subject, based on World Gold Council analysis, with chemical processing having the highest share of 46%, followed by pollution control (29%), manufacturing or regeneration of catalysts (15%) and fuel cells (10%). The key attribute that regulates the widespread use of gold nanocatalysts is its high rate of operation under mild conditions, thereby minimizing the operating costs of chemical plants. Gold nanocatalysts also have low activation energy and moisture improved behaviour. Oxidation of carbon monoxide is achieved in diverse temperature ranging between 90 and 400 K.

Au nanoparticles have hemispherical shapes and reaction sites serve as the perimeter interfaces around the particles. On the edge and phase sites of Au surfaces, CO gets adsorbed and oxygen adsorbs on the support surfaces. In the interface field, both then react. CO is oxidized more readily than hydrogen by using Au, MgO or MnOx at 20 °C. In a prototype air purification unit that extracts CO from the air at room temperature, the gold nanocatalyst Mintek, South Africa, is commercially used. It is intended for use in restaurants, hospitals, offices and hotels. In oxidizing carbon monoxide, gold nanocatalysts are often used to extract it from the hydrogen feed applied in fuel cells, making the fuel cells more effective. Nanoparticles of titanium dioxide have photocatalytic properties and have therefore been used to create self-cleaning surfaces that minimize current emissions. When exposed to UV radiation, it is also a strong oxidizing agent. This helps the degradation into less toxic species of VOCs, nitrous oxides and other contaminants. An aluminosilicate molecular sieve, otherwise known as a zeolite, is another widely used structure in separation and catalysis. It is a porous crystalline solid that is well described. To oxidize hydrocarbons, such as toluene and benzaldehyde, nanosized zeolites (10–100 nm) have been developed. There are two main reasons why these zeolites make oxidation eco-friendly. Firstly, as the reaction is caused by visible light, there is a decrease in the consumption of energy. Secondly, pathways of low-energy reaction are followed that help to reduce unnecessary secondary photoreactions and thus increase the target product's yield.

## 2.5 Risk Assessment and Sustainability of Nanotechnology in Pollution Management

Nano-science research has documented that non-absorbents, nanocatalysts, bioactive nanoparticles, nanopowder, nanotubes, magnetic nanoparticles and nanoparticles are typically used to address many of the environmental problems after remediation. Nanotechnology has many benefits that help to improve the existing remediation methods, and this provides a new approach that is better than the current ones, due to the high surface area to volume ratio of the nanoparticles that enables them to absorb maximum quantities of contaminants, and their incredibly small size often enables quantum effects to come into play. Because of their increased surface area per unit mass, nanoparticles are more reactive than bulk ones (Isa et al. 2019). Nano-science has been used in a number of scientific, environmental, industrial and medical applications.

Nanotechnologies are expected to make a significant contribution to minimizing the impact of pollution using nanoparticles and nanomaterials in the environment, and are considered to be integrated into various materials to enhance distinct properties (National Research Programme (NRP) 2017). It is a promising step to control pollution from their source or restrict the process to a certain extent, so the strategies used by nanoparticles to control pollution in the following ways:

1. Some nanoparticles such as zerovalent ions act as a reduction agent by donating electrons to pollutants, thus reducing them to a less toxic material.
2. Without subsequent release, nanoparticles can be designed with specificity and affinity for a particular contaminant, which helps to absorb the contaminants.
3. There are internal voids in nanomaterials like dendrimers that allow them to trap minute substances (Triano et al. 2015).
4. To degrade organic and inorganic pollutants, low quantities of energy may be used (Isa et al. 2019).

5. The bacterial cell wall is destroyed by some nanoparticles which interfere with DNA synthesis (Raj Kumar and Gopinath 2016).
6. Nanomaterials prevent hazardous intermediates and products from forming and prevent pollutants from being released at production sites (Pandey et al. 2016).

By replacing currently used hazardous materials, nanotechnology aids the development of environmentally friendly materials. In the fabrication of computer monitors, the use of carbon nanotubes as a replacement for cathode ray tubes (with lead as one component) was suggested. By removing the use of harmful heavy metals and dramatically reducing material and energy requirements, while providing improved efficiency, CRT monitors using carbon nanotubes prevent pollution. In photovoltaic cells, the efficiency of nanoporous silicon was studied. Its porous structure contributes to quantum confinement and an increase in the bandgap, while higher internal quantum efficiency benefits from increased light absorption. Organic solar cells were produced using $TiO_2$ nanoparticles coated with an organic dye to transform light into energy by a method similar to photosynthesis in search of an alternative to Si photovoltaics. Although the conversion efficiency is only 10%, using simple technology, this type of cell can be produced from cheap, low-purity materials. By using nanostructured electrode materials such as lithium cobalt oxide, iron fluoride, cobalt chloride, rubidium oxide and nickel phosphide, the ALISTORE European Network of Excellence in Advanced Lithium Energy Storage Systems has been working on increasing the power output of rechargeable lithium batteries from the current 200 to 300 Wh/kg. To have actual control over the form, size and location of nanoscale metals used in a catalyst, nanofabrication methods are followed. The ones used in cars include platinum group metals (PGM). Investigators indicated that a nanofabrication-based automotive catalyst would cause a highly reduced loading level of PGM. This will decrease the quantity of energy consumed, enhance environmental sustainability and contribute to the sustainable use of resources.

As it can be regenerated, the adsorbent used is economical. The decrease in pH is useful in reversing metal adsorption on CNTs and the recovery rate is nearly 100% at pH below 2. It was found that the nano-adsorbent can be regenerated and reused many times without affecting the adsorption power of the adsorbent. The key advantage of carbon nanotubes is that the iron oxide adsorbent can be conveniently isolated by applying a magnetic field. Because of the low cost and high stability, nano-alumina particles are used to remove metals such as Pb, Cr, Cu, Cd and Hg from waste water. Nanoparticles known for specific properties can easily eliminate metals and other contaminants from waste water. The removal of different chemical species from waste water is simple due to the large number of active sites in nanoparticles. The challenge of extracting heavy metals from waste water is that there is no technology available that can extract metals from waste water effectively, but the use of nanotechnology offers a strong solution for waste water management.

Due to its lower operating pressure and high flow rate, nanofiltration is also cost-effective compared to reverse osmosis (Sharma and Sharma 2012). As monovalent ions are partly permeable and show absolute impermeability against bivalent salts

(Sharma and Sharma 2012), nanofiltering membranes help to extract hardness from the water to a large extent. Total dissolved solids (TDS) and hardness can be reduced by nanofiltration in addition to colour, odour and heavy metal ion removal. The high efficiency of membrane filtration helps to remove from the water the various forms of contaminants and can also achieve the optimal standards for water purification (Zekić et al. 2018). Nanofiltration is a process of treatment of the high-pressure membrane, but less energy is consumed compared to reverse osmosis because of low drive pressure (Zekić et al. 2018).

The advantages of photocatalysis are high availability, limited toxicity, cost-effectiveness and established characteristics of the material (Bai et al. 2017). Due to its supermagnetic ultraviolet photocatalytic activity, low human toxicity, high stability and low cost, $TiO_2$ nanoparticles are commonly used as a photocatalyst. Furthermore, when combined with other nanoparticles, the efficacy of $TiO_2$ as a photocatalyst is strongly increased. The emphasized value of bifunctional property is polymeric nano-adsorbents used in the removal of organics and heavy metals, whereby the inner shell branches have the ability to adsorb organics, while the outer shell adsorbs heavy metals (Yang 2019). Zeolites are a modern, flexible, low-cost nanomaterial that can be used for the treatment of waste water and desalination of water (Sutisna et al. 2017). Zeolites have a charged, permeable and thick polyamide active layer that provides a high surface area characterized by hydrophilic pores that form possible pathways for water flow in combination with their porous structure (Yurekli 2016).

## 2.6 Challenges and Recommendations

### 2.6.1 Challenges

The key drawbacks to nanoparticle direct use relate to the human and environmental hazards that accompany their use, which have not yet been fully established. An additional hindering factor may be the disposal of the spent adsorbents. The expense of both planning and deploying these innovations is another significant factor to consider. While many of the base nanoparticles are relatively inexpensive to acquire or prepare, the changes necessary to maximize the adsorption potential of the target contaminants, as in the case of many of the studies alluded to above, add to the complexity and economic feasibility of pilot-scale, and probably large-scale, applications. Therefore, in addition to the based studies on large-scale applications, the need for comparative testing between different adsorbents is important to allow for faster development of the technology for potential applications (Zhou et al. 2016).

Overall, magnetic nano-adsorbents have shown strong potential for fast removal of hydrocarbon wastes. However, the fact that the performance of such applications depends on a variety of factors affecting the rate of removal, such as the ease of synthesizing the nanocomposite, the ease of implementation and the cost of the material, may have the effect of restricting the widespread use of such nanocomposites. It should be noted that there is a large gap in knowledge in the existing literature on the optimum conditions to which the various adsorbents, or their combinations, are ideally suited. Each adsorbent has its own particular collection of tailored treatment conditions to maintain high pollutant removal rates; however, their changed types or the combined use of multiple adsorbents could also have a significant impact on the efficacy of the removal of target contaminants under various operating conditions.

Even, when pilot-scale experiments and field implementations need to be scaled up for the systems, the lack of knowledge related to optimized operating conditions could easily lead to incorrect results and eventual failure of the treatment method. It can also lift maintenance costs while making various components of the device unfeasible. Equally significant, it is necessary to use naturally polluted environment instead of synthesized environment that does not represent the diverse components present in natural environments when designing a study dealing with the removal of persistent contaminants using nanomaterials. High production costs and possible health risks are included in the drawbacks of carbon nanotubes, and the complex multistage production process for polymeric nano-adsorbents is also a disadvantage.

It is known that nanoparticles are released into the environment (Wilson 2018), and through a variety of known and unknown sources, these nanoparticles can enter the environment. Research aimed at developing an approach that understands how nanoparticles spread into the environment and their possible toxicity due to their activity has shown that nanoparticles may interact with the biological system when inhaled due to their small size, solubility and wide surface area (USEPA 2017), which may end up binding to proteins in the blood, thus suppressing the immune system. Most nanoparticles require authentication of their effectiveness and safety in the field due to the negative impact exerted by nanoparticles, as they have been successfully demonstrated on the laboratory scale, and this may probably be the reason why few nanotechnological applications for environmental clean-up have been commercialized for the time being. At this time, many risks associated with nanoparticles are not identified, but as more is learned about their transformative behaviour, the number of potential hazardous ones may increase over time.

### 2.6.2 Recommendations

The need to develop analytical tools for environmental measuring and monitoring of manufactured nanoparticles cannot be overemphasized, because there are currently not many standard methods for easily detecting and monitoring nanoparticles in the environment. In environmental systems, for measuring nanoparticles, there are only a few quantitative analytical techniques, and most of these are time-consuming and need expensive equipment and expertise. The occurrence and fate of nanoparticles after they are released into the environment show a gap in information and awareness, since there is no legislative obligation to track environmental nanoparticles, or other particles. In different environments, certain models and extrapolations seek to measure the quantity of nanoparticles. These models, however, are based on estimates of nanoparticles released into the environment and have not been calibrated using specific field measurements.

In addition, more research is required to assess the impacts of nanoparticle on the full ecosystem. Findings from ecotoxicological studies indicate that, under certain environmental conditions, animals are influenced by certain nanoparticles. The tests, however, were performed using elevated pristine nanoparticle concentrations. The authors suggested future studies to evaluate the exposure to nanoparticles that are functionalized since several nanoparticles created are functionalized, which changes their behaviour. Significant processes that have not been extensively studied are changes by environmental factors such as oxidants, light and microorganisms, resulting in biological or chemical modifications or breakdown of the functionalized surface or surface coated with natural compounds. Moreover, contained in a matrix, most nanoparticles are released and not as single nanoparticles. The study of nanoparticle forms in which organisms in the environment and man may be exposed to them is significant.

Furthermore, it is advisable to develop engineering applications with nanobiotechnology for onsite bioremediation. For remediation, there is a need to create smarter nanomaterials. New coatings or functional classes, for instance, may increase nanoparticle mobility. The capability to carry out multiple functions like catalysing multiple pollutant reactions on the same particle or interacting with hydrophilic and hydrophobic contaminants could be more advanced in nanomaterials.

## 2.7 Concluding Remarks

Nanotechnology provides an important alternative solution to current environmental pollution remediation, and superior performance is attributable to the novel properties of nanoparticles. The most recent techniques used in the remediation of polluted environment were discussed in this chapter. Such approaches are deemed to be the most successful and cost-effective till date.

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3

# Soil Microbiome: A Key Player in Conservation of Soil Health Under Changing Climatic Conditions

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## Abstract

Plants are frequently subjected to a range of biotic and abiotic stress conditions in the environment that restrict their development and growth. Improving agricultural output and productivity to feed the world's rapidly growing population would be a big challenge if chemical fertilisation were reduced. Agricultural intensification places enormous strain on the soil's ability to influence its functions, resulting in long-term large-scale environmental degradation and productivity loss. Soil health conservation is essential for sustainable agriculture and a fundamental indicator of agro-ecosystem sustainability. However, soil resources, particularly climate change, are presently facing significant threat threats significant from anthropogenic activities. Agriculture, climate, and agriculture's survival are all facing increased problems and complexity as a result of environmental changes. Soil microbiome is necessary for the preservation of the earth's natural soil cycling processes associated with organic matter breakdown, nutrient cycling, and agricultural yields, and is thus critical to human health-being. Examining the significance of soil microorganisms and their related activities in a range of ecological and microenvironmental niche zones is thus not only relevant, but also critical. Modern genomic techniques showed significant prospects for distinguishing uncultivated diversity and discovering changes in the microbial community associated with susceptible and disease-tolerant plants and discovering how climate change impacts microbes. Advances in next-generation

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sequencing (NGS) platforms, gene editing technologies, metagenomics bioinformatics techniques enable us to unfold hidden web interactions and core microbiomes to effectively deploy the microbiome to improve agricultural absorption of nutrients and resistance to abiotic and biotic stresses. Because of our negligence to maintain and conserve microbe's population, we are reducing certain constituents of the microbial population that are economically and environmentally important due to degradation caused by time changes in temperature and climate. In this study, we include a broader explanation of microbial communities' dynamics, as well as their relationships with various plant hosts and the environment. This awareness will be crucial for better predicting the roles of ecosystems in a changing environment.

**Keywords**

Microbiome · Climate change · Plant–microbe interaction · Agriculture sustainability · and Biotic–abiotic factors

## Abbreviations

| | |
|---|---|
| ACC | 1-Aminocyclopropane-1-carboxylic acid |
| AM | Arbuscular mycorrhizal |
| C | Carbon |
| CAS | Cell Alive System |
| Ccamk | Calcium/Calmodulin-Subordinate Protein Kinase |
| cm | Centimeter |
| $CO_2$ | Carbon dioxide |
| DGGE | Denaturing Gradient Gel Electrophoresis |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic acid |
| FACS | Fluorescence-activated cell sorting |
| FAO | Food and Agriculture Organization |
| GOS | Galacto-oligosaccharides |
| HTS | High-throughput sequencing |
| IPCC | Intergovernmental Panel on Climate Change |
| $M_R$ | Relative formula of mass |
| N | Nitrogen |
| Otus | Operational taxonomic units |
| PVOH | Poly(vinyl alcohol) |
| SIP | Steady isotope probing |
| UN | United States |

## 3.1 Introduction

The key research concerns of today's study are environmental conservation, global warming mitigation, and increasing food production to fulfill world food needs. The UN FAO has assessed that the global food supply could be increased by approximately 70% by 2050 sequentially to take care of an extended total population of about ten billion. As a conclusion, the global food supply must continue to increase sustainability in the face of rising competition for natural resources, notably land and water, as well as rivalry for food and biofuel, as well as the requirement to function in a carbon-constrained economy. Expanded anthropogenic exercises have a significant impact on climate (Malla et al. 2019) overall and economic horticulture specifically (Fang et al. 2018). The idea of "reasonable escalation" has been utilized to depict the difficulties of expanding the profitability of horticultural land to create more food in climate benevolent courses in the time of evolving atmosphere (Chowdhary et al. 2018). Mechanical upheaval, rapid population growth, and the expansion of enlightened zones have marked a watershed moment in nature and man's interaction with his current situation, affecting every aspect of human life. However, the Intergovernmental Panel on Climate Change is working on a solution. The shifting limits of the atmosphere have an impact on both small and scale big-scale species (plants), and it is a major global issue affecting life on the planet. Changes in the structure, abundance, size, and usefulness of plant-related microbial species are also influenced by changes in the environment. Environmental change is accepted to grant both immediate and aberrant consequences for plant–soil–microorganism co-operations (Bojko and Kabala 2017), by modifying the network structure, relative plenitude, and capacity, as the soil network taxa fluctuate significantly in their physiology, development rates, and temperature affectability. It is often assumed that indirect effects of climate change on soil microbial networks mediated by plants are more grounded than direct repercussions for shaping the subsurface microbial network. As microbial community structure and composition vary, so does the pace at which the important and explicit processes are regulated, and this in turn has an impact on ecosystem functioning. In spite of the abundance of research on plant species' migration in response to climate change, most studies fail to characterize soil microbial communities' capacity to alter their range in order to retain their link with the soil and plant community.

There are millions of fungus, billions of bacteria, and other microorganisms living in the soil. Microbes in the soil play an important role in supplement cycling and shielding plants from the damaging effects of abiotic and biotic stresses. Serious farming techniques result in increased crop production, but they can have negative effects on the organic and physical qualities of soils. Farming methods alter the diversity and synthesis of soil microbial networks, and these altered networks have an impact on agrarian ecosystems that are currently poorly understood. On frequently plowed and non-plowed soils, soil microbial networks have different effects on plant efficiency, supplement obtaining, and biological system functioning. (Kumar et al. 2015). In horticultural frameworks, macronutrients are commonly given through synthetic composts. In contrast with the compound and engineered manures, the uses of bio-fertilizers and bio-pesticides improve the development of plants, along these lines helping in ecological manageability and harvest efficiency. Plant development advancing rhizobacteria, a gathering of the assorted rhizospheric organic organisms, produces an assortment of bioactive compound substances that other than advancing the development of plants shield the plants from microorganisms (Dubey et al. 2019b). Most of the accessible writing affirmed that the rhizospheric communications at the plant organism levels are mediated either straightforwardly or by implication by root exudates. Notwithstanding, the ongoing progressions in the high-throughput innovation improved the comprehension of mainstream researchers to concentrate such sorts of communications at the gross network level. For this reason, it is necessary to conduct functional level (what they are doing) analyses of plant–microbe interactions in order to identify the signals involved in interspecies interactions. In the current audit, we have endeavored to examine how environmental change impacts soil microbial networks and plant–microorganism connections straightforwardly or in a roundabout way.

## 3.2 Soil Microbiome

One of the most important aspects of biodiversity on the planet is soil. Fungi and bacteria like the Streptomyces family are included in the list of terrestrial organisms. The soil microbiome consists of bacteria and fungus. The soil microbiome's well-being will affect the robustness of the plants associated with it. A considerable lot of the terrible illnesses in crops or different conditions are connected with changes in the soil microbiome. In addition, both the soil microbiome and the plants associated with it are affected by a large number of developments produced in the soil composition. Plant-associated microorganisms are also being studied as potential allies in the battle against disease and pests. Numerous microbes have long been recognized for their ability to aid plants in keeping healthy, whether through improved nutrition or disease protection. Plants have a perplexing relationship with soil microbiomes that are helpful for plant development, nourishment, stress resilience, and threaten plant microorganisms.

## 3.3 Function of Soil Microbiome for Improving Soil Health Under Changing Climate

Microorganisms, the most assorted and lavish gathering of life forms establish over 60% of the Earth's biomass (Bar-On et al. 2018). The function of microorganisms in continuing the energetic respectability and harmony of the biosphere is basic, as the means of life is dependent upon the supported and microbial parleyed change of issue, both in the amphibian just as earthbound conditions. Organisms have a horde of capacities, and they assume a basic part in supportability and biogeochemical cycling. They additionally make medications and drugs; channel the wastewaters and assume a part in bioremediation. The puzzling nature of the soil is attributed to fluctuated network size and species wealth; this baffling nature of the soil is ascribed to fluctuated network size and species riches. According to current worldwide estimates, soil contains roughly 4–5 × $10^{30}$ microbial cells, which is 10 times more than the number found in the oceans. Apart from viruses. Soil microorganisms including microbes, archaea, and other organisms play an assorted and frequently unequivocal part in the working of a biological systems, for example, driving the cycling of significant components (for example, N, C, and P). This cycling of components other than molding the structure and capacity of environments likewise improves the soil with the capacities that can offer shifted types of assistance to the individuals (Aislabie et al. 2013). Soil microbiome alongside their partnered capacities decides the efficiency of agrobiological systems. As of now referenced, economical farming depends on soil well-being and the variety of organisms, in this way the current day research must concentrate on overseeing soil microbiome. The utilization of advantageous microorganisms (that improves the well-being and nature of the plant, and helps in reusing of yield buildups that too with lower ecological impacts) are the essential practices for reasonable energy and food creation. It is assessed that a larger part (= 90%) of the microbial variety stays to be investigated. This epic unexplored variety compares to secret stashes of improved and inventive biotechnological advancements and applications in the fields of energy, agribusiness, synthetic compounds, mining, materials, food, drugs, and ecological assurance. The identification of the key environment driving components is one of the difficult assignments and controlling these drivers to create proper advantages is significantly additionally requested. Researchers around the world are currently faced with the challenge of maximizing the functions of microbiomes within the limitations of natural and anthropogenic activities, including new strains of pests and pathogens, climate change, and the use of chemical fertilizers, as these activities continue to threaten stable agricultural productions (Callaway 2016). Accomplishing the objective, in the setting of multiplying the worldwide food interest constantly 2050, requires the speedy and moment arrangements by conveying the soil microbiome to build the protection from different burdens (biotic and abiotic) and to improve plant supplement take-up. (Gamuyao et al. 2012) offer some of maybe a couple of occasions to discuss the management challenges of horticulture under environmental change, not many unexploited. A superior keen of the complex connections linking the plant genotypes, microbiome structures, and diverse ecological components offer fundamental data in economical farming.

## 3.4 Characteristics of the Microbiome of Soil

Plant microbiomes are assorted, comprising of inconvenient microorganisms, potential endophytes, and helpful symbionts. Historically, the microbial variety has been isolated and refined and tested in various nutritional medium and production conditions. Different plant nourishing and administrative prerequisites are satisfied by microbial exercises inside, by all accounts, and in proximate soil environmental factors. These wholesome necessities essentially incorporate nitrogen, phosphorous, and iron. Likewise, components, for example, nitrogen, phosphorous, and iron additionally can manage plant development by invigorating the creation of plant development controllers. To screen the potential microbes engaged with plant development advancement, culture-based techniques were regularly used (Taulé et al. 2012). Simple plate tests or culturing microorganisms in broth to uncover plant beneficial properties are examples of these procedures. They are likewise used to locate the hereditary segments behind these useful aggregates and their portrayal. Notwithstanding, the way of life subordinate methodologies miss most of the non-cultural microbial variety in the microbiome. Furthermore, relatively few studies have shown that plant-mediated recruitment of the rhizospheric microbiome is involved.

The specific disclosure of the microbial populace in the rhizosphere and on the surface of the root is exclusively subject to the examining and sequencing strategies utilized, and this represents a troublesome test. Besides, the ways and agent numbers for extricating microorganisms from rhizospheric tests are additionally pivotal in this examination. There are a few components, including plant have genotype, soil type, and development rehearses (examined in detail in the accompanying areas), that are key drivers in molding the rhizospheric microbial network. By utilizing the steady isotope probing (SIP) procedure, (El Zahar et al. 2008) found that the root exudates of different plants *Triticum aestivum*, *Zea mays*, *Brassica napus*, and *Medicago truncatula* are engaged with molding the rhizospheric bacterial network. The rhizospheric microorganisms acclimatizing root exudates were isolated by breaking down just the DGGE profile of [13C] DNA (fixed by plants from a controlled 13 $CO_2$ climate), though the life forms using soil natural issue were investigated utilizing [12C] DNA. This research discovered that some bacteria, such as the order *Sphingobacteriales* and the species *Myxococcus*, can only use root exudates from all plants, but bacteria from the order *Sphingomonadales* can use carbon sources from both root exudates and soil organic matter. Critically, a portion of the bacterial gatherings (class *Enterobacter* and request *Rhizobiales* as generalists) are ordinarily present in each of the four plant species. This outcome infers that a few microscopic organisms can have wide host endurance capacity, beating host particularity restrictions, albeit a few microbes can fall into the severe wholesome prerequisite classification, which is pulled into and upheld by explicit host root exudate mixes.

In the assessment of the whole microbiome, the hidden effort was started with the exposure of a protected 16S rRNA quality progression and its application and Polymerase Chain Reaction in the ID of microorganisms. Until this point, there are intensive redesigns achieved with this methodology, regarding metagenomics, to look at and grasp the microbiome in a widely inclusive perspective in a short period. As of late, these mechanical movements were generally assessed and analyzed the extent that prospects, hindrances, and solicitations. These procedures consolidate starting with whole metagenome testing, followed by cleansing, segment, and sequencing, finally data assessment and comprehension. In particular, the sequencing development is encountering fast unforeseen development, as it gives wide and all-around viewpoints on metagenomics, and HTS or forefront sequencing listened to today. The 454 Roche Sequencer, the HiSeq 2000, and the AB SOLiD System are paired with these HTS techniques (Yergeau et al. 2014). Plus, other advanced sorts, for example, deoxyribonucleic corrosive/Ribonucleic corrosive stable isotope examining and DNA shows (Phylo Chip and helpful quality groups), also have promising features in the trial of microbiomes, especially their viable parts. Starting at now, there is a change from metagenomics to metatranscriptomics, as the last answers the assortment and utilitarian part of the microbiome, instead of simply showing the assortment, like the past. It was also recently considered that the microbiome's functional adaptability and function-based diversity are more likely to be dominating elements in niches than sheer variety.

In metatranscriptomics approaches, RNA-SIP, quantitative opposite record PCR, and correlative DNA investigation combined with pyro-sequencing give progressed practical experiences into microbiome exercises in the soil and rhizosphere. In particular, the significance of RNA-SIP was underlined in future examinations for worldly investigation of the progression of root-inferred carbon and the separation of essential and optional microbial utilizers, which have higher paces of marking than their qualities and need not rely upon cell division, not at all like DNA-SIP (Uhlik et al. 2013). By defeating the overall limitations of quantitative PCR and microarray innovation in investigating the quality demeanor of an intricate network, these trendsetting innovations face colossal difficulties. These difficulties incorporate choosing either mRNA or rRNA alone as per an examination objective, accomplishing more extensive inclusion of a biological RNA pool, expanding the affectability of sequencing to arrive at environmentally significant information. (Peiffer et al. 2013) have indicated the critical network contrasts among 27 maize innate lines (hereditary variety in solitary animal variety) with basic improved populaces in the corn rhizosphere. They likewise incorporated a pilot study to choose appropriate groundwork sets (out of four recently detailed sets) and found the V3–V4 locale of the 16S rRNA quality (preliminaries 515F and 806R) to be generally reasonable, because of enhanced classifiable arrangements just as its diminished intensification of maize plastid-related groupings. This endeavor infers the fundamental thought of preliminary choice before playing out any wide metagenomics or metatranscriptomics examines. Metaproteomics, however, has a completely new approach that focuses on the complex functional nature of the microbiome and entails extracting the metaproteome from experiments and using MS for fingerprinting peptides (Kolmeder and De Vos 2014). In the early stages of enhancement, both metatranscriptomics and metaproteomics face various difficulties due to analyzing imperatives (eliminating humans and muds) and acquisition of knowledge examining and allotting bunches of orthologous gatherings of proteins from metagenomic and metaproteomics (existing and future) information are another significant cycle driven methodology and should be supplemented by different procedures to decide the variety and practical relatedness of the rhizospheric microbiome.

After the acquaintance of sub-atomic methods with breaking down the entire network of microscopic organisms, the incredible plate tally peculiarity surfaced to established researchers. Hereafter, practically all microbial network studies will include sub-atomic fingerprinting strategies, alongside the way of life subordinate methods that are as yet important for more profound comprehension of individual portrayals and are valuable for examining collaborations with have plants. That even though there are numerous specialized developments in HTS that lead to shrewd and more extensive comprehension of the microbiome phylotypes and capacities, (Dini-Andreote and Van Elsas 2013) have underscored its prevention in testing biological theories and the current need for a change in outlook from HTS (or comprehensive endeavors) to investigations of key inquiries concerning yet-unexplored plant–soil–microbiota frameworks, particularly toward a phenotypic variety of the rhizospheric microbiome on both spatial and worldly levels.

## 3.5 Factors Determining the Composition and Role of Soil Microbiome

In the unpredictable and dynamic plant root communication with the microbiome, both biotic and abiotic factors assume basic functions for microbiome structure, extravagance, and variety. Biotic components, for example, have genotypes, cultivars, formative stages, nearness to root, and root engineering, and abiotic factors, for example, temperature, soil pH, occasional variety, and the presence of rhizospheric stores, go about as synthetic signs for organisms and impact the microbiome network structure and capacity. In any case, the degree to which both abiotic and biotic components add to microbial networks is not completely perceived.

## 3.6 Direct Impacts of Climate Change on Soil Communities and Plants

Climatic change influences the working and the overall wealth of the microbial networks present in the soil. These microorganisms frequently show a serious extent of minor departure from the premise of their development rates, physiology, and affectability towards evolving temperature. In their research, Deangelis et al. (2015) found that long-term warming of forest soil leads to alterations in microbial populations in temperate forest soils. Subsequently, with expanding 5 °C temperature in mild timberland changes the overall bounties of microorganisms dwelling in the soil, for example, microscopic organisms, and thusly, expanded the bacterial to contagious proportion. For processes they mediate, global factors like global warming impact respiration rates of soil bacteria in a direct manner. Thus the function of raised temperature in the digestion of the microorganisms has gotten extensive consideration as of late (Gao et al. 2018).

In any case, a few inquiries are still should be responded to that (I) what is the effect of changing microbial network on various capacities, for example, deterioration of old and new natural issue and; (II) how dampness, temperature, and their collaboration, influence just specific microbial networks like methanogens, inside a network (III) which instruments drive the net environment reaction of microbial exercises to environmental change. Subsequently, we suggest the following methodologies like control of the microbial network and factorial warming along with temperature inclinations, (for example, height) or dampness for comprehending postulations questions. In this way, another helpful methodology for investigating these inquiries is to utilize equal transfers of soil and plants alongside climate slopes (Gao et al. 2018).

## 3.7 Climate Change Secondary Impacts on Plants and Soil Microbiome

Environmental change adjusts plant phenology and dispersion of organisms along these lines; plant species conveyance is influenced in light of environmental change. Numerous examinations neglect to clarify the capacity of soil-related microorganisms to move their reaches to keep up the negative or positive connection between the soil microbiome and plant. Soil microorganisms are considered as helpless dispersers subsequently, they react to environmental change at various rates when contrasted with that of the plant. In any case, we realize that the differential dispersal capacities among microorganisms and plants can modify plant profitability and foundation of the new plant species just as the collaborations among them. Those plants that build up effectively in the new ranges are known to deliver more significant levels of plant guard-related mixes like polyphenols (Agrawal and Weber 2015). Expansion in the general temperature of the earth or foreseen environmental change impacts crop species, creepy crawly bugs, weeds, and yield infections. Weeds are known to cause about 34% loss of harvests bookkeeping, 18% because of bugs, and 16% to infections. Environmental change seems to can expand the huge negative effect that bugs, weeds, and illness previously existed in the agrarian framework. Some foreseen impacts likewise include: a few weed animal categories advantage more than crops from higher temperatures and expanded $CO_2$ levels.

- Warmer temperature expands events of creepy-crawly bothers by quickening their life cycles, which frequently diminishes time consumed in weak time on earth arranges.
- Warmer temperatures expand winter endurance and advance toward the north extension of the scope of weeds, microorganisms, and bugs.

- Longer developing seasons permit bug populaces to increment since more ages of vermin can be delivered in a solitary developing season.
- Moisture and temperature stress related to a warming atmosphere leaves crops more defenseless towards various sorts of sicknesses change predominance of illness and reach will likewise influence the creation of domesticated animals.

## 3.8 Determination of Microbiome by Host Genotype

For fungi and bacteria, host specificity is well-understood in terms of interactions with plants. Rhizobium-legume symbiosis has been extensively investigated and demonstrates extremely host-specific interactions in the classical instance. The rhizospheric microbial communities of maize roots, pea, wheat, and sugar beet, *M. truncatula*, and *Arabidopsis* have been studied using automated ribosomal intergenic spacer. Microbes in the root's endophyte compartment were shown to respond to the host genotype in a minor but observable way, according to newly launched high-throughput investigation on *Arabidopsis*, the model plant. The microbiomes of wheat, peas, and oats (*Avena sativa*) were studied in a comparable bulk soil for four weeks. Curiously, the microbiomes were discovered to be not the same as one another, with a significant change yet to be determined of prokaryotes and eukaryotes between various plant species. Oat and pea applied solid choice on eukaryotes, though choice by wheat was a lot more fragile (Turner et al. 2013). Like current maize inbreds, it was discovered that the rhizosphere microbial community composition of plants grown in five different environments and approximated for the microbiome at flowering time had heritable variation. Even if the genetic component of maize allele has a role in microbiome variation, it is still speculative at this point in time. An ongoing trial with the foundation of rhizosphere networks in three cultivars of potato (*Solanum tuberosum*) filled in two unmistakable field destinations uncovered that just 4% of operational ordered units were reliant on the host genotype by 40% soil-explicit plenitude. Strangely, potato cultivars indicated contrasts in organisms having a place with the groups of microorganisms that have been read widely for their capacity to control plant microorganisms, and in another examination, it was demonstrated that plant age and genotype of yam (*Ipomoea batatas*) additionally impacted the root microbiome (Marques et al. 2014). Also, the structure and capacity of the rhizospheric bacterial network related to *Arabidopsis* at four diverse plant improvement stages (seedling, vegetative, shooting, and blossoming) were investigated and indicated that there were no huge contrasts in bacterial network structure. Curiously, the microbial network at the seedling stage was discovered to be unmistakable from the other formative time focuses. Intriguingly, rice (*Oryza sativa*) freak lines of a typical beneficial interaction pathway quality, calcium/calmodulin-subordinate protein kinase (CCaMK), had a critical effect on the rhizospheric microbiome while testing under both paddy and upland field conditions. That review indicated a critical diminishing in the number of inhabitants in class A proteobacteria (an ecologically omnipresent gathering) in a latent homozygous (R) CCaMK freak under both tried conditions because of a significant move in the number of inhabitants in its segment orders: *Sphingomonadales* and *Rhizobiales*. Similarly, there was an expanded bounty of Anaerolineae (*Chloroflexi*), Clostridia (*Firmicutes*), and a subpopulation of Actinobacteria (*Saccharothrix* spp., unclassified *Actinosynnemataceae*) in R plants under paddy and upland conditions.

In another examination, Shakya et al. (2013) indicated that a high rate (over 90%) of Operational Taxonomic Units (OTUs) explicit to tested develop *Populus deltoides* trees from two watersheds of North Carolina and Tennessee in two seasons (spring and fall) had the predominant phyla Proteobacteria (56.1%), Actinobacteria (17.5%), and Acidobacteria (10%). In any case, the predominance of Proteobacteria was supplanted by Actinobacteria from spring to fall in the Tennessee examples. The center rhizosphere OTUs were inside the requests Burkholderiales and Rhizobiales. This examination further endeavored to research the effect of host genotype and aggregate on network structure, where over 40% variety of elements was factually unexplained and just roughly 20% was fundamentally contributed in two environments of the tree subterranean: rhizosphere and endosphere. This investigation didn't legitimately clarify any host-explicit difference in rhizospheric bacterial network structure, rather proposing a roundabout impact of changing soil properties (through rhizodeposits and exudates) that demonstrated a measurably critical effect on the rhizospheric microbiome. These investigations (Shakya et al. 2013) suggest open-finished conversation starters. What are the particular qualities/alleles that control microbial networks? What's more, what are the particular host factors that are associated with the organization of the microbial populaces? Consequently, the theory that microbial network synthesis could be connected legitimately to have genotype requires further examination dependent on a more extensive scope of plant genotypes.

## 3.9 Alteration of Host Pathways Signaling

In light of biotic and abiotic stresses, plants initiate complex jasmonic corrosive and salicylic corrosive flagging pathways that lead to limited and foundational safeguards. The variety in the salicylic corrosive and jasmonic corrosive flagging guard pathways influences the plenitude, variety, and creation of the normal bacterial microbiome of Arabidopsis, and vulnerable genotypes have a higher wealth of microbial networks. It was indicated that the actuation of a plant's jasmonic corrosive guard pathway altogether modified the rhizosphere microbial network (Carvalhais et al. 2013). Then again, the creation of a solitary exogenous glucosinolate fundamentally adjusted the microbial network on the underlying foundations of transgenic Arabidopsis. As of late, the principal proof of the enrollment of helpful root microorganisms after over-the-ground herbivory and pathogenic bacteria assault appeared: airborne aphid taking care of and pathogenic microbial assault expanded the number of inhabitants in the nonpathogenic rhizobacterium *Bacillus subtilis* in the rhizosphere of sweet pepper (*Capsicum annuum*) and Arabidopsis plants. These investigations uncover another sort of connection and bring up the issue of how different herbivory/microorganism assaults would influence the colonization of root-related organisms. These investigations indicated that even a minor adjustment in plant roots could have significant repercussions for soil microbial networks. In any case, the substance sign that triggers the expanded colonization under airborne herbivory has not been found at this point.

## 3.10 Alteration in Root Secretions

Rhizodeposition speaks to around 11% of net fixed carbon and 27% of carbon distributed to roots. This rhizodeposition contains both low-and high-$M_r$ mixes. The low-$M_r$ mixes are more bountiful in exudates and incorporate amino acids, natural acids, phenolic mixes, basic sugars, and other little auxiliary. The amount of root exudates produced by various plant species and genotypes varies widely and may be affected by a wide range of conditions, including nutrient levels, disease, stress, and even the microbiome itself. This variety in synthetics, for example, chemotactic or flagging particles to coordinate changes in microbial pieces, may impact the creation and elements of microbial networks. The emission of sugars and sugar alcohols is managed by plant formative stages, which may assist with arranging the collection of the rhizospheric microbiome on roots. Also, it was presumed that particular formative stages in plants may emit various phytochemicals (Chaparro et al. 2014). The particular function of root exudates in the forming of the rhizosphere is additionally affirmed by indicating various gatherings of regular mixes got from plant root exudates synergistically changing the root microbiome. The known parts of cucumber (*Cucumis sativus*) root exudates *p*-coumaric corrosive and vanillic corrosive demonstrated differential impacts on the soil microbiome: *p*-coumaric corrosive expanded the pathogenic contagious taxa that corrupt the *p*-coumaric corrosive, while vanillic corrosive advanced the plant development advancing rhizobacteria *Bacillus* spp. Microbiome arrangement was additionally influenced by changing the piece of root exudates. In particular, this was finished by expanding the phenolic mixes as contrasted and sugars by making an ATP-restricting tape carrier freak in Arabidopsis. It was additionally upheld by the decrease of phenolic exudates in transgenic rice (restraint of Phe alkali lyase quality articulation) that brought about diminished microbial networks. The previously mentioned investigations upheld the idea that plant root discharge may assume a solid part in molding the rhizospheric network structure and capacity.

As of late, plant–microorganism communication examines were done with explicit plants and organisms, and low-$M_r$ natural acids in the root exudates, for example, l-malic corrosive, citrus extract, and fumaric corrosive were appeared to go about as chemo-attractants to set up root colonization. These examinations propose that biotic or abiotic stress systems may adjust the emission of natural acids in the root exudates and pull in explicit microorganisms and modify the structure and synthesis of the microbiome (Yergeau et al. 2014). Carboxyl acids, for example, malic corrosive and citrate, are appropriate carbon hotspots for some microorganisms. Carbon improvement of the rhizosphere, particularly carboxylate discharge and fermentation at the root surface, may strongly affect organizing rhizospheric microbial networks.

## 3.11 Targeted Engineering of Plant Microbiomes

The root microbiome is the essential determinant for the turn of events and development of a plant by aiding security against biotic anxieties, supplement take-up, and abiotic stress resilience (Dubey et al. 2019a), and control of this microbiome can additionally upgrade these highlights. Different organisms can associate with one another in the rhizosphere as utilitarian consortia (Fig. 3.1). For instance, *Arbuscular mycorrhizal* (AM) growths and PGPR in model fields supplemented each other as to different restricting supplements and biological system capacities. The rhizosphere microbiome may be altered via soil conditioning (including typical soil amendments) or by the addition/activation of signal molecules or substrates (such as root exudates) to induce or attract the desired microbiota. In such a manner, a few investigations have been completed to empower the utilization of explicit exudates and substrates in field preliminaries (Pascale et al. 2020). Soil can likewise be immunized with key microbial strains to change the structure of microbial networks. For instance, consortium immunization of the plant-advantageous bacterium

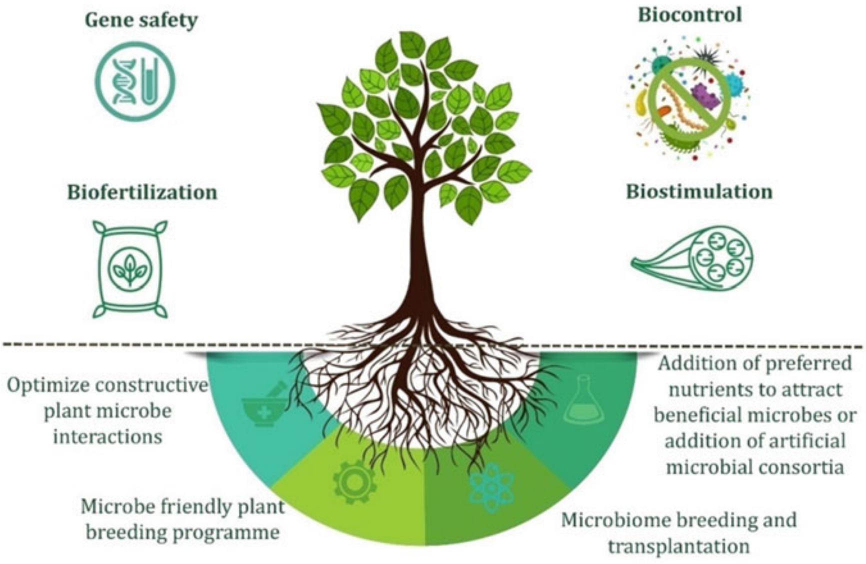

**Fig. 3.1** New era for applying Soil Microbiome Engineering

*Pseudomonas libanensis* TR1 and the AM parasite *Claroideoglomus claroideum* BEG210 into the sunflower rhizosphere, intending to outfit the capability of plant development elevating microbiota to lift pressure resistance, brought about improved plant development under metal and saltiness stress alone or in the mix (Ma et al. 2019). Chili plants grown with bacterium cultures of *Bacillus amyloliquefaciens* and *Acinetobacter* sp. UQ202 were shown to develop more quickly and had a greater resistance to soil-borne infection by Phytophtheria capsici (Syed-Ab-Rahman et al. 2019). The amount of flavonoids, antioxidants, and P in the harvested pods rose when *Agrobacterium* sp. 10 $C_2$ was used on bean plants. At 15 days after vaccination, the bacterial network in the soil around the plant changed to hold a few types of PGPR, for example, *Actinomycetes* spp. what's more, *Brevibacterium* spp.

The seed microbiome has additionally been appeared to improve germination, plant endurance, and execution, and can be acquired overages (Mitter et al. 2017). Co-vaccinating the rhizosphere of tomato seedlings with *Pseudomonas stutzeri* and *Stenotrophomonas maltophilia* supported plant development, and both radiated diffusible mixes (e.g., dimethyl disulfide) that are dynamic against the leaf microorganism *Botrytis cinerea* (Rojas-Solís et al. 2018). The microorganisms incorporated as endophytes could multiply and colonize the people to come. Similar to root exudates, roots operate as gatekeepers to select beneficial bacteria that may enter as endophytes from the rhizosphere. Curiously, plants likewise remove microscopic organisms into the rhizosphere, yet the instruments by which microorganisms (pathogenic or something else) enter and leave the plant holobiont require further examination. Likewise, roots can devour related organisms straightforwardly and use them as nitrogen sources, proposing that the presence of microbial biomass in the rhizosphere additionally adds to plant development way vaguely, albeit further examinations will be important to decide if explicit microorganisms are favored by plants for utilization.

Plant breeding efforts in recent years have focused on developing crops that are more resistant to pests and pathogens and climate change. Nonetheless, plants are normally unfit to safeguard themselves against both biotic and abiotic stresses at the same time because the particular pathways are typically hostile.

## 3.12 Developing Areas in Microbiome Engineering

Microbiome designing can have a further effect on producing higher-yielding and stronger harvests. These incorporate soil added substances (counting new natural soil changes and root exudates), artificial microbial consortia, microbiome reproducing and transplantation, the rearing and utilization of "microorganism benevolent" crop cultivars (have intervened microbiome designing), and mixes of these.

## 3.13 Utilizing Organic Soil Amendments and Root Exudates to Attract and Maintain Beneficial Microbiomes

Natural soil corrections incorporate the utilization of yield build-ups, natural squanders, manure, peat, and biochar, yet in addition include bio-fumigation, for instance, for illness concealment through soil fungistatic (Bonanomi et al. 2018). Naturally, altered soil ordinarily harbors a possibly better operational microbiome with more certain microbial exercises and more practically interrelated species than do soils where compound manures were applied. To have a better understanding of soil health, it will be important to classify the functional groupings of bacteria linked with certain organic additions. Adjusting and advancing the utilization of natural alterations for explicit soil/crop mixes would set up supportable and solid soil well-being and its microbial biological system administrations, bringing about equivalent or preferred yields over synthetic composts alone.

Salicylic acid and other root exudate signaling molecules have a significant impact on the makeup and dynamics of the rhizosphere microbiome. This offers the chance of utilizing these atoms to balance microbiomes way deliberately. The root exudates of plants, such as coumarins, are a source of phenolic compounds, which play a function in the conditioning of the root microbiota and help plants get more accessible iron from the soil. Different investigations propose that coumarins restrain soil-borne microorganisms while not influencing the useful rhizobacteria (Voges et al. 2019). Benzoxazinoids are another class of maize root exudates that have a function in forming the bacterial and contagious microbiome in the rhizosphere, bringing about insurance against herbivore creepy crawly assaults. Future research should examine the role of exudates such as coumarins, malic acid, camalexin, and benzoxazinoids in influencing the microbiome. It's important to identify which exudates attract different microorganisms since this will allow for the specialized usage of these chemicals to attract and sustain PGPR. Distinguishing which exudates pull in which organisms is a significant assignment ahead because this will empower the particular utilization of these mixes to draw in and look after PGPR. For instance, the enrollment of a useful microbiome to a soil that helped plants to develop under saline conditions has as of late been accomplished by the expansion of the root exudate and ethylene antecedent, 1-aminocyclopropane-1-carboxylic corrosive (ACC), before plant development (Liu et al. 2019). Plants under biotic or abiotic stress radiate ACC into the soil to forestall the biosynthesis of overabundance ethylene that would some way or another reason extra pressure to plants. When ACC deaminase is added to the soil 2 weeks before planting, it attracts and proliferates microbial communities that help cleave ACC into ammonia and α-ketobutyrate. Ethylene levels in plants are reduced when the previously recruited 'ACC-hungry' microbiota is swiftly degraded when the plants are under stress later on. At the point when plants are under pressure at a later stage, the recently pulled-in "eager for access microbiome rapidly corrupts ACC exudates, consequently empowering more ACC exudation and diminishing ethylene levels in plants. A drawn-out objective of this methodology is to break down and approve the effects of ACC soil changes on the soil microbiome and yields of different harvests under different pressure conditions.

## 3.14 Artificial Microbial Consortia

AMCs, also known as synthetic microbial consortia, are akin to synthetic biology in that they can recreate the microbiome of a plant. It is conceivable to create AMC that contains different capacities for plant development advancement. This can conceivably understand a portion of the disadvantages of conventional microbial bio-fertilizers, such as host inconsistency, incapable seriousness with indigenous microorganisms, and inadaptability to the neighborhood climate (Hart et al. 2018). The different advances engaged with planning the ideal AMC incorporate choosing the beginning of the organisms, acquiring and refining the center microorganisms, enhancing the microbial co-operations as per their similarity, and evaluating the viability of these consortia(Kong et al. 2018). A bacterial consortium comprising of *Comamonas testosterone*, *Pseudomonas putida, Enterobacter cloacae*, and *Citrobacter freundii* has been accounted for to upgrade phosphate portability and improve yield profitability by old twofold. In addition, a co-operation between the diazotrophic N-fixing bacterium *Azotobacter vinelandii* and the mycorrhizal growth *Rhizophagus irregularis* has been appeared to fundamentally improve root investigation under field conditions. Prompting improved supplement take-up in wheat. AMC likewise assumes a part in stress resistance, and a consortium containing *Pseudomonas putida* KT2440*, Sphingomonas* sp. OF178, *Azospirillum brasilense* Sp7, and *Acinetobacter* sp. EMM02 prompted improved dry spell pressure resilience in maize. Since the rhizosphere microbiome can change plant development by emitting specific phytohormones, an ongoing report planned two manufactured microbial networks that comprised of bacterial strains demonstrating ACC deaminase movement. Vaccination of these manufactured microbial consortia brought about the antimicrobial movement against *F. oxysporum f.* sp. *lycopersici,* decreased side effects on tomato plants, and upgraded development on a helpless substrate.

## 3.15 Microbiome Breeding and Transplantation

Microbiome reproducing can be accomplished dependent on the rule that the host plant shows qualities that favor gainful microorganisms through ages. When the microbiome is selected by the host, the bacteria that are permitted to interact with the host and passed vertically to their offspring are selected in an indirect manner. Using this approach, one of the host's phenotypes that is highly influenced by the microbiome is repeatedly propagated. This strategy depends on proliferating one aggregate of the host which is fundamentally influenced by the microbiome. A multigenerational exploratory framework was as of late used to choose for microorganisms that impact late or early blossoming in *Arabidopsis thaliana*. More than ten ages, the four most appropriate microcosms were chosen dependent on the ideal early-or late-blooming aggregate. Plants immunized with "late-blossoming microbiomes" showed expanded inflorescence (Panke-Buisse et al. 2015). This microbiome reproducing and transplantation technique has potential in various applications, including the rearing of soil microbiomes to elevate development or to withstand biotic and abiotic stresses, for example, soil-borne illnesses, saltiness, or dry spell spells. A better selection method, quantification of generational change to track microbiome intergenerational changes, stability assurance of selected microbial strains, practical microbiome transplantation techniques, and field trials assessing their functionality for improved crop production should be the focus of future research.

Microbiome transplantation can likewise be fruitful without microbiome rearing and even outside of the soil. Using the pathogen Pseudomonas syringae as a model, Wicaksono and colleagues (2018) investigated whether the process by which the medicinal Manuka plant (Leptospermum scoparium) generates antibacterial oils could be recreated in different models. To replicate this antimicrobial activity, wound inoculation of kiwi fruit with manuka plant microbiomes might be used to boost plant defense against *P. syringae* infection. Essentially, the function of plant-selected rhizobacteria in infection obstruction was surveyed by looking at the root microbiome and metagenomes of *Ralstonia solanacearum*-safe and—powerless tomato assortments, and flavobacteria were discovered to be more bountiful in the rhizosphere of the safe plant. Disease symptoms were similarly controlled in susceptible plants by transplanting a microbiome rich in Flavobacterium from the resistant variety's rhizosphere.

## 3.16 Microbiome Preservation

The definite portrayal of regular microbial networks by the trial method is basic for credible microbiome-related biological investigations related to network move and shrinkage with reality. The microbial community and metabolome recovered from preserved samples are strongly influenced by the collection technique, length and temperature of sample transit, sample archiving method, storage temperature, storage duration, and sample retrieval method for analysis. Unfortunately, only the analytical and instrumentation parts of quality control are treated seriously, whereas the control processes for sampling and transportation of samples are typically overlooked or not taken very seriously. Data from microbiome research might be skewed by errors in sample collection and storage, which can lead to a reduction in microbial viability and metabolite loss during transit and storage. Inappropriate testing and capacity of test deceive the information making fluctuation in consequence of microbiome-related investigations and are likewise liable for diminished practicality of microbiota and loss of metabolite during capacity and transportation. Along these lines, there emerges a requirement for a point-by-point depiction of the examining and conservation systems. The target of examining is to gather a piece of test from a natural specialty, little enough in volume to be advantageously moved and taken care of in the lab, to keep up properties of the climate it is gathered from. Tests for unblemished microbiome safeguarding ought to be conveyed at a temperature underneath 4 °C and in obscurity at the earliest opportunity, in the wake of inspecting. Once in the research facility, tests must be moved to a fridge as quickly as time permits. Deferral in the example handling may bring about the modification or move of the microbiota of the test. When analyzing samples, it's best to maintain the

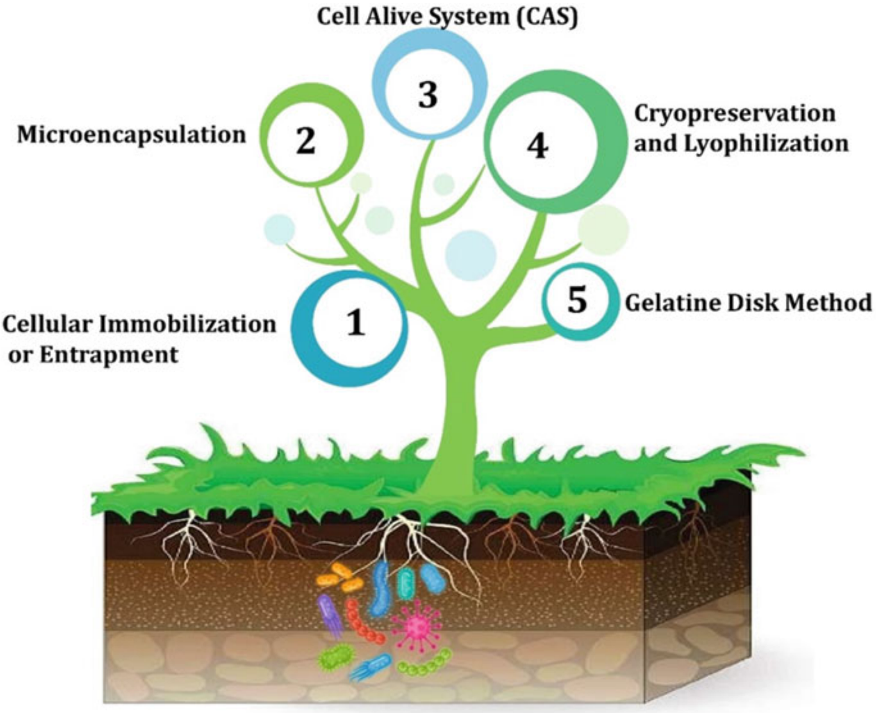

**Fig. 3.2** Different strategies for soil microbiome preservation

same ratios or concentrations of the components of interest as in the original environment if possible. In order to ensure effective analysis, it is essential that the samples be handled and processed in a manner that does not alter their composition. This requires the examples to be dealt with and treated so that no huge changes in synthesis happen which may hamper the legitimate investigation. During protection, it is prescribed to keep up the inventiveness of the example, however, much as could be expected and no addition, misfortune, or weakening must occur (Fig. 3.2).

For DNA extraction, samples that have been maintained at room temperature for a long period of time are regarded inappropriate because of changes in the microbiome landscape of the samples. Prior investigations detailed, the capacity conditions don't firmly influence the microbial network structure of the examples (Roesch et al. 2009). The investigation of gut–microbiome variety, completed by Roesch et al. (2009), has uncovered that after 72 h stockpiling of tests at room temperature results just in the unobtrusive changes. It has been shown that temperature and conditions of storage have a significant impact on maintaining the microbiome's dynamic structure. In contrast with natural examples, microbiome-related examinations are more common in the human microbiome. Various examination papers have been distributed as of late on the best way to gather, transport, store and cycle fecal materials, oral washings, vaginal and skin swabs for microbiome investigation. Sample homogenization, abrupt freezing, storage at room temperature and in 95 percent ethanol have all been researched for their effects on cryoprotection and sample storage conditions. Itemized depiction of conditions and approaches of inspecting and test handling for microbiome examines have been talked about elsewhere (Song et al. 2016). Scientists are as yet upgrading the impacts of various stockpiling conditions on microbial network vacillation in various examples. To fill the information hole, in this article, we additionally looked into changed approaches and their centrality for the drawn-out conservation of flawless microbiome tests from various sources.

## 3.17 Methods of Microbiome Preservation

### 3.17.1 Cell Alive System (CAS) Technique for Intact Microbiome Preservation

Conventional microbial protection procedures have certain constraints and do not show promising outcomes as far as keeping up the feasibility and usefulness of the microbiome during long haul conservation of flawless examples. A tale strategy, the Cell Alive System (CAS), which began in Japan is the only scope of modern coolers created to improve the conservation technique in the food industry, particularly for the safeguarding crude fish things. The CAS-freezing procedure depends on an extremely straightforward rule that the turning movement of water during freezing forestalls the arrangement of gem cross-sections. What’s more, turning movement additionally limits the point of solidification of water, which helps longer freezing impact and lessen the time needed to accomplish the excessively cool status of water. In this framework, the presence of exchanging or wavering attractive fields and mechanical vibrations incite uniform cooling and the least estimated ice precious stone development in the protected examples. This cycle keeps up the characteristic surface and forestalls cell and sub-atomic harm in saved materials and further professes to impact the tissue endurance rate. Because of this component and value, in organ banking CAS has been drawing in additional consideration of life science investigates in current time. Morono et al. (2015) did a near examination on the impacts of CAS and diverse freezing conditions on the practicality of microbial cells from protected natural examples and unadulterated *E. coli* cells. They found that examples saved in the CAS cooler indicated more feasibility than the examples saved utilizing other freezing procedures. Besides, CAS freezing innovation has been affirmed as an all-around improved instrument for long-haul protection of remote ocean residue tests for geo-microbiological contemplates. Braun et al. (2016) utilized CAS freezing procedure to save sub-ocean bottom dregs tests, for bio-particle-based microbial bounty examines and accomplished agreeable outcomes. Trembath-Reichert and co-creators likewise effectively utilized CAS freezing for remote ocean silt protection, for fluorescence-activated cell sorting (FACS), and 16S rRNA-based microbial network investigation. CAS gives better outcomes when contrasted with different procedures following half-year stockpiling of sub-ocean bottom dregs tests (Morono et al. 2015). It is fitting here that CAS can be utilized as a device for unblemished example and microbiome protection. With appropriate adjustments to this strategy, specialists may have the option to accomplish expanded capacity period and practicality.

### 3.17.2 Cryopreservation and Lyophilization in Microbiome Preservation

Cryopreservation and lyophilization both are notable techniques for the long haul protection of microbial societies. Utilization of super-low temperature with various cryoprotectants for the protection of microorganisms is a conventional practice in each microbiological research center. Cryoprotectants are a gathering of synthetic substances, which keep the arrangement of the cross-section from water atoms during conservation. Kerckhof et al. (2014) talked about the science, nature, and instrument of activity of various gatherings of cryoprotectants in detail, however, it is not the topic of this survey. Quickly, super-low temperature gives high soundness and practicality to the microbial cells, while cryoprotectants decrease cell lysis by forestalling the arrangement of ice-precious stones during the freezing cycle. Today, a scope of good cryoprotectants is accessible on the lookout. Glycerol and Dimethyl Sulfoxide (DMSO) are notable cryoprotectants that are as of now accessible in the market. Glycerol goes through vitrification measures by framing hydrogen bond with water atom present in the capacity medium. There are no ideal strategies, which can be similarly applied for a wide range of organisms and cells. Various microorganisms act diversely regarding feasibility and soundness with various cryoprotectants and more work is should have been done. Various organisms act distinctively as far as reasonability and steadiness with various cryoprotectants and more work is required to have been done around here. Various classes of polysaccharides are additionally utilized as cryoprotectants for longterm conservation and transport of the clinical examples from various gatherings. If there should arise an occurrence of Lactobacillus, amino acids assume a significant part as cryoprotectants. They increment the versatility of unsaturated fat acyl of bacterial cell layer and increments cell suitability. Nyanga et al. (2012) read up various capacity conditions for conservation of yeast culture and found that protection of yeast on rice cake gives better outcomes. It is accepted that starch present in rice responds with yeast and structures glassing structure, which may shield the yeast cells from harm. Galactooligosaccharides (GOS) made out of various Galactose units (2–9 glucose monomeric units) are a rising cryoprotectant. As a cryoprotectant, GOS is getting more consideration because of its prebiotic properties.

### 3.17.3 Gelatine Disk Method: Preservation of Sample

During transportation lyophilization is a generally acknowledged strategy for long-haul stockpiling of microbial examples and for transportation reason. Be that as it may, because of the high cost of instrumentation numerous little labs and universities cannot bear the cost of this strategy. Stamp et al. concentrated on factors that influence the bacterial endurance capacity in the wake of drying and proposed straightforward bacterial conservation strategy utilizing gelatine circle. This strategy gives great outcome for the effective protection of various microorganisms, but, be that as it may, did not perform well for delayed conservation of *Vibrio and Neisseria* species. Obara et al. changed and normalized gelatine circle strategy given by Stamp for effective conservation of *N. gonorrhoeae* with stable morphology and antibiogram design for a considerable length of time. By utilizing this procedure, they kept up with b-lactamase action of penicillinase-delivering *N. gonorrhoeae* for a considerable length of time. They likewise observed that the gelatine plate technique permits endurance of particular organic entities as long as one year at −20 °C capacity. In another strategy, Kulkarni and Chitte effectively saved thermophilic bacterial spores on channel paper (Kulkarni and Chitte 2015). As per their review, spores of thermophiles can be saved at different temperature from 4 to −20 °C for very nearly one and a half year. There is no immediate report on the utilization of gelatine plate strategy for conservation of flawless microbiome yet; because of their ability to keep up with feasibility and practical solidness of various bacterial strains, analysts could expect this procedure as a potential option for long-haul safeguarding of microbiome.

### 3.17.4 Cellular Immobilization or Entrapment

Cell immobilization or ensnarement in the gelling lattice is another option of long-haul conservation of microbial suitability and usefulness. Arrangement of miniature beads or globules with various gelling operators like alginate and Acacia-gum is a notable technique for microbial cell capture. A few components such as size, surface, porosity, the surface to volume proportion, and the synthetic nature of gelling specialists affect cell practicality and usefulness during safeguarding. This strategy is getting acceptable consideration in the probiotic industry because of the high survivability and usefulness of the ensnared cells contrasted with other conservation techniques and continued delivery in the gut after admission. *Bifidobacterium* and *Enterococcus*, two important probiotics, have been successfully kept using this approach. Epitome followed by drying is a decent choice yet freeze-drying didn’t give a great reaction because of osmotic lopsidedness and oxidative pressure made during freeze-drying measure. Fluidized bed drying gives a better reaction as far as practicality and usefulness. Besides, expansion of cryoprotectants and cancer prevention agents in gelling grid gives long haul suitability and usefulness to captured cells. It is likewise seen that the utilization of biopolymers like Acacia-gum and Pullulan is superior to inorganic polymers. Point-by-point depiction about these is accessible by (Alonso 2016). Despite the fact that these approaches are often employed to preserve pure cultures of bacteria, they may also be utilized to preserve consortiums and mixed microbiomes. There are two sophisticated methods of encapsulation: electrospinning and electrospraying, both of which are described here.

### 3.17.5 Electrospinning and Electrospraying (Microencapsulation) in Microbiome Preservation

Numerous microbial cells show affectability to miniature ecological changes. In order to preserve them using standard preservation procedures, it would be very difficult and dangerous. Microencapsulation is a superior preservation strategy for these types of bacteria and is often used to get around these problems. Microencapsulation strategy for safeguarding turns out better for such kinds of microorganisms and is utilized to defeat such challenges. High voltage Electro hydrodynamics measures like electro-showering and electrospinning are giving acceptable outcomes in the safeguarding of organisms as of late. Electrospinning and Electrospraying are novel microencapsulation strategies used to keep up the practicality and usefulness of microorganisms. For the most part, the two advances are known as "sister advances" because the method of working of both the advances is practically comparative with just distinction the inconsistency of the polymer arrangement. A high viscosity polymer solution is required for electrospinning, but a low viscosity polymer solution is required for electrospraying. This produces polymer fibers with microbial cells encapsulated at the nano- and microscales, respectively. A few investigations in the ongoing past have applied the microencapsulation method for the protection of probiotic microbes. In their examination, López-Rubio et al. (2012) effectively utilized the electrospinning technique for the conservation of *Bifidobacterium* cells. As an encapsulating substance for bacterial cells, Polyvinyl-alcohol (PVOH) was utilized because of its GRAS feature and the ease with which bacterial cells may be recovered during viability tests. Another investigation completed by Liu et al. featured that electro-turning interceded Pluronic F127 dimethacrylate (FDMA) sinewy hydro-gel material permits microbial cells to live for over 7 days at 4 °C and over 2 months at −70 °C alongside holding their metabolic action.

In terms of cell encapsulation or trapping, electrospinning and electrospraying are deemed better. Because the physical nature of polymers and the applied voltage at jet-point fibers and droplets may be altered, a variety of forms and sizes can be achieved. In addition, the high surface-to-volume ratio and high porosity of the beads and fibers generated by both methods facilitate the flow of nutrients and metabolites. By changing the physical idea of polymers and applied voltage at fly point strands and beads' fluctuating size and shapes can be gotten. Furthermore, the two offer a few different focal points like high surface to volume proportion and high porosity of created dots and strands that make supplement and metabolite trade simpler (López-Rubio et al. 2012). In contrast to different strategies, non-association of temperature in these cycles decreased the protein-denaturation from unforgiving temperature conditions thusly expanding the phone suitability and usefulness during conservation. Food-grade polymers and the use of gelling ingredients with variable protein and carbohydrate properties make the approach more appealing. Although both the strategies are utilized with restricted gatherings of microorganisms and need to investigate their possibilities and safeguarding effectiveness in prospects utilizing a wide scope of organisms giving trouble with other protection techniques.

#### 3.17.5.1 Prospective Contribution from Genome to Phenome on the Host of the Soil Microbiome

The rhizospheric microbiome can affect plant development and improvement, as their associations are coevolved and coadapted after some time and space. Microbiomes change in creation, variety, and plenitude as per numerous elements. As a result, the microbiome undergoes changes as a result of the host's exposure. Overall plant production is influenced by the interaction between the soil microbiota, edaphic conditions, and the host. Enhanced microbial richness and diversity confer disease suppressiveness on the soil microbiome, in addition to serving as a predictor of soil quality. Microbiomes of disease-suppressive soils (against *Rhizoctonia solani*) of sugar beet were examined and compared by Mendes et al. (2011). Using a PhyloChip-based metagenomic method, the soils were then treated to reduce suppressiveness or blended to produce six different soil types. Even though there was no critical contrast found in the quantity of OTUs (more than 30,000 on the whole), the wealth of specific classes, which corresponds to the soil suppressiveness, indicated huge contrasts between the distinctive soil types. The major microbial segments of soil concealment found in this investigation incorporate the g-and b-proteobacteria (*Pseudomonadaceae, Burkholderiaceae,* and *Xanthomonadales*) and the Firmicutes *(Lactobacillaceae*), particularly more plentiful during *R. solani* disease, inferring the chance of a host-instigated microbiome to battle pathogenic assault. This examination was additionally reached out by culture-subordinate techniques, where one of the major *Pseudomonadacea*e bunch individuals was demonstrated to be hostile against *R. solani* disease and followed the key hereditary component as a nonribosomal peptide synthetase quality.

This examination (Mendes et al. 2011) is especially exhaustive, as it applied simultaneous investigation utilizing both culture subordinate and—free methodologies and showed the capacity of a sympatric soil microbiome to build Arabidopsis development under dry season conditions. Against the show of a solitary bacterial application to battle dry spells, this examination unwound the significance of the soil microbiome all in reducing dry season pressure. The examination considered breaking down both the dry season reaction qualities in the host and the atomic profile of the soil microbiome associated with the cycle. In the microbiome examination, there were 33 genera in the center microbiome of *Arabidopsis* soil that was at that point answered to be essential for the center microbiome of this species. Among them, the 14 OTUs were all the more profoundly bountiful in the *Arabidopsis* microbiome contrasted and other nonsympatric (pine [*Pinus* spp.] and maize) soil microbiomes. These 14 OTUs also dispersed species including *Micromonospora, Streptomyces, Bacillus, Hyphomicrobium, Rhizobium, Burkholderia,* and *Azohydromonas* spp. Moreover, it was reasoned that different soil microorganisms assume a function in improving the host's capacity to detect and react to the dry season. Another research, by Badri et al. (2013), shows the crucial effect of various soil microbiomes (from different hosts) on the herbivorous metabolome of *Arabidopsis*. The creation of phenolics, amino acids, sugars, and sugar alcohols (segments of the leaf metabolome tried) is altogether changed by the use of different soil organisms, thus impacting the taking care of herbivores.

Specifically, there was a positive connection saw between the creation of amino acids and the decrease in herbivory. This permits us to theorize that such an association and effect from subterranean and over-the-ground plant organs may not exclusively be associated with herbivory yet additionally in controlling other multitrophic connections with microorganisms, creatures, and neighboring plant species.

In a negative feedback process (the Janzen-Connell effect), the soil microbiome seems to have an important role in the dynamics of plant communities, resulting in the close presence of powerful and diversified competitors who share an ecological niche. This impact originates from the negative effect of soil-borne microbes and hunters, which restricts the foundation of a differing plant network, while the positive input is from having mutualists, consequently depicting the two impacts beginning at the multidimensional expense of destructiveness and mutualism (Bever et al. 2012). There are both positive and negative soil network criticism exercises assuming pivotal parts in the foundation of plant populace structure. A few examinations have exhibited the unavoidable part of certain endosymbionts (either *Arbuscular mycorrhizal* growths or diazotrophs) in the underlying foundation of species in another climate or network transformation because of positive criticism, yet likely prompting colorful species strength as opposed to building up a different plant. Nonetheless, this positive criticism will have a possible task to carry out in a farming framework, where single monoculture crops are utilized rather than a different animal varieties populace. Interestingly, through negative input, the remainder of entire soil microbial networks from local environments can help accomplish the rebuilding of local plant networks. The plant variety was very much reestablished in tall grass grassland by microbial-interceded negative input from local plant soil. In any case, a different report investigated the microbial network structure and synthesis (Rosenzweig et al. 2013), and there were no connective examinations between the soil microbiome and biological rebuilding ventures. Henceforth, these criticism instruments ought to be broken down alongside microbiome structure and capacity by coupling metagenomics, which will improve our insight into the mark microbiome engaged with such components. Furthermore, the negative criticism is practically fundamental to environment rebuilding and designing, which is a genuine worldwide worry because of the weight of a dangerous atmospheric deviation and other developing anthropogenic exercises upsetting the honesty of numerous biological systems. Positive feedback from the soil community may potentially bestow host-specific symbionts on endangered species. To preserve microbiomes, new preservation methods must be developed after recognizing their importance as a whole.

## 3.18 Sustainable Agriculture and Food Safety Due to Consequences of the Soil Microbiome

New ideas on agriculture are needed to feed the world's current population of 6.9 billion people. A global green revolution is a term used to describe the process of expanding food production and improving food quality in order to maintain population expansion without sacrificing environmental safety (Gupta 2012). Manageable agribusiness improvement is expected to moderate these issues. A definitive objective of supportable horticulture, as indicated by the U.S. Public Research Council, is to create cultivating frameworks that are gainful, productive, energy-saving, ecologically solid, rationing of common assets, and that guarantee sanitation and quality. It is our view that the most encouraging methodology to arrive at this objective is to substitute perilous agrochemicals (compound manures and pesticides) with ecologically amicable arrangements of gainful microorganisms, which could improve the nourishment of yields and animals and present insurance from biotic (microbes and bugs) and abiotic (counting contamination and climatic change) stresses. There is huge writing accessible for the identification, confinement, and use of microorganisms as a significant substitute for compound contribution for crop insurance (Doornbos et al. 2012). Expanding the soil microbial species wealth was demonstrated to be an indicator of plant well-being and efficiency. The valuable impacts and components of microorganisms on plant well-being and wellness and their use in farming are broadly contemplated and reported (Wu et al. 2013). The potential microbial secludes are figured utilizing distinctive natural and inorganic transporters either through strong or fluid maturation innovation. They are conveyed as individual strains or combinations of strains through seed treatment, biopriming, seedling plunge, or soil application. Further advancement of microbial detaches and the planning cycle is required through the broad exploration to present them in manageable horticultural practices. After discovering that the whole microbiome is an important and indispensable part of the plant host, the metaorganism, identifying healthy and functionally diverse microbiomes and how to use them to improve crop yield is another big and important challenge.

## 3.19 Conclusion

The age of microbial networks with altered (valuable) exercises can possibly upgrade crop well-being, through combatting plant infections and decreasing the utilization of composts. To arrive at this objective, an essential agreement in regard to the working of the plant microbiome through organism microorganism and plant-organism communication is expected, as well as a more profound comprehension of the dirt microbial local area structure over the long haul (long haul studies) and its pliancy and reaction to the ecological changes. Additionally, since individual organisms are key to the guideline of microbial local area construction and solidness, more thorough examinations exploring local area elements utilizing these singular microorganisms and their dirt microbial networks would help with propelling the field. This information could serve to completely comprehend the effect that these cornerstone microorganisms have on crop yields, infection opposition, and worldwide supplement cycles, yet additionally to uncover systems for microbiome designing.

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# Anaerobic Digestion for Climate Change Mitigation: A Review

# 4

Ashwani Kumar, Ashu Rani, and Mamta Choudhary

## Abstract

Since fossil sources for fuel and platform chemicals will become limited in the near future, it is important to develop new concepts for energy supply and production of basic reagents for the chemical industry. One alternative to crude oil and fossil natural gas could be the biological conversion of $CO_2$ or small organic molecules to methane via methanogenic archaea. Nowadays, the anaerobic digestion of energy crops has been widely applied in developed countries. It is reported that as high as 80–90% of the biogas plants in Germany used energy crops alone or with other waste biomass as substrate. Biogas production represents a fascinating process for the recovery of nutrients and renewable energy from various organic waste streams. The process is of interest for the production of value-added chemicals by mixed cultures and can also be applied in combined bioenergy production systems. Biochar is generated as a by-product of waste biomass pyrolysis, which is featured with a high proportion of carbon and porous structure and has been widely used as a soil amendment in agroecosystems. In Japan, about two million tons of rice husk are produced annually in the rice threshing process. Part of the rice husk is used in composting but a big fraction was still not properly used. Open burning of rice husks could cause serious climate change issues due to air pollution and has been prohibited by environmental regulations in Japan.

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**Keywords**

Anaerobic digestion · Bioaugmentation · Co-digestion · Direct interspecies · Psychrophilic anaerobes · Metagenomics · Microalgal biomass

## Abbreviations

| | |
|---|---|
| ACF | Accumulation continuous flow system |
| AD | Anaerobic digestion |
| AF | Anaerobic filter |
| AFP | Antifreeze proteins |
| AH | Anaerobic hybrid reactor |
| AHS | Anaerobic hybrid septic |
| AMBR | Anaerobic migrating blanket reactor |
| AnIBPR | Anaerobic immobilized bio-plate reactor |
| AnMBR | Anaerobic membrane bioreactor |
| AnSMBR | Anaerobic submerged membrane bioreactor |
| ASBR | Anaerobic sequencing batch reactor |
| BES | Bioelectrochemical systems |
| COD | Chemical oxygen demand |
| CPH | Cationic polymer equipped hybrid |
| DIET | Direct interspecies electron transfer |
| EGSB | Expanded granular sludge bed reactor |
| GAC | Granular activated carbon |
| HRT | Hydraulic retention time |
| HUSB | Hydrolytic up-flow sludge bed |
| LCA | Life cycle assessment |
| LCFA | Long-chain fatty acid |
| OLR | Organic loading rate |
| PTF | Polyurethane foam trickling filter |
| SBR | Sequencing batch reactor |
| UASB | Up-flow anaerobic sludge bed reactor |
| USFA | Unsaturated fatty acid |
| VFAs | Volatile fatty acids |
| $\Delta \acute{G}$ | Gibbs free energy |

## 4.1 Introduction

Energy, which is a crucial and vital ingredient for the modern development of economic activities of our society, is largely a derived from fossil fuels causing accumulation of greenhouse gases (Kumar 2001, 2018, 2020a, b, c, 2021, Zandi et al. 2018; Kumar et al. 2018a, b, 2020, 2022a, b; IPCC 2021). About 600 Tg of methane is produced largely (around 70%) per year due to the activity of methanogenic archaea (Conrad 2009). Thus, methanogenesis is important for the global carbon cycle. However, methanogens can be used for several applications, e.g., energy supply and the production of high-value compounds in the chemical industry (Enzmann et al. 2018). The potential contribution of anaerobic digestion to GHG reduction has been computed for the 27 EU countries on the basis of their 2005 Kyoto declarations and using life cycle data. Anaerobic digestion (AD) has been used widely as a form of energy recovery by biogas production from the organic fraction of municipal solid wastes (OFMSW) (Seruga et al. 2018).

The methanogens are also used in microbial electrosynthesis using $CO_2$ and electrical power to generate methane. As a result, anaerobic digestion (AD) has been emerging as an efficient and viable solution for fuel production from biowastes to alleviate environmental pollution and produce green energy (Wang et al. 2021a, b). Anaerobic digestion (AD) originated from dung, wood, sawdust, grass, and other plants, and paper waste (PW) is mainly composed of lignocellulosic matter and can be applied in AD (Ali et al. 2019; Li et al. 2020). However, the intrinsic lignocellulosic structure retards the decomposition of organic matter and negatively affects the biodegradation process (Abraham et al. 2020). Roy and Kumar (2013) reviewed the challenges faced during lignocellulose bioconversion and latest technologies to overcome one of the major hurdles in the process—the pretreatment procedure. The bioconversion process is however very complex and still requires many innovations.

## 4.2 Anaerobic Digestion

The process of composting is a source of greenhouse gases (GHG) that contribute to climate change. However, anaerobic digestion (AD) involves a consortium of microorganisms that convert substrates into biogas containing methane for renewable energy. Tilche and Galatola (2008) reported that anaerobic digestion has experienced several waves of technological development starting from wastewater treatment systems in the 1970s and showed promise as an alternative energy source in the 1980s. Subsequently, it became a standard for treating organic matter-rich industrial wastewater, and more recently returned to the market for its energy recovery potential, making use of different biomasses, including energy crops. Theuer et al. (2020) reviewed anaerobic digestion of livestock manure for the production of biogas. It provides a number of benefits creating interest in the technological application in many countries all over the world (Tabatabaei and Ghanavati 2018). They suggested several benefits of anaerobic digestion. Firstly, it produces methane ($CH_4$) that can be used for the generation of electricity, heat, and fuels, and the production of biogas remains independent of weather conditions. Secondly, it reduces greenhouse gas emissions from livestock husbandry (Meyer-Aurich et al. 2012; Agostini et al. 2015) at relatively low mitigation costs (Kalt et al. 2020; Scholz et al. 2011) by substituting fossil fuels, avoiding $CH_4$ and nitrous oxide ($N_2O$) emissions from manure storage, replacing synthetic fertilizers, and decreasing

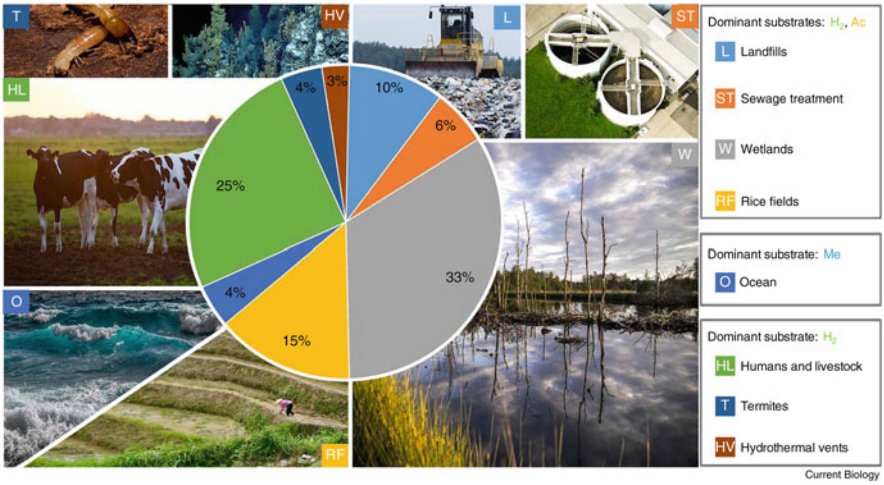

**Fig. 4.1** Sources of biological methane emissions to the atmosphere. Methanogens are found in a wide range of habitats, where they actively contribute 70% of the 500–600 Tg of methane introduced to the atmosphere each year. Percentages are the contributions of each habitat to the biological portion of the global methane emission budget. The ecology of each habitat largely determines the dominant substrates and probably selects for the types of methanogens found therein. Hydrogenotrophic methanogens are found in $H_2$-rich habitats, aceticlastic methanogens in acetate (Ac)-rich habitats, and methylotrophic methanogens in habitats rich in methyl compounds (Me). Source: Lyu, Z., Shao, N., Akinyemi, T., & Whitman, W. B. (2018). Current Biology, 28 (13). 727–732. Reproduced with permission License number 4970610534657 date 16.12.2020 from RightsLink

$N_2O$ emissions after field application of digestates (Massé et al. 2011). Thirdly, anaerobic digestion of livestock manure improves organic fertilizer quality compared with undigested manure due to a better availability of important crop nutrients such as ammonium, phosphate, and potassium and simultaneously improves soil structure and increases the soil organic matter content (Arthurson 2009). Moreover, biological degradation during anaerobic digestion can decrease the concentrations of weed seeds (Baute et al. 2016), pathogens (Fröschle et al. 2015), and antibiotics (Massé et al. 2014). Conceptually, the microbial processes of AD can be described by the sequential steps of hydrolysis, acidogenesis, acetogenesis, and methanogenesis (Bitton 2005; Venkiteshwaran et al. 2015).

Anaerobic digestion is carried out in a variety of modes (i) batch or continuous; (ii) single, double, or multiple steps (staged digesters); and (iii) vertical or horizontal treatment units. Different mixing methods are used. For “dry” (high solids) or “wet” digestion (low solids) concentration, a simple digester design consists of a single, suitably shaped, static, or mixed digester which operates under selected conditions (Venkiteshwaran et al. 2015). Different substrates can be used for AD (Fig. 4.1).

## 4.2.1 Pretreatment Methods

### 4.2.1.1 Wastewater Treatment

EU Water Framework Directive, the Oslo-Paris Convention, and oil operators in Norway are committed to the "zero discharge" of pollutants into the sea (Fakhru'l-Razi et al. 2009). According to Deng et al. (2021), the average limits of oil and grease discharge and chemical oxygen demand (COD) are 10 and 100 mg/L, respectively, in China.

Although oil and gas production is one of the most important industrial activities of modern civilization, the knowledge of microbial ecology and microbial interactions determining the efficiency of plants for oil-produced wastewater is limited (Deng et al. 2021). Microbial characteristics are important to the performance of oil-produced biological treatment processes using plants. They performed 16S rDNA amplicon sequencing of microbes that inhabited the plants from oil and marine associated environments. Biological wastewater treatment processes are regulated by many factors, such as local conditions, influent characteristics, reactor design, and operational parameters (Fig. 4.2).

Microbial communities determine the efficiency of biological treatment processes (Jiménez et al. 2018). Unlike information about microbial composition in municipal wastewater treatment plants investigated in many studies, information about the microbial composition characteristics, especially microbial origin, of oil-produced wastewater is limited (Deng et al. 2021).

The sequencing of 16S ribosomal (RNA) gene from ashore oil-water (AOW) microbial community indicated bacteria affiliated within the genera *Desulfovibrio*, *Flexistipes*, *Pseudomonas*, *Novispirillum*, *Halanaerobium*, *Sphaerochaeta*, *Acholeplasma*, *Marinobacterium*, and *Marinobacter* but often cannot explain microbial function variation (Deng et al. 2021).

### 4.2.1.2 Microbial Pretreatment

Roy and Kumar (2013) reviewed pretreatment methods. Wang et al. (2021a, b) reported that microbial pretreatment to lignocellulosic biomass for anaerobic digestion (AD) has achieved increasing attention. Microbial community analyses showed that microbial responses to oxygen varied significantly with microbial consortium, which consequently caused different AD performances.

## 4.3 Methane

Methane ($CH_4$) accounts for 14% of total global greenhouse gas emissions and is the second largest contributor to global warming (Intergovernmental Panel on Climate Change 2007). However, the atmospheric methane concentration has more than doubled since the start of the industrial era (Lyu et al. 2018). Methane is produced in the rumen and hindgut of animals by a group of Archaea known collectively as methanogens, which belong to the phylum Euryarchaeota (Hook et al. 2010). Ruminant livestock represent the single largest anthropogenic source of the potent

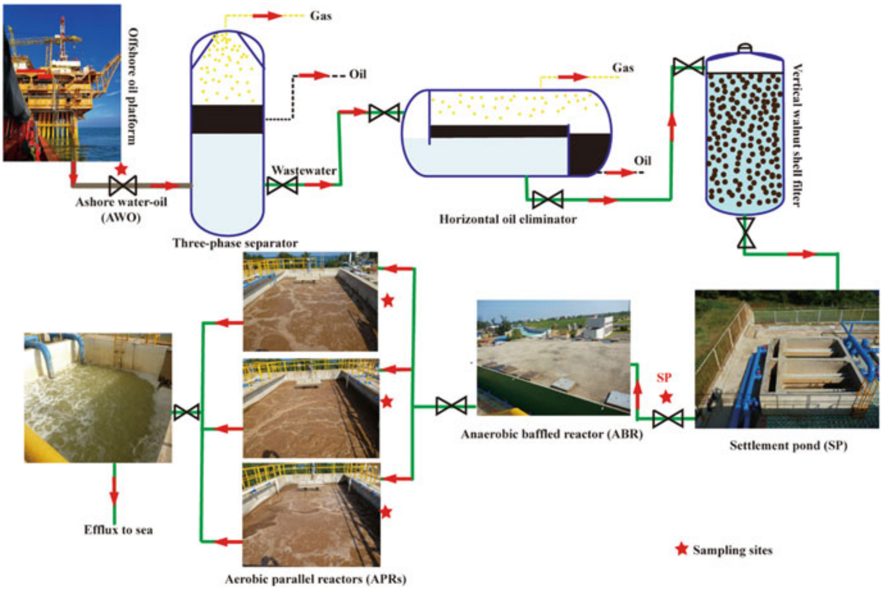

**Fig. 4.2** A schematic overview of the full-scale offshore-produced water treatment plant, red stars showing the sampling sites. Wastewater from three separators is settled in a horizontal oil eliminator with an upper oil slick, and then the lower water phase is filtrated through a vertical walnut shell filter and flows into a settlement pond (SP). These processes are followed by downstream biological treatment processes with a maximum treatment capacity of 1000 $m^3$/day. Wastewater flows into an ABR and is treated for 12 h; then the effluent from the ABR is treated in three APRs (APR1, APR2, and APR3) for 12 h. Finally, treated water is discharged to sea. Source: Deng S, Wang B, Zhang W, Su S, Dong H, Banat IM, et al. (2021) Elucidate microbial characteristics in a full-scale treatment plant for offshore oil produced wastewater. PLoS ONE 16(8): e0255836. 10.1371/journal.pone.0255836. Reproduced from Copyright: © 2021 Deng et al. as an open access article distributed under the terms of the Creative Commons Attribution License

greenhouse gas methane, which is generated by methanogenic archaea residing in ruminant digestive tracts (Shi et al. 2014). The majority of methane in nature is derived from acetate (Galagan et al. 2002). Methanogenesis, the biological production of methane, plays a pivotal role in the global carbon cycle and contributes significantly to global warming (Lyu et al. 2018). However, methane production for biogas components is good for the environment protection and greenhouse gas mitigation under controlled conditions.

Besides the canonical methanogenesis pathways described below and syntrophic interactions of methanogens and bacteria, methanogens are capable of methane generation from additional substrates (see review by Kurth et al. 2020). Kurth et al. (2020) provided extended list which is presented in Fig. 4.3.

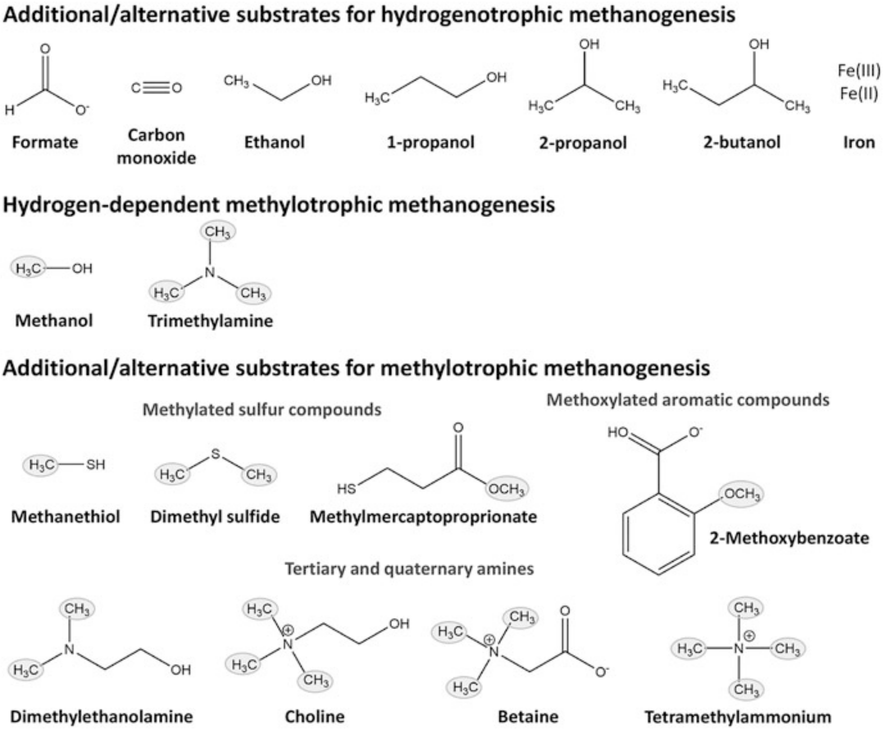

**Fig. 4.3** Extended substrate range of methanogens. 2-Methoxybenzoate is only one example for methoxylated aromatic compounds that can be used for methanogenesis (Mayumi et al. 2016). Source: Kurth, J.M., Op den Camp, H.J.M. & Welte, C.U. Several ways one goal—methanogenesis from unconventional substrates. Appl Microbiol Biotechnol 104, 6839–6854 (2020). https://doi.org/10.1007/s00253-020-10724-7. This is an open-access article distributed under the terms of the Creative Commons CC BY license, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited

## 4.4 Methanogens

Methanogens are a relative diverse group of archaea and can be found in various anoxic habitats (Garcia et al. 2000). Methanogens are the only known microorganisms capable of methane production, making them of interest when investigating methane abatement strategies (Hook et al. 2010). Methanogens belong to the Euryarchaeota of Archaebacteria and can convert inorganic-organic compounds into methane and carbon dioxide. The final product, biogas, is a mixture of methane ($CH_4$), carbon dioxide ($CO_2$), and other trace gases and is considered to be a suitable future replacement for fossil oil (Ren et al. 2008). Among livestock, methane production is greatest in ruminants, as methanogens are able to produce methane freely through the normal process of feed digestion. Much research has been directed toward methane abatement strategies to be used in ruminants and has been reviewed elsewhere (Shibata and Terada 2010). Abatement strategies are often limited by the diet fed, the management conditions, physiological state, and use of the animals.

Methanogens are biocatalysts, which have the potential to contribute to a solution for future energy problems by producing methane as a storable energy carrier (Enzmann et al. 2018). According to Pfeifer et al. (2021), methanogens thrive in habitats from hot vents in the deep oceans to ice-cold permafrost soils; in rice field soils, freshwater, and marine sediments; as well as in the intestine and oral cavity of animals. Chaudhary et al. (2018) reported methane Archaea from the human skin microbiome, and they are proposed to play a role in ammonia turnover. Methanogens use a limited range of substrates, including $CO_2/H_2$, formate, acetate, and methyl compounds (Hook et al. 2010). Shi et al. (2014) studied methane yield phenotypes linked to differential gene expression in the sheep rumen microbiome. Only a few rumen methanogens have been cultivated or characterized in detail, and their respective contributions to $CH_4$ production under in vivo conditions in livestock remain poorly defined (Buddle et al. 2011). It was reported that almost a third (28%) of anthropogenic $CH_4$ emissions are due to enteric fermentation in livestock (Yusufa et al. 2012) which will rise further due to an increased worldwide demand for meat, milk, and other animal products. Measurements of ruminant $CH_4$ emissions are mainly from animal trials in which the effects of particular diets or inhibitors of $CH_4$ formation were assessed (Martınez-Fernandez et al. 2014).

Acetate is the major source of methane in nature (Wang et al. 2011). The majority of investigations have focused on acetotrophic methanogens for which energy-conserving electron transport is dependent on the production and consumption of $H_2$ as an intermediate, although the great majority of aceto-autotrophs are unable to metabolize $H_2$. The presence of cytochrome c and a complex (Ma-Rnf) homologous to the Rnf (*Rhodobacter* nitrogen fixation) complexes is distributed in the domain. Bacteria distinguish non-$H_2$-utilizing *Methanosarcina acetivorans* from $H_2$-utilizing species suggesting fundamentally different electron transport pathways. The membrane-bound electron transport chain of acetate-grown *M. acetivorans* provides an understanding of acetotrophic methanogens.

### 4.4.1 Phylogeny and Habitats of Methanogens

Enzmann et al. (2018) reported that the methanogenic archaea belonged exclusively to the phylum Euryarchaeota. Earlier the methanogens were classified first into five orders, namely, Methanococcales, Methanobacteriales, Methanosarcinales, Methanomicrobiales, and Methanopyrales, but two more orders Methanocellales and Methanomassiliicoccales were added to it (Iino et al. 2013). Hydrogenotrophic methanogenesis from $H_2$ and $CO_2$ is found in almost all methanogenic orders with the exception of the Methanomassiliicoccales (Enzmann et al. 2018). Methane formation from acetate, called aceticlastic methanogenesis, can be found only in the order Methanosarcinales. In addition to phyla Euryarchaeota, two new phyla,

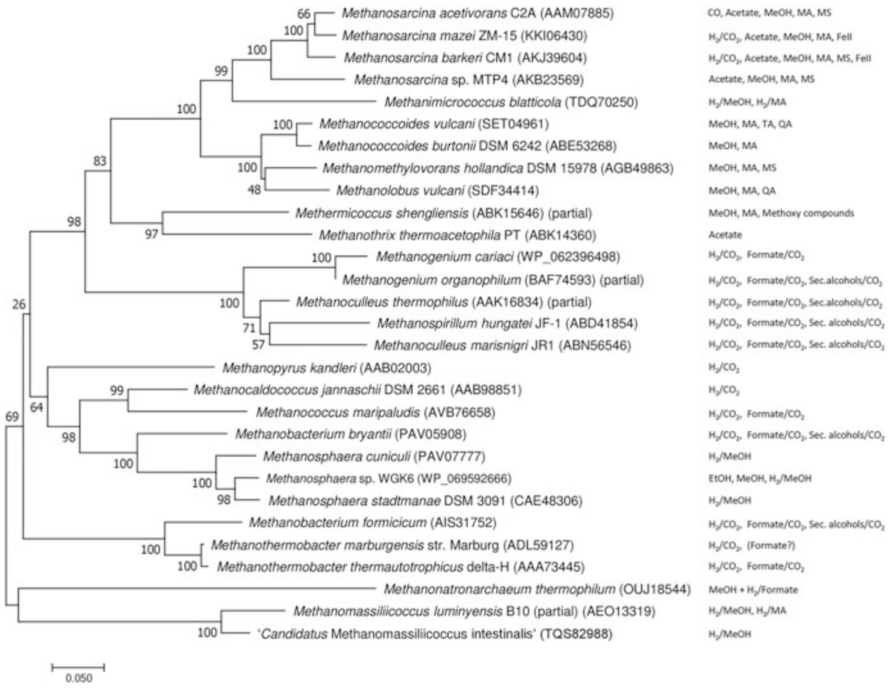

**Fig. 4.4** Evolutionary relationships of methyl-coenzyme M reductase (subunit A) of different methanogens. The evolutionary history was inferred using the neighbor-joining method. The optimal tree with the sum of branch length = 3.29201331 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Dayhoff matrix-based method and are in the units of the number of amino acid substitutions per site. The analysis involved 29 amino acid sequences. All ambiguous positions were removed for each sequence pair. There were a total of 583 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al. 2019). MA, methylamines; MS, methylated sulfur compounds; TA, tertiary amines; QA, quaternary amines. Source: Kurth, J.M., Op den Camp, H.J.M. & Welte, C.U. (2020) Several ways one goal—methanogenesis from unconventional substrates. Appl Microbiol Biotechnol 104, 6839–6854 . https://doi.org/10.1007/s00253-020-10724-7. This is an open-access article distributed under the terms of the Creative Commons CC BY license, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited

namely, Bathyarchaeota (Evans et al. 2015) and the Verstraetearchaeota (Vanwonterghem et al. 2016), have been postulated. Genome sequences from both phyla indicate a methylotrophic methane metabolism in these—as of yet uncultivated—potential methanogens.

Kurth et al. (2020) compared evolutionary relationships of methyl-coenzyme M reductase (subunit A) of different methanogens (Fig. 4.4).

Enzmann et al. (2018) reported that in biogas plants, due to hydrolysis of complex polymers to sugars and amino acids, followed by fermentation and acetogenesis, acetate, $H_2$, and $CO_2$ are produced as substrates for methanogenesis. Therefore, hydrogenotrophic and aceticlastic methanogens are prevalent in mesophilic biogas plants, often dominated by species of *Methanosarcina* (*Methanothrix* at low acetate concentrations) or *Methanoculleus* However, under certain conditions syntrophic acetate oxidation may be the dominant path toward methane (Westerholm et al. 2016).

## 4.5 Methanogenesis

Methanogenesis is a microbial-related process relevant to methane production, i.e., acidification, hydrolysis, and methanogenesis. The microbial community has the populations of key acidogens (e.g., *Longilinea* sp.) and methanogens (e.g., *Methanosaeta* sp.). According to Sowers (2009), biological methanogens are prokaryotic single-cell microorganisms, classified as methanogenic Archaea. The first photosynthetically active life developed in the Archean, about 2.4 billion years ago (Knuesting et al. 2020). These microorganisms require highly reduced, anaerobic conditions for growth. Methanogenesis is ubiquitous in environments including deep subsurface rocks, submarine hydrothermal vents, freshwater and marine sediments, ruminants, and even the human colon.

Although most of the methane ($CH_4$) produced is oxidized to carbon dioxide ($CO_2$) by methane-consuming organisms, substantial quantities (ca. 1014 g/year) escape into the atmosphere where it acts as a potent greenhouse gas (Thauer et al. 2008). Methanogenesis is the terminal step in biomass degradation in many anaerobic environments and plays a central role in the global carbon cycle.

Most methanogens produce $CH_4$ by reducing $CO_2$ with hydrogen gas ($H_2$) (Deppenmeier 2002). However, some *Methanosarcina* species such as *M. barkeri* and *M. mazei* also are capable of using a variety of other substrates, including carbon compounds such as methanol, methyl sulfides, and methylamines (Deppenmeier 2004) and acetate, which accounts for ca. 2/3 of global $CH_4$ production (Ferry 1992).

The dominant source of $CH_4$ emissions from livestock is from ruminants (Naqv 2011), where $CH_4$ is formed as a by-product of feed fermentation in the forestomach (rumen) by $CH_4$-producing archaea, known as methanogens (Boone et al. 1993). Thauer et al. (2008) focused on the energy metabolism of methanogenic archaea growing on $H_2$ and $CO_2$, with the emphasis on differences between methanogens with and without cytochromes. The pathways are coupled to the generation of an electrochemical sodium ion gradient and an electrochemical proton gradient. Both ion gradients are used directly for ATP synthesis via membrane integral ATP synthases. The function of the abovementioned systems and their components in the metabolism of methanogens is described in detail by Enzmann et al. (2018). Molecular hydrogen is mainly produced by carbohydrate fermentation and then used by methanogens to produce $CH_4$ in the rumen (Ma 2019). Rumen methanogenesis mainly involves three types of methanogenic metabolic pathways, which includes hydrogenotrophic (reduction of $CO_2$ coupled to the oxidation of $H_2$), methylotrophic

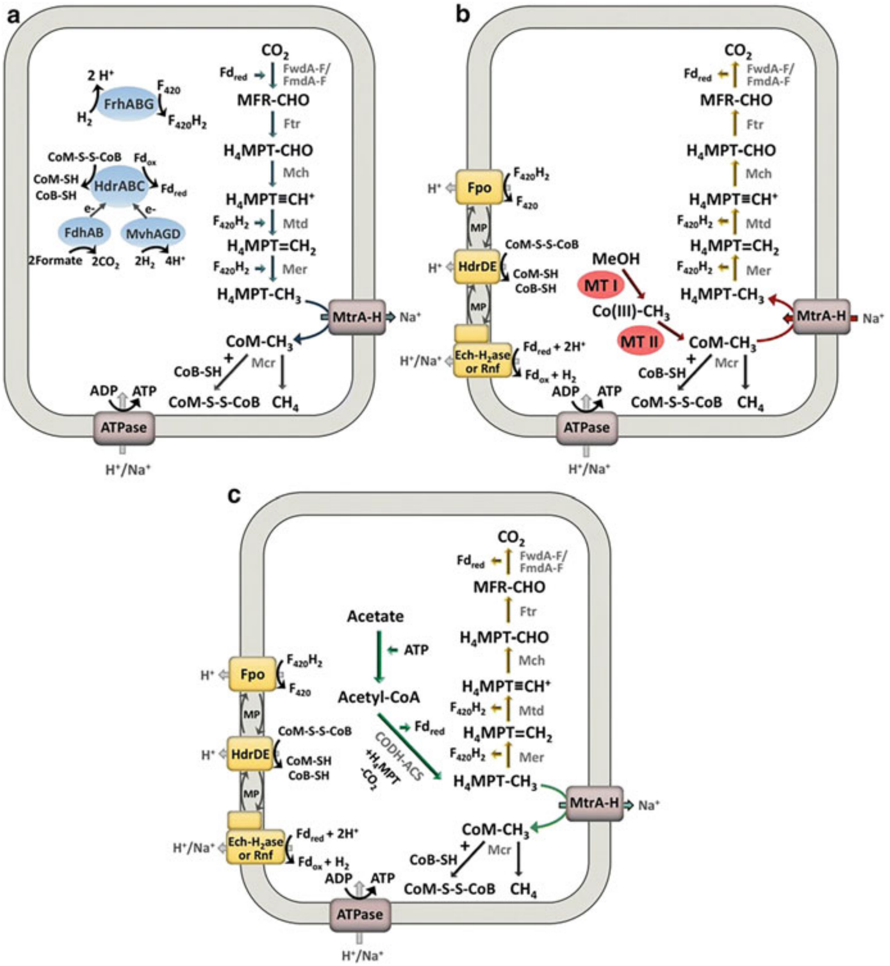

**Fig. 4.5** Kurth et al. (2020) depicted three methods of methanogenesis: (**a**) hydrogenotrophic, (**b**) methylotrophic, and (**c**) aceticlastic methanogenesis pathways. The ferredoxin electron carrier is a two-electron carrier. Some methanogens use a H4MPT derivative called tetrahydrosarcinopterin (H4SPT). The Na+/H+ translocation stoichiometry is not represented in the figure. FwdA-F/FmdA-F, formylmethanofuran dehydrogenase; Ftr, formylmethanofuran-tetrahydromethanopterin formyltransferase; Mch, methenyl-tetrahydromethanopterin cyclohydrolase; Mtd, methylenetetrahydromethanopterin dehydrogenase; Mer, 5,10-methylenetetrahydromethanopterin reductase; MtrA-H, tetrahydromethanopterin S-methyl-transferase; McrABCDG, methyl-coenzyme M reductase; FrhABG, coenzyme F420-reducing hydrogenase; HdrABC, soluble heterodisulfide reductase; MvhAGD, F420-nonreducing hydrogenase; FdhAB, formate dehydrogenase; FpoA-O, F420H2 dehydrogenase; HdrDE, membrane-bound heterodisulfide reductase; Ech-H2ase, energy-converting hydrogenase; Rnf, Na + −translocating ferredoxin; NAD+, oxidoreductase complex; ATPase, ATP synthase; CODH-ACS, acetyl-CoA decarbonylase/synthase; MTI and MTII, methyltransferase; CoB, coenzyme B; CoM, coenzyme M; H4MPT, tetrahydromethanopterin; MFR, methanofuran; Fd, ferredoxin; F420H2, reduced coenzyme F420; MP, methanophenazine; CO(III), cobalamin binding protein. Source: Kurth, J.M., Op den Camp,

(conversion of methyl group-containing compounds), and acetoclastic pathways (Rother and Krzycki 2010). Three main methanogenesis pathways (hydrogenotrophic, methylotrophic, and aceticlastic) share the core pathway of methanogenesis yet also differ in many aspects of their biochemistry and physiology (Fig. 4.5).

### 4.5.1 Hydrogenotrophic Archaea

According to Lyu et al. (2018), methanogenesis is an anaerobic respiration that uses oxidized carbon such as $CO_2$ as a terminal electron acceptor and generates methane as the final product of metabolism. $H_2$ + $CO_2$ and formate are converted to $CH_4$ via the $CO_2$-reducing pathway, while methanol and methylamines are metabolized by the methylotrophic pathway. They are the predominant source of methanogenesis in deep marine sediments, termite hindguts, and human and animal gastrointestinal tracts, which altogether contribute a third of biologically generated methane emissions. Thus, methanogens are common in habitats that are poor in other electron acceptors, such as $O_2$, $NO_3$, $Fe^{3+}$, and $SO_4{}^{2-}$. Deppenmeier (2004) reported that members of the genus *Methanosarcina* are strictly anaerobic archaea, deriving their metabolic energy from the conversion of a restricted number of substrates to methane. During hydrogenotrophic methanogenesis, $H_2$ is oxidized to $H^+$, and $CO_2$ is a terminal electron acceptor and generates $CH_4$. The $H_2$-dependent $CO_2$ reduction proceeds via carrier-bound $C_1$ intermediates which become stepwise reduced to methane (Fig. 4.5a).

### 4.5.2 Methylotrophic Methanogens

Methylotrophic methanogens are common in marine and hypersaline, sulfate-rich sediments where they utilize methylated compounds such as trimethylamine, dimethyl sulfate, and methanol. New members of this group have recently been found in habitats such as the bovine rumen. Methanomassiliicoccales occur in a large variety of anoxic habitats including wetlands and animal intestinal tracts especially in ruminant animals. They likely are among the major methane producers. Considering the role of methane as potent greenhouse gas, resolving the methanogenic nature of a broad range of putative novel methylotrophic methanogens and assessing their role in methane emitting environments are pressing issues for future research on methanogens (Söllinger and Urich 2019) (Fig. 4.5b).

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**Fig. 4.5** (continued) H.J.M. & Welte, C.U. Several ways one goal—methanogenesis from unconventional substrates. *Appl Microbiol Biotechnol* 104, 6839–6854 (2020). https://doi.org/10.1007/s00253-020-10724-7. This is an open-access article distributed under the terms of the Creative Commons CC BY license, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited

### 4.5.3 Aceticlastic Methanogens

Aceticlastic methanogens split acetate to form $CH_4$ and $CO_2$. Biological methanogenesis from acetate is one of the most important processes for the maintenance of the carbon cycle on Earth. Galagan et al. (2002) analyzed the complete genome sequence of an acetate-utilizing methanogen, *Methanosarcina acetivoran*s C2A. *Methanosaeta* are obligate acetoclastic methanogens that are known to use only acetate or acetate plus electrons obtained via direct interspecies electron transport (DIET) (Venkiteshwaran et al. 2015). Acetoclastic methanogens are the dominant methane producers in anaerobic digesters, rice fields, and wetlands. *Methanosarcina mazei* belongs to the group of aceticlastic methanogens and converts acetate into the potent greenhouse gases $CO_2$ and $CH_4$. The aceticlastic respiratory chain involved in methane formation comprises the three transmembrane proteins Ech hydrogenase, F420 nonreducing hydrogenase, and heterodisulfide reductase. The energy-conserving transmembrane enzyme system used in the aceticlastic pathway of methanogenesis has been referred to as Fd/heterodisulfide oxidoreductase. The electron flow from Fdred to heterodisulfide reductase in *Methanosarcina mazei* has been reconstructed in recent years (Kurth et al. 2020) (Fig. 4.5c).

## 4.6 Improvement in Methane Production

### 4.6.1 Nano-Biochar

Zhang and Wang (2021) suggested catalytic use of biochar-supported nano-zerovalent iron (nZVI-BC) on anaerobic co-digestion (co-AD). Methane production potential ($R_0$) and daily methane production rate ($G_m$) of sewage sludge and food waste were enhanced with the catalyst as compared to normal biochar.

### 4.6.2 Bioaugmentation

The plant residues obtained after the phytoremediation process can be used in anaerobic digestion (Das and Kumar this volume). However, such residues still contain high concentrations of HMs which inhibit the digestion efficiency of plant residues. Mao et al. (2021) reported that bioaugmentation is an effective method to improve the degradation efficiency and methane yield of plant residues rich in HMs. They isolated cellulose-degrading anaerobic bacteria from cow dung, *Paracoccus* sp., termed strain LZ-G1 which degraded cellulose and simultaneously adsorbed $Cd^{2+}$. Thus, bioaugmentation provides an easy and a feasible method for the actual on-site treatment of HM-rich phytoremediation residues.

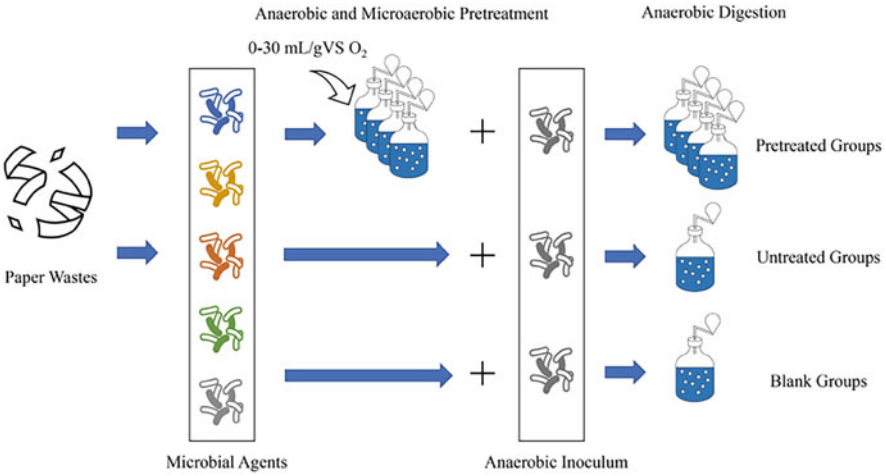

**Fig. 4.6** Schematic diagram of the experimental setup. Song, C., Li, W., Cai, F., Liu, G., & Chen, C. (2021). Anaerobic and Microaerobic Pretreatment for Improving Methane Production From Paper Waste in Anaerobic Digestion. *Frontiers in Microbiology*, *12*, 1520. https://doi.org/10.3389/fmicb.2021.688290. At Frontiers, the entire content of all present and past journals is immediately and permanently accessible online free of charge and published under the CC-BY license, which permits unrestricted use, distribution, and reproduction in any medium, provided the original authors and the source are credited

### 4.6.3 Ultrasound Pretreatment

Zerrouki et al. (2021) investigated the use of ultrasound pretreatment as potential technique to solubilize organic matter and fermentation of fruit juice effluents in anaerobic batch reactor.

### 4.6.4 Micro-Oxygenic Treatment

Zhen et al. (2021) reported that micro-oxygen pretreatment, i.e., supplying a small amount of oxygen during microbial pretreatment of kitchen waste, shortened the fermentation cycle and improved the gas production efficiency of anaerobic fermentation. Recently, anaerobic and micro-aerobic pretreatment has attracted attention as it overcomes the obstacles of biogas production. In micro-aerobic condition, the stimulated microbes showed higher hydrolysis and acidogenesis activity (Xu et al. 2014). Microbial community analysis by Song et al. (2021) showed that *Clostridium* sensu stricto *1* and *Clostridium* sensu stricto *10* possessed high relative abundance after anaerobic pretreatment by straw-decomposing inoculum (SI), while *Bacteroides* and *Macellibacteroides* were enriched after micro-aerobic pretreatment by sheep manure (SM) which were all contributable to the cellulose degradation (Fig. 4.6). Zhen et al. stated that after the micro-aerobic pretreatment, *Firmicutes* and

*Bacteroidetes* were the predominant phyla during the AD of rice straw (Zhen et al. 2021).

## 4.6.5 Role of Temperature

### 4.6.5.1 Mesophilic and Thermophilic Temperature

Jiang et al. (2021) worked on in situ hydrogen biomethanation technology to upgrade biogas production. They reported that efficiency of biomethanation relies on various parameters, e.g., temperature, gas supplement, and hydrogenotrophic methanogens. They further reported that the hydrogenotrophic methanogenesis was performed better at thermophilic condition, while the dominant archaea genera *Methanobacterium* and *Methanothermobacter* performed better at mesophilic and thermophilic temperature, respectively. Jiang et al. (2021) concluded that the highest $CH_4$ content (greater than 90%) was obtained when $H_2$ and $CO_2$ were feeding at ratio of 4:1 and *Methanothermobacter* was dominant.

### 4.6.5.2 Psychrophilic Temperature

Tiwari et al. (2021) presented a future outlook on psychrophilic anaerobic digestion (AD). In this technique, the low temperature acclimated microbial biomass are used to overcome thermodynamic constraints through expression of cold-adapted enzymes obtained by genotypic and phenotypic variations. They suggested that bioaugmentation with psychrophilic strains could reduce start-up time and ensure daily stable performance for wastewater treatment facilities at low temperatures (Fig. 4.7).

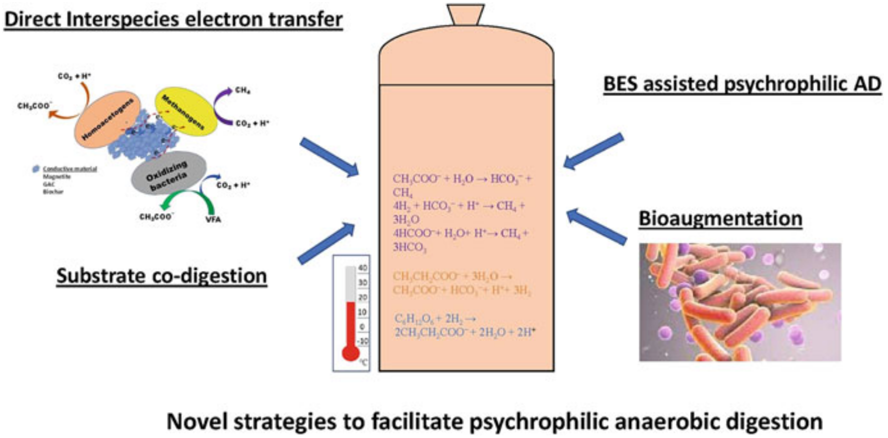

**Fig. 4.7** Novel strategies to facilitate psychrophilic anaerobic digestion. Source: Tiwari, B. R., Rouissi, T., Brar, S. K., & Surampalli, R. Y. (2021). Critical insights into psychrophilic anaerobic digestion: novel strategies for improving biogas production. Waste Management, 131, 513–526. 10.1016/j. Reproduced with license no. 5126880444981 from RightsLink dated 13 August

### 4.6.6 Effects of Silver Nanoparticles

Grosser et al. (2021) investigated the effects of nanoparticles on performance and stability of anaerobic digestion of sewage sludge in four reactors. They included sewage sludge (control reactor), and remaining ones were fed with sewage sludge with the addition of the following additives: (1) silver nanoparticles (NPs reactor), (2) ionic silver ($AgNO_3$ reactor), and (3) diluent used for nanoparticles (DIS reactor). They reported a fivefold increase in the number of *Methanosarcina* genus in Ag-NPs reactor compared to the control reactor.

## 4.7 Biotechnology of Archaea

According to Pfeifer et al. (2021), biotechnology can be defined as any technological application using biosystems, organisms, or derivatives thereof, to manufacture or modify bioproducts or to develop and engineer processes for specific application. Extracellular electron exchange in *Methanosarcina* species and closely related Archaea plays an important role in the global carbon cycle and enhances the speed and stability of anaerobic digestion by facilitating efficient syntrophic interactions (Holmes et al. 2019). During recent years, genetic tools for methanogens have been improved, opening a new field of research on these important microorganisms. Archaea represent a novel domain of life distinct from bacteria and eucarya (formerly known as eukaryotes). Nayak and Metcalf (2017) described the development of a Cas9-mediated genome-editing tool that allows facile genetic manipulation of the slow-growing methanogenic archaeon *Methanosarcina acetivorans* (see also Neumann et al. 2020).

Hassa et al. (2018) have reported that taxonomic profiling of biogas-producing communities by means of high-throughput 16S rRNA gene amplicon sequencing provided high-resolution insights into bacterial and archaeal structures of AD assemblages and their linkages to fed substrates and process parameters (Figs. 4.8 and 4.9).

They further characterized specific marker genes (Fig. 4.6) for direct and immediate insights into microbial community compositions and the phylogenetic relationship of community members by their PCR amplification from whole community (metagenomic) DNA. A widely and commonly used approach for microbial community profiling without prior cultivation is the analysis of the 16S small subunit ribosomal RNA (rRNA) gene sequence (Lebuhn et al. 2014; Simó et al. 2014).

### 4.7.1 Synthetic Genes for Industrial Products Production

The rapid autotrophic growth of the methanogenic archaeon *Methanococcus maripaludis* on $H_2$ and $CO_2$ makes it an attractive microbial chassis to inexpensively produce biochemicals (Lyu et al. 2018). Lyu et al. (2018) reported that a synthetic gene encoding geraniol synthase (GES) derived from *Ocimum basilicum* was cloned

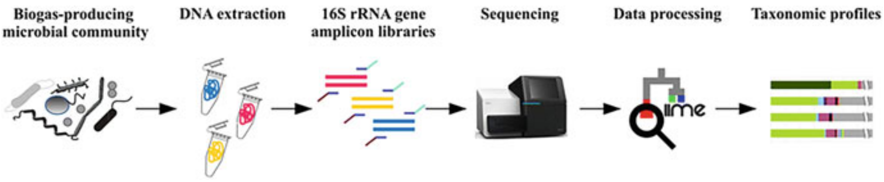

**Fig. 4.8** Schematic overview on taxonomic profiling of biogas-producing microbial communities applying 16S rRNA gene amplicon sequencing. After extraction of whole community DNA, 16S rRNA gene amplicon libraries were constructed and subsequently sequenced. Obtained sequences were processed with the program QIIME (Caporaso et al. 2010) to calculate taxonomic community profiles. Source: Hassa J, Maus I, Off S, et al. 2018. Metagenome, metatranscriptome, and metaproteome approaches unraveled compositions and functional relationships of microbial communities residing in biogas plants. *Appl Microbiol Biotechnol.*; 102(12):5045–5063. https://doi.org/10.1007/s00253-018-8976-7. **Open Access** This article is distributed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made

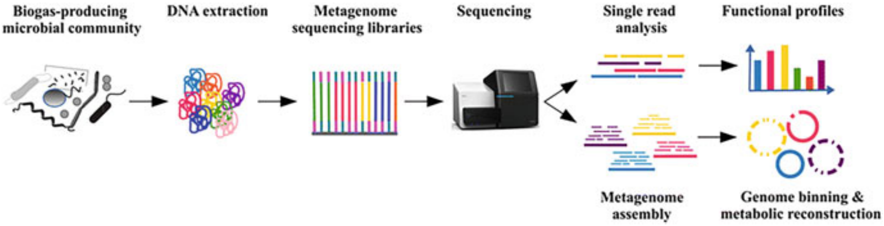

**Fig. 4.9** Hassa et al. (2018) depicted workflow for functional profiling of microbial biogas communities exploiting metagenome sequence data. After sampling at biogas reactors, total DNA was extracted for construction of whole metagenome shotgun libraries which were subsequently sequenced on high-throughput sequencing platforms. Resulting sequencing data were quality checked and functionally characterized based on single read sequences in order to deduce functional profiles of the underlying biogas community. Moreover, metagenome assembly followed by a binning approach was applied to compile MAGs, which were then analyzed for their metabolic potential. Source: Hassa J, Maus I, Off S, et al. 2018 Metagenome, metatranscriptome, and metaproteome approaches unraveled compositions and functional relationships of microbial communities residing in biogas plants. *Appl Microbiol Biotechnol.*; 102(12):5045–5063. https://doi.org/10.1007/s00253-018-8976-7. **Open Access** This article is distributed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made

into a *M. maripaludis* expression vector. Recombinant expression of GES in *M. maripaludis* during autotrophic growth on $H_2/CO_2$ or formate yielded geraniol at 2.8 and 4.0 mg $g^{-1}$ of dry weight, respectively. A conceptual model centered on the autotrophic acetyl coenzyme. Thus, a biosynthetic pathway alteration strategy can divert more autotrophic carbon flux to geraniol production (Lyu et al. 2018).

The discovery of a methanogen that can conserve energy to support growth solely from the oxidation of organic carbon coupled to the reduction of an extracellular electron acceptor expands the possible environments in which methanogens might thrive (Holmes et al. 2019), e.g., introduction of a bacterial esterase allowed *M. acetivorans* to grow on methyl esters (like methyl acetate and methyl propionate) (Lessner et al. 2010). Expression of the gene encoding 3-hydroxybutyryl-CoA dehydrogenase (Hbd) from *Clostridium acetobutylicum* resulted in formation of L-lactate (0.59 g/g methane) from methane with acetate as intermediate, possibly by Hbd exhibiting lactate dehydrogenase activity in the heterologous host (McAnulty et al. 2017). Thus, the principal possibility might exist to engineer *M. acetivorans* for industrial production.

Timmers et al. (2017) reported “trace methane oxidation” (i.e., “reverse methanogenesis”) in wild-type methanogens during net methane production. Heterologous expression in *M. acetivorans* of genes encoding methyl-CoM reductase from anaerobic methanotrophic archaea (ANME-1) resulted in a strain that converted methane to acetate three times faster than the parental strain (Soo et al. 2016). The same holds true for the production of other high-value products like amino acids or vitamins with methanogens, and due to their slow growth, a technical application is not yet developed (Schiraldi et al. 2002). However, new processes may emerge using heterologous methanogens.

## 4.8 Extracellular Electron

Anaerobic digestion (AD) of organic waste to produce biogas is a mature biotechnology commercialized for decades (Feng et al. 2021). Some of the recent advances are presented here. Extracellular electron exchange in *Methanosarcina* species and closely related Archaea plays an important role in the global carbon cycle and enhances the speed and stability of anaerobic digestion by facilitating efficient syntrophic interactions.

### 4.8.1 Mineralization

Iron corrosion is an electrochemical process involving oxidation of metallic iron [Fe(0)] to Fe(II) (anodic reaction; Eq. (1)) and reduction of external electron acceptors (cathodic reaction) (Fig. 4.10).

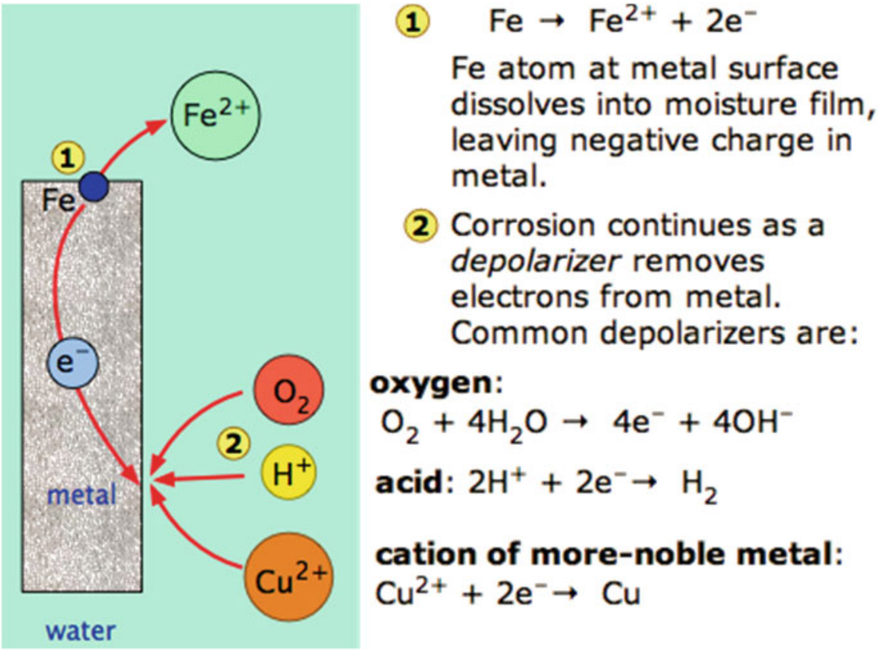

**Fig. 4.10** Electrochemical corrosion of iron. Corrosion often begins at a location (1) where the metal is under stress (at a bend or weld) or is isolated from the air (where two pieces of metal are joined or under a loosely adhering paint film.) The metal ions dissolve in the moisture film and the electrons migrate to another location (2) where they are taken up by a depolarizer. Oxygen is the most common depolarizer; the resulting hydroxide ions react with the $Fe^{2+}$ to form the mixture of hydrous iron oxides known as rust. (CC BY 3.0 Unported; Stephen Lower) https://chem.libretexts.org/Bookshelves/General_Chemistry/Book%3A_Chem1_(Lower)/16%3A_Electrochemistry/16.08%3A_Electrochemical_Corrosion

### 4.8.2 Biomineralization: Microbiologically Influenced Corrosion (MIC)

Biomineralization refers to biologically induced mineralization in which an organism modifies its local microenvironment creating conditions such that there is chemical precipitation of mineral phases extracellularly.

Microbiologically influenced corrosion (MIC) or biocorrosion that enhances corrosion under anoxic conditions via uptake of electrons from metallic iron [Fe (0)] has been regarded as one of the major causative factors (Kato et al. 2015). Iron-corroding microorganisms were enriched from rice paddy field soil using a sulfate-free freshwater medium supplemented with Fe(0) granules as the sole electron donor under an $N_2/CO_2$ gas atmosphere. Kato et al. (2015) demonstrated acetogenesis-dependent MIC in pure cultures of acetogenic bacteria. Besides this newly isolated acetogen *Sporomusa* sp. GT1 and *S. sphaeroides* enhanced iron corrosion by generating acetate with Fe(0) granules as the sole electron donor. The enriched

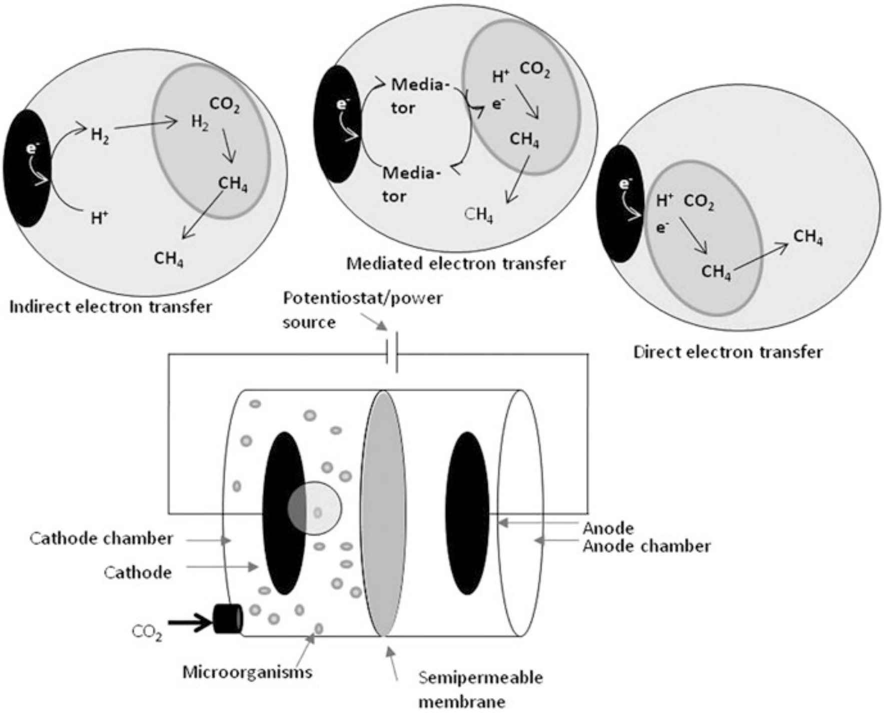

**Fig. 4.11** Extracellular electron transfer. Means of electron transfer within a separated, electromethanogenic system at the cathode: indirect electron transfer (IET), mediated electron transfer (MET), and direct electron transfer (DET). Enzmann, Mayer F., F., Rother, M. et al. Methanogens: biochemical background and biotechnological applications. AMB Expr 8, 1 (2018). https://doi.org/10.1186/s13568-017-0531-x. Reproduced under Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), Attribution 4.0 International (CC BY 4.0) which permits unrestricted use, distribution, and reproduction in any medium, provided appropriate credit is given to the original

communities with *Sporomusa* sp. and *Desulfovibrio* sp. produced larger amounts of acetate coupled with Fe(0) oxidation prior to $CH_4$ production. In addition to sulfate-reducing bacteria and methanogenic archaea in marine environments, acetogenic bacteria in freshwater environments, e.g. *Sporomusa sphaeroides* DSM2875 and *Acetobacterium woodii* DSM1030, have recently been suggested to cause MIC under anoxic conditions (Kato et al. 2015; Enzmann et al. 2018) (Fig. 4.11).

These observations demonstrated that growth of microorganisms utilizing Fe (0) as the sole electron donor occurred in the Fe(0) enrichments and that most of the reducing equivalents for the $CH_4$ generation were derived from Fe(0) oxidation (Enzmann et al. 2018).

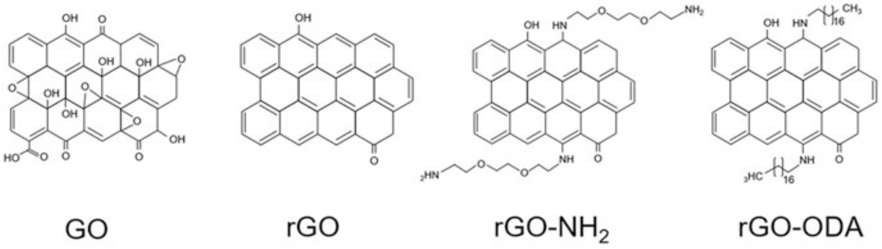

**Fig. 4.12** Graphene oxide (GO)-based materials, including GO, and reduced GO (rGO), to anaerobic microbial communities, can promote DIET. Source: Igarashi, K., Miyako, E., & Kato, S. (2020). Direct Interspecies Electron Transfer Mediated by Graphene Oxide-Based Materials. *Frontiers in Microbiology*, *10*, 3068. https://doi.org/10.3389/fmicb.2019.03068 Frontiers open access

### 4.8.3 Direct Interspecies Electron Transfer (DIET)

Conductive materials are known to promote direct interspecies electron transfer (DIET) by electrically bridging microbial cells (Igarashi et al. 2020). Previous studies have suggested that supplementation of graphene oxide (GO)-based materials, including GO, and reduced GO (rGO), to anaerobic microbial communities, can promote DIET (Igarashi et al. 2020) (Fig. 4.12).

Direct interspecies electron transfer (DIET) plays a crucial role in the anaerobic biodegradation process, in which electrons released from electron-producing microorganisms (e.g., *Geobacter* species) are transferred directly to electron-consuming microorganisms (e.g., *Methanosarcina* species) (Reguera et al. 2005; Lovley 2011; Kato et al. 2015; Kouzuma et al. 2015) DIET utilizes defined coculture system composed of electroactive microorganisms, such as *Geobacter* species and members of *Methanosarcinales methanogens* (Rotaru et al. 2014a, b). Rotaru et al. (2014a) used DIET coculture of *Geobacter metallireducens* and *Methanosarcina barkeri* to report basic characteristics of the model DIET coculture and determined that long-range electron transfer via pilin is necessary for DIET-mediated process of methane production by ethanol oxidation followed by acetate disproportion (Eqs. (4.1) and (4.2)) and DIET-mediated $CO_2$ reduction (Eq. (4.3)). Biodegradation of ethanol results in production of $CH_4$ and $CO_2$ (Eq. (4.4)).

Ethanol oxidation to acetate:

$$2C_2H_5OH + 2H_2O \rightarrow 2CH_3COOH + 8H^+ + 8e^- \tag{4.1}$$

$CH_4$ production by acetate disproportion:

$$2CH_3COOH \rightarrow 2CH_4 + 2CO_2 \tag{4.2}$$

$CH_4$ production by DIET-mediated $CO_2$ reduction:

$$CO_2 + 8H^+ + 8e^- \rightarrow CH_4 + 2H_2O \tag{4.3}$$

Overall reaction (Eqs. (4.1) + (4.2) + (4.3)):

$$2C_2H_5OH \rightarrow 3CH_4 + CO_2 \tag{4.4}$$

Igarashi et al. (2020) examined the DIET-promoting efficiency of GO on model coculture of *G. metallireducens* and *M. barkeri*. Amendment of GO induced methane production and ethanol consumption, while coculture without GO (Fig. 4.3) or monocultures of either microorganism in the presence of GO showed no methane production during the cultivation period tested. DIET brings a new opportunity to improve the efficiency of biogas technology as it may replace mediated interspecies electron transfer (MIET) by efficient electron transfer between exoelectrogens and electrotrophic methanogens, thereby enhancing yields and rates of biogas production. It is concluded that supplementation of Graphene oxide (GO) and reduced GO (rGO) to a defined DIET coculture composed of an ethanol-oxidizing electron producer *Geobacter metallireducens* and a methane-producing electron consumer *Methanosarcina barkeri* promoted methane production from ethanol (Igarashi et al. 2020). Ethanol, as the initial electron donor in the discovery of the DIET pathway, is now a “hot topic” in the literature (Feng et al. 2021).

## 4.9 Applications

The most successful application so far at the commercial scale has been anaerobic digestion, which has been widely adopted for waste treatment. Methane recovery from waste activated sludge (WAS) through anaerobic digestion is generally restricted by the poor degradability of WAS. Wu et al. (2021) suggested a novel sludge pretreatment technology by using the calcium hypochlorite ($Ca(ClO)_2$) in enhancing the methane production from WAS anaerobic digestion. In an anaerobic digester, methanogens work together with a consortium of other microorganisms to break down organic waste and produce methane-containing biogas as an energy product. Pure cultures of methanogens are capable of $H_2$ production from formate and methane production from coal. On the other side, genetically modified methanogens have been developed to produce methane from methyl esters, geraniol from $H_2$ and $CO_2$ or formate, isoprene from methanol, and acetate or lactate from methane.

According to Rother and Krzycki (2010) among the archaea, they are also the only known group synthesizing proteins containing *selenocysteine* or *pyrrolysine*. All but one of the known *archaeal pyrrolysine*-containing and all but two of the confirmed *archaeal selenocysteine*-containing proteins are involved in *methanogenesis*. Synthesis of these proteins proceeds through suppression of translational stop codons but otherwise the two systems are fundamentally different. Rother and Krzycki (2010) summarized the recent developments in selenocysteine-and pyrrolysine-related research on archaea and aimed to put this knowledge into the context of their unique energy metabolism (Fig. 4.1).

In the hydrogenotrophic pathway of methanogenesis, $CO_2$ is sequentially reduced to methane in seven steps via coenzyme-bound intermediates using $H_2$ as the electron donor (Fig. 4.1) (Rother and Krzycki 2010). If the methanogenic growth substrate is formate, it is first oxidized to $CO_2$ via (sometimes Sec-containing) formate dehydrogenase (FDH, Fig. 4.1) (Jones et al. 1979).

### 4.9.1 Sweet Sorghum as a Source of Hydrogen and Methane

Antonopoulou et al. (2008) reported exploitation of sweet sorghum biomass as a source for hydrogen and methane. They investigated fermentative hydrogen production from the sugars of sweet sorghum extract at different hydraulic retention times (HRT). The subsequent methane production from the effluent of the hydrogenogenic process and the methane potential of the remaining solids after the extraction process were assessed as well (Antonopoulou et al. 2008). They demonstrated that biohydrogen production can be very efficiently coupled with a subsequent step of methane production and that sweet sorghum could be an ideal substrate for a combined gaseous biofuel production.

### 4.9.2 Anaerobic Digestion

Kim et al. (2004) performed studies to overcome the low efficiency of anaerobic digestion of sewage sludge and food waste by combining temperature-phased digestion, sequencing batch operation, and co-digestion technology. They demonstrated that the temperature-phased anaerobic sequencing batch reactor (TPASBR) system for the co-digestion of sewage sludge and food waste resulted in enhanced volatile solid (VS) reduction and methane production rate (Kim et al. 2006). This study aimed to evaluate the performance of the unified high-rate anaerobic digestion (UHAD) system treating co-substrate of sewage sludge and food waste. They reported enhanced performance could be attributed to longer retention time of active biomass, faster hydrolysis, higher $CH_4$ conversion rate, and balanced nutrient conditions of co-substrate in the UHAD system.

### 4.9.3 *Clostridium butyricum*

Junghare et al. (2012) isolated a mesophilic alkaline tolerant fermentative microbe from estuarine sediment samples and designated as *Clostridium butyricum* TM-9A, based on 16S rRNA gene sequence. This TM-9A strain produced hydrogen efficiently from a range of pentose and hexose sugars including di-, tri-, and polysaccharides like xylose, ribose, glucose, rhamnose, galactose, fructose, mannose, sucrose, arabinose, raffinose, cellulose, cellobiose, and starch. Optimization of process parameters improved molar hydrogen yield of TM-9A strain (Junghare et al. 2012).

### 4.9.4 Reactor System

He and Osborne 2012 reported that hardened and insoluble fat, oil, and grease (FOG) deposits are the primary cause of sewer line blockages leading to sanitary sewer overflows (SSOs). They studied physicochemical characteristics of full-scale grease interceptors (GIs), the first "line of defense" against FOG buildup in sewer lines. They further assessed the physicochemical characteristics of two full-scale GIs pipes over a 1-year period. Statistically significant differences between bio-augmented and untreated cycles were detected for several chemical and physical properties. The treated cycles had lower BOD and COD at the grease interceptor outlet. Hu et al. (2018) studied effects of lipid concentration on thermophilic anaerobic co-digestion of food waste and grease waste in a siphon-driven self-agitated anaerobic reactor. High-strength lipid wastes FOG (fat, oil, and grease) normally could not be directly released to the collection system in many metropolitan areas (He and Osborne (2012)).

### 4.9.5 Biogas

Natural degradation of organic material results in the production of biogas by microorganisms under anaerobic conditions. Anaerobic digestion converts organic material into biogas, a renewable fuel that could be used to produce electricity and heat or as vehicle fuel. In recent years, anaerobic digestion (AD) of waste and residues from agriculture and industry, municipal organic waste, sewage sludge, etc. has become as one of the most attractive renewable energy pathway. Biogas typically consists of methane (50–75%) and carbon dioxide (25–50%), minor amounts of other gases, and water vapor (http://www.biogas-renewable-energy.info). Biogas is produced from complex organic material that is decomposed by microorganisms in the anaerobic digestion (AD) process. Anaerobic digestion of energy crops, residues, and wastes is of increasing interest in order to reduce the greenhouse gas emissions and to facilitate a sustainable development of energy supply (Weiland 2010; Dornelas et al. 2017).

For anaerobic digestion, two substrates have been taken into account: (1) agricultural resources and (2) municipal organic waste (Pertl et al. 2010). Einarsson and Persson (2017) reviewed potential for biogas production from crop residues and manure, accounting for key technical, biochemical, environmental, and economic constraints. Maragkaki et al. (2018) reviewed biogas production from sewage sludge by adding small amount of agro-industrial by-products and food waste residues. They investigated co-digestion of sewage sludge (SS) and food waste (FW), grape residues (GR), crude glycerol (CG), cheese whey (CW), and sheep manure (SM), in a small ratio of 5–10% (v/v). Weiland (2010)) suggested that the digestate from anaerobic fermentation is a valuable fertilizer due to the increased availability of nitrogen and the better short-term fertilization effect. Anaerobic treatment minimizes the survival of pathogens which is important for using the digested residue as fertilizer.

The various methods of biogas production can be classified in wet and dry fermentation systems. Most often applied are wet digester systems using vertical stirred tank digester with different stirrer types dependent on the origin of the feedstock. In comparison to the fossil reference system, the electricity production using biogas saves GHG emissions from 0.188 to 1.193 kg $CO_2$ eq per kWh (e) (Bacenetti et al. 2013).

According to Plugge (2017), it is important to have in-depth knowledge and understanding of the anaerobic microbiome. Anaerobic co-digestion of sewage sludge and other organic wastes at a wastewater treatment plant (WWTP) is a promising method for both energy and material recovery. Maragkaki et al. (2018) demonstrated improving biogas production from anaerobic co-digestion of sewage sludge with a thermal dried mixture of food waste, cheese whey, and olive mill wastewater.

Different physiological groups of microorganisms are involved as follows: hydrolytic bacteria, fermenting bacteria, organic acid-oxidizing bacteria, and methanogenic archaea, and these microorganisms degrade organic matter via cascades of biochemical conversions ultimately to biogas (Weiland 2010). Syntrophic relationships between hydrogen producers (acetogens) and hydrogen scavengers (homoacetogens, hydrogenotrophic methanogens) are critical to the process (Carballa et al. 2015).

The production of biogas by anaerobic digestion (AD) of agricultural residues, organic wastes, animal excrements, municipal sludge, and energy crops has a firm place in sustainable energy production and bioeconomy strategies, and biogas production has increased rapidly in many countries over the last 20 years. In biogas plants, complex polymers are hydrolyzed to sugars and amino acids, followed by fermentation and acetogenesis, and acetate, $H_2$, and $CO_2$ are produced as substrates for methanogenesis. Therefore, hydrogenotrophic and aceticlastic methanogens are prevalent in mesophilic biogas plants, often dominated by species of *Methanosarcina* (*Methanothrix* at low acetate concentrations) or *Methanoculleus* (Sundberg et al. 2013; Lucas et al. 2015). However, under certain conditions, syntrophic acetate oxidation may be the dominant path toward methane (Westerholm et al. 2016) (Fig. 4.13).

According to Theuer et al. (2020), efforts to integrate biogas plants into bioeconomy concepts will lead to an expansion of manure-based (small) biogas plants, while their operation is challenging due to critical characteristics of some types of livestock manure. Due to varying digester types, feedstocks, and process conditions, 16S rRNA gene amplicon sequencing showed differences in the taxonomic composition.

Wirth et al. (2021) suggested that biogas production through co-digestion of second- and third-generation substrates is an environmentally sustainable approach. They reported that co-digestions in anaerobic digestion experiments the combined

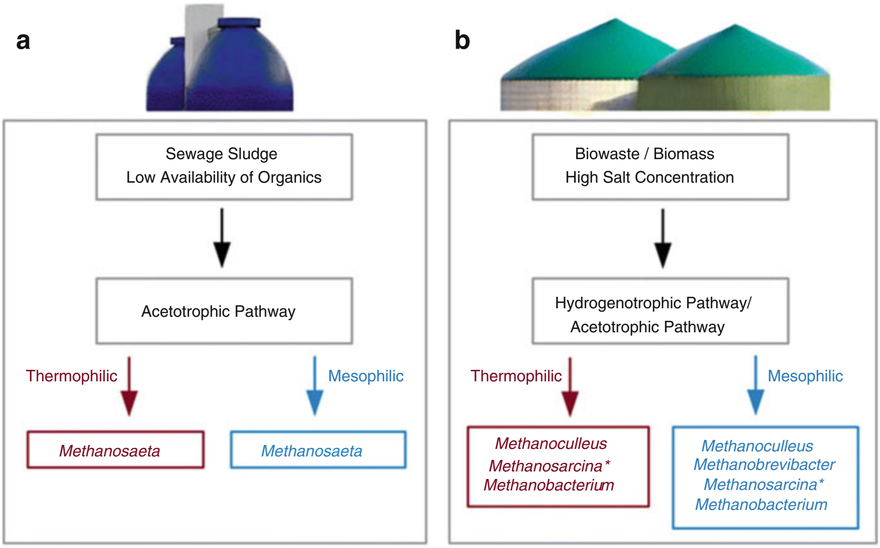

**Fig. 4.13** Metagenome, metatranscriptome, and metaproteome approaches unraveled compositions and functional relationships of microbial communities residing in biogas plants. Source: Hassa J, Maus I, Off S, et al. 2018 Metagenome, metatranscriptome, and metaproteome approaches unraveled compositions and functional relationships of microbial communities residing in biogas plants. *Appl Microbiol Biotechnol*.;102(12):5045–5063. https://doi.org/10.1007/s00253-018-8976-7. **Open Access** This article is distributed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made

substrates like green willow biomass, chicken manure waste, and microalgae biomass, the biogas yield was significantly higher as compared to the yield when energy willow was the sole substrate. Wirth et al. (2021) applied genome-centric metagenomics approach to gain functional insight into the complex anaerobic decomposing process. They enumerated the importance of *Firmicutes*, *Actinobacteria*, *Proteobacteria*, and *Bacteroidetes* phyla as major bacterial participants, while Methanomicrobia and Methanobacteria represented the archaeal constituents of the communities. The carbohydrate hydrolyzes the representatives of long-chain carbohydrate hydrolyzing microbes Bin_61: *Clostridia* is followed by action of hydrogenotrophic methanogen species *Methanoculleus* (Bin_10) and *Methanobacterium* (Bin_4). Wirth et al. (2021) reported that a sensitive balance between $H_2$ producers and consumers was shown to be critical for stable biomethane production and efficient waste biodegradation.

## 4.10 Discussion

Ma et al. (2018) suggested that anaerobic digestion, widely considered as a promising waste biomass disposal treatment approach, is attracting increasing interest in all corners of the globe. Production of biogas provides a versatile carrier of renewable energy, as methane can be used for replacement of fossil fuels in both heat and power generation and as a vehicle fuel (Weiland 2010). Messineo et al. (2019) reviewed the anaerobic digestion as important available route to recover energy from waste via production of biogas while reducing organic load and pollutants to the environment. The use of farming and agro-industrial wastes as co-substrate in anaerobic digestion can induce benefits related to the simultaneous treatment of different wastes. This co-digestion can significantly enhance the process stability as well as the biomethane generation.

The Conference on Sustainable Development of Energy, Water and Environment Systems (SDEWES) conferences of 2021 debated the issue of state of the art and future directions and priorities in the various areas of sustainable development. One of the important areas of sustainable development includes adoption of renewable energy sources by integrating heating, transport, industry, buildings, water, cooling, electricity, waste, wastewater, forestry, and agriculture systems (Østergaard et al. 2021). Wang et al. (2021a, b) discussed bioenergy potential from manure-generated biogas projects and the bottlenecks of AD technology. According to them, reducing energy input in AD projects and enhancing the efficiency of methanogenesis of livestock manure are key factors for achieving a high net output of biogas projects.

Thauer et al. (2008) suggested that although methanogenic archaea can reduce $CO_2$ with $H_2$ to methane, this does not take into account the fact that methanogens with cytochromes have considerably higher growth yields and threshold concentrations for $H_2$ than methanogens without cytochromes. These and other differences have been explained by Thauer et al. (2008) in his review. The methanogens with cytochromes, the first and last steps in methanogenesis from $CO_2$, are coupled chemiosmotically, whereas in methanogens without cytochromes, these steps are energetically coupled by a cytoplasmic enzyme complex that mediates flavin-based electron bifurcation (Thauer et al. 2008). Wang et al. (2011)) reported that overwhelming majority of methanogens capable of growth via conversion of the methyl group of acetate to methane do not metabolize $H_2$ suggesting they employ an electron transport pathway distinct from that proposed for the few acetotrophic methanogens in which $H_2$ is an obligatory intermediate, e.g., *M. acetivorans* which is acetotrophic methanogen incapable of metabolizing $H_2$.

Söllinger and Urich (2019) reported that Methanomassiliicoccales occur in a large variety of anoxic habitats including wetlands and animal intestinal tracts of ruminant animals, i.e., in the major natural and anthropogenic sources of methane emissions, respectively. Considering the role of methane as potent greenhouse gas, resolving the methanogenic nature of a broad range of putative novel methylotrophic methanogens and assessing their role in methane emitting environments are pressing issues for future research on methanogens (Ferry 1999; Kumar et al. 2018a, b, 2020).

Biogas production from sewage sludge volatile solids (VS) by anaerobic digestion slows down toward the end of the process, among inhibitory factors being pH increase upon ammonia accumulation, poorly digestible biomaterials, and high fixed solid (FS) content (Kouzi et al. 2020). According to Kouzi et al. (2020), the risk of hazardous compounds limits the utilization of sewage treatment plant sludge, biogas production being one of the most common applications (Chen et al. 2014).

However, the bioenergy conversion efficiency of this process is not ideal. They further suggested that another problematic aspect of anaerobic digestion is the nutrient-rich effluent which needs to be treated before discharge. According to Liu et al. (2018), the biological processes for treating municipal wastewater have been developed based on the philosophy of biological oxidation with high energy consumption and generation of waste sludge. Thus, the energy self-sufficient biological reclamation of municipal wastewater needs to be addressed urgently.

## 4.11 Conclusion

Biogenic methane is a significant greenhouse gas, and the conversion of organic wastes to methane is an important bioenergy process. *Methanosarcina* species play an important role in methane production in many methanogenic soils and sediments as well as anaerobic waste digesters. Extracellular electron exchange in *Methanosarcina* species and closely related Archaea plays an important role in the global carbon cycle and enhances the speed and stability of anaerobic digestion by facilitating efficient syntrophic interactions. The discovery of a methanogen that can conserve energy to support growth solely from the oxidation of organic carbon coupled to the reduction of an extracellular electron acceptor expands the possible environments in which methanogens might thrive. Progress has been made in the past few decades to identify key microorganisms influencing AD. Yet, more work is required to realize robust, quantitative relationships between microbial community structure and functions such as methane production rate and resilience after perturbations. Venkiteshwaran et al. (2015) suggested other promising areas of research for improved AD may include methods to increase/control (1) hydrolysis rate, (2) direct interspecies electron transfer to methanogens, (3) community structure-function relationships of methanogens, (4) methanogenesis via acetate oxidation, and (5) bioaugmentation to study community-activity relationships or improve engineered bioprocesses. For developing sustainable climate change mitigation technology, it is essential to develop better reactors for high yield of fuel production through methanogenesis with controlled conditions. RE technology policies need to be in place to ensure that the adoption of environmentally sustainable biogas energy production becomes popular all over the world. Combined heat and power (CHP) unit is a very sustainable energy source which can help us combat global warming and related climate change issues. The actual targets can be achieved by infrastructural growth and systemic perspectives.

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# Mitigation of Microbially Influenced Corrosion of Concrete Sewers Using Nitrite

# 5

Xuan Li and Guangming Jiang

## Abstract

Sewer systems are essential compartments of urban life, preventing human exposure to unhygienic sewage and sewage-borne diseases. However, microbially influenced concrete corrosion (MICC) occurs in sewers globally and ultimately leads to severe structural deterioration and early failure of those concrete structures. The MICC in sewers is mainly caused by hydrogen sulfide ($H_2S$) that occurs in the headspace gas. In sewers, $H_2S$ is produced due to the metabolism of sulfate-reducing bacteria (SRB) under anaerobic conditions in parts of sewers and is subsequently released into headspace sewer gas in the gravity sections of the sewer system. In gravity sewers, $H_2S$ in the headspace gas is further oxidized into sulfuric acid by sulfide-oxidizing microorganisms (SOMs) on concrete surfaces, leading to concrete corrosion. The remediation and replacement of corroding concrete sewers could cost billions of dollars annually, which becomes a huge financial burden worldwide. In recent years, advanced understanding of inhibitory and biocidal effects of nitrite or free nitrous acid ($HNO_2$, FNA), the protonated form of nitrite, on microorganisms, has led to the development of a series of nitrite-based applications in the bioremediation of sewer corrosion. These applications target at (1) reducing $H_2S$ production in anaerobic sewers, (2) mitigating the corrosion development of existing corroding sewers, and (3) implementing new concrete with nitrite admixture for long-term corrosion control with minimum maintenance. This chapter provides a comprehensive summary of the current status of nitrite-based applications in the remediation and control of concrete sewer corrosion.

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**Keywords**

Sewer · Concrete · Corrosion · Nitrite · Free nitrous acid

## 5.1 Introduction

As one of the most critical components of the urban infrastructure in modern societies, sewer networks collect and transport sewage to treatment plants, preventing human exposure to unhygienic sewage and related sewage-borne diseases. To ensure the durability and reliability of sewer infrastructures, concrete is widely used in the construction due to its high compressive strength (Söylev and Richardson 2008). Typically, concrete structures in sewers are designed to perform for 50–100 years with minimal maintenance (Monteiro and Kurtis 2003). However, severe structural deterioration and early failure of sewers due to corrosion occur worldwide, which shortens the expectancy of sewer service life by more than 50% (Monteiro and Kurtis 2003). The remediation cost for sewer corrosion is estimated to reach over $390 billion within the next 20 years in the USA (Gutiérrez-Padilla et al. 2010). Furthermore, sewer networks can be considered as a reactor where transformations of a broad range of chemicals occur through chemical and biological reactions. Some products from in-sewer chemical or biological processes, such as hydrogen sulfide ($H_2S$) and volatile organic sulfur compounds, are malodorous and harmful to human health. Concrete corrosion in sewers thereby increasingly triggers high economic expenses, as well as severe health and environmental concerns (Grengg et al. 2018).

Concrete corrosion is a complex process with various chemical and biological reactions between concrete and sewer gases (i.e., $CO_2$ and $H_2S$) in gravity sewers (Jiang et al. 2015). Hydrogen sulfide ($H_2S$), in particular, is ubiquitous in gravity sections of sewers. Through chemical and/or biological reactions, $H_2S$ consumes the alkalinity of concrete pipes, producing loose corrosion products such as gypsum and ettringite that have poor structural capacity. The corrosion process leads to weakened structure and eventual collapse of the concrete (Li et al. 2017). Since the biological oxidation rate is much higher than the chemical oxidation rate, microbially influenced concrete corrosion (MICC) is regarded as the main cause of sewer concrete deterioration (Hvitved-Jacobsen et al. 2013).

Nitrite has been recently applied in the wastewater field as an inhibitor or biocidal agent for sewer corrosion control and bioremediation. Promising results have been achieved with the application of nitrite at different phases of sewers. This chapter provides an overview of the sewer system, and concrete corrosion process, and summarizes the current status of nitrite-based applications in the remediation and control of concrete sewer corrosion.

## 5.2 Sewer System and Concrete Corrosion

### 5.2.1 Sewer System

#### 5.2.1.1 Overview of the Sewer System

The sewer system is mainly designed for wastewater collection and transportation among various wastewater infrastructures (Hvitved-Jacobsen et al. 2013). The applications of sewer systems prevent human exposure to unhygienic sewage and sewage-borne disease. The ancient sewers were built using brick and mortar. However, in modern society, concrete is considered a more cost-effective material and has been applied predominantly for constructing sewer pipes (Söylev and Richardson 2008). The total length of sewers in Australia is almost 117,000 km, and approximately 40% of this network is constructed of concrete (Cayford 2012). Based on the wastewater flow conditions, sewers can be further divided into rising mains and gravity flow regions (Fig. 5.1). The rising main sections are operated with no gas phase, pumping the wastewater to a higher altitude. Gravity regions are usually partially filled with wastewater and wastewater flows downstream due to gravity (Fig. 5.1). Buried deep beneath the ground, sewer pipes ideally serve for 50–100 years with minimal maintenance considering the property of concrete itself (Monteiro and Kurtis 2003).

With the broad range of pollutants and the high microbial presence in wastewater, sewers function as "microbial reactors" where substances are transformed and degraded through chemical and biological processes (Hvitved-Jacobsen et al. 2013). Sewer system contains five main components: wastewater, biofilms, sewer gas in gravity sewers, sewer sediments, and concrete sewer walls (Fig. 5.1). The concrete walls allow the development of biofilms and sediments, where most of the microbial processes occur. Wastewater and sewer air mainly provide the energy/nutrient source for these microbial processes (Hvitved-Jacobsen et al. 2013). In

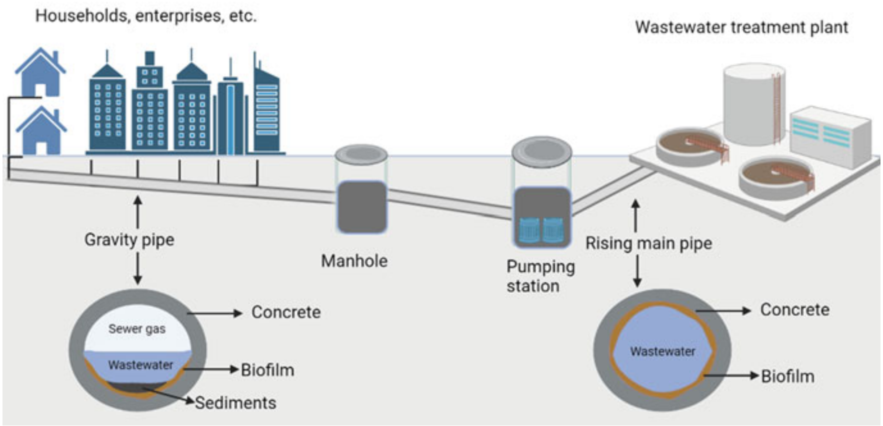

**Fig. 5.1** An overview of the sewer system

sewers under anaerobic conditions, the $H_2S$ and methane generations cause some main health concerns for sewer networks (Jiang et al. 2010; Lin et al. 2015). In partially filled gravity sewers, with the existence of both sewer air and wastewater, the microbial process can be complex. Due to the limitation of oxygen diffusion in anaerobic parts of the gravity sewer, sulfate may remain the main electron acceptor (Hvitved-Jacobsen et al. 2013). However, in aerobic parts, oxygen from the sewer air becomes the main electron acceptor, oxidizing $H_2S$ in the sewer air (Okabe et al. 2007).

#### 5.2.1.2 Sulfide in Sewers

Within the sewer system, the sulfur cycle is one of the most complex nutrient cycles. Various sulfur compounds exist and can be transformed into many oxidation states in wastewater through both biotic and abiotic processes (Suzuki 1999). Sulfate and sulfide are the most dominant species in wastewater with elemental sulfur present only as an intermediary or a product of highly localized conditions (Hvitved-Jacobsen et al. 2002). The concentration of dissolved sulfate is typically 20–200 mg/L in wastewater (Zhang et al. 2008). Underneath the wastewater, porous concrete pipes provide the habit for microorganisms (Hvitved-Jacobsen et al. 2002; Zhang et al. 2008). Due to the depletion of oxygen, the submerged areas become thermodynamically favorable for sulfate-reducing bacteria (SRB) to use sulfate as an electron acceptor for growth (Eq. (5.1)). Some SRB can also grow with sulfite or thiosulfate as electron acceptor (Hao et al. 1996). Hydrogen sulfide is thus produced by SRB, and this production is facilitated by high sulfate concentrations, low dissolved oxygen (DO), low flows, and long residence times (Ling 2013). The critical DO for sulfide production is below 0.5 mg/L; otherwise, SRB activity could be inhibited and sulfide can be chemically oxidized (Hao et al. 1996; Hvitved-Jacobsen et al. 2002).

$$SO_4{}^{2-} + 2C(\text{organic matter}) + 2H_2O \leftrightarrow 2HCO_3{}^{-} + H_2S(aq) \tag{5.1}$$

The buildup and accumulation of sulfide in wastewater would finally lead to its emission into the sewer atmosphere in gravity sections of sewers (i.e., gravity pipes, wet wells, and manholes). Among the sulfide species, hydrogen sulfide is the only species that can partition into the sewer gas (Ling 2013). The partition process of $H_2S$ is pH-dependent. The first and second dissociation of sulfide in aqueous solutions are described as Eq. (5.2) and Eq. (5.3), with $pK_{a1}$ value of 7 and $pK_{a2}$ ranging from 11.96 to 17.00 (Lens and Pol 2000). Lower wastewater pH could lead to more hydrogen sulfide emission (Hvitved-Jacobsen et al. 2002).

$$H_2S(aq) \leftrightarrow HS^- + H^+ \tag{5.2}$$

$$HS^- \leftrightarrow S^{2-} + H^+ \tag{5.3}$$

Aside from the sewage pH, other factors, such as higher sewage temperature and high turbulence intensity, can also promote the hydrogen sulfide emission from wastewater (Carrera et al. 2015). Hydrogen sulfide is a highly toxic, irritant, and flammable gas with a characteristic smell of "rotten eggs" and is one of the main compounds of the odor in sewers (Jiang et al. 2017). Although there are volatile organic compound (VOC) emissions from the wastewater to the sewer gas, the concentrations are actually low at ppb to ppm levels or even less (Wu et al. 2006; Chan and Hanaeus 2006; Huang et al. 2012). The smell of sewer gas is mainly caused by $H_2S$, which comes with serious Occupational Health and Safety (OH&S) concerns to sewer workers and odor complaints from the community (Jiang et al. 2015). More importantly, the emission of $H_2S$ also leads to the corrosion in gravity sewers, which significantly shortens the service life of sewer pipes (Jiang et al. 2016).

## 5.2.2 Concrete Corrosion in Sewers

### 5.2.2.1 Overview of Sewer Concrete Corrosion

$H_2S$ in the gas phase is the precursor to corrosion in sewers. As discussed above, $H_2S$ is generated in anaerobic sections of sewer pipe due to the metabolism of SRB and subsequently released into the headspace in gravity pipes, manholes, and pumping stations (Parker 1945; Jiang et al. 2015). This acidic gas could react with alkaline concrete pipes ($pH > 13$ for new concrete) and initiates the corrosion. The corrosion process initiates when the intact concrete with high pH (ca. 13) reacts with $H_2S$ and $CO_2$ directly, lowering the pH to ca. 9 (Hvitved-Jacobsen 2001). Then sulfide-oxidizing microorganisms (SOMs) colonize on the concrete surface and further reduce the pH of concrete to below 3 (Sun et al. 2015). Once SOMs are well developed on concrete surfaces, MICC predominately contributes to the corrosion of concrete sewers (Li et al. 2020a). Depending on the surface pH, various SOMs have been detected in the corrosion layer. With pH ranging from 5 to 9, *Thiothrix* spp., *Thiobacillus plumbophilus* (formally as *Sulfuriferula plumbophilus*), *Thiomonas* sp., and *Halothiobacillus neapolitanus* have been detected (Okabe et al. 2007; Santo Domingo et al. 2011). *Thiothrix* spp. and *Thiomonas* spp. have the ability to grow chemoorganoheterotrophically or chemolithoautotrophically using either organic carbon or the inorganic sulfur species $H_2S$, $S_2O_3^{2-}$, and $S^0$ as electron donors. In comparison, *Halothiobacillus* sp. are obligate chemolithoautotrophic sulfur-oxidizing bacteria (Li et al. 2017). With pH lower than 5, acidophilic SOMs dominate the concrete surface, including *Acidithiobacillus* spp., *Acidiphilium* spp., *Mycobacterium* spp., and *Ferroplasma* spp. (Li et al. 2017). The sulfuric acid generated by SOMs reacts with the alkaline components of concrete, leading to the mass loss of concrete (Hvitved-Jacobsen 2001) (Fig. 5.2).

Both abiotic and biotic corrosion processes heavily depend on the water content of the concrete (Joseph et al. 2012; Ward et al. 2011; Wells et al. 2012). Within the sewer, concrete corrosion mostly occurs in the regions just above the sewage flow level and at the ceiling of the headspace, respectively, being referred to as the "tidal region" and the "crown region" (Fig. 5.2). The tidal regions are areas where the concrete of the sewer is frequently wetted by wastewater, while the crown regions

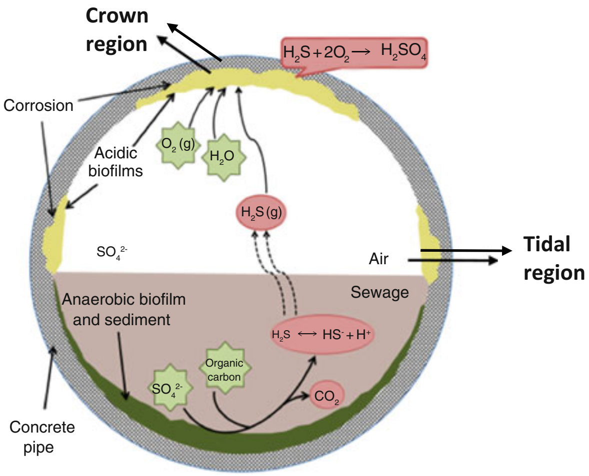

**Fig. 5.2** A diagram of a cross section of a gravity concrete sewer pipe showing the major transformations occurring that lead to the acid formation in the upper aerobic biofilms and the onset of sewer corrosion, adapted from Li et al. (2017)

are exposed to the headspace of sewer gas and only very occasionally (during flooding) are in contact with the wastewater (Fig. 5.2). For crown regions, with high relative humidity (RH) (60–100%) in the sewer gas, moisture will condensate on the crown region, as the walls of sewer pipe usually have a lower temperature than the sewer atmosphere due to cooling from the surrounding soil (Joseph et al. 2012, Ward et al. 2011, Wells et al. 2012). In the tidal regions, sewer walls are frequently wetted by wastewater flooding, which will increase the water content of the concrete in those regions.

#### 5.2.2.2 Corrosion Layer Conditions

Once the corrosion is well developed, on the corroding concrete surface, a soft (cottage cheese like) moist layer forms comprising largely of crystalline gypsum and other sulfur species (Cayford 2012). The microbial biofilms exist within the corrosion layer together with corrosion products (Fig. 5.3). Due to the paucity of nutrients innately present in concrete, the development of microorganisms mainly relies on the sewer gases and wastewater. The diffusion of gases (i.e., $CO_2$, $H_2S$) provides volatile substrates and nutrients for the development of SOM (Jiang et al. 2014). And some exudates from microorganisms' metabolization and wastewater flooding (especially in tidal regions) can also be used as nutrients for the biofilms in corrosion layers (Nica et al. 2000).

The microbial biofilm is dense near the surface of the corrosion layer where the diffused oxygen and $H_2S$ levels are high. Sulfuric acid is generated by SOM in the biofilm, and this diffuses through the corrosion layer toward the surface of the intact concrete (Okabe et al. 2007). With the acid diffusion, the gypsum ($pH < 3$) and ettringite zones ($pH > 3$) are formed and as they are expansive, together with iron mineral precipitation, cause cracking of the intact concrete (Fig. 5.3).

Corrosion significantly reduces the service life of sewers, which ultimately leads to severe structural deterioration and early failure of sewers. Since the pipes typically

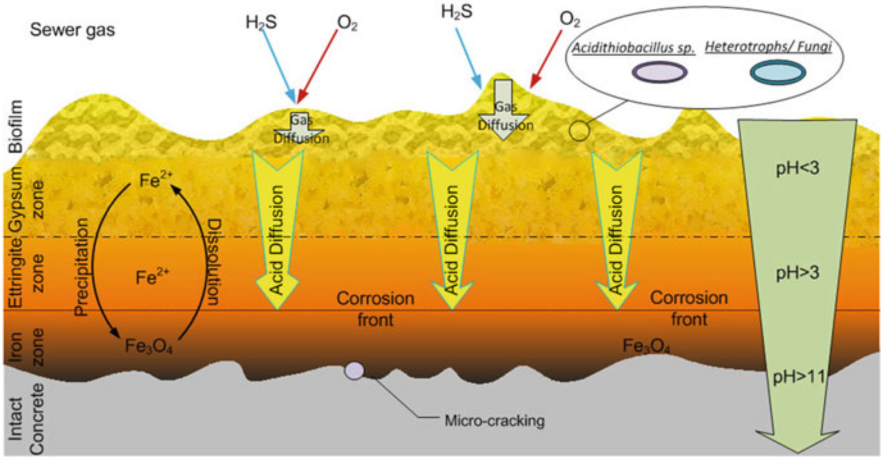

**Fig. 5.3** A conceptual model for the sewer concrete corrosion layers (Li et al. 2017)

run below streets or developed property, sewer replacement and rehabilitation are difficult and cause a huge financial expenditure (Pikaar et al. 2014). Annual rehabilitation costs were estimated to reach over €450 million in Germany, £85 million in the UK, and over $12 billion in the USA, respectively (Grengg et al. 2018). As occurrences and severity of corrosion are exacerbated in the coming years, much more expenditure is expected for the rehabilitation and control of concrete corrosion, which is a significant financial pressure on wastewater utilities and associated governmental agencies.

## 5.3 Applications of Nitrite in Sewer Systems

### 5.3.1 Reducing $H_2S$ Production in Anaerobic Sewers

The antimicrobial effect of nitrite has been known for decades in the food industry. In the last decade, increasing evidence has shown that nitrite is inhibitory to the metabolism of a variety of key microorganisms in the wastewater system (Duan et al. 2020). In particular, strong biocidal or inhibitory effects have been observed with sewer biofilm, including critical micrograms for $H_2S$ production (i.e., SRB). The sulfide control effect of nitrite was firstly tested through laboratory studies, where a continuous addition of nitrite at 20–140 mg-N/L for 25 days reduced the sulfide production by >90% (Table 5.1). The sulfide production ratio gradually reached the pre-addition condition after 2.5 months, suggesting a long-lasting effect of nitrite (Mohanakrishnan et al. 2008). Following this, the dependency of the inhibitory effect on nitrite levels and the exposure time was investigated with a range of nitrite concentrations (40, 80, 120 mg-N/L) and exposure time up to 24 days (Jiang et al. 2010). With continuous dosing of 80–120 mg-N/L of nitrite for 24 days, the sulfide

**Table 5.1** A summary of dosage and results of nitrite or FNA dosing in reducing sulfide production in sewers

| Chemicals | Condition | Dosage | Frequency | Reduction ratio | Recovery | Reference |
|---|---|---|---|---|---|---|
| Nitrite | Laboratory | 20–140 mg-N/L | Continuous dosing for 25 days | >90% | 2.5 month | Mohanakrishnan et al. (2008) |
| | Laboratory | 40 mg-N/L | Continuous dosing for 24 days | About 80% | About 60 days | Jiang et al. (2010) |
| | | 80, 120 mg-N/L | | >95% | | |
| | Real sewer | 100 mg-N/L | Continuous dosing for 3 days | >90% | 3 weeks | |
| FNA | Laboratory | 0.26 mg-N/L | 12 h/5 days | >80% | N/A[a] | Jiang et al. (2011a) |
| | Laboratory | 1.54 mg-N·h/L | 6–8 h/week | 88%–99% | N/A | Nguyen and Marano (2016) |
| | Real sewer | 0.26 mg-N/L | 8–24 h/10 days | >80% | N/A | Jiang et al. (2013) |
| FNA + $H_2O_2$ | | 0.26 mgN/L FNA with 60 mg/L $H_2O_2$ | 8–24 h/10 days | >80% | N/A | |

*N/A* not applicable

production activity was inhibited by more than 95%, and the recovery took about 60 days to reach the pre-addition condition (Jiang et al. 2010). This lasting effectiveness was believed to be due to the decreased or suppressed growth of SRB in biofilms during the dosing period, and the recovery of these populations required 1–2 months (Jiang et al. 2010). However, a field investigation was carried with much shorter dosing period, where similar reduction of SRB activity was achieved in a real sewer at the city of Gold Coast, Australia (Table 5.1). 100 mg-N/L of nitrite was dosed continuously for 33 hours, where the sulfide production was completely suppressed during dosing and the recovery took about 3 weeks (Jiang et al. 2010). This relatively short dosing period suggests that the lasting sulfide control effect was likely due to a biocidal rather than an inhibitory effect.

A follow-up study further investigated the lasting sulfide control effects achieved by a short-term dosing of nitrite under different pH and nitrite concentrations. In this study, the viable fraction of microorganisms in the anaerobic sewer biofilm reduced by 65–75% after 6–24-h treatment with 0–120 mg-N/L nitrite under pH levels of 5–7, suggesting a strong biocidal effect achieved by nitrite (Jiang et al. 2011b) (Table 5.1). More importantly, the level of the biocidal effect showed a much stronger correlation with the free nitrous acid (FNA) concentration, which is well described by an exponential function, than with the nitrite concentration or with the pH level, suggesting that FNA is the actual biocidal agent (Jiang et al. 2011b). FNA ($HNO_2$) is the protonated form of nitrite, of which the concentration is determined by the nitrite concentration and pH as Eq. (5.4).

$$\text{FNA as HNO}_2\text{-N} = \text{NO}_2{}^{-}\text{-N}/\left(\text{Ka} \times 10^{\text{pH}}\right) \tag{5.4}$$

where Ka is the ionization constant of the nitrous acid, $\text{Ka} = \text{e}^{-\frac{2300}{\left(270+T\left(^{\circ}\text{C}\right)\right)}}$.

This is a significant finding as it implies that the level of FNA can be controlled by adjusting either the nitrite concentration or the pH. It is important to note that, for the same nitrite concentration, the FNA concentration would increase by ten times when pH is lowered by one unit (Anthonisen et al. 1976). The strong biocidal effect of FNA leads to the possibility of intermittent dosing of FNA as a measure for controlling sulfide in sewers. For a successful application of intermittent dosing, three parameters, i.e., dosing concentration, exposure time (dosing duration), and the dosing interval (repetition frequency), are critical. A comprehensive evaluation of FNA concentration ranging from 0.18 to 0.36 mg-N/L, exposure time of 12–96 h, and a dosing interval of 4, 8, and 12 days was conducted under laboratory conditions (Jiang et al. 2011a). It was demonstrated that FNA as low as 0.26 mg-N/L was able to suppress sulfide production after an exposure of 12 h, and with a dosing interval of 5 days. The average sulfide production under such a dosing scheme was reduced by >80% (Table 5.1). This study demonstrated the feasibility and effectiveness of interment dosing, which is more economically applicable. Furthermore, the inactivation kinetic parameters of sewer biofilm by FNA were evaluated with a 2-fraction Bayesian model (Jiang and Yuan 2014). The study determined the most cost-effective dosage (FNA concentration times the exposure time) as 1.4 mg-N·h/L for the 80% control efficiency of hydrogen sulfide (Table 5.1).

The long-term effectiveness of FNA dosing for control of sulfide production was further tested in a full-scale rising main sewer located in the city of Gold Coast, Australia, for 6 months (Jiang et al. 2013). One single dose of FNA or FNA + $H_2O_2$ for 8–24 h reduced the sulfide production by 80% for 10 days, confirming the effectiveness of intermittent dosing of FNA or FNA + $H_2O_2$ (Table 5.1). Furthermore, no biofilm adaptation or resistance to FNA was observed during the 6-month trial. Instead, successive dosing achieved better control efficiency which was thought to be due to repetitive weakening of the biofilms, suggesting an additional benefit for a long-term application (Jiang and Yuan 2013).

Besides nitrite or FNA, different types of chemicals have been applied in sewers to prevent sulfide production, including oxygen, nitrate, ferric/ferrous salts, and magnesium hydroxide. Oxygen and nitrate have been added into sewers to oxidize sulfide chemically and/or biologically to reduce the sulfide concentration (Zhang et al. 2008). Ferric/ferrous salt dosing targets at precipitating with sulfide to insoluble metal sulfide, and ferric salts also inhibit sulfide production by sewer biofilms (Jiang et al. 2015). Magnesium hydroxide ($Mg(OH)_2$) elevates wastewater pH, typically to 8.5–9.0, and thus reduces the transfer of hydrogen sulfide from water to the sewer air as well as sulfide productions (Gutierrez et al. 2009). However, all these chemicals require continuous dosing to achieve effective control, resulting in large chemical consumption and cost. The general cost of chemical dosing using these chemicals is estimated to be 0.04–0.18 $\$/m^3$-wastewater in sewers. In comparison to these chemicals, the cost of intermittent FNA or FNA + $H_2O_2$ dosing in sewers is much less at 0.01–0.03 $\$/m^3$, confirming the intermittent FNA/nitrite dosing as a cost-effective and environmentally friendly strategy for sulfide control in sewers (Duan et al. 2020).

### 5.3.2 Mitigating the Corrosion Development of Existing Corroding Sewers

The bactericidal effect achieved by FNA (formed by nitrite under acidic conditions) on sewer biofilms provides a possibility of controlling the MICC development in corroding sewers. Once MICC is well established on concrete surfaces, the corrosion layer is under acidic conditions with a pH lower than 3 (Sect. 5.2). This acid corrosion surface provides a perfect condition for the formation of FNA. A recent laboratory investigation sprayed sodium nitrite solution on corroding concrete surfaces to achieve a surface FNA concentration of 100–1000 ppm (Table 5.2). After the spray, the $H_2S$ uptake rate (SUR) of coupons reduced by 84–92%, with no obvious recovery for up to 12 months, suggesting a long-lasting effect in inhibiting the corrosion development (Sun et al. 2015). This was attributed to the biocidal effect of the FNA (free nitrous acid, the protonated form of nitrite ($HNO_2$)), which formed on the acid corrosion surface by the nitrite solution. The biocidal effect was also evident by the sharp decrease of viable cells and ATP level by 75% and more than 98%, respectively (Table 5.2).

**Table 5.2** A summary of results for nitrite application on corroding concrete

| Chemicals | Conditions | Dosage | Mitigation effect | Recovery | Reference |
|---|---|---|---|---|---|
| Nitrite | Corroding concrete surface in laboratory corrosion conditions | FNA concentration of 100–1000 ppm | SUR reduced by 84–92% | No recovery for up to 12 months | Sun et al. (2015) |
| Nitrite | A solution containing scraped corrosion layers ($pH < 3$) | 15.2 mg $HNO_2$-N/L | Viable cells decreased by 75%, ATP reduced by >98% | No recovery for up to 700 h | |
| Nitrite | Corroding concrete surface exposed in a real sewer manhole | FNA concentration of 100–1000 ppm | SUR and sulfate concentration in the corrosion layer reduced by 30%, 6 months after the spray | 15 months after the spray | Li et al. (2022) |

In comparison to the laboratory conditions (i.e., controlled $H_2S$ concentration, temperature, and relative humidity), environmental conditions in real sewers are generally more dynamic. To further validate the effectiveness of spraying nitrite on corrosion control of concrete, a follow-up study was conducted in a real sewer manhole at the city of Gold Coast, Australia (Li et al. 2022). Two types of concrete were exposed to the corrosive conditions within a sewer manhole for 21 months, while nitrite spray was conducted at the 6th month of the exposure. Due to the nitrite spray, both the sulfide uptake rate (SUR) of coupons and sulfate content in the corrosion layer reduced by about 30%, 6 months after the spray, demonstrating the corrosion mitigation effect of nitrite. However, the reduction of SUR and sulfate content in the corrosion layers became <10%, 15 months after the spray (Li et al. 2022). The application of nitrite on coupons inside the real sewers showed a faster recovery of SOMs activity compared to the laboratory. This could be attributed to the dynamic sewer conditions with a high range of fluctuation of $H_2S$ concentration and temperature, and also the potential wash-off of nitrite on the corrosion surface. Thus, an annual application of nitrite on corroding surface was recommended.

Besides nitrite spray, to mitigate the MICC of corroding sewers, physical and chemical treatments have been applied by (1) reducing the biological sulfide-oxidizing activity through removing the corrosion layer by high-pressure washing (Nielsen et al. 2008), (2) increasing the surface pH and deactivating the sulfide-oxidizing bacteria by application of magnesium hydroxide (Sydney et al. 1996), and (3) surfacing coating using silver bearing zeolite, epoxy, or polymer fiber (Berndt 2011). The surface washing effect was found to be temporary with a quick recovery of SOMs activity within 30–40 days after surface washing in a real sewer (Nielsen et al. 2008). The application of magnesium hydroxide and surface coating both require a surface preparation before the application, which raises operational difficulties. In contrast to these techniques, nitrite spray requires no surface preparation before application, and the environmental and health concerns are limited as nitrite is regularly used as a substrate for denitrifying organisms in wastewater systems (Li et al. 2020a).

### 5.3.3 Increasing the Corrosion Resistance of Nitrite Admixed Concrete

The inhibitory effect of nitrite on SOMs was demonstrated by spraying a nitrite solution on corroding concrete surfaces (Sect. 3.2). However, this approach requires a continuous reapplication of nitrite on concrete due to the recovery of SOMs activity. Thus, nitrite admixed concrete, which contains calcium nitrite as an admixture during casting, was proposed to achieve a long-lasting effect with minimum maintenance. In a real sewer manhole, the corrosion loss of nitrite admixed concrete was reduced by 30% constantly for 18 months, compared to that of concrete without the nitrite admixture (Li et al. 2020b). The sulfide uptake activity was also reduced by 30%, confirming an inhibitory effect of nitrite on SOMs. Next-generation sequencing (NGS) further revealed a negative correlation between the calcium nitrite admixture in concrete and the abundance of SOMs. This field study for the first time demonstrated that using nitrite admixed concrete is a promising strategy to mitigate the MICC in sewers.

On nitrite admixed concrete, surface washing showed a synergistic mitigation effect on MICC control (Li et al. 2021). Conventionally, surface washing was considered as a temporary mitigation approach on Ordinary Portland Cement (OPC) concrete where the sulfide-oxidizing activity reached the pre-washing level in 30–40 days and 60–140 days after surface washing in a real sewer and laboratory chambers, respectively (Nielsen et al. 2008; Sun et al. 2016). This is majorly attributed to the quick recovery/re-establishment of SOMs on OPC concrete surfaces (Nielsen et al. 2008, Sun et al. 2016). However, on nitrite admixed concrete, the synergetic effect of calcium nitrite admixture in concrete and one single wash could lead to about 45 and 58% reduction in corrosion loss for concrete with 1 and 4% of calcium nitrite admixture, respectively, in a real sewer manhole for 10 months (Li et al. 2021). This combined use of nitrite admixed concrete and surface washing further provide a long-lasting effect on MICC mitigation.

To provide long-lasting corrosion mitigation, various techniques have been applied to increase the corrosion resistance of concrete. Water to cementitious materials ratio (w/c) is the primary factor that controls the properties of concrete (Gutiérrez-Padilla et al. 2010). Generally, decreases in the water/cement ratio of concrete mixtures lead to improvement of the mechanical properties and durability of concrete (Roy et al. 2001). Chemical acid immersion tests showed that concrete with lower w/c had lower corrosion loss (Mehta 1986). However, in some studies, concrete with a high w/c ratio (up to 50%) also yielded less mass loss after immersion in sulfuric acid (Hewayde et al. 2007). Changing concrete mix design through the addition of admixtures, alternate binders or alternate aggregate can also chemically improve corrosion resistance. Current admixtures mainly focus on changing the physiochemical properties of concrete, such as adding polymers to reduce the permeability of concrete (Vincke et al. 2002). Supplementary cementitious materials (SCMs), including silica fume (SF) and fly ash (FA), have been applied commercially to improve the mechanical properties and sustainability of concrete. SF, the by-product of ferrosilicon alloy production, is an ultrafine powder consisting of spherical silicon dioxide ($SiO_2$) particles. FA, generated by coal combustion, is another fine-particle residue that includes silicon dioxide ($SiO_2$) and calcium oxide (CaO). With the fine particles and the pozzolanic characteristic, SF and FA reduce the porosity of concrete which results in denser concrete microstructure, higher structure performance, and reduced sulfuric acid penetration into the core of the concrete (Roy et al. 2001). Replacing 30 and 5% of cement with FA and SF resulted in good resistance to $Na_2SO_4$ solution (Roy et al. 2001). However, the increased chemical resistance cannot guarantee its resistance to MICC. For instance, the addition of SF enhanced the concrete corrosion loss with *Thiobacillus* sp. (a type of SOMs) (Fisher et al. 2008).

Calcium aluminate cement (CAC) showed up to six times higher corrosion resistance than OPC-based concrete in sewers (Kiliswa 2016). Recent studies revealed that this higher corrosion resistance is attributed to the higher acid neutralization capacity, the formation of an aluminum hydroxide gel under acid attack, and the possible bacteriostatic effect due to the higher Al content (Grengg et al. 2018). Nevertheless, the use of CAC concrete was limited as CAC concrete was not resistant to highly acidic conditions (pH 1–2) (Herisson et al. 2014). Although several investigations have found a high acid resistance and potential antimicrobial properties in geopolymer concrete, there is no record of research evaluating the performance of geopolymer concrete related to MICC environments (Grengg et al. 2018). Antibiotic-loaded fibers and biocides such as tungsten and triclosan showed inhibitory effects on the growth of SOMs under laboratory conditions; however, these chemicals are toxic to the environment (De Muynck et al. 2009).

In contrast, the application of nitrite admixed concrete is environmentally friendly and has no adverse effect on concrete mechanical properties. The 28-day compressive strength of calcium nitrite admixed concrete with four different dosages (i.e., 1–4% weight of the cement) meets the structural requirement (Li et al. 2020c). Negligible changes in the community of anaerobic sewer biofilm developed on concrete in wastewater were observed due to the nitrite admixture (Li et al. 2020c). The leaching of nitrite into the sewer environment from the nitrite admixed concrete was slow, leading to a fairly low concentration of nitrite (ppb level) in wastewater (Li et al. 2020c). Furthermore, nitrite is regularly used as a substrate for denitrifying organisms in wastewater systems. Thus, the leached nitrite can be easily degraded within the sewer, which limits if any environmental and health concerns. Therefore, the addition of calcium nitrite into concrete is environmentally friendly to wastewater infrastructures with promising MICC mitigation effects.

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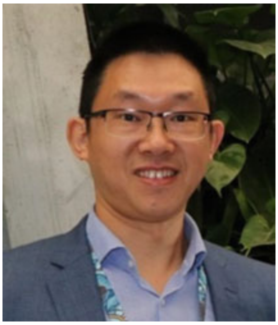

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# 6

# Metabolic Engineering and Synthetic and Semi-Synthetic Pathways: Biofuel Production for Climate Change Mitigation

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## Abstract

The Green Revolution has fueled an exponential growth in human population since the mid- twentieth century. Due to population growth, food and energy demands will soon surpass supply capabilities. Plant genetic engineering has the potential to overcome some of these challenges. As an example, bioenergy crops can be genetically improved for producing more biomass, being more resilient to stresses like drought conditions and pathogens. Besides they can synthesize cell wall materials with reduced recalcitrance toward deconstruction processes. This strategy has been put forward for the environmentally sustainable production of fuels and chemicals currently derived from petroleum refining. Industrial crops produce feedstocks for fabricating fiber, biopolymer, and construction materials. These crops offer the potential to reduce our dependency on petrochemicals that currently serve as building blocks for manufacturing the majority of our industrial and consumer products. Recent findings that plant metabolic pathways can be reconstituted in heterologous hosts and metabolism in crop plants can be engineered to improve the production of biofuels have given a new hope for molecular biological approaches in improving food and biofuel production. The

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de novo engineering of genetic circuits, biological modules, and synthetic pathways is beginning to address these crucial problems and is being used in related practical applications.

**Keywords**

Electron transport · Photosynthetic process · Transmembrane complex · Synthetic biology · Climate change · Metabolic engineering

## 6.1 Introduction

Climate change impacts—higher temperatures, extreme weather, drought, increasing levels of carbon dioxide, and sea level rise—are jeopardizing the quality and quantity of our food supplies (Kumar et al. 2018a, b, 2020). Pingali et al. (2012) said that a Green Revolution version 2.0 is necessary to feed 11 billion population by 2100 (Gerland et al. 2014). This will require new designer crops which are precision engineered for optimal yield environmental interactions, microbiome, and soil. The goals of the synthetic biology are robust, predictable, and rapid engineering of biology align well with this vision (Cameron et al. 2014a, b). According to Mortimer (2019) to date, much synthetic biology research has focused on microbial engineering.

## 6.2 Systems and Synthetic Biology

Mortimer et al. (2019) suggested that out of several definitions of synthetic biology the one emerging from the Engineering Biology Research Consortium (EBRC; www.ebrc.org) is most appropriate and is quoted here: —“Synthetic biology aims to make biology easier to engineer… It can be thought of as a biology-based toolkit that uses abstraction, standardization, and automated construction to change how we build biological systems and expand the range of possible products”. Synthetic biology (synbio) is the construction of biological components, such as enzymes and cells, or functions and organisms or their redesign to perform new functions. Synthetic biology was founded as a biophysical discipline that sought explanations for the origins of life from chemical and physical first principles. Modern synthetic biology has been reinvented as an engineering discipline to design new organisms as well as to better understand fundamental biological mechanisms (Bartley et al. 2017). Plant synthetic biology is an emerging field that combines engineering principles with plant biology toward the design and production of new devices (Liu and Stewart 2015).

A conceptual scheme describing the integration of systems and synthetic biology. In the upper panel, a “cloud” of systems biology integrates top-down and bottom-up approaches to exploit the omics data (Fig. 6.1). Synbio is controversial because its

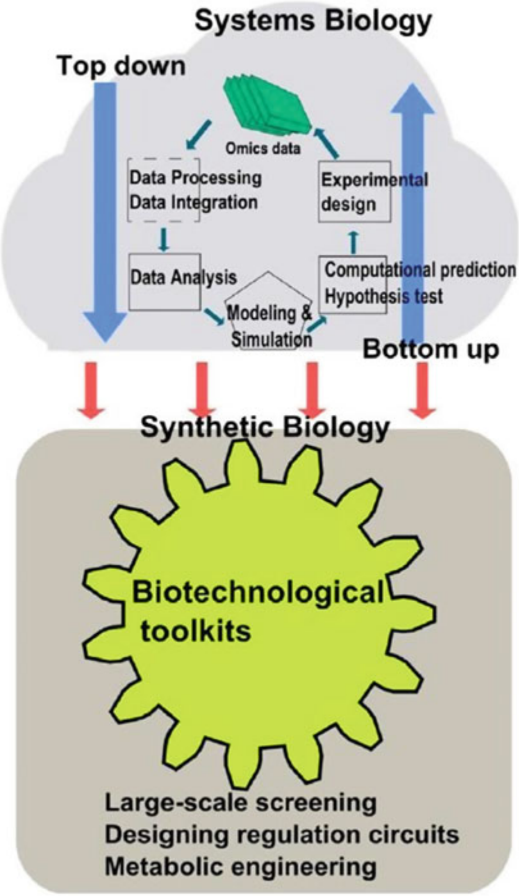

**Fig. 6.1** A conceptual scheme describing the integration of systems and synthetic biology. In the upper panel, a "cloud" of systems biology integrates top-down and bottom-up approaches to exploit the omics data. The knowledge from systems biology can guide the design of precision engineering for synthetic biology. The lower panel highlights that the key element of synthetic biology is to develop versatile biotechnological tools for its various applications (Source: Kalluri, U. C., Yin, H., Yang, X., & Davison, B. H. (2014). Systems and synthetic biology approaches to alter plant cell walls and reduce biomass recalcitrance, 1207–1216. https://doi.org/10.1111/pbi.12283. Reprinted under license number 5143261483992 dated 6 September)

purpose is to build artificial biological systems that don't already exist in the natural world.

This field is growing rapidly after the gene editing tool CRISPR-Cas9, first used in 2013, that locates, cuts, and replaces DNA at specific locations became available (Neumann et al. 2020). Besides, development of Registry of Standard Biological Parts, which catalogs over 20,000 genetic parts or BioBricks that can be ordered and used to create new synthetic organisms or systems, has also facilitated synbio. But synthetic biology is still controversial because it involves altering nature, and its potential and risks are not completely understood (https://blogs.ei.columbia.edu/2019/08/14/synthetic-biology-help-environment/).

The term "genetic rescue" has been used in conservation for several decades; Revive & Restore uses an expansive definition that includes the use of advanced biotechnologies. With the increasing affordability of genomic sequencing and the advent of CRISPR-Cas9 gene editing technology, there is an opportunity to develop a suite of innovative new genetic rescue applications. Genetic Rescue Toolkit that has the potential to advance and complement conventional conservation practice is now available (https://reviverestore.org/what-we-do/genetic-rescue-toolkit/).

The engineering concept of the design–build–test–learn (DBTL) cycle can underpin the application of synthetic biology to organismal engineering (Fig. 6.2) (Liu and Stewart 2015; Petzold et al. 2015; Mortimer 2019). The convergence of next-generation sequencing and synthetic biology, along with progress in analytic technologies such as mass spectroscopy, opens the door to the creation of large, reliable libraries of pure natural products for biofuels as well as drug discovery (Mitchell 2011). This emerging field should play an important role in future agriculture for traditional crop improvement, but also in enabling novel bioproduction in plants. Numerous carbon dioxide removal (CDR) strategies have been proposed (Williamson 2016; Kumar 2018a, b; Minx et al. 2018). Liu and Stewart (2015) presented design cycles of synthetic biology as well as key engineering principles, genetic parts, and computational tools that can be utilized in plant synthetic biology.

## 6.3 The CRISPR/Cas Revolution

Repair of DNA double-strand breaks (DSBs) plays a critical role in the maintenance of the genome (Sonoda et al. 2006). The vital roles of RNA in regulating DNA repair have started to emerge and reflect their importance in maintaining genome integrity by signaling DNA repair cascade via a mechanism not understood yet (Raina et al. 2021). Raina et al. (2021) further reported conserved and crucial role of RNA molecules, RNA processing enzymes, and other factors in DNA repair. It appears that most of the genome gets transcribed, but many of these transcripts do not code for proteins. These transcripts are called noncoding RNAs (ncRNAs), and some of these ncRNAs remain associated with chromatin in a sequence-specific manner to control many cellular pathways such as gene expression. di Fagagna (2014) suggested that small ncRNAs acting at the site of DNA lesions signal the presence of DNA damage in the cell, and on the genes involved in their biogenesis to achieve accurate DNA repair.

CRISPR (clustered regularly interspaced palindromic repeat)-associated (Cas) system can be used as easily programmable tools for genome engineering (Kumlehn et al. 2018). This was possible due to discovery of molecular effectors that (i) can be targeted easily to specific sequences in a genome or transcriptome, (ii) possess intrinsic nuclease activity, or (iii) are modified to derivatives that deliver defined biochemical reactions or properties to genomic loci or transcripts (Kumlehn et al. 2018). Earlier investigations have shown that double-strand breaks (DSBs) in the nuclear DNA can trigger two independent endogenous DNA repair pathways: nonhomologous end joining (NHEJ) and homologous recombination (HR), which can frequently result in small or large chromosomal changes (Sonoda et al. 2006). HR plays a dominant role in any DSB repair in yeast, whereas NHEJ significantly contributes to DSB repair in vertebrates. Advances in the engineering of site-specific nucleases, such as zinc- finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), have allowed more reliable targeted plant gene editing

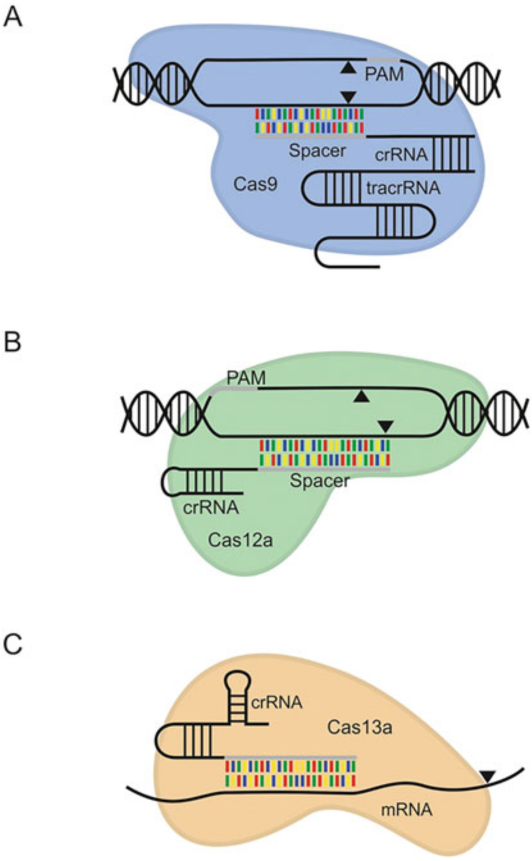

**Fig. 6.2** Schematic representation of the three CRISPR Class 2 single-protein effectors, Cas9, Cas12a, and Cas13a. (**a**) After hybridization of the small RNAs, tracrRNA and crRNA, a complex with Cas9 is formed. The crRNA contains a 20-nucleotide guide sequence (Spacer) and mediates sequence-specific DNA binding at the recognition site upstream of the protospacer adjacent motif (PAM). Subsequent to Cas9 recruitment to the target DNA, a sequence-specific double-strand break (DSB) is induced. (**b**) In contrast to Cas9, Cas12a requires only a single crRNA for DNA targeting, and the 23–25-nucleotide recognition site is located downstream of the PAM sequence. The Cas12a nuclease-induced DSB possesses 5 nt single-strand DNA overhangs, 18–23 nucleotides distal and downstream to the PAM in the spacer region. (**c**) The Cas13a-mediated RNA-specific cleavage activity requires one single crRNA. Binding of the Cas13a/crRNA complex to its target RNA is mediated by the guide sequence of the crRNA. RNA cleavage occurs at the catalytically active sites of Cas13a located on the outer protein surface. (**a**–**c**) All three single-protein effectors can be specifically directed to almost any target DNA/RNA by exchanging the specific recognition sequences of the crRNA. Kumlehn J, Pietralla J, Hensel G, Pacher M, Puchta H. The CRISPR/Cas revolution continues: From efficient gene editing for crop breeding to plant synthetic biology. J Integr Plant Biol. 2018;60(12):1127–1153. https://doi.org/10.1111/jipb.12734. Reproduced with RightsLink license number 5143280375691 dated 6 September 2021

(Carroll, 2011; Neumann et al. 2020). ZFN- induced double-strand breaks are subject to cellular DNA repair processes that lead to both targeted mutagenesis and targeted gene replacement at remarkably high frequencies. TALENs target one nucleotide (instead of three) at the target site, making TALENs highly specific (Christian et al. 2010). However, difficulties of protein synthesis, design, and validation needed for TALENs and ZFNs are some of the constraints for the widespread implementation of these nucleases for regular use.

CRISPR/Cas is the most promising and efficient genome-editing technique other than the nucleases. CRISPR/Cas was discovered in bacteria or archaea as a type II prokaryotic adaptive immune system, which provides bacteria immunity against invading phages (Kumawat et al. 2019). The mechanism of immunizing bacteria against viral attack starts with the incorporation of protospacer, which are small fragments of a foreign sequence in the host chromosome at the proximal end of the CRISPR array (Jinek et al. 2012). The protospacer consists of identical repeats; the transcription product of these repeats results in the generation of precursor CRISPR RNA (pre-crRNA). Subsequently, their enzymatic cleavage leads to the formation of crRNA, which has the ability to complementarily base pair with the protospacer sequence of the invasive viral target (Jinek et al. 2012; Kumawat et al. 2019). After recognition of target and complementary base pairing, Cas9 nuclease digests the target sequence and directs the silencing of viral sequences (Raina et al. 2021). In bacteria, there are three types of CRISPR/Cas systems known to date. The type II system, transactivating crRNA (tracrRNA), which is complementary to the pre-crRNA, in the presence of Cas9 tracrRNA helps in the maturation by processing with the ds-RNA-specific ribonuclease RNase III (Jinek et al. 2012; Kumawat et al. 2019). For efficient genome editing, single-guide RNAs (sgRNAs) are synthesized by combining the tracrRNA and crRNAs in which 5′ sequence of sgRNA binds to the target sequence and 3′ sequence binds to the Cas9 nuclease (Kumawat et al. 2019). The targeted mutagenesis by CRISPR/Cas9 is achieved by generating the sgRNAs complementary to the desired site, which allows binding of Cas9 to the desired site (Raina et al. 2021). The Cas9 enzyme subsequently cleaves the DNA at the desired site, resulting in the DSB, which is repaired by the HRR or NHEJ pathway leading to small indels (Raina et al. 2021).

Earlier CRISPR/Cas9 systems reported for plants could only modify one or a few target sites. However, Ma et al. (2015) reported a robust CRISPR/Cas9 vector system, utilizing a plant codon optimized Cas9 gene, for convenient and high-efficiency multiplex genome editing in monocot and dicot plants. Yu and Zhao (2019) reported that the CRISPR/Cas system used was derived from the prokaryotic type II CRISPR system of *Streptococcus pyogenes*, which includes precursor CRISPR RNA (pre-crRNA), transactivating crRNA (tracrRNA), and Cas9 nuclease. The tracrRNA is involved in the maturation of pre-crRNAs into crRNAs (Yu and Zhao 2019). Type II system utilizes a single protein for target recognition and cleavage. Dual tracrRNA/crRNAs are engineered as single-guide RNAs (sgRNAs) while using CRISPR/Cas for genome editing. sgRNAs retain two critical features, 5′ sequence that is complementary to the target DNA and 3′ sequence that binds to the Cas9 protein. DSBs generated can be repaired by nonhomologous end joining

(NHEJ) and homology directed repair (HDR) to create gene knockout or gene knock-in type of modifications, respectively (Fig. 6.2) (Rong and Golic 2020).

The unique genomic site-specific DNA double-strand breaks (DSBs) are mostly repaired via nonhomologous end joining (NHEJ), a highly mutagenic mechanism of partial end-resection and religation of the free ends (Fig. 6.2). Kumlehn et al. (2018) reported various Cas9/12 enzymes available for the simultaneous application of various enzyme activities, to multiple genomic sites. This allows to redirect plant metabolism in a multifunctional manner and pave the way for a new level of plant synthetic biology.

Kumlehn et al. (2018) further described that in order to find foreign DNA, the Cas9 nuclease requires this gRNA, which is formed by the crRNA and a tracrRNA and a short G-rich sequence motif, termed protospacer adjacent motif (PAM) positioned next to the gRNA- specific part of the target sequence (Fig. 6.2a). After formation of the Cas9/gRNA complex, the DNA is scanned for the PAM sequence. The effector protein is able to target simultaneously multiple loci. Upon PAM identification, the complex separates the DNA double strand and checks whether the spacer on the crRNA is complementary to the sequence next to the PAM site. Once the hetero-duplex is formed, the nuclease domains of Cas9 are activated and induce a blunt-end DSB three base pairs (bp) upstream of the PAM (Kumlehn et al. 2018; Fig. 6.2)

Secondary structure formation of the gRNA plays a crucial role in Cas cleavage activity, as the gRNA 5′ terminal part must be capable of base pairing with the target DNA and also be capable of binding between Cas endonuclease and gRNA which is essentially dependent on two-dimensional (2D) structures formed within the gRNA 3′ terminal scaffold (Kumlehn et al. 2018; Fig. 6.3) (Ma et al. 2015). Online tools, such as RNAfold (rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi) and MFOLD, are available to predict the most likely occurring secondary structure variants of gRNAs in silico. Taking advantage of this opportunity, substantial interactions between the target-compatible 5′ terminal part of the gRNA and its 3′ scaffold can largely be avoided.

According to Kalluri et al. (2014), synthetic biology builds on a systems biology knowledge base and leverages high-precision tools for high-throughput assembly of multigene constructs and pathways, precision genome editing, and site-specific gene stacking, silencing, and/or removal. Shih (2018) suggested that we have had the ability to modify organisms with recombinant DNA and molecular biology techniques for several decades, and synthetic biology is distinct in its motivation and perspective in providing a bottom-up understanding of biological systems as it has mainly focused on understanding the underlying principles that control biological processes in order to design and modify organisms for engineered applications. Thus, synthetic biology separates itself from traditional molecular biology in its drive to understand systems. However, the nascent field of plant synthetic biology has much to gain from these advances (Ro et al. 2006; Peralta-Yahya et al. 2011).

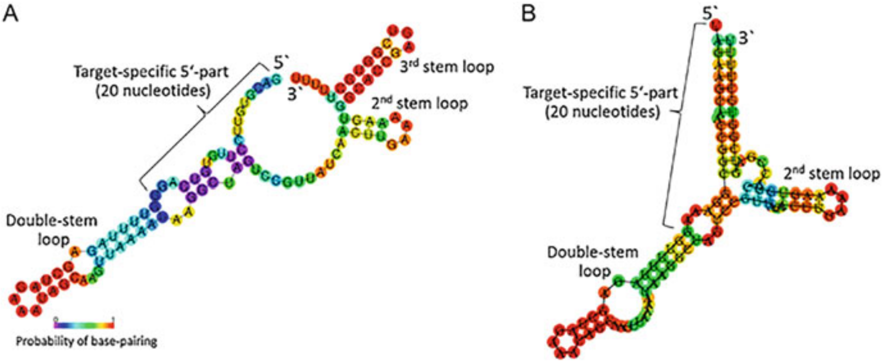

**Fig. 6.3** Secondary structure of single-guide RNAs. (**a**) Example of guide RNA with high functionality, due to limited base pairing of the target-specific part and to the presence of all three stem loop structures required to correctly interact with Cas9 endonuclease. Probability of the individual bases to pair is indicated by a color code. (**b**) For comparison, a guide RNA with severely compromised functionality, owing to a high number of paired nucleobases of the target-specific part and to the absence of an essential stem loop structure (namely, the third one according to **a**). The structure models were generated using the RNAfold online platform (rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi). Kumlehn J, Pietralla J, Hensel G, Pacher M, Puchta H. The CRISPR/Cas revolution continues: From efficient gene editing for crop breeding to plant synthetic biology. J Integr Plant Biol. 2018;60(12):1127–1153. https://doi.org/10.1111/jipb.12734. Reproduced with RightsLink license number 5143280375691 dated 6 September 2021

## 6.4 The Role of Synthetic Biology in Atmospheric Greenhouse Gas Reduction

According to DeLisi (2019), it will require a full battery of approaches including greenhouse gas emission control, alternative energy sources, adaptation, and the development of technologies for removing atmospheric greenhouse gases. The last of these is important for multiple reasons, including a centuries-long rate of clearance of $CO_2$ and some other trace gases from the atmosphere by natural processes (https://www.bu.edu/eng/2019/11/05/can-synthetic-biology-address-climate-change/).

As it turns out, planting trees is not the only way forestry can help in climate mitigation (Winchester and Reilly 2019). Presently, emission reduction efforts have largely focused on decarbonizing the two economic sectors responsible for the most emissions, electric power and transportation. Other approaches aim to remove carbon from the atmosphere and store it through carbon capture technology, biofuel cultivation, and massive tree planting (Kumar 2018a, b; Kumar et al. 2018a, b, 2020).

DeLisi (2019) suggested that systems and synthetic biology (SSB) can modulate the fast carbon cycle and thereby mitigate climate change (Cameron et al. 2014a, b; Hugh et al. 2018; Kumar 2020). The cost of generating electricity by renewable energy technologies such as wind and solar power is economically competitive with that of coal (https://www.lazard.com/perspective/levelized-cost-of-energy-and-levelized-cost-of-storage-2018/).

Some of the techniques of land management via systems and synthetic biology are available (DeLisi 2019). These include development of engineered soil microbes that would convert organic carbon into stable carbonate engineered plants with increased root to shoot ratio, amplification of processes controlling soil mineral absorption and mineral carbonation, and pigment modification of selected tree species. Equally importantly are new methods to engineer plants with reduced requirements for fertilizers, increased yields, more efficient machinery for nitrogen fixation and photosynthesis, and reduced need for water, which can enable the use of currently nonarable land for plant growth (see Jez et al. 2016).

## 6.5 Synthetic Biology Tools to Engineer and Control Microbial Communities

Synthetic biologists use engineering approaches, including computational models and modular DNA "parts," to rationally engineer living organisms (Khalil and Collins 2010). Advancements in genetic engineering (including CRISPR/Cas systems for efficient gene deletions, insertions, and transcriptional control (Reguera 2011) and rapid methods to assemble DNA fragments (Chao et al. 2015) enable modular components to be interconnected to build metabolic pathways and construct biological circuits to control cellular behavior (Brophy and Voigt 2014 and see review by Mccarty and Ledesma-Amaro 2019).

Like engineers, the synthetic biologists have adopted the concept interchangeable components. In microbial synthetic biology, this was pioneered by the BioBrick standards (https://biobricks.org/), and more recently a plant standard has been developed based on type IIS restriction endonuclease assembly (Patron et al. 2015; Mortimer 2019) (Fig. 6.1). As the cost of DNA synthesis has plummeted, the use of synthetic genes (e.g., codon optimized for expression in different species) is now within reach of the average research laboratory (Mortimer 2019).

Three different endonuclease systems have been successfully deployed in plant systems: zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced palindromic repeats (CRISPR)—CRIPSR-associated (Cas) proteins such as CRIPSR-Cas9 (Neuman et al. 2020). CRISPR-based systems in particular are expanding rapidly, with new functions added via gene fusion with the endonuclease, such as gene repression (Liang et al. 2017). Recent advances in which whole metabolic pathways can be introduced with a single transformation event hold great promise (Shih et al. 2016a, b; Fuentes et al. 2016). Combinatorial supertransformation of transplastomic recipient lines (COSTREL) takes advantage of both plasmid and nuclear transformation (Fuentes et al. 2016; see review by Mortimer 2019).

### 6.5.1 Applications of Plant Synthetic Biology

The use of synthetic genes (e.g., codon optimized for expression in different species) is now within reach of the average research laboratory. However, in the future, it is likely that plant synthetic biology will become increasingly important for commercial agriculture also.

### 6.5.2 Production of Functional Biomaterials

According to Mccarty and Ledesma-Amaro (2019), the compartmentalization of tasks, modular tenability, and intercellular signaling mechanisms between members of synthetic microbial consortia make them an ideal platform to produce complex, functional biomaterials. Biomaterial production necessitates fine-tuned control of gene expression and pattern formation. Advancements in synthetic biology tools for spatial programming will enable synthetic consortia to produce materials of defined sizes, shapes, and patterns (Figs. 6.2 and 6.3).

Plant natural products (PNPs) are widely used as pharmaceuticals, nutraceuticals, seasonings, pigments, etc., with a huge commercial value on the global market (Liu et al. 2017).

In an excellent review, Liu et al. (2017) summarized the recent progress of microbial cell factory engineering, identification of novel biosynthetic pathways, and the production of PNPs on an industrial scale. Firstly, two tentative strategies based on biological -omics and synthetic biology technologies for gene mining from the biosynthetic pathways of PNPs are introduced. This is followed by improvement of precursor supply, enzyme activity, and product transport. Finally, several milestone examples of industrial PNP production in microbial cell factories are showcased (Fig. 6.3) (Kumar et al. 2014; Kumar 2015; Kumar and Gupta 2018; Gajraj Randhir et al. 2018; Bhansali and Kumar 2018; Kumar et al. 2018a, b; Hirani et al. 2018). A schematic summary of microbial cell factory design, optimization, and industrial production presented by Liu et al. (2017) is reproduced here (Fig. 6.4).

### 6.5.3 The Potential of Synthetic Microbial Consortia in Bioprocesses of the Future

The potential of synthetic microbial consortia in bioprocesses of the future given by Mccarty and Ledesma-Amaro (2019) is presented in Fig. 6.5.

### 6.5.4 Synthetic Antibody Could Prevent and Treat COVID-19

Using a mouse model, researchers have recently shown that a synthetic antibody could neutralize SARS-CoV-2. This could help prevent infection as well as treat COVID-19 in those who already have it. SARS-CoV-2, the new coronavirus, gains

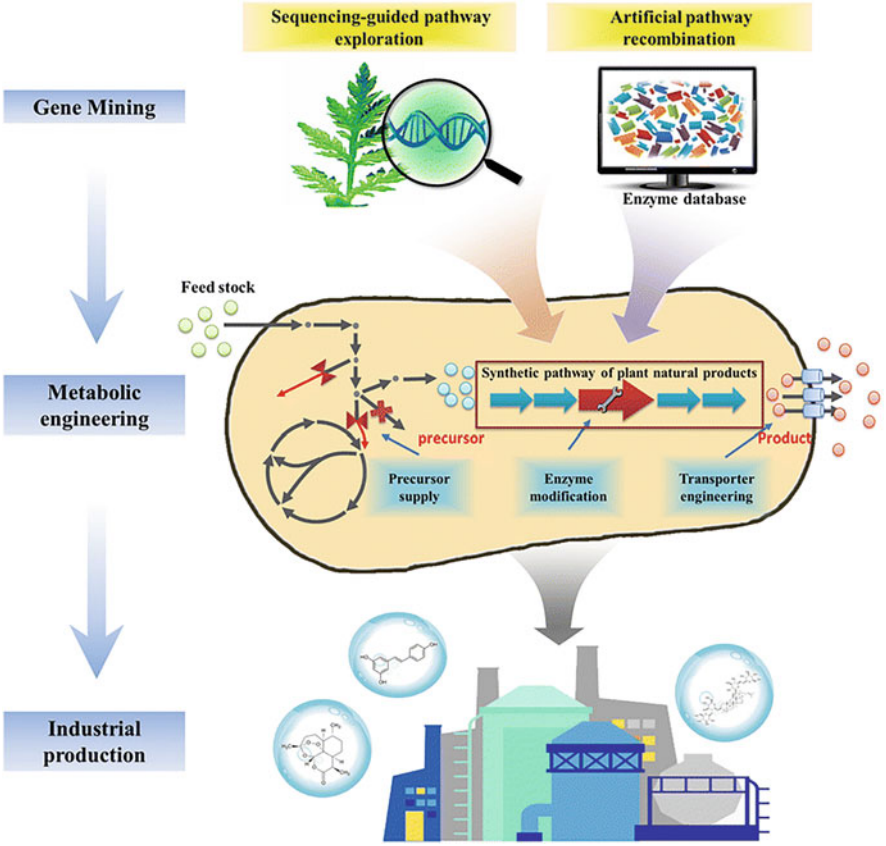

**Fig. 6.4** A schematic summary of microbial cell factory design, optimization, and industrial production. The mining of biosynthetic pathways of plant natural products by sequencing-guided pathway exploration and artificial pathway recombination. Optimization of microbial cell factories by improving precursor supply, enzyme modification, and transporter engineering. Industrial-scale production of three natural products (artemisinin, resveratrol, and carotenoids) is shown as an example. Source: Liu X, Ding W, Jiang H. Engineering microbial cell factories for the production of plant natural products: from design principles to industrial-scale production. *Microb Cell Fact.* 2017;16(1):125. Published 2017 July 19. doi:10.1186/s12934-017-0732-7 Permission Open access

entry into cells in the body using a receptor called angiotensin-converting enzyme 2 (ACE2). ACE2 is present on the surface of cells in the airways and the lungs. After a person inhales viral particles, spike proteins on the outside of the virus bind to this receptor, which allows the virus to enter cells and cause disease. Other coronaviruses, including the virus behind the 2002 SARS outbreak, also bind to the ACE2 receptor. However, it seems the new coronavirus binds to it more tightly, perhaps underlying its higher infectiousness. Researchers from Tulane University in New Orleans, LA, have now developed an antibody that stops the virus from attaching to the ACE2 receptor, ultimately preventing infection. In a paper on the

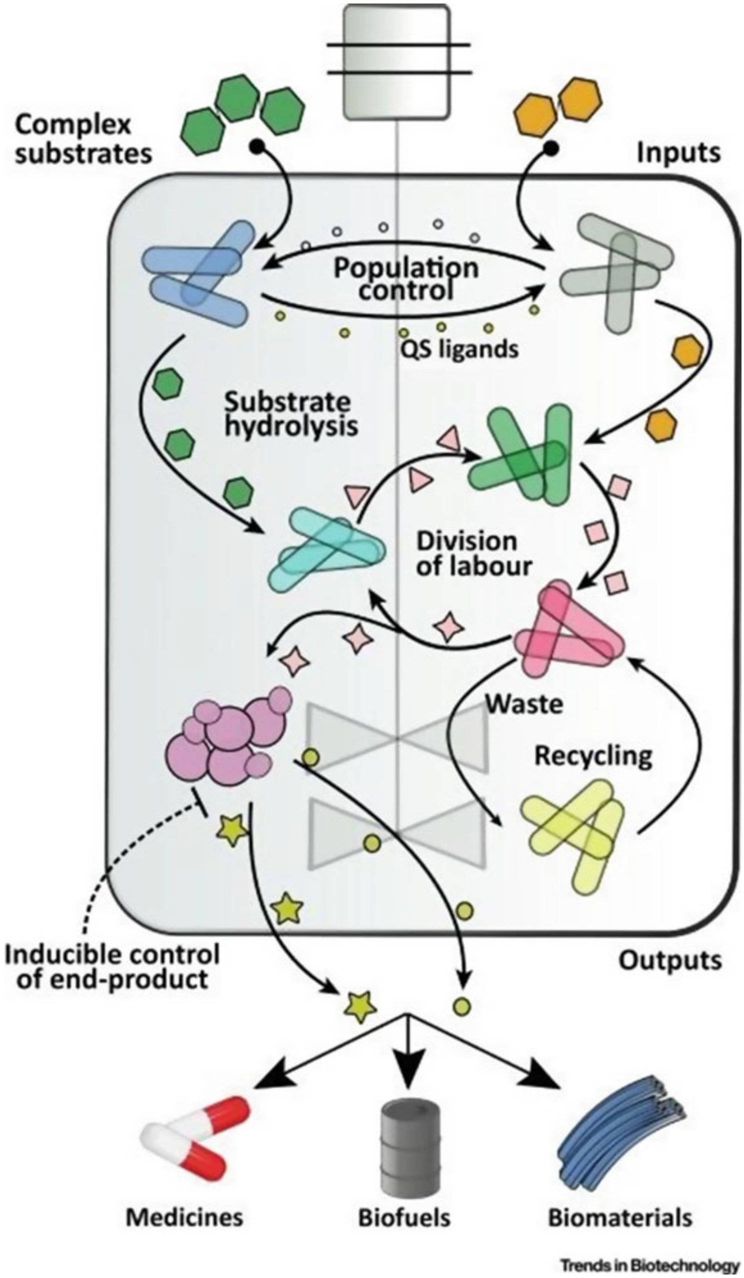

**Fig. 6.5** The Potential of Synthetic Microbial Consortia in Bioprocesses of the Future. Source: Mccarty and Ledesma-Amaro (2019). Synthetic Biology Tools to Engineer Microbial Communities for Biotechnology. *Trends in Biotechnology*, *37*(2), 181–197. 10.1016/j.tibtech.2018.11.002. This is an open-access article distributed under the terms of the Creative Commons CC-BY license, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. You are not required to obtain permission to reuse this article

preprint server bioRxiv, the researchers say that healthcare professionals could use the antibody both before and after a person has had exposure to SARS-CoV-2. It could be especially beneficial for people who cannot receive a vaccine for health reasons. https://www.medicalnewstoday.com/articles/synthetic-antibody-could-prevent-and-treat-covid-19

### 6.5.5 Artemisinin

Artemisinin is acknowledged as an effective pharmaceutical compound for the treatment of malaria (Barbacka and Baer-Dubowska 2011), a serious disease that in 2015 alone affected 214 million people (African 88%, South-East Asia Region 10%), causing 438,000 deaths (WHO 2015). By engineering the genotype and carbon flux of *S. cerevisiae*, the yield of artemisinic acid reached 0.65 g/L in flask fermentation, starting from an initial 0.11 g/L (Ro et al. 2006). However, when the recombinant strain was cultured in a well-controlled fermenter, in the presence of extractive solvent isopropyl myristate (IPM) 25 g/L, artemisinic acid was produced. Therefore, the production of artemisinic acid has been improved from 0.1 to 25 g/L in engineered *S. cerevisiae* through in vivo carbon flux rewiring and optimization of fermentation conditions (Paddon et al. 2013) achieving the semi-synthesis of artemisinin (combined with one-step photochemical catalysis) at an industrial scale.

### 6.5.6 Resveratrol

Resveratrol is a polyphenolic compound found in several plant species, such as bushberries, peanuts, cranberries, and grapes. Resveratrol has been proved to decrease the risk of heart disease, diabetes, and cancer (reviewed in Chun-Fu et al. 2013). Beekwilder et al. first introduced $4CL_2$ (4-coumaroyl-CoA ligase) from *Nicotiana tabacum* and STS (stilbene synthase) from *Vitis vinifera* into *E. coli*, and produced 16 mg/L resveratrol from 4-coumaric acid (Beekwilder et al. 2006).

## 6.6 Renewable Energy

### 6.6.1 Biomass and Biofuels

The Green Revolution has fueled an exponential growth in human population since the mid- twentieth century. Due to population growth, food and energy demands will soon surpass supply capabilities. Kumar et al. (2018c, 2020) reported that rates of world consumption of fuel and population growth show a constant increase. As per data of BP (British Petroleum World Energy Statistics 2019), total primary energy consumption (oil, natural gas, coal, nuclear energy, hydroelectric, and renewable energy) in 2018 was 13,864.9 million tons of oil equivalent. Total oil consumption was 4662.1 million tons, while renewable energy consumption was still low, at

561.3 million tons. Therefore, it is necessary to expand the use of renewable energy sources in order to address the limitations of fossil fuels (Kumar et al. 2020).

Plant genetic engineering has the potential to overcome some of these challenges. As an example, bioenergy crops can be genetically improved for producing more biomass, being more resilient to stresses like drought conditions and pathogens. Besides they can synthesize cell wall materials with reduced recalcitrance toward deconstruction processes (Bailey-Serres et al. 2019; Ralph et al. 2019; Lin and Eudes 2020). This strategy has been put forward for the environmentally sustainable production of fuels and chemicals currently derived from petroleum refining (Rosales-Calderon and Arantes 2019).

### 6.6.2 $C_3$ and $C_4$ Plants

Plants with $C_3$ photosynthesis such as poplar *Populus* spp. and interspecific hybrids of *Populus* spp., and willow (*Salix*) also generate high yields of biomass, but take longer to grow and have higher contents of lignin, making the polysaccharides less accessible; thus, the biomass quality is lower.

According to Byrt et al. (2011), several $C_4$ grasses with $C_4$ photosynthesis, such as Aleman grass (*Echinochloa polystachya*), elephant grass (*Pennisetum purpureum*), foxtail millet (*Setaria italica*), miscanthus (*Miscanthus giganteus*), sweet sorghum (*Sorghum bicolor*), sugarcane, energycane (*Saccharum complex*), and switchgrass (*Panicum virgatum*), are ideal energy crops because they possess the following traits: high conversion efficiency of light into biomass energy, high water use efficiency, and high leaf level nitrogen use efficiency (see also Taylor et al. 2010).

The main feedstocks for bioethanol are sugarcane (*Saccharum officinarum*) and maize (*Zea mays*), both of which are $C_4$ grasses, highly efficient at converting solar energy into chemical energy, and both are food crops (Byrt et al. 2011). **Biotechnology for $C_4$Rice** $C_4$ plants are unique in possessing two types of photosynthetic cells. $C_4$ plants have "Kranz" anatomy that has both mesophyll cells (M), in which $CO_2$ is fixed by $C_4$ acids, and bundle sheath cells (BS), where ribulose bisphosphate carboxylase oxygenase (RuBisCO) fixes $CO_2$ derived from the $C_4$acids of the mesophyll cells. This spatial separation of initial $CO_2$ fixation in M cells and subsequent refixation in BS cells is generally associated with Kranz anatomy, but can also occur in single cells (Kumar et al. 1983, 1984; Bender et al. 1985; Neumann et al. 2020). $C_4$ photosynthesis is an effective solution for overcoming the catalytic inefficiency of RuBisCO (Christin and Osborne 2013). Transfer of $C_4$ photosynthetic cDNA sequences for maize phosphoenolpyruvate carboxylase (PEPC), pyruvate orthophosphate dikinase (PPDK), and NADP-malate dehydrogenase (MDH) has all been reported and expressed in the M of cells of rice (reviewed in Miyao et al. 2011; Hibberd and Covshoff 2010; DiMario et al. 2016). Genes encoding the $C_4$ pathway enzymes and most of the metabolite transporters have now been identified. Ermakova et al. (2020) reviewed the building of biochemistry for $C_4$ rice. The key genes encoding the entire biochemical pathway of the NADP-ME type $C_4$

mechanism (Fig. 6.6) are available for $C_4$ rice project. Rice with its increased photosynthetic potential as envisaged may provide additional source of biofuels through genetic manipulations and/or use of synthetic biology.

## 6.7 Lignocellulosic Biofuels

Plant cell walls are virtually the only source of cellulose for the paper industry and will be a great source of sugars for the predicted lignocellulosic biofuels era (Somerville et al. 2010; Kumar 2018a, b; Kumar 2020). The primary cell wall, consisting mostly of cellulose, hemicelluloses, pectin, and proteins, demarcates each plant cell. Secondary cell walls, composed mostly of cellulose, hemicelluloses, and lignin, are deposited between the plasma membrane and primary wall in specialized cell types following cessation of cell enlargement (Handakumbura and Hazen 2012) According to Yang et al. (2013), lignocellulosic biomass is composed mostly of secondary cell walls built with polysaccharide polymers that are embedded in lignin to reinforce the cell wall structure and maintain its integrity. During plant development, deep reductions of lignin cause growth defects and often correlate with the loss of vessel integrity that adversely affects water and nutrient transport in plants. The lignin polymer is the product of oxidative coupling of phenolic metabolites, normally p-hydroxycinnamyl alcohol monomers (the so-called monolignols) (Vanholme et al. 2012).

Cellulose biosynthesis occurs at the plasma membrane, and various soluble and membrane proteins have been reported to play a role in this pathway (Endler and Persson 2011). The lignin polymer is the product of oxidative coupling of phenolic metabolites, normally p-hydroxycinnamyl alcohol monomers (the so-called monolignols).

## 6.8 Lignin Biosynthesis

Lignin, in the secondary wall, is the major cause of lignocellulosic biomass recalcitrance to efficient industrial processing (Vanholme et al. 2012). Several review articles have provided a detailed view of the lignin biosynthesis pathway in the past (Zhao and Dixon 2011). Lignin, a polyphenolic heteropolymer of monolignols, is correlated with recalcitrance of biomass. Yang et al. (2013) reported that cell differentiation into vessel or fiber cells starts independently. Both are regulated by independent master transcription factors that rapidly share the same regulatory network to control the expression of many genes involved in the biosynthesis of the three major secondary cell wall components: cellulose, xylan, and lignin (Fig. 6.6). Lignin is considered to be the main inhibitory factor for pulping, forage digestibility, and efficient enzymatic hydrolysis of plant cell wall polysaccharides, but it cannot be easily removed (Roy and Kumar 2013; Chanoca et al. 2019).

The shikimate pathway, which is present in bacteria, yeasts, and plants, but not in animals, is the entry pathway toward a plethora of phenolic compounds. This plastid-

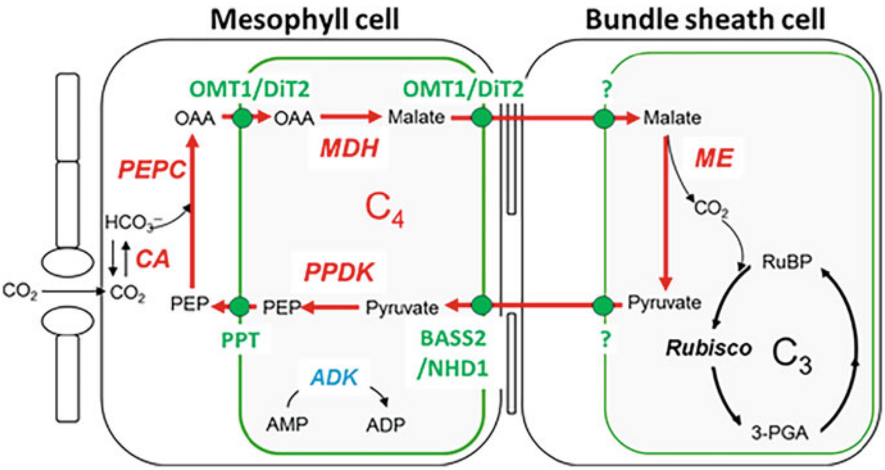

**Fig. 6.6** Enzymes and transporters included in the construction of $C_4$ rice. These include carbonic anhydrase (CA), PEP carboxylase (PEPC), malate dehydrogenase (MDH), NADP-malic enzyme (ME), and pyruvate Pi dikinase (PPDK). Adenylate kinase (ADK) has been added to ensure AMP is converted to ADP. The required transporters are oxoglutarate/malate transporter (OMT1), PEP/phosphate translocator (PPT), dicarboxylate transporter (DiT2), pyruvate/sodium symporter (BASS2), and sodium/proton antiporter (NHD1). Malate importer and pyruvate exporter in bundle sheath chloroplasts are not yet confirmed. Other abbreviations used are OAA for oxaloacetate and PEP for phosphoenolpyruvate. Source: Ermakova, M., Danila, F.R., Furbank, R.T. and von Caemmerer, S. (2020), On the road to $C_4$ rice: advances and perspectives. Plant J, 101: 940–950. https://doi.org/10.1111/tpj.14562. Attribution 4.0 International (CC BY 4.0)

localized pathway is highly transcriptionally and posttranslationally controlled (Tzin and Galili 2010). Genetic engineering of lignin, a major recalcitrance factor, improves saccharification and thus the potential yield of biofuels (Wang et al. 2015).

According to Vanholme et al. (2010), lignin is made primarily from three monolignol units: *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol, which are synthesized in the cytosol, and then transported to the apoplast. Monolignol radicals are generated, and these are then incorporated by combinatorial radical coupling into the lignin polymer, forming *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) subunits (Vanholme et al. 2010) (Fig. 6.6). The result is a highly heterogeneous polymer. Plant breeding programs have succeeded in developing lower-lignin plants, e.g., *brown midrib* (*bm or bmr*), which have up to ~20% reduction in lignin compared to wild type (Li et al. 2008; Kumar et al. 2018a, b).

## 6.9 Metabolic Engineering

Kalluri et al. (2014) reported recent breakthroughs in synthetic biology approaches to reduce biomass recalcitrance. Lignin engineering efforts are primarily focused on modifications of lignin composition, often with introduction of novel lignin forms in plants (Vanholme et al. 2012a), to reduce cross-linking with wall components resulting in easier extractability, digestibility, and recalcitrance of usable wall polysaccharides.

Genetic engineering has been used to decrease the lignin content and to change the lignin composition of switchgrass (*Panicum virgatum* L.) to decrease cell wall recalcitrance to enable more efficient cellulosic biofuel production (Fu et al. 2011; Xu et al. 2019). Transgenic switchgrass derived by the downregulation of a lignin biosynthetic enzyme, caffeic acid 3-O-methyltransferase (COMT), was shown to have higher sugar release and ethanol yield due to reduced lignin content and syringyl/guaiacyl (S/G) lignin monomer ratio, yet maintain similar biomass yield as non- transgenic switchgrass plants (Fu et al. 2011; Baxter et al. 2014, 2016).

Yang et al. (2013) described synthetic biology tools in *Arabidopsis* to rewire the secondary cell network by changing promoter-coding sequence associations. This resulted in reduction in lignin and an increase in polysaccharide depositions in fiber cells while preventing vessel collapse. They introduced a new strategy to boost transcription factor expression in native tissues (Fig. 6.7).

Zhang et al. (2014) selected the C4H gene, encoding for the second enzyme in the lignin biosynthesis pathway, as a target gene to control the flux through that pathway and consequently the production of monolignols. They presented two compatible approaches:

1. To narrow down lignin biosynthesis into vessels and
2. To increase secondary cell wall thickening. Both were used to generate healthy plants with increased sugar yield upon saccharification. This engineering allowed them to generate healthy plants with reduced lignin and enhanced cell wall deposition, which—after various pre-treatments—exhibit improved sugar releases from enzymatic hydrolysis as compared to wild type.

## 6.10 Bio-Based Platform for Industrial Products

Industrial crops produce feedstocks for fabricating fiber, biopolymer, and construction materials. These crops offer the potential to reduce our dependency on petrochemicals that currently serve as building blocks for manufacturing the majority of our industrial and consumer products. Lin and Eudes (2020) reviewed the metabolites synthesized in plants which can be used as bio-based platform chemicals for partial replacement of their petroleum-derived counterparts. They presented plant metabolic engineering approaches aiming at increasing the content of these metabolites in biomass.

Lin and Eudes (2020) emphasized the recent advances in the manipulation of the shikimate and isoprenoid biosynthetic pathways promoted production of coproducts in bioenergy crops (Figs. 6.8 and 6.9). Isoprenoids are a large and extremely diverse group of natural products, comprising over 70,000 compounds—approximately one-third of all known natural products (The Dictionary of Natural Products Online: http://dnp.chemnetbase.com/intro/). Their roles include stabilization of cell

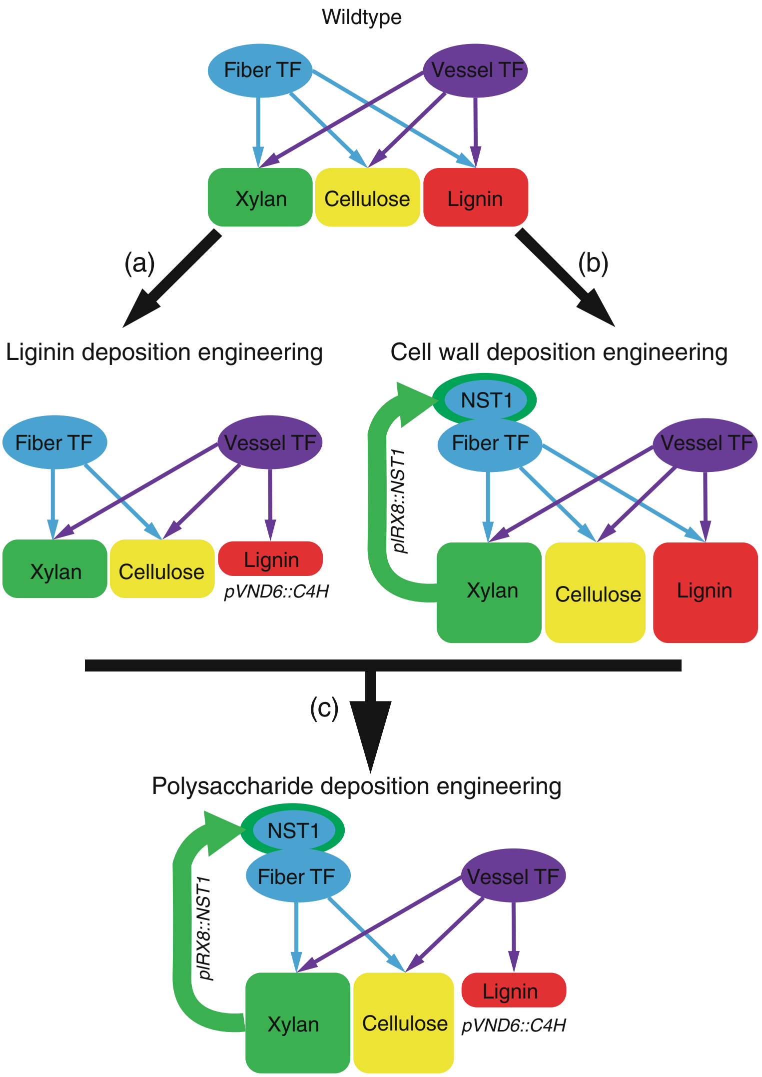

**Fig. 6.7** Model of secondary cell wall engineering. (**a**) Lignin engineering: Lignin biosynthesis is dependent of the *C4H* gene expression, which is under the control of a vessel-specific promoter (*pVND6*), a promoter that is independent of the fiber regulatory network. (**b**) Cell wall artificial positive feedback loop (APFL): The *IRX8* secondary cell wall promoter (*pIRX8*), known to be one of the downstream induced targets of the *NST1* transcription network, was used to express a new copy of *NST1*. When secondary cell wall transcription factors (comprising *NST1*) are expressed, they induce their downstream network and *pIRX8* used to control the expression of the new *NST1* allele. Consequently, it will further enhance the accumulation of NST1 transcription factor, which over-induce secondary cell wall gene expression including *pIRX8* promoter, causing an increase in secondary cell wall deposition compared to wild-type plants. (**c**) The APFL was combined with the lignin engineering, allowing enhancing preferably polysaccharide deposition, because lignin biosynthesis is under control of *pVND6*, which is not regulated by *NST1*. (Source: Yang, F., Mitra, P., Zhang, L., Prak, L., Verhertbruggen, Y., Kim, J. S., Sun, L., Zheng, K., Tang, K., Auer, M.,

membranes (hopanoids and sterols), cell wall biosynthesis (dolichols), modification of tRNAs, antioxidant activities, carriers for electron transport, hormones, photosynthetic and non-photosynthetic pigments, intracellular signal transduction, extracellular signaling molecules, defense molecules, vitamins, protein-targeting components, virulence factors, etc. Such products may represent a valuable option for enhancing the commercial value of biomass and attaining sustainable lignocellulosic biorefineries.

## 6.11 Biochemicals Derived from the Shikimate Pathway

The shikimate pathway, which is confined to plastids in plants, is responsible for the synthesis of aromatic amino acids that are precursors to secondary metabolites such as pigments, hormones, alkaloids, and phenylpropanoids including lignin (Maeda and Dudareva 2012). In microbes, the shikimate pathway has been exploited for the production of aromatic chemicals which are otherwise derived from petroleum-based benzene, toluene, and xylene (Lee and Wendisch 2017) Nevertheless, most aromatic compounds used for industrial applications are still synthesized chemically due to the inefficiency of current biological production methods. Several metabolic steps from these engineering approaches developed in microbial systems have been successfully implemented in plants, which open new avenues for the production of shikimate-derived metabolites in bioenergy crops (Fig. 6.8) (Lin and Eudes 2020). The intermediates of the shikimate pathway have the potential to generate bio-replacements for commonly fossil fuel-derived aromatics.

## 6.12 Biochemicals Derived from the Isoprenoid Pathways

Terpenes and terpenoids are the largest and most diverse class of specialized metabolites $>80{,}000$ compounds (Christianson 2017). In general, the structural diversity of terpenes depends on the number of isoprene ($C_5H_8$) moieties that constitute them. With the exception of steroids ($C_{27}$), terpenes can be subdivided into hemi- ($C_5$), mono- ($C_{10}$), sesqui- ($C_{15}$), di- ($C_{20}$), sester- ($C_{25}$), tri- ($C_{30}$), tetra- ($C_{40}$), and polyterpenes ($C_{5n}$, $n > 8$), based on the number of isoprene units (Ashour et al. 2010). In contrast to terpenes, which comprise solely hydrocarbons, terpenoids are more structurally diverse and consist of oxygen-containing or chemically modified terpene analogs (Tong 2013). All the terpenes and terpenoids described are named—isoprenoids. Several isoprenoids are considered as potential

**Fig. 6.7** (continued) Scheller, H. V., & Loqué, D. (2013). Engineering secondary cell wall deposition in plants. *Plant biotechnology journal*, *11*(3), 325–335. https://doi.org/10.1111/pbi.12016. Reproduced with license number 5143270940671 dated 6 September 2021 through RightsLink)

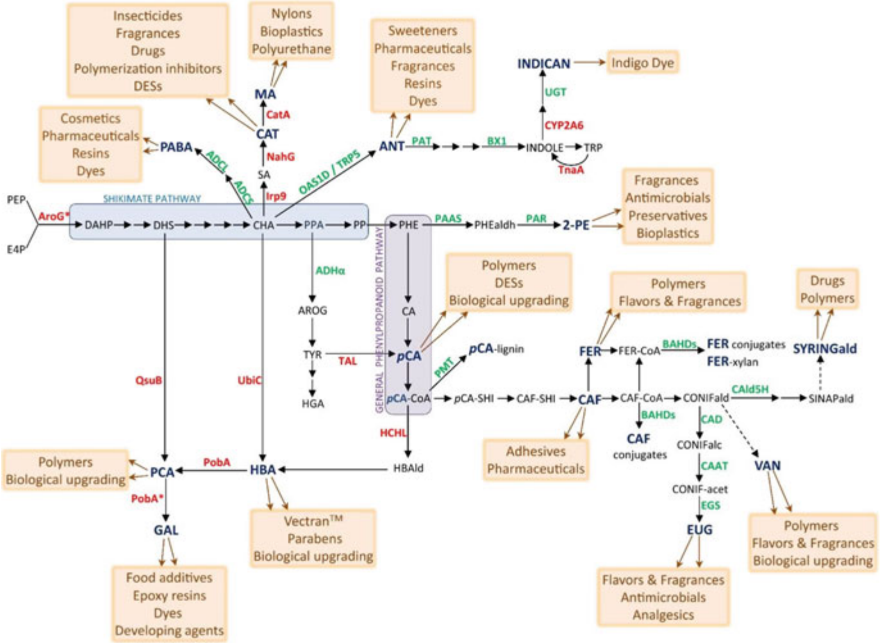

**Fig. 6.8** Proposed metabolic steps for the synthesis of chemicals derived from the shikimate and general phenylpropanoid pathways. Enzyme names are indicated in the case of steps that have been the object of metabolic engineering. Green and red fonts are used to denote enzymes from plant and non-plant origins, respectively. Asterisks indicate that a mutant version of the enzyme was used. ADCL, 4-amino-4-deoxychorismate-lyase; ADCS, 4-amino- 4-deoxychorismate synthase; ADHα, arogenate dehydrogenase alpha; ANT, anthranilate; AroG, 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP) synthase; AROG, arogenate; BAHDs, BAHD transferases; BX1, indole synthase; CAAT, coniferyl alcohol/acetyl-CoA transferase; CAD, cinnamyl alcohol dehydrogenase; CAF-CoA, caffeoyl-CoA; CAF, caffeate; CAld5H, coniferaldehyde 5-hydroxylase; CatA, catechol 1,2-dioxygenase; CAT, catechol; CA, cinnamate; CHA, chorismate; CONIFalc, coniferyl alcohol; CONIFald, coniferaldehyde; CONIF-acet, coniferyl acetate; CYP2A6, cytochrome P450 monooxygenase; DAHP, 3-deoxy-D-arabino-heptulosonate 7-phosphate; DES, deep eutectic solvents; DHS, 3-dehydroshikimate; EGS, eugenol synthase; EUG, eugenol; E4P, erythrose 4-phosphate; FER-CoA, feruloyl-CoA; FER, ferulate; GAL, gallate; Hbald, 4-hydroxybenzaldehyde; HBA, 4-hydroxybenzoate; HCHL, hydroxycinnamoyl-CoA hydratase–lyase; HGA, homogentisate; Irp9, salicylate synthase; MA, muconic acid; NahG, salicylate hydroxylase; OAS1D, rice anthranilate synthase α-subunit; PAAS, phenylacetaldehyde synthase; PABA, p-aminobenzoate; PAR, phenylacetaldehyde reductase; PAT, phosphoribosyltransferase; pCA-CoA, p-coumaroyl-CoA; pCA-SHI, p-coumaroyl- shikimate; pCA, p-coumarate; PCA, protocatechuate; PEP, phosphoenolpyruvate; PHEald, phenylacetaldehyde; PHE, phenylalanine; PMT; p-coumaroyl-CoA/monolignol transferase, PobA, 4-hydroxybenzoate 3-monooxygenase; PPA, prephenate; PP, phenylpyruvate; QsuB, 3-dehydroshikimate dehydratase; SA, salicylate; SINAPald, sinapaldehyde; SYRINGald, syringaldehyde; TAL, tyrosine ammonia-lyase; TnaA, tryptophanase; TRP, tryptophan; TRP5, Arabidopsis feedback-insensitive anthranilate synthase α-subunit; TYR, tyrosine; UbiC, chorismite pyruvate-lyase; UGT, indoxyl glucosyltransferase; VAN, vanillin; 2-PE, 2-phenylethanol (Source: Lin, C., Eudes, A. Strategies for the production of biochemicals in bioenergy crops. *Biotechnol Biofuels* **13,** 71 (2020). https://doi.org/10.1186/s13068-020- 01707-x. This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution, and reproduction in any

bioproducts with commercial value for the production of nutraceuticals, flavors/scents/colors, biofuels, and biopolymers (Lin and Eudes 2020).

Isoprenoids are all derived biosynthetically from the five-carbon precursor isopentenyl pyrophosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP), for which the ratio is controlled by IPP isomerase (IDI/IPI). The biosynthesis of IPP or DMAPP in plants occurs via two spatially distinct pathways, which are the cytosolic mevalonate (MVA) pathway (McGarvey and Croteau 1995) and the plastidial methylerythritol phosphate (MEP) pathway (Fig. 6.9).

MVA pathway is generally present in germane to Archaea and in most eukaryotes, fungi (*Saccharomyces cerevisiae*), plant cytoplasm, and some bacteria, while the MEP pathway is germane to Bacteria and exists in most cyanobacteria, algae, plant chloroplasts, and some protozoa (Li et al. 2020). In addition, it has been suggested that both pathways are antagonistically regulated by the circadian clock, which may be exploited as an engineering strategy to orchestrate gene expression of the two pathways for production of desired products (Li et al. 2020).

With recent advancements in plant synthetic biology, implementation in crops of multigene isoprenoid metabolic pathways to develop biofuels and bioproducts at industrial scale is becoming attainable (Tatsis and O'Connor 2016; Wright and Nemhauser 2019).

The amount of specialized isoprenoids in plants is low and has to be improved through genetic engineering (Moses et al. 2013). Therefore, in order to produce sustainably bioactive isoprenoids at higher titers, boosting the common precursors within the MVA or MEP pathways via plant metabolic engineering represents an attractive option. The biosynthetic pathways of isoprenoids are known to be highly regulated, and studies have been conducted to regulate MVA or MEP pathways (Hemmerlin et al. 2012; Banerjee and Sharkey 2014). For terpenoid biosynthesis, IDI/IPI and several other enzymes in the MVA pathway (HMGR, HMGS, MVK, and PMK/PVK) and the MEP pathway (DXS, DXR, and HDR) are rate- limiting enzymes (Cordoba et al. 2009 Wang et al. 2019; see review by Li et al. 2020) (Fig. 6.2). Vickers et al. (2014) reviewed systems and synthetic biology tools for overexpression experiments using various MEP and MVA pathway genes in plants. The simplest approach is to overexpress or repress target synthase genes (Vickers et al. 2014). The entire cytosolic MVA pathway was inserted onto the tobacco chloroplast genome and homoplastic transgenic plants were regenerated. These plants had increased levels of mevalonate, carotenoids, squalene, sterols, and triacylglycerols compared with control plants, and it is reasonable to presume that these plants can also be engineered to produce high levels of volatile isoprenoids (Kumar et al. 2012).

**Fig. 6.8** (continued) medium or format, as long as you give appropriate credit to the original author (s) and the source, provide a link to the Creative Commons license, and indicate if changes were made)

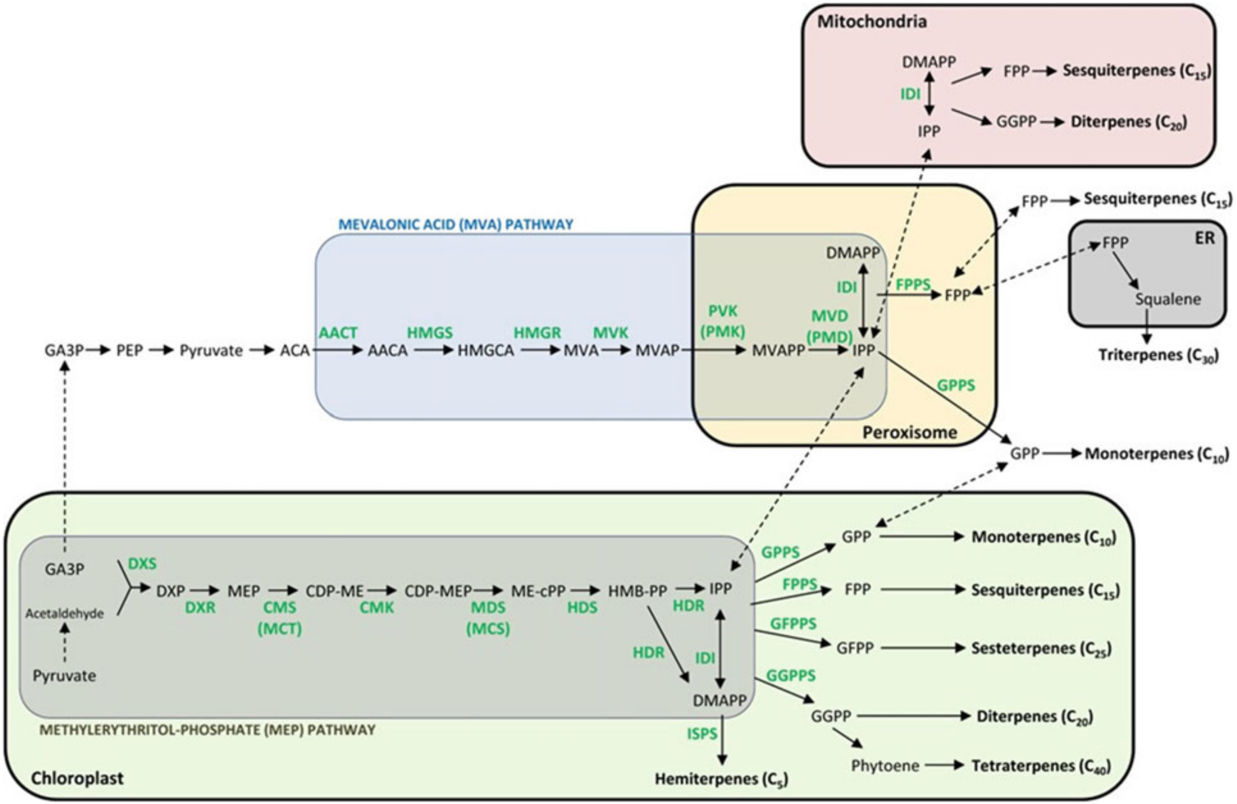

**Fig. 6.9** A depict of isoprenoid biosynthesis from cytosolic MVA and plastidial MEP pathways. AACA, acetoacetyl-CoA; AACT, acetoacetyl-CoA thiolase; ACA, acetyl-CoA; CDP-ME, 4-diphosphocytidyl-2-C-methylerythritol; CDP-MEP, CDP ME 2-phosphate; CMK, CDP-ME kinase; CMS/MCT, CDP-ME

synthase; DMAPP, dimethylallyl diphosphate; DXP, 1-deoxy-D-xylulose 5-phosphate; DXR, DXP reductoisomerase; DXS, DXP synthase; ER, endoplasmic reticulum; FPP, farnesyl diphosphate; FPPS, farnesyl diphosphate synthase; GA3P, glyceraldehydes-3-phosphate; GFPP, geranylfarnesyl diphosphate; GFPPS, geranylfarnesyl diphosphate synthase; GGPP, geranylgeranyl diphosphate; GGPPS, geranylgeranyl diphosphate synthase; GPP, geranyl diphosphate; GPPS, geranyl diphosphate synthase; HDR, HMBPP reductase; HDS, HMB-PP synthase; HMB-PP, 1-hydroxy-2- ethyl-2-(E)-butenyl 4-diphosphate; HMGR, HMG-CoA reductase; HMGCA, 3-hydroxy-3-methylglutaryl-CoA; HMGS, HMG-CoA synthase; IDI/IPI, IPP isomerase; IPP, isopentenyl pyrophosphate; ISPS, isoprene synthase; MCS/MDS, ME-cPP synthase; ME-cPP, ME 2,4-cyclodiphosphate; MVA, mevalonate; MVAP, mevalonate-5-phosphate; MVAPP, mevalonate 5-diphosphate; MVD/PMD, MVAPP decarboxylase; MVK, MVA kinase; PEP, phosphoenolpyruvate; PVK/PMK, MVAP kinase; MEP, 2-C-methyl-D-erythritol-4-phosphate (Source: Lin, C., Eudes, A. Strategies for the production of biochemicals in bioenergy crops. *Biotechnol Biofuels* **13,** 71 (2020). 10.1186/s13068-020-01707-x This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made.)

## 6.13 Agri-Waste to Value-Added Products

Bioconversion of agri-food waste to value-added products is very important toward zero waste and circular economy concepts. Microbes are valuable sources for a range of bioactive molecules, including microbial pigment production through fermentation and/or utilization of waste. Usmani et al. (2020) reviewed some of the recent advancements made in important bioengineering technologies to develop engineered microbial systems for enhanced pigments production using agri-food waste biomass/by-products as substrates in a sustainable way. The use of microorganisms either aerobically or anaerobically could be a sustainable and eco- friendly solution for food waste management by generating biofuels, electrical energy, biosurfactants, bioplastics, biofertilizers, etc. (Sharma et al. 2020; Kumar et al. 2022 in press) (see also Kumar et al. 2022a, b, c, d in press).

## 6.14 Discussion

Significant improvements in genetic engineering and plant synthetic biology have emerged in recent years and are expected to support rapid, precise, and robust engineering of plants (Shih et al. 2016a, b). Besides agricultural yield for the purpose of food production, modern society also requires the use of crops as a clean energy source in the form of biofuels. Shih et al. (2016a, b) reviewed recent advances made in the field of plant synthetic biology, specifically in genome editing, transgene expression regulation, and bioenergy crop engineering, with a focus on traits related to lignocellulose, oil, and soluble sugars. Because the bulk of synthetic biology has focused on microbes, plant synthetic biology has inherent and specific challenges.

*E. coli* has been the predominant chassis of choice for the synthetic biology community, but plants have different physiologies, metabolisms, life cycles, and environmental niches, making it difficult to expect that all synthetic biology tools can be broadly transferable between plants. Although synthetic biology has greatly increased the throughput of DNA assembly, the low transformation efficiency of plants is a major bottleneck that will be needed to advance the field, especially to develop high-throughput assays in non-model systems (Shih et al. 2016a, b).

Genetic improvement of crops started since the dawn of agriculture and has continuously evolved in parallel with emerging technological innovations. Plant metabolic engineering is still at a development stage and faces several challenges, in particular with the time necessary to develop plant-based solutions to bio-industrial demands. The recent success of several metabolic engineering approaches applied to major crops is encouraging, and the emerging field of plant synthetic biology offers new opportunities. Some pioneering studies have demonstrated that synthetic genetic circuits or orthogonal metabolic pathways can be introduced into plants to achieve a desired function. The combination of metabolic engineering and synthetic biology is expected to significantly accelerate crop improvement (Pouvreau et al. 2018).

Synthetic biology concerns the engineering of man-made living biomachines from standardized components that can perform predefined functions in a (self-) controlled manner (Ausländer et al. 2017). In particular, the in vitro design, synthesis, and transfer of complete genomes into host cells point to the future of synthetic biology: the creation of designer cells with tailored desirable properties for biomedicine and biotechnology (Ausländer et al. 2017).

The construction may be in the native or a non-native setting. Though the scientific community often uses the phrase "genetic engineering," in practice, genetics was never engineered. Synthetic genetics is a subdiscipline of synthetic biology that aims to develop artificial genetic polymers (also referred to as xeno-nucleic acids or XNAs) that can replicate in vitro and eventually in model cellular organisms. This field of science combines organic chemistry with polymerase engineering to create alternative forms of DNA that can store genetic information and evolve in response to external stimuli.

The term—xeno-nucleic acids, abbreviated XNA, has grown in popularity to the point that it has become a catch-all phrase for almost any unnatural nucleic acid, raising the question: what is XNA and how does it differ from chemically modified DNA? The XNA can be used to safeguard synthetic biology organisms by storing genetic information in orthogonal chromosomes. Anosova et al. (2015) provided a structural perspective on known antiparallel duplex structures in which at least one strand of the Watson–Crick duplex is composed entirely of XNA.

All genetic polymers, like RNA shown here, are composed of building blocks called nucleotides that contain a sugar moiety (black), phosphodiester linkage (red), and nucleobase (green). The identity of the sugar determines whether the polymer is DNA, RNA, or XNA (Nguyen et al. 2020).

The budding yeast *Saccharomyces cerevisiae* is a popular cell factory platform, with several genetic and molecular tools available to facilitate the production of compounds of interest for the chemical and pharmaceutical industry. The synthesis of increasingly complex molecules, in turn, require successful construction of large and complex heterologous pathways, which impose an added metabolic burden by competing for required cellular resources with native metabolism. Competition for a common pool of ATP, cofactors, coenzymes, and precursors between native and heterologous pathways can adversely affect growth, lengthen fermentation, and decrease product titers and yields. One way to avoid this problem is to activate the production pathway after the major growth phase

Biomaterial production necessitates fine-tuned control of gene expression and pattern formation. Advancements in synthetic biology tools for spatial programming will enable synthetic consortia to produce materials of defined sizes, shapes, and patterns.

## 6.15 Future Directions and Concluding Remarks

CRISPR/Cas9 gene editing technology has successfully introduced modifications at target DNA sequences in many plant species including *Arabidopsis*. The plant DNA damage response under stressful conditions is a key process influencing plant growth and development in response. Characterizing various novel interactions between DNA repair proteins in response to stress will open new avenues for crop improvement under climate change and resulting stresses. Furthermore, with the advent of CRISPR-Cas9 screens, it will be exciting to identify novel genes involved in DNA repair in plants not otherwise possible by classical genetics. Besides this, it is important to understand the link between DNA repair and chromatin dynamics. DNA repair proteins and processes require access to the DNA damage, which requires extensive chromatin remodeling and epigenetic modifications at the site of the DNA damage (Raina et al. 2021). After the target gene is edited, the CRISPR/Cas9 construct needs to be removed to ensure genetic stability and to gain any regulatory approval for commercial applications. However, removal of the transgenes by genetic segregation, backcross, and genotyping is very laborious and time-consuming. Yu and Zhao (2019) developed the methods which allow fast and effective isolation of transgene-free T2 *Arabidopsis* plants with the desired modifications at the target genes.

The goals of the synthetic biology discipline: robust, predictable, and rapid engineering of biology align well with this vision (Cameron et al. 2014a, b). To date, much synthetic biology research has focused on microbial engineering (with the vast bulk of that being the model organism *Escherichia coli*). Plant synthetic biology has necessarily been more limited and has faced different challenges, such as life cycle length. However, advances in synthetic biology can play an important role in it and will help develop pathways for biofuel production (Bhansali and Kumar 2018; Kumar 2020).

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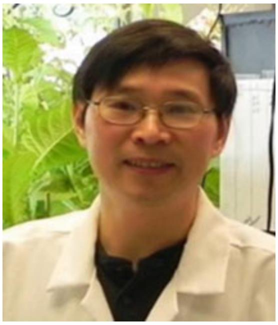

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# Handmade Paper Industry: A Green and Sustainable Enterprise and Its Challenges

# 7

Nisha, Raman Singh, and Kuldeep Singh

## Abstract

Due to stringent environmental pollution, development, and implementation, green technologies have a challenge for technology developers. The handmade paper industry emerged as a green and sustainable enterprise for the high demanding paper industry. The paper industry uses raw materials containing cellulose fibers, generally wood, recycled paper, and agricultural residues. Nonwood raw materials (bagasse, cereal straw, etc.) comprise approximately 60% of cellulose fibers. The industrialized process for paper manufacturing involves many stages like raw material preparation, pulping, screening, chemical recovery, bleaching, stock preparation, and paper-making—the paper industry facing challenges of availability of raw material and environmental protection.

Due to the pressure on forests, conventionally wood-based resources became scarce and uneconomical. Their scarceness has already led to a decline in capacity utilization in the Indian paper industry. Researchers try to enhance the level of sustainable development, economy, and societies. Therefore, the handmade paper industry must be developed to meet the increasing demand for paper products in an environment-friendly way. The handmade paper industry has untapped potential for environment-friendly products and production systems. Therefore, in social, environmental, and economic scenarios, papermakers must find new

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market opportunities while balancing their sustainability and profitability. This chapter describes sustainability and challenges for the paper industry and explains how to face this situation.

**Keywords**

Sustainability · Handmade paper · Forest · Pulp · Aromatic extraction · Area · Separation Process

## 7.1 Introduction

Paper manufacturing is a typically diversified and growing process industry that produces different types of paper. In India, the manufacturing of handmade paper starts in third century B.C., and this traditional knowledge is preserved through generations of craftsmen popularly known as "Kagzis." The word "Kagaz" meaning paper has its origin from "Kagzis." In India, the handmade paper industry is growing, and handmade paper products are gaining demand in national and international markets. Handmade paper has several uses including decorative, envelops, notebooks, etc. (Fig. 7.1). Thus, the handmade paper industry has achieved a commercial venture status and a sound business proposition (Ragauskas et al. 2006).

**Fig. 7.1** Products made from handmade paper

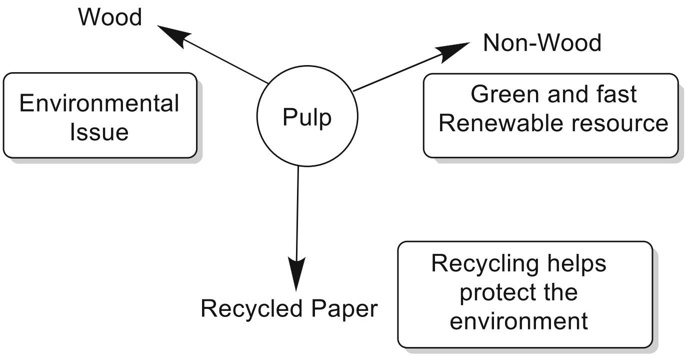

**Fig. 7.2** Sources of pulp in pulp and paper industry

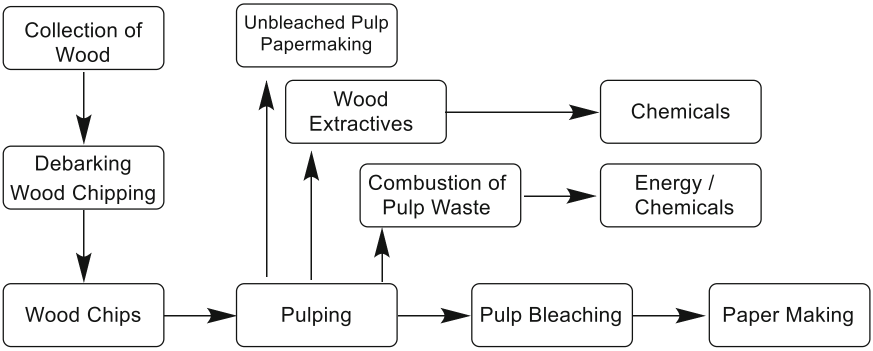

**Fig. 7.3** Pulp mill operations

Wood pulp is used to manufacture papers in the early nineteenth century in Europe. The paper-making process consumes many trees, harmful chemicals, and a considerable amount of water, resulting in water and soil contamination. Generally, raw materials for pulp and paper-making are cellulose fiber from wood, agricultural residues, and recycled paper (Fig. 7.2).

The industry depends on woody raw material, and future growth is not sustainable (Ashori 2006). Some developing countries use nonwood such as bagasse, cereal straw, bamboo, jute, and sisal as raw materials to produce cellulose fibers. In general, the necessary process for paper manufacturing is a two-step process – pulp preparation, and then the pulp is converted into paper. However, to reach the final step of paper-making, the pulp is processed in several stages. These stages are raw material preparation, pulp manufacturing, pulp washing and screening, chemical recovery, bleaching, stock preparation, and paper-making (Fig. 7.3). The harvested wood is processed to remove the lignin from wood pulp, either mechanically or chemically. The pulp is bleached. At this stage, pulp is processed based on the type and grade of required paper.

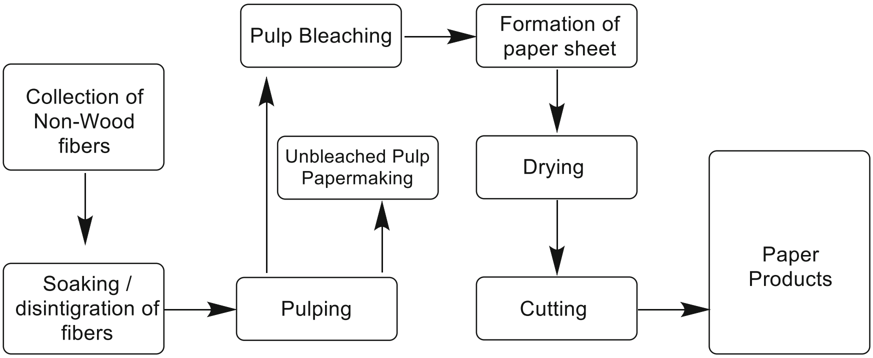

**Fig. 7.4** Handmade paper production workflow

The concept of making eco-friendly papers emerged in response to the environmental perspectives. Handmade paper is an eco-friendly paper made from nonwood fibers. Making of handmade paper is free from all chemicals, and it is also a biodegradable product. The manufacturing of handmade paper does not require some new type of types of equipment or machine. Its manufacturing utilizes the same pieces of equipment used for standard paper-making of wood fibers (Fig. 7.4).

Due to the increasing use of papers and paper products, most industrial setups use recycled papers to produce pulp. Non-recycled paper is either landfilled or incinerated. Separate as well as integrated setups are possible for pulp mills and handmade paper mills. After manufacturing pulp in mills, fiber is used for further processing in making different grades of paper or cardboard. Demand for raw material and pulp regularly increases due to the growth of the pulp and paper industry (Abd El-Sayed et al. 2020) In recent years, nonwood fibers have been used as a raw material for paper-making as paper made from nonwood fibers has a fine quality over wood fibers. Additionally, nonwood fibers are also used to overcome the shortage of wood fibers in certain countries. Besides economical to wood pulp, the paper industry also faces some problems with the use of nonwood fibers. However, in the last few years, technology development has overcome its competitiveness to wood pulp. However, some countries focus only on nonwood fibers due to the limited supply of wood fibers.

Due to environmental considerations, and the quality of finished paper manufactured from nonwood fibers, countries with adequate wood supply show their interest to increase this trend. Due to environmental concerns such as depleting forest resources and disposal of agricultural residues, the demand for nonwood plant fibers for paper-making increases in Europe and North America. European countries have faced a shortage of short fibered hardwood pulp, and some nonwood fibers can replace it. The processes and technology are available in the countries already using these raw materials in the paper industry. Several countries are already using different nonwood fibers for paper-making. Straws are one of the largest sources of nonwood fibers. The other major contributors in this category are bagasse and bamboo (Holbrey and Rogers 2002; Ragauskas et al. 2006).

Nonwood plant fiber can be grouped in two categories:

1. *By-products or recovered products from other industries or agriculture*
   These substances are used as common nonwoods or hardwood substitutes. Common examples are agricultural waste such as cereal straws, bamboo (shorter fiber species), sugarcane bagasse, esparto, sorghum stalks, kenaf (whole stalk or core fiber), corn stalks, reeds and grasses, and horticulture waste.
2. *Specialty nonwoods or softwood substitutes*
   Crops such as cotton staple and linters; sisal; abaca; bamboo (longer fiber species), flax, kenaf bast fibers, and hemp; hesperaloe; etc.

   Wood pulp is still the major source for most of the paper manufactured, and unfortunately trees are used as the primary source of wood pulp. According to a survey in the manufacturing of 1 ton of paper, 277 eucalyptus or 462 bamboo plants are needed. Therefore, desertions of forests govern on a significant transformation in technology, and so papermakers found a renewed interest in nonwood fibers for the better quality of the paper. For the paper industry, this value addition of the paper manufacturing process opens its business to export in other countries. On the other hand, a survey shows that making one lakh greeting cards on handmade paper save 500 trees. Handmade paper has greater tensile, bursting, tearing, and double-fold strength as compared to conventional papers. Handmade paper is affordable, eco-friendly, and biodegradable. It can be recycled, and is excellent for writing, printing, and utility.

## 7.2 National/International Demand

Indonesia, Malaysia, and the Philippines have emerged as *handmade paper giants*. The Indian handmade paper industries have considerable potential to complete the increasing demand for handmade paper products. To maximize the production of handmade paper, the industry utilizes the paper recycling process in an environmentally sound way. Undoubtedly, the use of eco-friendly paper offers a self-sustainable society.

Handmade paper has been used in many ways. For example, common uses are office stationery, drawing and documentation sheets, writing pads, conference folders, and computer printouts. It is customarily used for printing certificates and degree awards by several universities and institutions. Fancy products and diaries, photo frames, paper bags, and a variety of other decorative and showcase products are made from handmade paper. The export market for Indian handmade paper and its products has been in exceptional growth, especially in exporting to developed countries like the USA, Germany, Europe, Australia, etc.

The countries like Germany, Sweden, the USA, Japan, and Italy use approximately 73%, 69%, 49%, 60%, and 45% of waste paper annually, while in India, it is very low, about 20%. The major handmade industries are located in Northern India, Rajasthan and New Delhi, and also in southern India, Tamil Nadu, Pondicherry, and Hyderabad. Several small- and medium-sized handmade paper industries have been developed and established nearby Hyderabad. The world's largest center of the handmade paper is situated in Sanganer near Jaipur in Rajasthan. In India, training on research and production of handmade paper-making is provided by well-established and recognized institutes, viz., M.R. Exports Vijayawada; Jabalpur Chamber of Commerce & Industry for EDP; Shri AMM Murugappa Chettiar Research Centre, Chennai; and Kalpana Handmade Paper Industries, Sanganer, Jaipur (Hirsch et al. 2006).

## 7.3 Composition of Wood

The pulp is a significant component used worldwide in the manufacture of paper. It also has increasing importance in the wide variety of cellulose products in the food, pharmaceutical, and textile industries. Globally, wood provides approximately 90% of pulp production and the remaining 10% originates from annual plants. Wood is an organic material. The major elements of wood are carbon, hydrogen, and oxygen. Besides these elements, it contains a lower quantity of nitrogen and other inorganic elements such as sodium, potassium, calcium, magnesium, and silicon.

Table 7.1 shows the elements of wood. These elements constitute macromolecules known as polymers, which form the cell wall. This cell wall of wood is composed primarily of cellulose, hemicellulose, lignin, and small amounts of extractives. Table 7.2 summarizes the typical composition of wood resources (Ramage et al. 2017).

**Table 7.1** Composition of wood (elements present in the wood)

| Element(s) | Content (%) |
|---|---|
| Carbon (C) | 49 |
| Hydrogen (H) | 6 |
| Oxygen (O) | 44 |
| Nitrogen (N) | <1 |
| Other inorganic elements (Na, K, Ca, Mg, Si) | <1 |

**Table 7.2** Composition of wood

| Component | | Content (%) | |
|---|---|---|---|
| | | Softwood | Hardwood |
| Cellulose | | 40–44 | 40–44 |
| Hemicellulose | | 30–32 | 15–35 |
| Lignin | | 25–32 | 18–25 |
| Different building blocks of lignin | p-Coumaryl alcohol | 0.5–3.5 | Trace |
| | Coniferyl alcohol | 90–95 | 25–50 |
| | Sinapyl alcohol | 0–1 | 50–75 |

### 7.3.1 Cellulose

Celluloses are present in softwoods as galactoglucomannans and arbinoglucuronoxylan, minor amounts of arabinogalactan, xyloglucan, and other glucans. The predominant hemicelluloses for hardwoods are glucomannan and glucuronoxylan, with lesser amounts of galactans and glucans.

### 7.3.2 Hemicellulose

Hemicellulose is a heteropolymer with side chains. It is composed of pentoses (xylans), alternating units of mannose and glucose (mannans or glucomannans), or galactose units (galactans). The amount of sugar monomers can change quite dramatically for different feedstock sources.

### 7.3.3 Lignin

Of the three major biopolymers that constitute wood, lignin is distinctly different from the other macromolecular polymers. Lignin is an amorphous polyphenolic polymer that is synthesized by enzymatic dehydrogenative polymerization of 4-hydroxyphenyl propanoid units (Fig. 7.5).

The cellulose content of most of nonwoods is comparable with that of woods commonly used for paper-making. The nonwoods have lower lignin content, making delignification relatively easy and environment-friendly by consuming less chemicals.

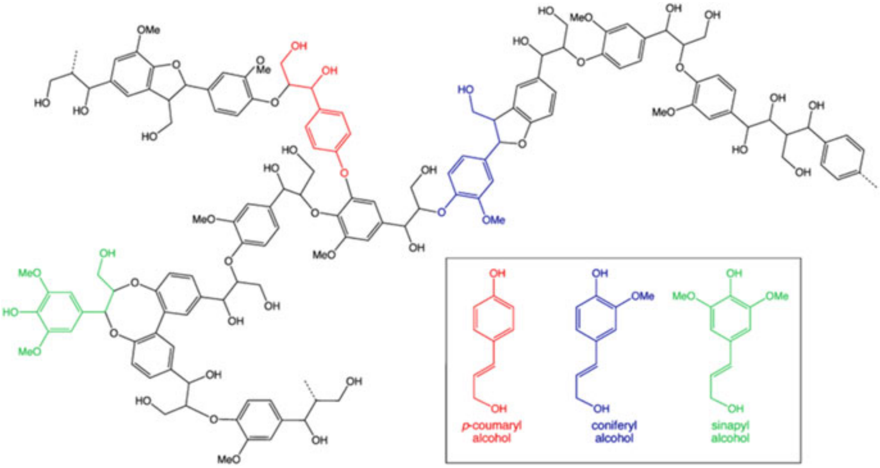

**Fig. 7.5** Structure of lignin

**Advantages of Using Nonwood Fiber (Rodríguez et al. 2008; Rousu et al. 2002):**

- A smaller content of lignin than wood
- Does not require chipping
- Does not require debarking
- Speedy growing fiber resource
- Nonwood pulp produced at low temperatures
- Require a lower quantity of chemicals such as caustic soda
- Require only a simplified process to industry
- Because of the thin structure, easier impregnation of cooking chemicals
- Does not present shives
- Feasible manufacturing processes even to a smaller factory
- The easier beating of nonwood pulp fibers
- Easier bleaching than wood fibers
- Require lesser refining energy for achieving the same degree of freeness
- Require low chemical for bleached to achieve high brightness
- Require less energy in pulping than wood fibers
- Economic benefits to food crops due to pulping of the nonwood fiber materials.

**Some of the Characteristic Features of Handmade Paper Are Mentioned Below:**

- Manufactured almost all types of papers such as printing and writing papers, linerboard, corrugating medium, newsprint, tissue, and specialty papers
- Produce a wide variety of fancy color decorative wraps
- Gives an elegant and smooth surface for writing
- The indestructibility and excellent strength for performance
- High tensile bursting, tearing, and double-fold strength as compared to mill made paper
- Produce unmatched texture of drawings used by artists or engineers

## 7.4 Easily Availability of Machine/Equipment

Rag chopper, beaters, pulp tanks, calender machine, agitator, hydraulic press, cylinder mold, Vat power drives machine, iron box, etc. are major tools and types of equipment used for handmade paper-making. All these machines and equipment can be easily made by many Indian manufacturers, thus supporting local development in other areas.

## 7.5 Disadvantages of Using Nonwood Fiber (Bajpai 2018)

Though nonwood fibers have many advantages over wood fibers, it has some disadvantages too:

- Constant availability and annual supply of fibers to mills.
- Most nonwoods are annual plants.
- Require storage of the harvested crop over several months due to short harvesting seasons.
- Need special attention to collection, transportation, and storage of some raw materials like agricultural residues to avoid moisture content.
- Consumption of more chemicals for some of nonwood fibers like straw, thereby reducing strength of the pulp.
- Face difficulty in handling of agricultural residues than wood chips due to their bulkiness, so reducing the pulp yield.
- Increase the cost of transport due to their low density and high volume than wood.
- Required large storage capacity for continuous pulp production.
- Has high ash content of nonwood plants (1–20%) while in softwoods and hardwoods <1%.
- Limitations in paper quality and drainage problem of pulp due to shorter length of nonwood fiber.
- Shorter length of fiber causes slow drainage, reducing the processing speed and lengthening of the drying section, thereby significantly increasing energy and cost of the process.
- Presence of high silica content in nonwood fiber causes dissolution in black liquor, so increasing viscosity of black liquor.
- Removal of moisture content from nonwood fiber significantly increases the capital investment.

## 7.6 Challenges for Handmade Paper Manufacturing Process

The processing of raw materials has different types of negative and positive impact on the environment depending upon the technologies used by manufacturer. Among all the processing steps, recycling of waste paper is a major positive factor to decrease the load on trees, thereby known as reducing the environmental impact. The negative impact on environment is the leakage of harmful compounds at the outputs of the manufacturing process units.

### 7.6.1 Challenges for Raw Material

Environmental issues that arose from deforestation for raw material supply became the main challenges. Other challenges are global trade flows and collection systems for recovered paper. Moreover, the recovered paper should be optimized as raw materials and encouraged as a source of renewable energy. Other than recovered paper, nonwood plant fibers are prominent to use to address the environmental concern. In this kind of raw materials, the cellulose content is generally around 50%, close to wood. For instance, cotton, hemp, sisal, and kenaf, other nonwood fibers, are becoming increasingly crucial in pulp and paper-making. They are available abundantly and regularly from different sources. The old conventional methods to make pulp fiber are very complicated chemical processes that affect the environment. However, pulp obtained from recycled paper is an alternative process that requires less energy and a low quantity of chemicals (Cabalova et al. 2011).

### 7.6.2 Strengthening of Handmade Paper

Softwood kraft or sulfite pulp is added to typical nonwood pulps or hardwood substitutes to provide the strength to the paper. Specialty nonwood pulp may be used for producing 100% nonwood paper or blended with waste paper pulp in the furnish depending on the quality and grade of paper. Due to the superior properties of specialty nonwoods to softwoods, it may be blended in a lower amount with softwood or wood pulp to produce lightweight and ultralightweight printing and writing papers. These specialty pulps can produce specialty papers such as currency, cigarette papers, tea bags, dielectric paper, etc. Thus, possible combinations of wood pulp, nonwood pulp, and recycled waste paper pulp are made to optimize the best possible conditions for meeting the specific customers' requirements.

### 7.6.3 The Degree of Difficulty in the Performance of Fiber Materials During Pulping

The performance of fiber material can be estimated by the following factors (Ashori 2006; Iskalieva et al. 2012; Li 2013; Lönnberg et al. 1996):

1. **Economical viability of pulping**—Whether the fiber contents and forms are readily available, and the overall pulping process is economical.
2. **Delignification and fiber dissociation**—The lignin content is one of the deciding factors in difficulty on the pulping process's degree.
3. **Adaptability of pulping methods**—The pulping method should be convenient for stock preparation and quickly adaptable.
4. **Quality and performance of pulp**—The color, degree of bleaching difficulty, drainability, and beating pulp performance.

### 7.6.4 Challenges in Pulping

In general, pulping of nonwood fibers is easier as compared to wood fibers. Due to low lignin content in nonwood fibers, its cooking requires fewer chemicals during the cooking process. Raw materials are cooked at high temperatures and low pressure so that lignin can be separated from the fibers. The main cooking processes used for nonwood fibers are soda, sulfite, and sulfate processes, while for wood, kraft or sulfate is preferred (Sridach 2010).

Most nonwood plants are high in silica content. This high content of silica causes many problems. During the pulping and chemical recovery process, the presence of silica and ash in spent liquors of nonwood fiber creates difficulty in drainage, causing problems in the smooth formation of paper-making. These spent liquors contain high concentrations of inorganics so that traditional systems cannot treat it. The cellulose, hemicellulose, lignin, and extractive contents are fundamental to pulping. Papermakers have done a significant development by using lime alkali oxygen pulping to solve this problem. The addition of lime solves the problem due to silica. Calcium silicate is formed on the addition of lime to the cooking liquor, which is insoluble in water. Thus, the presence of silica in the pulp becomes an improvement during the peroxide bleaching. Low yield is another problem during the pulping of the nonwood plant. However, some nonwood fibers have high annual yields per hectare. Kenaf has about two times higher the average annual yield per hectare than that of fast-growing softwood fibers (Liu et al. 2018).

The pulping of nonwood materials is favored over wood fiber in several ways. However, there are many disadvantages too. Handmade paper-making is a nonpolluting process that has advantages over the conventional process of paper-making (Jain et al. 2017; Muraleedharan and Perumal 2010).

## 7.7 Environmental Effect

As the making of paper has an impact on the environment, its consumption is often discouraged. The conventional methods for the manufacturing of paper use cellulose from felling trees, thereby increasing global warming. These conventional methods also use various chemicals that release toxic substances into the environment, thereby enhancing environmental pollution. $CO_2$ coming from burning fuels and black liquors generates electricity and steam used to run the plant. During the chemical pulping process, some gases are formed which pollute the environment, such as $SO_2$ and $NO_x$. The emission of both these gases contributes to acid rain (Sixta 2008). Mills switched from heavy fuel oil to low sulfur and sulfur-free fuels for electricity generation and heating, replaced fuel oil with natural gas, and implemented careful process-control techniques, all of which helped. Table 7.3 shows the primary pollution sources during the manufacturing of paper (Gavrilescu et al. 2012) (Fig. 7.6).

Paper recycling has a significant effect on the sustainable development of the paper industry. Environmental issues have been addressed by different organizations using suitable alternatives for wood pulp. In the USA and Europe, ~40% of the recovered paper is included in paper-making. However, paper industries in China used a mixture of vegetable pulps and waste paper as raw materials. Due to environmental issues, the industry was also forced to use the recovered paper as raw material.

During the paper manufacturing process, a considerable quantity of wastewater per ton of paper is generated. Therefore, to overcome water pollution, this resulting

**Table 7.3** Pollution sources in producing pulp and paper

| S. No. | Process step | Pollutants |
|---|---|---|
| 1. | Pulp bleaching | Air emissions |
| | | Dissolved material |
| | | Residual chemicals |
| | | Wastewater |
| 2. | Pulp manufacture | Air emissions |
| | | Wastewater |
| 3. | Pulp drying | Air emissions |
| 4. | Paper manufacture | Dissolved material |
| | | Residual chemicals |
| | | Solid wastes |
| | | Wastewater |
| 5. | Pulp washing and screening | Dissolved material |
| | | Residual chemicals |
| | | Wastewater |
| 6. | Wood preparation | Solid wastes |
| | | Wastewater |

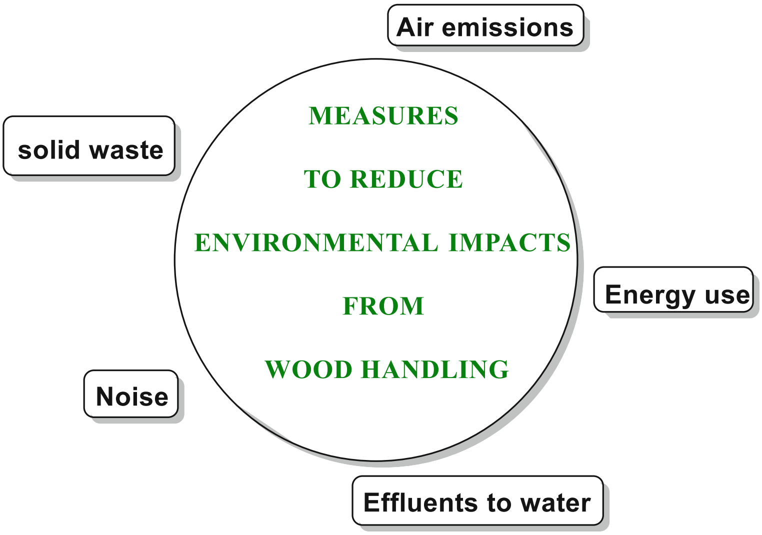

**Fig. 7.6** Measure to reduce environmental impact from wood handling

waste paper must be processed in a wastewater treatment plant by the handmade paper industry.

The handmade paper production uses completely nonwood and recycled fibers, thus utilizing non-forest raw materials. Therefore, it also consumes less energy, conserves natural resources like trees, and saves the environment from global warming. Hence, this is more advantageous to conventional paper production processes, which are based on forest entirely. Due to depletion in forest, there is

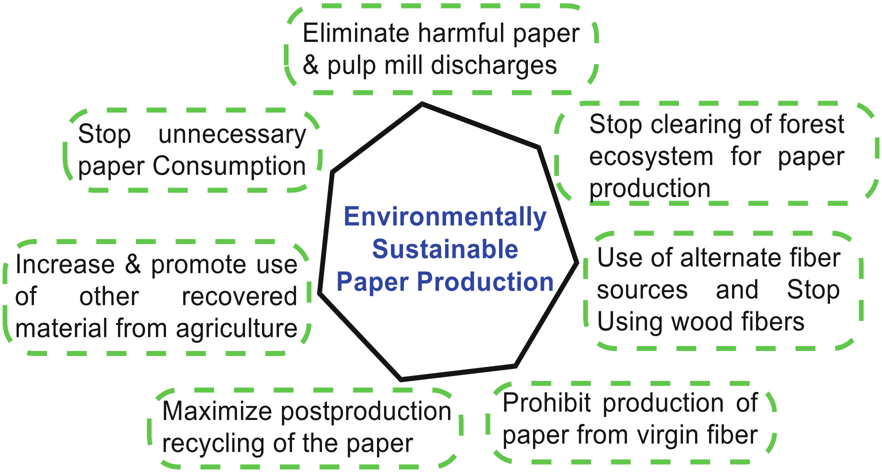

**Fig. 7.7** Objectives for environmentally sustainable paper production

more demand for handmade paper production units. Hence, economic optimization and environmental protection also encounter researchers' minds to attract complete attention in this area (Cabalova et al. 2011) (Fig. 7.7).

The enhancement in raw material can be done using biomass materials and agricultural residues such as straws, rice husks, and grasses. The industry may initiate itself to grow, specifically the nonwood biomass materials for handmade paper production. The Indian handmade paper industry uses cotton rags, equally white, and colored or waste paper in this direction. The population is increased day by day, and rapid development decreases the forest area paying the environment to a high cost, which is the primary concern of the world (Jain et al. 2017; Rani et al. 2011; Subramanian and Kumar 1995).

## 7.8 Economic Effect

In the current scenario, the handmade paper industry has become a growing paper industry. Later in the past, this sector has around 42 production units, and nowadays, production units are increased magnificently to more than 3000. In India, handmade paper units are spread over the country. Some of these are situated in the districts of Kurukshetra (Haryana), Kalapi (Jhansi, Uttar Pradesh), Sanganer (Rajasthan), Pune (Maharastra), Mahaboobnagar (Andhra Pradesh)), and some clusters in West Bengal *(Khadi Village Industry Corporation, India).*

The paper industry is one of the core industries concurrent with basic human needs such as educational material production, billing papers, carry bags, etc. Thus, this improvement brings well-being to society. However, the use of information technology and digital media platforms has been increasing day by day globally, but it cannot affect the demand for paper. Its consumption is also increasing. In developed countries, per capita, paper consumption is higher. Among these, the top five highest per capita paper consumption countries are Austria, Finland, Germany, the USA, and Sweden. However, per capita consumption of paper in India is below the global average of 55 kg, approx. 10 kg per capita (Jain et al. 2017).

Currently, the handmade paper industry has its craft paper as well as other converted product units. Today, the demand for handmade paper products, such as fancy, gift, and decorative items, photo albums, stationery items, packaging items and carry bags, drawing papers, greeting and invitation cards, and business cards, are increasing in the market day by day due to showing their significant benefit to the environment. Thus, the handmade paper's value-added products provide a strong impetus to the national and international markets to invest them. It is a paradigm for many other traditional small-scale industries that could successfully save the country at challenging times of recession. Crops and agricultural waste also contribute intensively to the production of biofuels. The pulp and paper industry in the USA processes approximately 108 million tons of wood annually to produce pulp and paper sustainably (Hubbe and Bowden 2009).

Sustainability in the paper industry remains controversial. The reason is a high capital-intensive paper-making process, which consumes a higher quantity of cellulose, energy, and water. However, sustainability is tried to be balanced by using various economic, social, and scientific factors. And all these factors become a challenge for the beneficial outcome of the paper industry. The paper industry comprises several small, medium, and large enterprises. Different mills have variable sustainability, depending on their location. It also depends on their stakeholder's potentials, that is, how they are going to find accessibility in markets. Paper and board are high-quality, safe, recoverable, and reusable materials for the paper and pulp industry (as secondary fibrous raw materials) or biofuel production. Suppose all paper products are tried to be produced in a responsible manner where resources can be used without harming the environment, then the whole paper-making process could be sustainable. Papermakers have adopted energy- and resource-saving measures that indirectly contribute to environmental performance (Finger and Schuler 2004; Hubbe and Bowden 2009).

## 7.9 Societal Impact

The manufacture of handmade paper is traditional art (Ghorbannezhad et al. 2011). It plays a vibrant role in becoming one of the critical export-oriented sectors of India. Paper plays an essential and fundamental part of our society, whether used for writing, printing, education, wrapping paper for gifts, carrying bags in daily life, or offices. Therefore, due to the increasing demand for paper, more production of paper is required. Nowadays, the production of paper is around 300 million tons. Thus, the handmade paper industries have more social responsibility. Besides this responsibility, paper industries work more sustainably among natural resources and economics (Jain et al. 2017).

Primarily rural areas are growing increment in the school-going children, increasing consumption/demand for paper production. However, modern digitalization and information technology use have increased, but it had not reduced paper demand. Therefore, there is a suitable demand for paper in India. The demand for paper in printing and writing can also be found emergent by rising in the modern retail sector, increasing documentation, and a jump in consumers.

## 7.10 Significance of Handmade Paper

Initially, the handmade paper was designed for decorative purposes. Nowadays, it has found uses as writing paper, craft paper, packaging, etc. Table 7.4 lists the advantages of the handmade paper unit over a standard integrated paper unit.

## 7.11 Future Research Areas

Nowadays, paper becomes an essential and fundamental part of society worldwide in everyday life. The handmade paper production reaches up to approximately 300 million tons each day worldwide. And approximately 90% of this paper is produced from mature pulpwood. Due to the increasing demand for paper, millions of trees are cut down every day. Therefore, researchers have looked into additional natural, biodegradable, and nonwood resources to complete the demand of finished paper due to the limited supply of raw materials. The researchers have investigated different kinds of agricultural nonwood lignocellulosic by-products.

In recent years, significant problems such as shortage of resources, contamination of the environment, and technical equipment forced the papermakers to face difficulties in developing the paper industry (Lönnberg et al. 1996). However, nonwood fibers have outstanding physical and optical properties of fine papers, but they account only a small fraction of the raw material throughout the world. Due to a shortage of wood resources, some developing countries use about 60% of the cellulose fiber coming from nonwood materials from the available by-products such as corn straw, bagasse, reed, grass, jute, flax, bamboo, and sisal (Ashori 2006).

**Table 7.4** Advantages of handmade paper unit over normal integrated paper unit

| Sl no | Description of resources | Handmade paper unit | Normal integrated paper unit |
|---|---|---|---|
| 1. | Consumption of resources like raw materials | Low | High |
| 2. | Water consumption per ton cubic meter | 150 | 250 |
| 3. | Electricity consumption | Low | More |
| 4. | Chemical consumption | Low | High |
| 5. | Pollution | Low | High |

In general, nonwood fiber mills are usually low-capacity plants. Therefore, their recovery systems are not good enough, neither technically nor economically. Hence, significant research is required in this field. In Asian countries, the pulp and paper-making industries are in the phase of developing their process. Some use different nonwood raw materials, such as cereal straw, reeds, grasses, and sugarcane bagasse for pulping and paper-making. Therefore, pulping methods required more attention to high-speed development (Bajpai 2015).

Upgradation of the handmade paper industry can be done by accumulating and developing a few specific market segments of new capacity units. However, the handmade paper industry's sustained growth will require further research in several key areas.

## 7.12 Conclusions

Handmade paper-making is a heritage art of India that creates something novel and useful with very few resources. India and other developing countries struggle with the scarcity of raw material, power resources, and lack of funds. The growth of the handmade paper industry is producing employment and also motivating an eco-friendly way of paper production. The handmade paper-industry is helping to manage waste, and it uses waste as a resource. The handmade paper industry is making a difference socially by promoting sustainable living. The industry encourages the reduction, recycling, and reuse of wastes as raw material for new products. Paper recycling for the manufacturing of handmade paper saves the natural wood raw stock, saving trees, generating less pollution, and saving the environment. It requires less total energy than producing virgin paper. It also decreases the overall capital cost to the handmade paper unit and reduces water consumption. Also, it has a favorable impact on the carbon dioxide balance and the greenhouse effect. All these benefits motivated researchers to overcome these drawbacks. Moreover, there is no doubt that the handmade paper industry is going to play an essential role in sustainability, waste management, and employment generation in the future.

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# 8

# Bioremediation Approaches and the Role of Microbes in the Bio-sustainable Rehabilitation of Polluted Sites

Ishani Joardar and Subhasish Dutta

## Abstract

Rapid agricultural progression, urbanization, and intensive industrialization are among the major contributors to land and water contamination. Given scenario imposes a serious urgency in the sustainable abatement of these effluents without further threatening the ecosystem. Bioremediation of organic and inorganic adulterants follow both ex situ and in situ engineered techniques for alleviating the toxic remnants through aerobic and anaerobic means. Bioventing, biosparging, bioreactors, composting, bioaugmentation, rhizofiltration, bio-simulation, bioleaching, biosorption, phytoremediation, etc. are among the conventional approaches followed for the degradation of pollutants. One of the advantages is that the bioremediation process can adapt to its site-specific conditions and bio-transform into its innocuous counterparts. The objective of this book chapter is to briefly understand the role of microbial ecology, analyze the preexisting and contemporary bioreclamation technologies and their field efficacy, and discuss the limitations of this process.

## Keywords

Bioremediation · Contaminants · Microbes · Environmental pollution

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## 8.1 Introduction

Large amounts of waste are emitted into the environment daily owing to the ever-increasing human activities, global industrial revolution, agricultural practices and their respective emission into the nearby waterbodies.

There is an increasing need to protect the ecosystem from these harmful counterparts through innovative, eco-friendly pollution abatement technologies. Keeping in mind bioremediation is considered as one of the emerging technological advancements for the decontamination of polluted aquatic and terrestrial environments.

Bioremediation involves the usage of microorganisms like fungi, bacterium, algae, yeast, or recombinant microbial components for the decontamination of polluted water and soil environments without emitting any secondary wastes (Kumar et al. 2011). By these microbial consortia, natural disintegration of the contaminants takes place. Inception and stimulation of such a microbial consortia take place through several methods, viz., the addition of nutrients, carbon sources, growth substances, and electron donors/acceptors and controlling the environment following which the reaction takes place.

Emerging technologies and advances like in situ, biofilter, bioreactor, and genetically engineering-assisted processes have contributed to the rapid growth of this domain. Utilizing the harmful contaminants as a nutrient or energy source by the microbial flora instead of culminating it as propagators of ailment is the goal of bioremediation (Naik and Duraphe 2012).

These hazardous wastes can be divided into metals, nonmetals, inorganic and organic compounds, and metalloids. Organic substances include petroleum hydrocarbons and aliphatic, aromatic, alicyclic, and polycyclic aromatic hydrocarbons. Other organic pollutants include halogenated and non-halogenated contaminants, insecticides, agricultural effluents, and explosives. Inorganic pollutants which are decontaminated into their nontoxic counterparts include Ag, As, Al, Be, Cu, Cd, Cr, Fe, Hg, Ni, Pb, Sb, Se, and Zn. Besides microbial flora, higher plants are also used in this process (Kumar et al. 2011; Naik and Duraphe 2012).

Bioremediation is not an elixir; instead, it is a natural process that is an efficient substitute to methods like incineration, composting, physical removal, usage of synthetic chemicals for the removal of wastes from aquatic ecosystems, adsorbents, etc.

## 8.2 The Principle of Bioremediation

According to definition, bioremediation involves the usage of living organisms preferably microorganisms like bacteria, fungi, and algae to disintegrate toxic substances into their harmless counterparts. The interaction of both plants and microbes in the root zone is an important factor from low pH conditions (Dwivedi 2012).

Any form of organic or inorganic waste conversion must be controlled into an innocuous form or below a certain concentration limit as stated by regulatory bodies (Mueller et al. 1996). Moreover, the enzymes that are involved must be able to react efficiently with the components into their harmless counterparts (Biswas 2015).

Bioremediation is effective under optimal environmental conditions which permits proper microbial growth and its degeneration, the conditions of which will be discussed further.

### 8.2.1 Factors Affecting Bioremediation

The factors rely on conditions like the capability of a microbial population in degrading the pollutants, nutrients, environmental factors, type of the soil, oxygen content, etc.

#### 8.2.1.1 Nutrients and Environmental Requirements

With the help of bio-stimulation, essential nutrients can be added to the soil for improving, modifying, and stimulating the indigenous microorganisms so that they become capable of bioremediation. These nutrients form the foundation of the enzymes to efficiently break down the contaminants (Harekrushna and Kumar 2012).

The microbial cells require enough optimal levels of carbon (50%), nitrogen (14%), phosphorus (3%), sodium (1%), sulfur (1%), etc., among others. Both sulfur and phosphorus contribute to 70% of the remaining essential nutrients. And carbon is the basic constituent of most living forms and is thus required in quantities larger than other elements. Microorganisms are present in the soil environment readily; however, they should be present in the required amounts for it to support bioremediation. Both growths of the microbial species and their activity are highly dependent on the pH, moisture of the environment, and optimal conditions which must be achieved. A pH of 5.5–8.8 should be maintained, and if the soil environment is too acidic, it is suggested to rinse the pH by the addition of lime. Temperatures of around 15–45 °C are an optimum condition favoring microbial activity as the rate doubles for every 10 °C rise in the temperature. To enhance solar warming and moisture retention, the plastic covering can be used in the harsh summer, autumn, and late spring seasons. Moreover, the soil should have 25–28% of moisture and water holding capacity for the microbes to work on it properly. Many times, gypsum or any other organic compost can be added. If the soil has lower permeability of soil, it will hamper the water movement, soil nutrients, and oxygen content; thus, such a soil environment won't be appropriate for any in situ bioremediation technique. Tilling or sparging is essential to increase the amount of oxygen that is present in the soil. Hydrogen or magnesium peroxide can also be added to it to manually increase the oxygen content (Naik and Duraphe 2012; Srivastava et al. 2014).

#### 8.2.1.2 Energy Sources

One of the most important parameters that affect bacterial activity is the availability and the capability of the reduced organic substances to serve as potential energy sources. Whether the pollutant will act as an effective source of energy for the microorganism is a function of the average oxidation state of carbon that is present in it. Generally, higher oxidation states indicate lower yields of energy which on the other hand provide lower energy stimuli for the degradation of the microorganisms.

The outcome of the entire process of bioremediation depends upon the microbial factors, proper substrate concentration, and environmental factors (as described above) like pH, temperature, nutrition, moisture content, etc. The overall molecular structure and concentration of the pollutants heavily contribute to the kind of microbial transformation that occurs and whether the compound serves as a primary, a co-metabolic, or a second substrate in the process of bioremediation (Srivastava et al. 2014).

#### 8.2.1.3 Bioavailability and Bioactivity

Bioactivity indicates the operating condition of the microbiological processes, and the rate at which the microbial cells enter the process of bioremediation and convert the contaminants into their harmless counterparts is referred to as bioavailability. One of the limitations regarding the widespread usage of bioremediation methods is many times to which the contaminant is available in the microbial population. The bioavailability of many agents lessens with time which is a consequence of weathering and aging. Many organic contaminants do not enter the bioactive and the aqueous phase of the soil readily. Their bioavailability can be increased by the usage of surfactants, chelators, emulsifiers, and dispersants. Increased concentration of microbes and the elevated microbial conversion capacities never indicate a higher biotransformation rate when the mass transfer is taken as a limiting factor (Boopathy et al. 1998). This happens to be the factor for most of the polluted soil and sediment environment. In a case where explosives were buried in the soil for microbes to contaminate them and undergo bioremediation, they failed to degrade even after 50 years. But after the treatment with rigorous mixing of them with soil and microbes and breaking the soil fragments induced the biodegradation rapidly (Chekroun et al. 2014). This factor is largely dependent on physicochemical factors as a result of the slow mass transfer of any degrading microbes. Bioactivity indicates the operating condition of the microbiological processes, and the rate at which the microbial cells enter the process of bioremediation and convert the contaminants into their harmless counterparts is referred to as bioavailability. Increasing the bioactivity rates simply assists the operating capacity of the microbes and improves the system conditions (Macaulay and Rees 2014).

## 8.3 Methods of Bioremediation

There are two methods of biotechnology: in situ and ex situ bioremediation.

### 8.3.1 In Situ Bioremediation

This is defined as the biological treatment implemented in the removal of harmful chemicals which is situated on the subsurface. The methods which fall under bioremediation are:

**Biosparging** It is defined as the injection of air pressure which is below the level of groundwater oxygen concentrations and enhancing the rate by which contaminants undergo biological degradation with the help of naturally occurring bacteria. Since this process enhances the mixing which occurs in the saturated zone, it increases the exposure of soil to the groundwater. This process is easy to install and low cost since injection points can be conveniently installed and provides ample flexibility in the designing and the construction of the same (Kensa 2011).

**Biopilling** Biopilling refers to the full-scale technology where soils are dug out and mixed with modified soil and transferred onto a treatment chamber and bioremediation under forced aeration. The end products of this process are carbon dioxide and water. Biopills, which are a composition of both farming and composting, consist of a treatment chamber, aeration chamber, irrigation system, and nutrition inlet, to encourage microbial activity. Wells are used to supply air and nutrients, and the low airflow helps in providing the oxygen which is needed while lowering the volatilization and the emission of contaminants in the atmosphere (Kensa 2011; Tyagi et al. 2011).

**Bioaugmentation** In this process, exogenic microorganisms are found from the outside soil environments and are capable of degrading contaminants present in them. It has been mainly used in the treatment of municipal wastewater. It has been found that the addition of bacterial cultures helps in enhancing the rate of transformation (Tyagi et al. 2011).

### 8.3.2 Ex Situ Bioremediation

This method involves the excavation or digging out of the groundwater to promote microbial degradation. The process takes place at elevated temperatures of 55–65 °C.

The methods which fall under this are:

**Bioreactors** These slurry or aqueous reactors help in the ex situ treatment of the contaminated soil and water environment which is dug and pumped from a contaminated habitat. The containment vessel and apparatus help in the formation of a three-phase mixing environment for increasing the rate of bioremediation of the water-soluble and soil-bound contaminants as a liquid slurry of the polluted soil and biomass which is capable of degrading the target toxic components. In a bioreactor system, the extent and rates of biodegradation are more efficient than in situ or any solid-phase destruction system since they undergo controlled environments of operation. Temperature, moisture, pH, nutrient levels, and agitation are all factors that can be controlled in both continuous and fed-batch reactors. It results in the enhanced microbial activity of the same. Many kinds of bioreactors are available which include under bed, membrane, biofilm, and airlift bioreactors, and sequential batch biofilm bioreactors (Robles-González et al. 2008; Singh 2014).

**Land farming** In this method of ex situ bioremediation, the polluted soil environment is dug out and spread over a bed and is periodically inclined until all the contaminants have completely degraded. In this way, aerobic breakdown of the pollutants takes place under indigenous microorganisms. Land-farming techniques have been adopted by many oil companies for the treatment of soils contaminated by oil. As compared to other methods, land farming is comparatively simpler, inexpensive, and efficient in the case of biodegradable pollutants in lower concentrations (Kensa 2011; Juwarkar et al. 2014).

**Composting** It is a thermophilic, aerobic treatment procedure where the contaminated substance is mixed with a bulking agent for the treatment of agricultural wastes, municipal solid, and sewage sludge wastes. The optimum temperature recorded for composting using microorganisms is 40–70 °C. This method is used for the treatment of soils contaminated by solvents, petroleum hydrocarbons, chlorophenols, pesticides, herbicides, nitroaromatic derivatives, and polycyclic hydrocarbons. With the help of composting, the number of residues on top of the soil can be reduced considerably (Juwarkar et al. 2014).

### 8.3.3 Phytoremediation

The term phytoremediation has been derived and obtained from two Latin words "phyto" and "remedium" which means plant and filtering or restoring, respectively. Thus, this is an approach through which plants play a major role in alleviating the pollution caused by many contaminants or recalcitrants present in the natural environment. The plants which act as biofilters metabolize substances that are in turn obtained from nature. They remove the pollutants present in both soil and water. Nowadays, phytoremediation is done by plants that are found easily in genetically engineered plants (Flathman and Lanza 1998). Many scientists have suggested that transgenic plants are safer and widely applicable for phytoremediation. *Arabidopsis thaliana* is one such plant that has been genetically engineered and modified to express two genes *ars*C and g-ECS1 which codes for arsenite reductase (modifies arsenate and arsenite) and glutamylcysteine synthetase which produces a thiol that detoxifies arsenite, respectively. Other high-energy compounds which can be treated by this method include GTN, TNT, RDX, TETRYL, and HMX9. Many tobacco plants are also genetically modified into expressing bacterial pentaerythritol tetanite (PETN) reductase which modifies these plants so that they take up high energy and degrade them accordingly to nonexplosive counterparts (Agnello et al. 2016). The plant families which assist in successful phytoremediation are Asteraceae, Brassicaceae, Caryophyllaceae, Cunoniaceae, Cyperaceae, Euphorbiaceae, Fabaceae, Flacourtiaceae, Lamiaceae, Poaceae, Violaceae, etc. Phytoremediation can be divided into three major categories depending on their mode of action: phytoextraction or phytoaccumulation, phytostabilization or phyto-immobilization, and phytotransformation or phytodegradation (Sharma and Pandey 2014).

#### 8.3.3.1 Phytoextraction or Phytoaccumulation

In this process, heavy metals (As, Cd, Pb, Zn, petroleum hydrocarbons, and any radionuclides) are accumulated by the plants into the parts of its root and part of the plants which are above the ground. The hyperaccumulator plants absorb the metals and then accumulate them into a confined space. This is also used for the methods of phytomining where metal extraction takes place by harvesting hyperaccumulator plants like *Sedum alfredii*, *Rumex crispus*, and *Viola baoshanensis* (Chaney et al. 2020; Bhatnagar and Kumari 2013).

#### 8.3.3.2 Phytostabilization or Phyto-immobilization

Generally, metals like Cd, Cr, Cu, Ni, Pb, and Zn are treated by this method with the help of plants like *Anthyllis vulneraria* and *Festuca arvernensis*. In this process, the plants decrease the potency and the migration of the pollutants to the contaminated soil environment. It also allows the conversion of the bioavailable forms of pollutants into the unavailable forms, thus ensuring that it doesn’t enter the biological system (Mani and Kumar 2014).

#### 8.3.3.3 Phytotransformation or Phytodegradation

In this process, the plants degrade and metabolize the organic pollutants, xenobiotic substances, explosives, nitrates, etc. which are present in the soil; moreover, enzymes that are secreted by the plants are also used for the breakdown of contaminants outside the plants. The plants which are used in this process are *Elodea canadensis*, *Pueraria*, etc. This method generally helps in the breakdown of organic compounds. When this process occurs within the plant body, the pollutant or recalcitrant uptake is dependent on factors like properties of the contaminants like hydrophobicity, polarity, and solubility, and also the plant characteristics (Subramanian et al. 2006; Singh and Singh 2017). Other methods include rhizofiltration where the roots of the plants exude, absorb, concentrate, and precipitate the metals (mainly As, Cd, Pd, and Zn) and phytovolatilization where the plants take up heavy metals like mercury or other heavy metals which are available in the soil and volatilize it from the region of its foliage (Verma and Sharma 2017; Verma et al. 2006).

## 8.4 Microbes That Assist in the Bioremediation Processes

Microorganisms are available everywhere and can be isolated from any kind of environmental condition. Since there are different kinds of contaminants that are present, a large variety of microorganisms are required for an effective remediation. The first patent for a successful bioremediation agent was filed in 1974 for a strain of *Pseudomonas putida* (Prescott et al. 2002) for the degradation of petroleum. In the following years, e.g., in 1991, nearly 70 microbial genera were used for the degradation of petroleum compounds, and almost a similar count was followed again for the successive two decades (Glazer and Nikaido 2007).

1. **Aerobic and anaerobic microbes**: Some general aerobic microbes which are used for bioremediation are *Alcaligenes*, *Mycobacterium*, *Pseudomonas*, *Rhodococcus*, *Sphingomonas*, etc. for the biotransformation of harmful contaminants. *Acinetobacter* and *Acidovorax* under both aerobic and anaerobic conditions have been used for the removal of polychlorinated biphenyl (Wang et al. 2016). Textile dyes can be efficiently removed by microaerophilic and aerobic environments by *Pseudomonas entomophila*. It was incubated for 120 h and remediated 93% of reactive black textile dye having a concentration of 500 mg/L (Khan and Malik 2016). Similarly, a bacterium consortia of *Pseudomonas* sp. and *Providencia rettgeri* were used for remediating four azo dyes (Disperse Red 78, Disperse Red 81, Reactive Black 5, and Reactive Orange 16) (Lade et al. 2015). Anaerobic processes take place in the absence of oxygen. These kinds of microbes are rarely used for bioremediation. However, there is a growing interest in the anaerobic bacteria that are used for the transformation of the polychlorinated biphenyls in the river water, trichloroethylene, and chloroform compounds. Anaerobic methods are rarely used as textile dye degradation under this condition was found to generate aromatic amines.
2. **Bacterium and genetically engineered microbes (GEMs) involved in the bioremediation**: The bacteria which help in the biotransformation of organic compounds are *Azotobacter* sp., *Bacillus* sp., *Flavobacterium* sp., *Mycobacterium* sp., *Nocardia* sp., *Pseudomonas* sp., *Streptomyces* sp., and *Xanthomonas* sp. The compounds which are treated by these bacteria range from complex aromatics, hydrocarbons, cresol, phenols, anthracene, PCBs, diazinons, cycloparaffins, etc. Other heavy metals like Cu, U, Cd, Pb, Ni, Cr, Zn etc. are also degraded by bacteria like *Bacillus* sp., *Pseudomonas aeruginosa*, *Aerococcus* sp., *Aeromonas* sp., *Citrobacter* sp., *Rhodopseudomonas palustris*, etc. (Biswas 2015). Some of the other studies included bacteria like *Lysinibacillus* sp. (removal of Remazol red dye), *Brevibacillus parabrevis* (removal of Congo red dye), *Pseudomonas pulmonicola* (malachite green dye), *Pseudomonas putida* (crystal violet), *Micrococcus glutamicus* (Reactive Green 19A), and *Pseudomonas desmolyticum* (Direct Blue 6 dye) (Singh et al. 2020). In a recent finding where strains of bacteria were isolated and screened from petroleum-contaminated soils of Iran, it was noted that *Citrobacter sedlakii* had the degrading capacity of 69% of 5% v/v of kerosene and *Enterobacter hormaechei*

had the ability to degrade 48% and 42% of 5% v/v kerosene, respectively (Ghoreishi et al. 2017). Other new age bioremediation processes include the usage of a variety of genetically engineered bacteria, e.g., *Escherichia coli* JM109 (for the removal of Acid Red-laden wastewater and Direct Blue 71), *Xenophilus azovorans* KF46, *Pseudomonas luteola*, *Klebsiella pneumoniae* RS-13, *Clostridium perfringens* for azoreductase-induced dye degradation, *Burkholderia cepacia* (Agent Orange removal), *Sphingomonas* sp. CDS-1 (organophosphate and carbamate), and *Pseudomonas* sp. enzymes like Atz (A, B, and C) and TriA for the degradation of atrazine. Other genetically engineered bacterium species are *Burkholderia xenovorans* strain LB400 (Aroclor 1242 removal), *Comamonas testosteroni* VP44 (4-chlorobenzoate removal), *Pseudomonas putida* BH pS10-45 (for the removal of phenol), *Ralstonia* sp. KN1-10A (trichloroethylene removal), *Pseudomonas fluorescens* HK44 (naphthalene removal), *Escherichia coli* BL21AI-GOS (organophosphate removal), *Cupriavidus necator* JMP134-ONP (nitrophenol removal), *Sphingomonas paucimobilis* 551 (phenol removal), *Rhodococcus erythropolis* strains (phenol removal), etc. (Singh et al. 2020).

3. **Fungal microorganisms that are involved in bioremediatio**n: Fungi have the ability to mineralize xenobiotic components into $CO_2$ and $H_2O$ with the help of nonspecific ligninolytic and efficient oxidative enzyme systems (catalase, peroxidases, laccases, cytochrome P450 monooxygenases, etc.) for the decolorization and degradation of wastewater contaminated with synthetic dyes, heavy metals, oil spills, leather tanning effluents, etc. It undergoes either an enzymatic or nonenzymatic process where the former consists of intracellular enzymes (cytochrome P450, etc.) and extracellular enzymatic processes (manganese peroxide, lactase, etc.). The nonenzymatic process consists of biosorption, biomineralization, biodegradation, stripping, etc. Fungi obtained from extreme environments like Antarctica's polar ice caves or other deep sea biosphere are among the unique category of extremophilic fungi that assist in bioremediation of large amounts of heavy metals, dyes, or oil spill under high stressful conditions. The fungi which aid in the biotransformation and bioaccumulation of organic molecules and complex hydrocarbons are *Coprinellus radians*, *Cryptococcus* sp., *Fomitopsis pinicola*, *Gloeophyllum trabeum*, *Penicillium simplicissimum*, *Phanerochaete chrysosporium*, *Pleurotus ostreatus*, etc. Some fungi that utilize heavy metals are *Rhizopus arrhizus*, *Stereum hirsutum* etc. for the degradation of Cd, Cu Pb, Ag, Hg, and Zn (Biswas 2015; Kumar et al. 2011; Majeau et al. 2010). Fungi play a major role in the removal of complex recalcitrants like leather tanning pollutants, textile synthetic dyes, industrial emissions, pesticides, POPs, PAHs, PPCPs, and other heavy metals from wastewater and groundwater. In textile dye bioremediation, *A. niger* was used for the complete decolorization of azo red dye with 1000 mg/L of dye concentration at a pH of 9. Besides, *A. niger* has been used for the treatment of municipal waste. Its hydrolytic enzymes catalyze the production of biogas, volatile fatty acids, organic fertilizers, and other biomethanation techniques so that complex polymeric products can be easily hydrolyzed. In textile dye bioremediation, *A. niger* was also used for the

complete decolorization of azo red dye with 1000 mg/L of dye concentration at a pH of 9 (Deshmukh et al. 2016). White rot fungi, namely, *Bjerkandera adusta*, *Phanerochaete chrysosporium*, *Pleurotus* sp., by virtue of their ligninolytic enzymes (peroxides and laccases) aid in the removal of organic pollutants (pesticides, etc.) (Voběrková et al. 2017; Yang et al. 2013). Other white rot fungi include *T. versicolor* and *Lentinus tigrinus* for the removal of soils contaminated with cresolate (mostly found in petroleum oil spills). It has also been found that increasing the production of laccase from the cultures of *T. versicolor* and *P. ostreatus*, 3000 and 2700 U/L, promoted better bioremediation of pyrene and phenanthrene (Yang et al. 2013). In *Rigidoporus* sp., another type of white rot fungus, FMD21 was used for the bioremediation of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (Dao et al. 2019). Marine fungi as compared to the other categories of fungi possess more tolerance to complex hydrocarbons. They have the ability to withstand high salinity, pH, and other extreme environments than terrestrial ones, the enzymes derived from which aid in the removal of heavy metals and crude oil components and thus help in both bioremediation and nanotechnology. In an estuary which was contaminated with complex phenolics, lactase enzyme obtained from *Trichoderma viride* NFCCI-2745 assisted in its complete removal (Divya et al. 2014). In a very recent study, two isolates of *Trichoderma harzianum* were used in the bioremediation of heavy metals. In the first isolate, Mn and Pb had the highest rate of removal, 84% and 85% respectively; as for the second isolate, the biodegradation percentage was 87% for Mn and 84% for Pb (Hussain and Mutlag 2021). In another study, ten fungal strains, which included six *Aspergillus* species, two *Fusarium*, and one each to *Acremonium* and *Penicillium* obtained from the Indian marine and mangrove coastline, had the ability to biodegrade *n*-alkane by 95% and the crude oil content by 77% making it a potential microbe for its development as a bioremediation agent (Barnes et al. 2018).

4. **Algae that are involved in bioremediation of organic compounds and heavy metals:** *Chlamydomonas* sp., *Chlorella* sp., *Cylindrotheca* sp., *Dunaliella* sp., *Euglena gracilis*, *Selenastrum capricornutum*, *Chlorella pyrenoidosa*, *Chlorella vulgaris*, *Lyngbya spiralis*, *Oscillatoria* sp., *Phormidium valderium*, *Spirogyra hyalina*, *Tetraselmis chuii*, *Volvariella volvacea*, and *Zoogloea* sp. These are some of the algae which are used for the utilization of heavy metals and organic components like naphthalene, DDT, phenol, benzene, toluene, chlorobenzene, toxaphene, etc. (Biswas 2015). Other groups of algae which are used for biodegradation and biosorption include *Cyanobacterium*, *Phormidium* (for removal of indigo blue dye and Remazol black B removal), *Nostoc linckia* (methyl red removal), and *Oscillatoria rubescens* (basic fuchsin dye removal) (Singh et al. 2020). In a recent study, *Spirulina* which is a part of animal food supplement is successfully used for recycling agro-industrial wastewater (Karthik et al. 2020). Microalgae have also been abundantly used in the bioremediation of polluted water, e.g., 37% reduction of both BOD and COD was observed in the Yamuna rivers of India during a 20-day study of its contaminated waters where the DO significantly increased by 116% (Shrestha et al. 2020). *Anabaena*, *Chlorella*,

*Cladophora*, *Oscillatoria*, *Spirulina* sp., *Scenedesmus*, and *Phaeodactylum tricornutum* have been reported to eliminate a considerate amount of heavy metals from acid mine drainage by acting as hyper-adsorbents or hyper-accumulators (Bwapwa et al. 2017).

## 8.5 Advantages and Disadvantages of Bioremediation

Being a natural process, it is accepted by the masses as a better and easier method of waste treatment without leaving much carbon footprint or pollution in the environment. Thus, the residues which are emitted usually do not contain any toxic or hazardous contaminant. It is inexpensive as compared to other waste clean-up processes that are presently available. Many hazardous substances can be easily converted into their detoxified forms and harmless products with the help of microorganisms or plants. This eliminates the further process of treatment and disposal of the waste residues which are emitted in the process. Destruction under or elimination of the target recalcitrant is possible by virtue of this method. Moreover, it can be easily performed on-site without bothering normal activities or rural environments. As a result, there is no threat to human or animal life, and also there is no need of transporting waste off-site. In the case of phytoremediation, the plants which are used can be easily monitored, altered, and genetically modified to achieve a particular trait. Naturally occurring microorganisms and plants like algae, fungi, etc. are used which are abundantly found and have high tolerance and natural abilities that help to preserve and protect the ecosystem. Genetically modified microbes speed up the procedure of bioremediation and recovery of the polluted site and have a high catalytic and utilization capacity using only a small cell mass (Amin et al. 2013; Kumar et al. 2018).

However, the substances which can undergo bioremediation are still limited. Not all contaminants are prone to complete degradation. While additives in the soil or aquatic environment enhance the functioning of one particular microorganism like bacteria or fungi, it might be toxic to other organisms which are inhabitant of that environment. Genetically modified organisms might get difficult to remove from the environment, and a lot of research is still needed in this domain. Extrapolation of the bench- and pilot-scale studies to full-scale field operations might get difficult. Processes like phytoremediation and biosparging often take a longer time than usual as compared to other processes (Kensa 2011; Amin et al. 2013; Kumar et al. 2018). Despite the outstanding research that has been done in the removal of crude oil and heavy metals from wastewater systems, there still remains some compounds like metal chlorinated compounds, radionuclides, etc. which fail to degrade by these methods. Land farming and bioventing possess environmental limitations and often fail to reach the space requirements even though they are highly economic and have considerable low operating costs. The genetically modified microbes (GEMs) have never been implemented in contemporary techniques or large-scale processes. It has also shown growth delay, fluctuation of other abiotic factors, and change in the other microorganism colony of the water system which is present in its natural habitat owing to the foreign strain of the added GEM (Abatenh et al. 2017). Further research is needed for the development of technologies which are applicable to sites which have multiple and complex mixtures of pollutants which are unevenly distributed in the environment. There are still concerns that the byproducts of bioremediation might be more persistent in the environment and its toxicity might be worse than that of the parent compound. Thus, regulatory uncertainty still remains to some extent regarding the performance efficiency of bioremediation.

## 8.6 Conclusion

Pollution of the environment surrounding us is nothing new, and the contamination of soil and aquatic environment has been growing alarmingly due to industrialization and wastes generated by various factories, the agriculture sector, and household wastes. Proper clean-up techniques must be implemented so that their emissions, end products, and residues on the other hand do not pollute the environment further. For this reason, bioremediation is used which is a unique, inexpensive tool for detoxifying, bio-transforming, and removal of contaminants, be it simple organic compounds or heavy metals. Compared to the other waste removal processes that are available, the advantages of this process outweigh the disadvantages that follow. This is the reason many sites choose to implement this technique for cleaning up the polluted environment. The challenge is to continue improving the research and the scientific processes for the further development of bio-remedial techniques to flourish commercially in an unhindered manner.

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# Recent Bioremediation Techniques for the Removal of Industrial Wastes

## 9

Surbhi Sinha

**Abstract**

Wastes from industries are the major root cause of environmental contamination. Industrial wastes comprise various toxic pollutants that can cause significant risks to live organisms and the environment. Thus, appropriate treatment techniques are mandatory for the removal of these harmful pollutants to safeguard the surrounding environment. Bioremediation is considered an apt technique as compared to physiochemical methods, which are costly, can cause pollution and eventually ruin the environment. Bioremediation methods have been acknowledged by the US Environmental Protection Agency as an environment-friendly technique that revives the polluted environment encouraging sustainable development. Hence, the present chapter addresses the recent bioremediation technologies along with their limitations. Moreover, the future perspective on bioremediation techniques is also discussed.

**Keywords**

Bioremediation · Electro-remediation · Industrial wastes · Phytoremediation

## 9.1 Introduction

Management of wastes has turned into a global issue as the development of industries and communities continues to increase. Rapid urban and industrial expansion has produced enormous quantities of solid and liquid wastes. Every year, approximately four billion tons of industrial and municipal wastes are generated

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**Table 9.1** Some of the examples of contaminants present in industrial wastes

| Contaminants | Sources | Applications | Side effects |
|---|---|---|---|
| Arsenic | Industries, agriculture | Car batteries, ammunition | Nausea, vomiting, abdominal pain |
| Methylene blue | Textile industries, printing industries | Dyeing, printing | Difficulty breathing, fever, vomiting |
| Benzene | Petroleum industries, plastic and rubber industries, agriculture | Plastics, resins, detergents, pesticides, drugs | Drowsiness, headaches, dizziness, tremors |
| Mercury | Waste incinerators, fuel combustion | Thermometers, barometers | Irritation, nervousness, headaches, insomnia |
| Phenols | Pharmaceutical, plastics, resins | Antiseptics, household cleaners | Nausea, rash, vomiting, swelling |
| Benzene | Automobile service stations, tobacco smoke, industrial emissions | Synthetic fibres, resins, rubber lubricants, detergents, drugs | Irregular heartbeat, headaches, drowsiness, dizziness |

worldwide, out of which about one billion tons are non-hazardous wastes (Nanda and Berruti 2021). India produces around 980 million tons of solid wastes annually, of which 290 million tons are industrial wastes (Swain et al. 2020). Various industries such as textile, dairy, pulp and paper, sugar mill, distilleries, oil palm, etc. discharge their wastes into the nearby waterbodies or land (Bisht et al. 2021; Sinha et al. 2019). Wastes released from various industries contain numerous organic and inorganic pollutants that may lead to severe environmental pollution. The organic contaminant comprises dyes, phenols, pesticides, polyaromatic hydrocarbon, chlorinated phenols and endocrine-disrupting chemicals, while inorganic contaminants include various toxic heavy metals, namely, chromium, cadmium, arsenic, mercury, etc. (Sinha et al. 2018). Table 9.1 lists some of the common examples of contaminants present in industrial wastes. Usually, industrial wastes are categorized into hazardous and non-hazardous wastes. Hazardous wastes comprise materials that are infectious, toxic, carcinogenic, dangerous, flammable, etc., while non-hazardous wastes are non-toxic and display no hazard. The decline of space and other issues of waste disposal have signalled the scientific groups globally towards seeking a rapid, low-cost and green technology for effective management of industrial wastes.

The bioremediation process is viewed as a propitious candidate for the treatment of industrial wastes, as it does not generate secondary pollution, is environment-friendly and is cheap, with simplicity in usage and wide applications (Singh and Sinha 2013). Moreover, the process is also approved by the US Environmental Protection Agency as a 'green' method to mitigate environmental pollution. The bioremediation method depends on the use of biological agents such as microbes or plants or other biological agents such as anaerobic or aerobic membrane bioreactors to detoxify the pollutants present in wastewater (Azubuike et al. 2016). Biological agents use their diverse metabolic pathways to treat the recalcitrant organic and inorganic pollutants, making water less hazardous and sustainable. Hence, the chapter presents the recent bioremediation techniques which can be utilized for the remediation of industrial wastes to safeguard the environment and human health.

## 9.2 Recent Bioremediation Methods for Mitigating Various Industrial Wastes

Mostly, physicochemical methods are utilized for the mitigation of industrial wastes. These methods are successful to some extent by reducing the carbon and nitrogen content of industrial wastes (Shah and Shah 2020). However, they are not capable of completely eradicating the wastes or pollutant. Another challenge in utilizing these physicochemical methods is the high cost concerning their modus operandi and the chemicals, apart from the secondary pollutants that are produced during various chemical purposes. Therefore, bioremediation techniques are desired for the removal of industrial pollutants.

While employing bioremediation techniques, it is important to make sure that the by-products that are generated after the process are non-toxic. The process of bioremediation depends on the following factors: chemical structure of the pollutant, the toxicity of the pollutant, its concentration, type and efficiency of biological agents and various environmental parameters. Commonly utilized bioremediation methods are described in further segments.

### 9.2.1 Microbial Bioremediation

It is an environment-friendly bioremediation method that utilizes the natural potential of microbes like bacteria, algae, fungi and yeast to detoxify the wastes. In this process, microbes utilize their enzymes to degrade the wastes into carbon dioxide, water and methane which eventually results in complete mineralization and removal of pollutants. A lot of enzymes such as azoreductase, laccase, lignin peroxidase, manganese peroxidase, etc. present in algae, fungi, bacteria and yeast have been confirmed to participate in the degradation of wastes (Bharagava et al. 2018). Table 9.2 lists some of the microbes utilized for the mitigation of various contaminants present in industrial wastes. The microbial bioremediation process thus primarily relies on the metabolic capacity of the microbes to transform the industrial wastes into non-toxic forms. The process can be employed both in situ and ex situ (Sharma 2020). Since microbial bioremediation is effectual only where the environment surroundings allow the growth and activity of the microbes, its implementations usually constitute the exploitation of different environmental factors so that microbes can grow and degrade the wastes at a higher rate. Generally, microbial bioremediation takes place under aerobic conditions, but sometimes anaerobic conditions also lead to the breakdown of industrial contaminants. Bioremediation by using microbes is not only green but also a cheap method to reduce the toxicity of industrial wastes.

**Table 9.2** Some of the recently utilized microbes for the removal of various contaminants present in industrial wastes

| Microbes | Contaminant removed | References |
|---|---|---|
| *Staphylococcus aureus* strain K1 | Hexavalent chromium | Tariq et al. (2019) |
| *Serratia* sp. | Oil sludge | Gidudu et al. (2020) |
| *Pseudomonas aeruginosa* | Furnace oil spillage | Marzan et al. (2017) |
| *Chlorella* sp. C2 | Power plant wastes | Chen et al. (2018) |
| *Chlorella* sp. and *Phormidium* sp. | Tannery wastes | Das et al. (2018) |
| *Fucus vesiculosus* | Cationic copper ions and anionic methyl orange | El-Naggar et al. (2021) |
| *Trametes versicolor* and *Irpex lacteus* | Fluoroquinolone | Čvančarová et al. (2015) |
| White-rot fungi | 2,3,7,8-tetrachlorodibenzo-*p*-dioxin | Dao et al. (2019) |
| *Chlorella vulgaris* | Atrazine, Molina | Hussein et al. (2017) |
| *Aeromonas hydrophila* | Chlorpyrifos | Shartooh et al. (2021) |

### 9.2.2 Genetically Modified Microbes for Enhanced Bioremediation

A large number of microbes present in the environment are utilized for abatement of pollutants, but many of these microorganisms display slower removal, due to the recalcitrant and persistent nature of the contaminants. Thus, it becomes necessary to enhance the efficiency of microbes for effective removal of different pollutants existing in industrial wastes. Genetic engineering is one such approach that can be utilized for developing novel microbial strains having exceptional features with wide array of bioremediation potency as compared to native microbes. A genetically modified microbe is one wherein the genetic make-up of the microbes is altered to enhance its bioremediation efficacy, and it was in the early 1980s that the cloning of the genes encoding catabolic enzymes, responsible for degradation of hazardous compounds, was started. Recently, molecular biologists from around the globe have constructed and utilized novel genetically engineered microbes for the bioremediation of different pollutants present in the environment. Table 9.3 lists some of the genetically modified microbes used for enhanced mitigation of pollutants present in industrial wastes.

### 9.2.3 Phytoremediation

Another inexpensive, environmentally friendly and socially acceptable in situ bioremediation process is phytoremediation. It is a process where the plants directly or indirectly are utilized for the removal of industrial wastes from soil or water to

**Table 9.3** Some of the genetically modified microbes used for bioremediation of contaminants

| Genetically modified microbe | Gene modified | Contaminant removed | References |
|---|---|---|---|
| *E. coli* | almA, xylE, p450cam | Oil spills | French et al. (2020) |
| *Deinococcus radiodurans* | MerH | Mercury | Gupta and Walther (2014) |
| *Bacillus amyloliquefaciens* | Laccase enzyme | Indigo carmine dye | Wang et al. (2017) |
| *Bacillus pumilus* | CotA-laccase enzyme | Acid red 1, acid blue 129 | Luo et al. (2018) |
| *Pseudomonas diminuta* | Phosphotriesterase enzyme | Organophosphorus chemical warfare agents | Bigley and Raushel (2019) |
| *Pseudomonas putida* | Enhanced green fluorescent protein | Methyl parathion and cadmium | Zhang et al. (2016) |
| *Pseudomonas putida* KT2440 | mpd and pytH genes | Organophosphates and pyrethroids | Zuo et al. (2015) |

protect the environment and human health. In the process, the plants accumulate the pollutants, metabolize them and promote the breakdown of the pollutants by using their metabolic energy (Farooqi 2021). Different strategies of phytoremediation employed for the removal of wastes are phytoextraction, phytostabilization, phytofiltration, phytodegradation, phytovolatilization and rhizodegradation (Chirakkara et al. 2016). Additionally, some microbes are known to be associated with plant roots, which can also be exploited to enhance the bioremediation efficacy of the plants. Plant growth-promoting bacteria (PGPR) present in plants are known to be beneficial for the phytoremediation process because these can cease the phytopathogens, endure the abiotic stress and reduce the toxicity caused by the wastes. Moreover, these PGPR have a high surface area, and also, they secrete diverse kinds of antibiotics, hormones, acids, etc. that help to promote plant growth (Ullah et al. 2015). Bacteria are also present in the inner tissues of the plants, known as endophytes, which can withstand high waste concentration and therefore, reduce the phytotoxicity, support the plant growth and improve the remediation efficiency of plants (Ma et al. 2015). Additionally, some fungi have been stated to safeguard the plant roots as well against toxic pollutants (Khan et al. 2014). However, the application of phytoremediation is restricted by the small size of the plants displaying limited remediation potential and inadequate capacity of the native plants to tolerate and degrade a broad range of pollutants. Table 9.4 lists some of the plants recently used for the bioremediation purpose.

### 9.2.4 Phytobial

Phytobial technique is an effective and green approach to remediate contaminants present in the industrial wastes. The process of phytobial uses plants along with microbes to mitigate the pollutants. Generally, the plants help in the uptake of the

**Table 9.4** Recently utilized plants for the removal of contaminants from industrial wastes

| Plants | Contaminant removed | References |
|---|---|---|
| Water hyacinth | Cadmium, arsenic | Nazir et al. (2020) |
| *Eichhornia crassipes* | Cd, Ni | Eid et al. (2020) |
| *Lemna minor* | Methylene blue | Imron et al. (2019) |
| *Lagerstroemia speciosa* | Methyl orange, methylene blue | Saraswathi et al. (2017) |
| *Eichhornia crassipes* | Ciprofloxacin | Yan et al. (2020) |
| *Myriophyllum aquaticum* | Tetracycline antibiotics and copper | Guo et al. (2020) |
| *Hydrilla verticillata* | Phenol | Chang et al. (2020) |
| *Raphanus sativus* | Phenol | González-Costas et al. (2020) |
| *Miscanthus sinensis* And. | Organochlorine pesticides | Mamirova et al. (2021) |
| *Eichhornia crassipes* | Organochlorine and Pyrethroid pesticides | Riaz et al. (2017) |

pollutants, while the microbes cause their degradation. Further, the process can be improved by utilizing specific bacterial strains capable of secreting myriad of plant growth-promoting substances such as siderophores, organic acids, biosurfactants and ACC deaminase that can convert pollutants into less toxic forms (Roy et al. 2015). The process has benefit of being implemented to large areas of contaminated sites. Additionally, the in situ employment of phytobial reduced the concentration of contaminants in the soil and thus helped in preserving the top soil. However, the technique has constraints of restricted plant root length, requirement of a lot of time for treatment procedure which may be several seasons, absence of discreet disposal methods and difficult recovery methods. Utilization of plants with deep roots, construction of transgenic plants and integration of phytobial with other bioremediation strategies can help in overcoming the above-mentioned problems (Selvi et al. 2019).

### 9.2.5 Electro-bioremediation Technique

Recently, the application of the electro-bioremediation method for the removal of industrial pollutants is gaining a lot of popularity. Electro-bioremediation is a hybrid technique that amalgamates bioremediation and electrokinetics for the remediation of various industrial wastes accumulated in soil and water. The concept of utilizing the electrokinetics phenomenon is to stimulate and mobilize the industrial pollutants and microorganisms for the removal process (Acuña et al. 2012). In this process, electro-kinetics comprises the application of different methods such as electrolysis, diffusion, electrophoresis, electroosmosis and weak electric field of about 0.2–2 V $\text{cm}^{-1}$ to the soil. The method can be utilized in situ and is especially effective for the removal of pollutants from the soil with less hydraulic conductivity, which is generally hard to remediate by conventional processes. Electro-bioremediation is a prospective technology that can be utilized both in the microbial phenomenon for the removal of pollutants and electrokinetics for the mobilization of pollutants present beneath the soil, nutrients and pollutant degrading microorganisms. It is an upcoming process that removes the contaminants existing in the heterogeneous matrix (Hassan et al. 2016). However, limitations like the selection of microbes, accumulation of calcium and magnesium ions on the electrode and formation of chlorine gas during the process restrict the application of electro-bioremediation for the removal of wastes from the soil (Vocciante et al. 2021). Thus, more research should be performed by the scientific community from all over the world to successfully apply this method for the bioremediation of industrial wastes.

### 9.2.6 Electrokinetic-Phytoremediation Technique

Associating phytoremediation and electrokinetics can be an outstanding approach for the removal of industrial wastes, as the process could significantly amplify the remediation process by increasing mass transfer. Administering electric field to the plant can intensify the removal capacity of the plants by the movement of wastes and nutrients resulting in increased uptake of plants (Dionísio et al. 2021). It is also stated by researchers that the plants subjected to small electric current were greener and generated higher biomass as compared to plants grown without an electric field (Li et al. 2019). Electrokinetic-phytoremediation is a sustainable, green technique and combines the merits of both phytoremediation and electrokinetics, but limitations like clogging of the electrode, alteration in soil pH and presence of chelating agents affect the efficiency of the process (Mao et al. 2015). Thus, further research is required for the successful implementation of this technology.

### 9.2.7 Microbial Fuel Cells for Bioremediation

A microbial fuel cell is an instrument that utilizes the biochemical energy of microorganisms to transform organic substrates into electrical energy. Usually, microbial fuel cells comprise cathode and anode linked by an exterior circuit and separated by a membrane. Microbes present in the anode chamber oxidize the organic substrate, producing protons that move to the cathode via the membrane and electrons that transfer through the anode to the external circuit generating electric current (Kumar et al. 2017). Bacteria are the most favoured microbes in a microbial fuel cell to produce electricity. This technology can utilize industrial wastes rich in organic content to generate electricity. Also, it can be utilized as biosensors and in secondary fuel generation. The process has numerous benefits such as direct electricity production, low cost, less generation of sludge, the minimum cost of operation, environment-friendly nature and resistance to environmental stress (Gude 2016). Even though the technology has a lot of advantages, restrictions like limited production of electricity, fluctuations in current and high internal resistance make it practically infeasible (Bruno et al. 2018). Thus, efforts should be made by the researchers to improvise the performance of the microbial fuel cells to utilize them effectively on a commercial scale.

### 9.2.8 Nano-bioremediation Technique

Nano-bioremediation is a sophisticated fast developing technique for the mitigation of industrial wastes from the environment by utilizing nanoparticles that are synthesized biologically. These bio-nanoparticles are quite substantial in removing the wastes or pollutants from the environment and do not generate any dangerous by-product during their synthesis. Generally, the process involves synthesizing nanoparticles with excellent remediation capability, using biological materials like microorganisms or plants. The nano-bioremediation technique has phenomenal merits such as its eco-friendly nature, cheap and rapid method of operation as compared to the conventional methods (Vázquez-Núñez et al. 2020). Thus, lately, a lot of work has been published by researchers from around the world for removing industrial wastes using the nano-bioremediation technique (Masum et al. 2019; Hamouda et al. 2019; Bhuyar et al. 2020). However, further study on their detailed mechanism for the removal of pollutants and sustainability is needed. The area of bioremediation combined with nanotechnology is quite new and not investigated much, but it displays great potential in the sector of biotechnology.

### 9.2.9 Constructed Wetlands

Constructed wetlands are an alternate and propitious technology for the mitigation of industrial wastes and pollutants from the environment. These wetlands rely on nature's system for the treatment of wastes. The concept here is to utilize natural matter such as sand, gravel, plants or microbes and naturally occurring methods under appropriate conditions for the removal of contaminants (Stefanakis 2020). The technique of constructed wetland has been marked as a sustainable, eco-friendly technology that offers several economic, environmental, technological and social benefits. It follows a decentralized approach and is especially suitable in areas where the application of traditional treatment processes is not economically feasible (Zhang et al. 2014). Moreover, the technique requires very little maintenance, manpower and minimal energy for its modus operandi. It is an upcoming technique that can be successfully used for the treatment of not only industrial but also domestic and municipal wastes. Since the constructed wetlands use biological agents and natural processes for the removal of wastes, it contributes a lot to effective sanitation, public health and protection of the environment and natural resources. Despite numerous advantages, the technique of constructed wetlands has some limitations which should be taken into consideration. One is that it requires a large land area as compared to other treatment processes. Another one is that if the design of the wetland is inaccurate, then it can cause odour issues (Omondi and Navalia 2020).

The process of constructed wetlands for a treatment system is a relatively new one whose potency is extensively accepted by researchers globally and thus is at the inquiry for additional developments both in developed and developing nations.

## 9.3 Limitations, Prospects and Conclusion

The process of bioremediation has come forth as a cheap and green alternate for the removal of industrial wastes in comparison to traditional methods which are costly and unsustainable. However, mostly the process is restricted to just biodegradable pollutants, and also there are some issues regarding the toxicity of the biodegradation products. Moreover, challenges such as lack of availability of pollutants to microorganisms, toxic effects of wastes on the biological agents, shortage of sufficient enzymes required for decontamination of wastes, refractory nature of the wastes/pollutants and insufficient biomass restrict their application. Furthermore, since the bioprocesses are extremely particular with the culture prerequisites, the implication of pilot-scale bioremediation study to successful large-scale operation becomes quite challenging. Additionally, molecular studies are known to improve the bioremediation process by producing genetically modified or transgenic biological agents, but ecological hazards, viz. disruption of exotic plants and abatement of biodiversity, make the transgenic plants and microbes unsuitable for environmental clean-up.

However, because of the versatility and adaptability of bioremediation technology, the prospect of applying it in various ways and forms is quite feasible in the future. Amalgamation of the bioremediation process with other remediation methods will not only result in enhanced mitigation of wastes but simultaneously will also diminish the entire cost and the limitations of individual process. Comprehensive research should be done on advanced bioinformatics and statistical tools for devising schemes that will remove industrial wastes in an eco-friendly manner. These tools can help in identifying microbes, their functions and their catabolic and metabolic pathways, in-depth, using novel algorithms, conquering the constraints related to the microbial culture-dependent approaches. Additionally, the research community should focus on isolating and characterizing indigenous microbes and plant species from the polluted area so that these will be able to tolerate and successfully degrade the industrial wastes. Furthermore, development of microbial consortia can lead to effective biodegradation of industrial wastes because of its metabolic diversities and synergistic effects. Scientific community, globally, should work more on synthesizing nanoparticles using different biological agents, as the nanoparticles enhance the surface area, thereby improving the degradation efficiency, cost and time.

In conclusion, industrial wastes are the prime cause of contamination and lethality in environment, and various processes of bioremediation are considered as a low-cost and environmentally friendly choice to manage and remove these perilous pollutants. Undoubtedly, due to the numerous benefits, the process of bioremediation is creating a way towards a greener and cleaner environment. However, comprehensive research on the several challenges of bioremediation processes is needed so that it can be successfully utilized economically and socially.

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# Pesticides: Indian Scenario on Environmental Concerns and Future Alternatives

# 10

Preeti Mishra and Vidya Patni

## Abstract

India has witnessed shrinking agricultural fields in comparison to yield pressures; hence intensified usage of chemical pesticides was the only solution during Green Revolution. Thus, high concentration of chemicals in the form of fertilizers and pesticides has caused a serious imbalance in environment: toxicity, abiotic stress, decreasing soil fertility, etc. India is the largest manufacturer of generic agrochemicals in the world. Its pesticide market is growing at a substantial rate. Plant- and microorganism-based agriproducts show biocontrol and pesticidal activity, showcasing them as alternatives to replace chemicals from agriculture industry. Nano-formulations and nano-encapsulated agriproducts are new candidates showing enhanced efficacy and target specificity without harming soil fertility. These are nonhazardous and can be employed with integrated pest management (IPM) strategies. The controlled release of biodegradable nano-pesticides is another emerging area which can be exploited further for sustainable agriculture.

## Keywords

Agriculture · Biopesticide · Pesticides · Nano-pesticides

## Abbreviations

| | |
|---|---|
| BIS | Bureau of Indian Standards |
| CAGR | Compound annual growth rate |

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| | |
|---|---|
| CSIR | Council of Scientific and Industrial Research |
| DBT | Department of Biotechnology |
| DDT | Dichlorodiphenyltrichloroethane |
| DST | Department of Science and Technology |
| eNAM | National Agriculture Market |
| FAI | The Fertiliser Association of India |
| FAO | Food and Agriculture Organization |
| FCO | Foreign and Commonwealth Office |
| FSSAI | Food Safety and Standards Authority of India |
| HIL | Hindustan Insecticides Limited |
| ICT | Information technology |
| INR | Indian rupee |
| IPM | Integrated pest management |
| MMT | Million metric tonnes |
| MoCF | Ministry of Chemicals and Fertilizers |
| MRL | Maximum residue limit |
| MT | Metric tonnes |
| NAPs | Nano-agriproducts |
| NGO | Nongovernmental organization |
| POPs | Persistent organic pollutants |
| PPQS | Directorate of Plant Protection, Quarantine, and Storage |
| $TiO_2$ | Titanium dioxide |
| UP | Uttar Pradesh |
| WHO | World Health Organization |

## 10.1 Introduction

Pesticide is any substance/mixture from chemical/natural sources, applied at the last stage in an agricultural practice to prevent/kill/repel pest(s) such as bacteria, fungi, insects, nematodes, etc. (FAO 2015). Pests pose different kinds of threats to crop plants and ultimately cause moderate to extreme yield losses. Population of the world is increasing exponentially, and for providing food security to all, efficient management of crop pests is the utmost priority. Cereals, vegetables, oils, and cash and fodder crops are the backbone of agricultural economy and affect the population of the world directly or indirectly.

Earlier two words (a) food security and (b) food safety were used differently as food security was related to developing countries, while safe food was a major concern for developed countries. Developing countries initially wanted higher yield anyhow, but now developed as well as developing countries are concentrating on safe and nontoxic food products. This change is mainly dependent on agricultural practices being used in fields before and after harvesting. Higher yield from a limited land was the major objective of Green Revolution. High yielding varieties, intensive agricultural practices, and effective pest control helped exponentially to achieve this goal.

In the decade of 1960, Green Revolution advocated increase in production of food and cash crops by using all means—chemical fertilizers, pesticides, insecticides, high-yielding varieties, etc. (Yadav and Anand 2019). In that period, scientists did not successfully evaluate the negative impacts of some of these strategies. Few negative impacts included impacts on environment, human beings, other living organisms, and nontarget subjects.

Food grain production in India was 80.6 million metric tonnes (MMT) in 1963–1964; it increased substantially by 285 MMT in 2017–2018 (FAI 2018). The approach certainly provided encouraging results in the country in terms of the overall food production, but at the same time, the problems associated with the widespread use of pesticides, and their long-term effects, were overlooked. These impacts were seen mostly in the countries of South-East Asia and India where the Green Revolution strategies were implemented (Raghuvanshi 2017). Now India has become self-sufficient in food production; it is the need of the hour to propagate practices favorable to sustainable agriculture.

Farmers protect commercial/cash crops at any cost due to large investments and market pressure. Few studies have shown that high usage of pesticides to control pests attacking commercially grown high-yielding varieties unexpectedly enhanced virulence in these crop pests (Sánchez-Bayo 2012). Hence, concern about their adverse effects on nontarget organisms, including human being, has also grown.
Only a meager amount of the sprayed pesticides is effective, as most of the quantity applied is released to soil, water bodies, atmosphere, and nontarget organisms. Extremely less quantity of pesticides reaches targeted pests in comparison to the pesticide applied (Pimentel and Burgess 2012).

Indian farmers avail loans for various agricultural practices. According to them, this investment is essential for better yield and further income. Higher usage of chemical fertilizers and pesticides can provide high income with low cost. Natural plant protectants and biopesticides are costlier than conventional approaches but lesser number of applications are required (Gupta and Dikshit 2010). Eco-friendly approaches like biocontrol and biopesticides are new and will take time to be adopted significantly in agriculture. However, in recent years, farmers are adopting IPM strategies which can effectively manage crop losses.

Eco-friendly approaches to manage diseases in crop plants need extensive research and promotion from government and private companies. It is time consuming and results are not meeting the expectations at many times. The approach distracts the farmers and they always remain in a dilemma between unethical use of banned pesticides and sustainable practices. It’s time to overcome all the hurdles and promote good agricultural practices for sustainable agriculture.

## 10.2 Consumption of Pesticides in India

In India, the crop losses have been recorded as more than INR 60 billion annually, by factors such as "weeds (33%), diseases (26%), insects (20%), birds (10%), rodents, and others (11%)" (Jayaraj et al. 2016). The recommended rate of application of pesticides should control/kill the most tolerant species for which "the pesticide is recommended" (Duke et al. 2017).

India is one of the most dynamic and largest generic manufacturers of agrochemicals in the world.

According to annual report of Directorate of Plant Protection, Quarantine, and Storage, production of pesticides was INR 186 metric tonnes in 2014–2015 and reached 217 MT in 2018–2019 with a CAGR of 3.83 (PPQS 2021).

Currently, biopesticide market of India is in growing stage and constitutes a small share. It has a huge potential for growth in the coming years. In India consumption of total indigenous pesticides (technical grade) in 2016–2017 was 29,372.03 MT; in 2017–2018, 19,686 MT; in 2018–2019, 26,013.67 MT; and in 2019–2020 again increased to 27,038.21 MT, and previous year it remained as 24,232.62 MT (PPQS 2021) (Table 10.1). This includes consumption of chemical and biopesticides in fields. The consumption of chemical pesticides has decreased during previous years (Fig. 10.1).

Maharashtra and Uttar Pradesh consume very high quantities of chemical pesticides. Punjab and Haryana instead of less agricultural area use exponential quantities of pesticides but have shown subsequent declining trend in the same (Devi et al. 2017). Northeastern states (Arunachal Pradesh, Assam, Manipur, Meghalaya, Mizoram, Nagaland, Sikkim, and Tripura) consume lesser amount of chemical pesticides in total which is very less in comparison to northern and southern Indian states. Sikkim is totally free from pesticide usage as its agriculture is totally organic farming based.

**Table 10.1** Status of year-wise consumption of different indigenous pesticides (chemical and biopesticides) in metric tonnes mT[a] (technical grade)

| S. no. | Name of pesticide | 2016–2017 | 2017–2018 | 2018–2019 | 2019–2020 | 2020–2021 |
|---|---|---|---|---|---|---|
| 1. | Insecticides | 11,542.65 | 3945.29 | 9478.05 | 10,656.56 | 6668.73 |
| 2. | Fungicides | 8789.28 | 9078.84 | 8962.21 | 9115.81 | 10,318.29 |
| 3. | Weedicides | 4075.28 | 2969.62 | 3998.45 | 4275.31 | 3296.73 |
| 4. | Rodenticides | 409.39 | 185.92 | 291.16 | 283.60 | 316.90 |
| 5. | Plant growth regulators | 110.19 | 33.03 | 85.34 | 90.04 | 96.59 |
| 6. | Biopesticides | 4385.79 | 3472.92 | 3195.94 | 2358.89 | 3535.38 |
|  | Total consumption of pesticides | 29,372.03 | 19,686.78 | 26,013.67 | 27,038.21 | 24,232.62 |

'000 metric tonnes (mT[a])

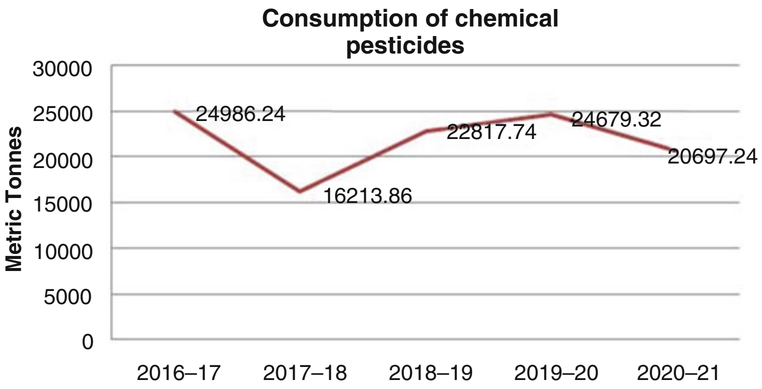


**Fig. 10.1** Consumption of indigenous chemical pesticides during 2016 to 2021 (PPQS 2021)

Hindustan Insecticides (India) Ltd. is the new name of Hindustan Insecticides Ltd. which took effect in 2018. The company is now dedicated to spread awareness among farmers through various training programs for safe and judicious use of pesticides. It is also educating agriculture workers for adoption of insightful integrated pest management practices. HIL trained 355 gardeners/nursery men for 200 h of skill development under Prime Minister's Kaushal Vikas Yojna (MoCF 2020).

## 10.3 Impact of Chemical Pesticides

### 10.3.1 Regulations and Quality Control

"The Insecticides Act, 1968" regulates registration of pesticides in India; before granting the registration, it requires data related to bio-efficacy and safety of human beings, wildlife, birds, domestic animals, beneficial parasites, and predators (Subash et al. 2018).

Only active principle and one metabolite of the pesticide formulation are used as markers by manufacturer companies, but this does not exclude the presence of adjuvants, which are cell penetrants. Mesnage et al. (2014) advised that all these factors related to adjuvants and other additives must be taken into account in the regulatory process.

Various initiatives like eNAM improved use of ICT, and other technologies can be encouraged. These can increase awareness among farmers and workers about newer techniques which may be more effective in agriculture (Krishna and Naik 2020).

### 10.3.2 Impact of Chemical Pesticides on Soil

Chemical pesticides and/or their transformation/byproducts remain in the soil and atmosphere for longer periods. In India and neighboring countries, chemical pesticides are found persistent in almost all components of the environment (air, water, and soil) for longer time periods (Yadav et al. 2015). In the 12th Stockholm Convention, few chemical pesticides like "aldrin, toxaphene, DDT, chlordane, dieldrin, endrin, heptachlor, hexachlorobenzene, and mirex" were identified as persistent organic pollutants (POPs) due to their accumulation in environment (Ashraf et al. 2013).

Organochlorines (DDT, aldrin, dieldrin, etc.) are commonly found persistent in the agricultural soil. These have been replaced successfully by organophosphate pesticides. Organophosphate pesticides are rapidly degradable by hydrolysis but have shown acute toxicity through their byproducts which is a matter of concern for India. According to recent annual report of the Ministry of Chemicals and Fertilizers, production of DDT has declined by 14.80% in India in the last 5 years. Atrazine, another harmful chemical, has shown 9.33% annual growth rate in production in India (MoCF 2021). Malathion has been consumed in agricultural fields at a constant rate during previous years.

According to Fig. 10.2, consumption of chemical pesticides has been retained during the last 5 years, while consumption of biopesticides has not gained the pace required for the country (PPQS 2021).

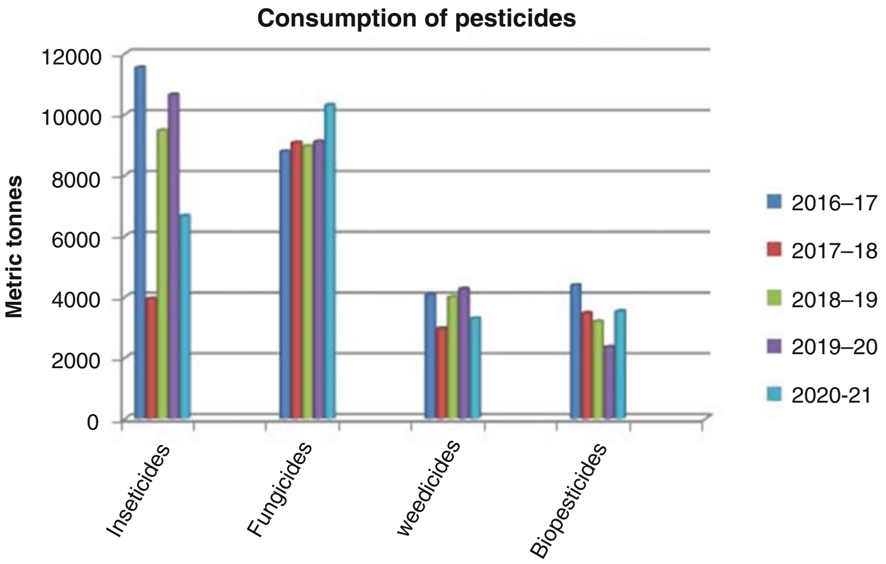


**Fig. 10.2** Consumption of chemical and biopesticides during the last 5 years (Source: Directorate of Plant Protection, Quarantine, and Storage)

Several banned organochlorine insecticides like DDT, aldrin, and lindane were detected in trace amounts in studied Palakkad soil of India (Gopalan and Chenicherry 2018). The soil transmits trace amount of insecticides to water bodies and food chain through grazing. Organophosphate levels, more than maximum residue limit (MRL), has been detected in agricultural soils of Idukki, Kerala (Jacob and Resmi 2015), and water bodies of Bijapur (Maharashtra) (Pujeri et al. 2011) and in Vidarbha (Maharashtra) (Lari et al. 2014).

A case study of southern India (carried out in the fields of Tamil Nadu, Karnataka, and Kerala states) cultivating banana reported high levels of chromium, nickel, copper, cobalt, and Manganese (Sahodaran and Ray 2018). The result indicated that excessive use of chemical fertilizers and plant protectants over a long period of time caused contamination of soil.

### 10.3.3 Impact of Chemical Pesticides on Water

Agricultural practices are contributing pollution not only to open water but also to the groundwater resources. Pesticide-contaminated water poses a great threat to human being and organisms pertaining it as their habitat. Major impact is evident on the aquatic plants and animals found in the habitat. Mahmood et al. (2016) stated that pesticide contamination causes low levels of dissolved oxygen in the water and hence causes various "physiological and behavioral changes in fish populations." Maurya and Malik (2016) observed accumulation of organochlorines and organophosphates in water, sediments, and fishes of Kali River (UP), India, due to influx from industrial, agricultural, and domestic wastes' dumping.

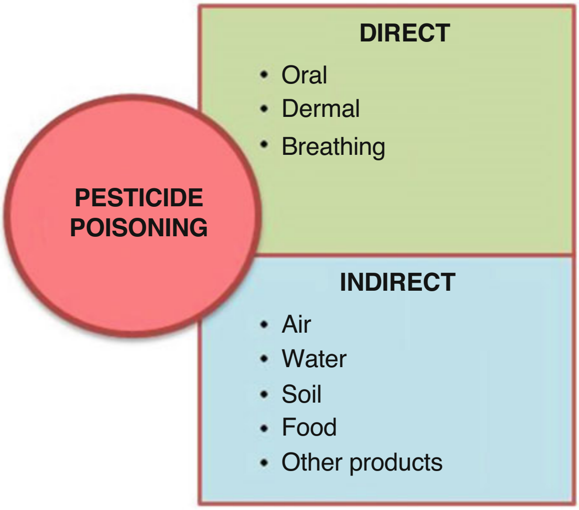
Kumarasamy et al. (2012) studied residues in water and sediments of Tamiraparani River Basin, South India. The study indicated that the accumulation and biomagnification of organochlorines in water can pose potential health hazards after consuming water and organisms from river. Many farmers of South Asian countries (including India) were using banned and toxic pesticides (rated by WHO) in their farms and were unaware of their negative impacts on the environment (Schreinemachers et al. 2015).

### 10.3.4 Chemical Pesticides and Health Hazards

Pesticide poisoning (accidental or intentional) is a major problem worldwide especially in developing countries. Humid environment favors breeding of insects and poses disease threats to standing crops. Agricultural workers are the victims of occupational health hazards as they are completely dependent on these practices.

Chintale et al. (2016) recorded more number of cases of organophosphate poisoning in month from August to December due to increased spraying of insecticides in the field. Pesticides are negatively impacting animal and bird community, bio-accumulating in food chain and hence impacting humans through consumption of meat, milk, and crops (Jayaraj et al. 2016).

**Fig. 10.3** Agents of pesticide poisoning



Humans get exposed to pesticides directly through contact, oral, dermal, and breathing, and indirectly through environment, soil, air, water, and products (Fig. 10.3). Intentional and unintentional exposures to pesticides are posing multiple threats to human health. Kim et al. (2017) suggested short-term effects of pesticide exposure: skin and eye irritation, headaches, dizziness, and nausea. He also stated some prevalent chronic impacts of same in the form of cancer, asthma, and diabetes due to pesticide exposure.

The first report of pesticide poisoning in India was documented from Kerala in 1958, where more than 100 people lost their lives due to consumption of parathion contaminated wheat flour (Kumar et al. 2013). Pandey et al. (2020) recently studied adverse effects of agrochemicals on female agri-practitioners.

One of the major reasons of pesticide poisoning in India is careless approach in handling pesticides and/or wearing of insufficient protective clothing. Pesticides and mixture of pesticide residues show many toxic effects on human health. Early symptoms due to poisoning of organophosphates are headache, hypersecretion, muscle twitching, nausea, diarrhea, vomiting, etc., while severe symptoms include tachycardia/bradycardia, bronchospasm/bronchorrhea, respiratory depression, seizures (esp. pediatric), etc. (Roberts and Reigart 2013).

Synthetic pesticides made up of hazardous chemicals can directly enter into an organism, or their byproducts accumulate and get biomagnified in the food chain. Fareed et al. (2013) have reported "adverse respiratory health and hematological alterations in agricultural workers" attributed to occupational exposure to organophosphates. Mittal et al. (2014) documented disorders in agriculture workers and community like cancer, reproductive abnormalities, neurological and behavioral disorders, premature hair graying, and even deaths due to "long-term exposure to pesticides in Malwa region of Punjab."

## 10.4 Alternatives to Chemical Pesticides

There is an increasing awareness about toxicity and environmental hazards being employed by chemical pesticides. Nature is competent enough to give solutions for the problems created by human beings on earth. The various chemicals being employed in agricultural fields are causing serious imbalance to nature.

### 10.4.1 Biopesticides

Biodiversity in the form of plants, fungi, bacteria, and other microorganisms are rich bioresources to balance nature in its own way. Singh et al. (2016) defined biopesticides as "naturally occurring biologically safe microorganisms that can be used to control and regulate outbreaks of pests in agriculture." Plants' secondary metabolites have shown significant potential for pesticidal properties against various pests (Koul 2016).

#### 10.4.1.1 Microbial Biopesticides

Microorganisms such as bacteria, fungi, algae, viruses, and protozoa are mostly used for the development of microbial pesticides (Costa et al. 2019). Mnif and Ghribi (2015) comprehensively explained biopesticidal isolates of bacteria like *Bacillus thuringiensis*, *Bacillus subtilis*, *Bacillus* sp., *Pseudomonas* etc. for their insecticidal, nematicidal, and fungicidal activity. Crampton (2017) reported that many strains of *Bacillus thuringiensis* produce different mixtures of proteins showing insecticidal activity on larvae infesting cotton. These different proteins kill specific species of insects. Fungi are the commonly used candidate as biopesticides because of their ability to control populations of many pathogenic insects and related genera (Becher et al. 2018).

Highly specialized virus family *Baculoviridae* has been used as biopesticide which represents DNA viruses showing pathogenic relationship with invertebrates (Haase et al. 2015). Microalgae, especially cyanobacteria, are considered as a biological agent (Costa et al. 2019). *Chlorella* was the first algae showing pesticidal activity producing chlorellin (Gupta et al. 2013).

Various state agriculture and horticulture departments (Kerala, Gujarat, Karnataka, Uttar Pradesh, Andhra Pradesh, Tamil Nadu, etc.) are involved in the production of microbial pesticides (Pathak et al. 2017).

#### 10.4.1.2 Biochemical/Botanical Pesticides

Plants have natural chemical defense line for pre- and postinfection activities. These chemicals are secondary metabolites like polyphenols, flavonoids, alkaloids, etc. Essential oils from plant families are distinctive bioactive compounds providing medicinal properties to the particular family. Defense-related compounds in plants like "secondary metabolites and essential oils" have been considered as a main source for formulating biopesticides (Lade et al. 2019). Biotechnology and molecular biology assisted in formulating biopesticides, and proved to be essential for incorporating changes in the desired way with high precision (Meena and Mishra 2020).

Biopesticides represent a range of bio-based substances helpful in controlling pests with a number of modes of action by inhibiting the growth, nutrition, development, and reproduction of pest or pathogen (Mnif and Ghribi 2015). Biopesticides comprising biocontrol efficacy have provided similar or in some cases better results than chemical pesticides, presenting them as an attractive alternative in conventional and organic farming (Gupta and Dikshit 2010).

It is now a well-established fact that biopesticides are nonhazardous and beneficial over chemical pesticides. But the government will have to provide enough resources for research and development in the field. Biopesticides' consumption in India was 3.2% overall in 2009–2010 (Subash et al. 2018).

Previous data shows that farmers are giving preference to chemical pesticides over biopesticides. Shelf life, slow release of biopesticide in the field, less efficacy, selection of matrix to pack the secondary metabolites or essential oil, and its degradation are major issues the biopesticide industry is facing. Awareness programs for farmers to selectively use chemical pesticides should be carried out by government and NGOs (nongovernmental organizations). Small-scale industry producing biopesticides should be given attention and resources.

A number of laboratories and units doing research on biological entities are functional in India, but units involved in commercial production are less in number (Mishra et al. 2020). Keswani et al. (2019) elaborated various constraints in biopesticides' promotion, registration, and commercialization in India. They insisted for the requirement of generous funding and administrative mechanisms for registration and marketing of biopesticides in India. In India, DBT, DST, CSIR, and many other agencies have launched schemes to promote research and development on biopesticides and their judicious use in designing IPM strategies.

Mishra et al. (2020) have extensively reviewed various agencies encouraging biopesticides in India, its status, production, and formulation technologies.

## 10.5 Nano-biotechnological Interventions for Crop Protection

### 10.5.1 Nano-pesticides

Nanoparticles are the revolutionary tools with variable physical and chemical properties. Nanoparticles may be synthesized using metal and nonmetal solutions. Nonmetal-based nanoproducts using silica, zinc, zinc oxide, etc. have significant properties providing solutions for controlling environmental pollution, animal diseases, plant diseases, etc. Nanoparticles are new and suitable candidate for the production of biopesticides (Al-Samarrai 2012).

Microorganism and plant extract-based nanoparticles are less toxic and effective against different pests of agricultural importance. Different chemicals being used to control crop diseases in combination with nanoparticles may be more effective. The nanoscale size changes chemical and physical properties of the final product and makes them unique in terms of conductance, efficacy, antimicrobial activity, etc. These properties can be exploited in plant disease management. The major concern in using nano-pesticides is target specificity. Nontarget organisms should not be harmed as they are beneficial in enormous ecological activities in the community.

Various nano-formulations have been prepared by scientists in India showing effective control of pests (Kamaraj et al. 2018).

### 10.5.2 Nano-encapsulation

Nanotechnology is a newer approach in the agricultural field and new to farmers constantly using chemical pesticides from decades. Nonhazardous eco-friendly biopesticides encapsulated or mixed in nano-formulations are comparatively safe and reliable. It is currently a new technology for plant protection. This approach is being used for formulation of nanoscale pesticides for delivery of specific pesticides into target host tissue. Nano-encapsulation is the process in which nanoscale particles are used for encapsulation of pesticides. This modification enhances the efficiency and mode of action of pesticide (Manjunatha et al. 2016). Metal nanoparticles especially of $TiO_2$ and Fe showed good results to adsorb whole and toxic residues of organochlorine pesticides (Rani et al. 2017). This facilitates the controlled release of bioactive compounds and can revolutionize the pesticide usage in India. Nano-encapsulation of secondary metabolites and essential oils in matrix enhances the reactivity of product on target pest.

Encapsulated neem oil using sodium alginate was found effective in soil against PEG (polyethylene glycol) coated nano-emulsion (Jerobin et al. 2012).

### 10.5.3 Nano-sensors

Nanotechnology can be proved as a vital tool for effective molecular management of diseases, defense induction in the host plant, disease detection, etc. Nano-sensors in the agricultural field can forecast presence of plant pathogens, nutrients in soil, weather, etc.

The sensors are providing innovative intervention to measure the signal due to any minor physical, chemical, and biological change in the soil. These sensors can also detect presence of and stress caused by microorganisms and other biotic factors. This will assist farmers to monitor status of nutrients and water in the soil. The devices will enable farmers to choose appropriate scheme for disease management in the field. Smart sensors can enhance productivity in agriculture by providing accurate information; thus, nano-sensors can make farmers self-sufficient to design integrated pest management for different diseases.

Various indigenous nano-sensors have been developed in India, few of which detect “perborate ions and hydrazine in water” (Gawas et al. 2018), mercury in environmental samples (Sowmyya and Vijaya Lakshmi 2018), arsenic in environmental samples sensitively (Vaishanav et al. 2017), and $Hg^{2+}$ ion (Shiva Prasad et al. 2018).

### 10.5.4 Regulation of Agri-Nanoproducts

Biosafety issues are posing a big challenge to nano-insecticides for agricultural applications especially in developing countries. Risk associated with using nanoproducts in agriculture is not easy to assess and moreover unexplored yet (Jain and Das 2021). Public awareness toward the risks is a serious issue. The studies being conducted on most of the agri-nanoproducts are not able to ensure the residual metal moieties in plants as well as soil. Comprehensive approaches to study starting point to end point are indispensible for agricultural systems of any country. Otherwise without proper research misinterpretation like genetically modified crops will prevail.

In a draft, DBT, Govt. of India, has accepted international guidelines for nano-agriproducts (NAPs) by European Union (DBT 2019). DBT in its report has described the use of nano-pesticides with or without carrier as "Safety studies on chemistry, bio-efficacy and residues, toxicity, packaging, and processing of molecules for registration, manufacture, or import of nano-pesticides should be conducted as per the regulatory aspect provisions under section 9(3) specified in the Insecticides Act, 1968. Nano-pesticides and plant growth regulators are also included in the act as per the guidelines of Central Insecticides Board and Registration Committee under the Ministry of Agriculture under Insecticides Act, 1968." DBT has emphasized on various in vivo and in vitro toxicity studies related to nano-pesticides before industrial production. These studies will help to understand biological responses and toxicity screening of nano-agriproducts at each level. Evaluation of nano-agriproducts should be carried out according to the guidelines of Insecticides Act, FCO, BIS, and FSSAI (Fig. 10.4). DBT (2019) has framed guidelines suggesting comprehensive stability testing, safety analysis, residue analysis, exposure risk, and hazard characterization of manufactured nano-agriproducts are compulsory depending upon the product type. In addition to these in vitro studies comprising in vitro digestion studies, in vitro genotoxicity testing with some other parameters needs to be taken into account. In vivo studies include in vivo genotoxicity testing, in vivo toxicity tests, in vivo repeated-dose 90-day oral toxicity study, etc. for safer use of these products (DBT 2019).

## 10.6 Conclusion and Future Prospects

India is a developing country with a growing population. Agriculture is facing enormous problems like weather, irrigation, poor soil health, pests, environment related, etc. affecting yield. Farmers were dependent on chemical fertilizers and pesticides on a large scale to increase the yield of crops and combating diseases. These are causing health hazards to the population residing in the area of application

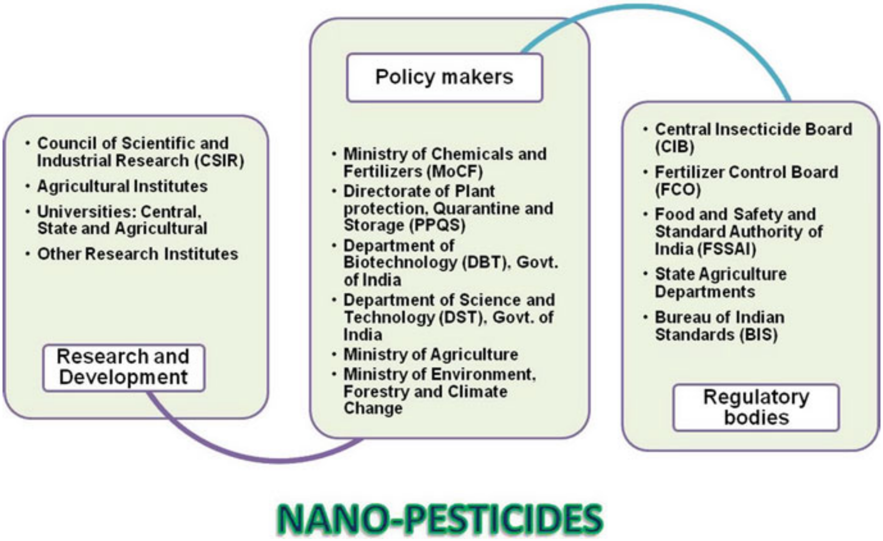


**Fig. 10.4** Stakeholders for nano-pesticide industry in India

like cancer, hormonal imbalance, cytotoxicity, nerve degradation, infertility, poisoning, etc.

Chemicals and their byproducts accumulate in soil and water and imbibe in the food chain, hence posing ecological imbalance. Pest resistance is another serious problem being enforced by chemical pesticides in the environment. Pesticide consumption in India has increased with a high growth rate. In comparison to the chemical pesticides, consumption of biopesticides in India was very low in the past years. But now farmers are attracted to organic farming in which chemicals' usage is prohibited, hence IPM and biopesticides are being practiced more.

In organic farming, crops suffer from diseases but substantial solution is not available. Nonhazardous biopesticides can pave the way and connect the agriculture with sustainability. Biopesticides are mainly of two origins: (a) microbial and (b) plant extracts. Pesticides of bacterial, fungal, and plant origin are nontoxic, eco-friendly, and nonhazardous. Nano-emulsion and nano-encapsulation of the pesticide principle from biological origin are future strategies to control the agricultural pests and improve the quality of crop plants. Various plants have been reported showing insecticidal, larvicidal, bactericidal, and fungicidal activity. Clay nanotubes are cost-effective alternatives to chemical pesticides. The controlled release of nano-pesticides is another excellent feature that could be exploited further. Specific polymers influence the controlled release of bioactive compound out of nanoscale matrix. Biopesticides and nano-pesticides with improved biodegradability, bio-efficacy, and bioavailability to the crop plants can transform the agricultural practice in India.

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# Part II

# Phytoremediation

# Phytoremediation: A Sustainable Solution to Combat Pollution

# 11

Kamakshi Saxena, Touseef Hussain, Raunak Dhanker, Parool Jain, and Shubham Goyal

## Abstract

Plants and microbes are the reservoirs of many structural and biological distinctive properties, which can be used to combat against environmental pollutants. They are the primary producers of ecosystems and transfer the primary productivity in the form of carbon energy to higher trophic levels in the food chain. They play a pivotal role in protecting the environment by reducing greenhouse gas emission, excess nutrients, heavy metal degradation, and other pollutants. They are found in every kind of habitat such as terrestrial, aquatic, and desert. A direct correlation has been identified among different plant and microbial communities, at different pollution levels, and different heavy metals in their habitats. Besides this, species composition of plants and microbes depends on different types of habitats and abiotic environmental factors. They consume excess amount of nutrients from the land and wastewater and take up $CO_2$ from the environment by the process of photosynthesis maintain the biological oxygen demand (BOD) and chemical oxygen demand (COD) of the habitat and restore the original

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conditions of natural habitat by reducing environmental and soil pollution. Further, their biomass can be used for bioenergy production, food production, and novel biochemical production for human and animal welfare. Further, their residual part can be utilized as fodder to cattle. The main aim of the article is to overview the advances in current and futuristic techniques for phytoremediation.

**Keywords**

Plants · Microbes · Bioremediation · Pollutants · Heavy metal · Advanced techniques

## 11.1 Introduction

The removal of heavy metal contamination has become a challenge for the environment, as various physical and chemical techniques used to remediate the toxicants are environmentally disruptive and have the chances of secondary contamination. Therefore, in the present scenario, bioremediation techniques offer an economic and environment-friendly approach as a suitable alternative to remediate the contaminants. The use of plants and their products, as well as microbes, is playing a pivotal role in phytoremediation (Guleri et al. 2020). The word phytoremediation is derived from the Greek word "phyton" meaning "plant" and "remedium" means "to correct." It helps in the removal of elemental pollutants to lower their availability in the soil. The environment has been continuously loading with the heavy metal pollutants as a result of the progression of urbanization and industrialization, which is significantly contributing to the deterioration of the environment health across the globe, hence becoming a major concern (Siddiqua et al. 2021; Ashraf et al. 2019). Other than plants, microalgae are the largest group of primary producers comprising 40–60% of aquatic diversity. They play predominant role in pollution reduction in aquatic ecosystems. Microalgae are single-celled and primary food source of second trophic organisms such as copepods (Dhanker et al. 2013). They are the best source to make the adsorbent model for the reduction in pollutants from their environment having several advantages over chemical adsorbent models (Guleri et al. 2020). Besides having prominent role in phytoremediation, they secrete many bioactive compounds, which act as sanitizer deterrents and the influence reproduction and growth rate of invertebrates (Dhanker et al. 2015) and aquatic stages of mosquito larvae (Dhanker et al. 2020). The anthropogenic sources, i.e., petroleum industries (Pitche 2016), agricultural practices (Hamzah et al. 2016), chemicals (Iqbal et al. 2016), mining (Chen et al. 2016), sewage (Farahat and Linderholm 2015), and electroplating (Muradoglu et al. 2015), are the chief originating medium for metalloids and heavy metals. However, the role of heavy metals in plant growth is indisputable, but it is restricted to a specific quantity. On this basis, they can be categorized as essential and nonessential heavy metals. Ni, Fe, Cu, Mn, and Zn are referred as essential heavy metals as they are essentially required up to a certain extent for various physiological and biochemical reactions (Cempel and Nikel 2016), while Pb, Cd, As, and Hg are referred as nonessential heavy metals with unknown functions in plants (Fasani et al. 2018). The excess amount of heavy metals results in alteration of physiological and biochemical processes and deteriorates the crop and human health (Haris et al. 2020; Rehman et al. 2017). This is a very alarming situation for the various components of environment, especially the heavy metal contamination in soil that may ultimately lead to the biomagnification. Most physical and chemical applications are expensive. Therefore, phytoremediation is the best suitable alternative for the removal of heavy metal contaminants by lowering their availability in the soil, hence preventing them to enter the food chain. This approach is plant-based, economic, easily available, and eco-friendly and reduces the risk of disposal. Sometimes, it is helpful in restoring the lost metals Ni, Fe, Zn, and Se to soil through phytomining and biofortification (Hussain et al. 2021). In this regard, plant selection for a specific metal is a key process for the final outcome. The main aim is to overview the advances in current and futuristic techniques for phytoremediation.

## 11.2 Need of Bioremediation of Heavy Metal-Infected Areas

### 11.2.1 Bioremediation

The process of bioremediation employs the use of microorganisms, which are present in nature or used deliberately to recover and repair the heavy metal-contaminated environment in the ecosystem. Bioremediation is an inventive and optimistic approach applied for retrieval and reduction in heavy metals in polluted sites. It is cost-effective and environment-friendly, with minimal health hazards and methodological way of revitalizing environment, polluted with toxic metals by making use of the characteristic biological mechanism of plants and microorganisms (Dhankar et al. 2020). In contrast to the conventional physical and chemical strategies, which are often costly, dangerous to life, and it is not much effective, when metals are at low concentration, and produce a substantial amount of sludge, which is toxic (Ekperusi and Aigbodion 2015). Blaylock et al. (1997) reported bioremediation as effective and economical, and he was able to save 50–65% of the cost of one acre Pb polluted soil by using bioremediation treatment in contrast to conventional methods such as landfill and excavation (Jain and Arnepalli 2016). Therefore, there is a need to pave a novel, systematic, cost-effective, and eco-friendly perspective for the retrieving inorganic metals (chromium, mercury, cadmium, and lead) added into the biosphere to shield the environment. The basic principle of bioremediation includes reducing the solubility of toxic heavy metals by altering pH, the redox reactions, and adsorption of hazardous waste from infected environment (Hussain and Dhanker 2021; Gadd 2010). Principally, bioremediation is a state of art used to neutralize and counteract heavy metal-contaminated soil and water, either ex situ or in situ. In this concern, microbes are considered as an important contrivance for retrieving metals as they can concentrate, remove, and make less soluble heavy metals from environment.

### 11.2.2 Need for Bioremediation in Heavy Metal-Affected Areas

During the last centenary natural processes, anthropogenic activities, unmanaged use of agrochemical, etc., have defiled areas of highly developed countries with high contamination of heavy metals. Heavy metal studies have defined its elements, which are present in nature possessing a high density and atomic weight, five times more than water. Toxic heavy metals are regularly present in natural waters but in tracer amounts, but many of them are toxic even when low. Nevertheless, the low levels of metals like arsenic, lead, cadmium, nickel, mercury, chromium, cobalt, zinc, and selenium are highly toxic and affect growth and development. Heavy metals if not properly assimilated in the body become injurious and start depositing in the body tissues. They make a pathway to enter the human body through water, air, food, or absorption through the skin surface when they are in contact with humans in pharmaceutical, manufacturing, agricultural, industrial, or residential areas (Dhankar et al. 2021). The chemical methods are cost-effective, which includes the use of heavy chemicals for washing such as leaching of heavy metals/mobilization into environment segments using chelating agents. Lately, the researchers shed light on heavy metal bioremediation as an emerging tool to conventional techniques. This could be done by new hopeful technology known as phytoremediation also called botanical remediation. On the basis of the mode of action, phytoremediation is further divided into subclasses such as phytostabilization, phytofiltration, phytovolatilization, and phytoextraction. Phytoremediation is a method for either converting or recovering toxic heavy metals into a less toxic form and/or get rid of these elements from the contaminated zone or downgrading organic substances and eventually mineralizing organic compounds into $CO_2$, $H_2O$, and $N_2$ gas, availing dead or living biomass. The method of bioremediation is applicable for both water and soil media through in situ and ex situ procedure. A major concern is adopting a relevant biosorbent in terms of economy and potential. Redox reaction is carried out by microbes and hence alters the bioremediation due to metal mobilization/immobilization (Ahamd et al. 2019).

### 11.2.3 Bioremediation Mechanism

The microorganism is ubiquitously present exhibiting resistance toward heavy metals and adapts themselves in a contaminated environment. On the cell wall, certain polymeric substances (present extracellularly) attach to heavy metals by phenomena like micro-precipitation or proton exchange of metals (Fomina and Gadd 2014). Due to the presence of amino, phosphoryl carboxyl, and sulfo groups, biomass surfaces exhibit a negative charge as potential ion exchange sites and metal sinks. Redox process, ion exchange, adsorption, precipitation, complexation, and electrostatic attraction are various channels involved in the bioremediation mechanism. Microbes may inculcate mobilization/immobilization of heavy metals through redox reactions and thus influence bioremediation. As, Fe, Hg, and Cr like heavy metals undergo oxide reduction processes (Glass 2000). Bioremediation process is hastened by changing an element from its stationary and insoluble form in sediments into its soluble and mobile phase. Many detrimental effects have been observed because of mobilization when metal ions are redistributed and released from their insoluble state from sediments into the soluble state (Fomina and Gadd 2014). Microbial metal immobilization serves as pool for metals by adopting different mechanisms (both in situ and ex situ) like bioconversion, bioaccumulation, biosorption, and/or intra/intercellular precipitation (as oxalates of Cd, Ni Zn, Cu, Co) employed in different ways (Comte et al. 2008; Mosa et al. 2016). Furthermore, due to impairment, an element can be easily drained from its aqueous phase in groundwater or wastewater (Klaus-Joerger et al. 2001; Ahluwalia and Goyal 2007). Cell wall and plasma membrane-like cellular inclusions act as fences and explore the entry of metal ions toward cells (Poirier et al. 2013). Bioaccumulation and biosorption are more attractive options to substitute traditional methods for bioremediation of heavy metals. Bioaccumulation sheds light to heavy metal uptake by living biomass (metabolism-dependent/active uptake) and is characterized by the absorption of contaminants by living cells/biomass (Haris et al. 2021). Biosorption is more superior in contrast to active bioaccumulation, as it is metabolized independently; however, it is largely dependent on the biomass/biosorbent type and contaminants present (Paliwal et al. 2012). Bioremediation of heavy metals in their metallic nanoparticles form, with the help of bacteria and the use of genetically engineered microbes as a part of bioremediation mechanism, has also been manipulated (Klaus-Joerger et al. 2001; Paliwal et al. 2012; Poirier et al. 2013; Mosa et al. 2016). Bioremediation serves many advantages, but also certain limitations, which should be kept into consideration while applying these methods (Table 11.1).

With the recent researches in the biotechnological field, genetically modified plants can be favorable in the phytoremediation approaches for making environment upstanding (Fasani et al. 2018). Futuristic studies should be highlighted on the combined use of more than one phytoremediation application for the successful interaction with heavy metal and ameliorated polluted area under field conditions.

## 11.3 Mechanism of Detoxification of Heavy Metals by Microbes

The society is dealing with many health and environmental challenges such as discharged household and industries, agricultural seepage, and other biotoxic substances containing practices. Hence, the emergence for the propelling need of cost-effective, eco-friendly, novel, and efficient solution to shelter the ecosystem for the amelioration of inorganic metals (Hg, Cr, Pb, and Cd) is present in the environment. In this context, current advancements in microbes-based heavy metal remediation have emerged as alternative promising conventional techniques. Heavy metals are nonbiodegradable and toxic to the microbes. The diverse forms of microbes have matured to generate potent mechanisms for detoxification to mitigate the detrimental effects of aforesaid metals. The microbes with bioremediation potential, especially in reference to environmental safety, have been underestimated for a long time.

**Table 11.1** Advantages and limitations of phytoremediation

| Advantages | Limitations |
|---|---|
| This is a passive technique and done on site | It is limited to shallow soils where heavy metal contaminants are localized to the surface only |
| The usage of low-cost affecting solar energy is done | This remediation is not acceptable by many regulatory agencies as it is in developmental stage |
| Environmental impact has been reduced and phytoremediation has added to improvement of landscape | Inadequate knowledge of farming, reproduction, and disease occurrence in plants used in phytoremediation |
| It is highly acceptable in public interest | As heavy metals are highly toxic, thus could be lethal |
| It supports animal life habitat | The plants used in metal remediation are limited in no. |
| It reduces dust and contaminants dispersal by wind | Phytoremediation is slower treatment process than the other traditional physicochemical methods |
| Leaching and mobilization of soil contaminants is lowered | Plants used at the contaminant site may not adapt toward climatic and environmental conditions |
| Harvested plants or their parts that have metals | |
| are easy to accomplish with existing technology | Compounds released by plants to increase the mobility of the metals can be leached into groundwater |
| Harvested biomass can be of economic importance | Decontaminated area must be large for the application of cultivation techniques |

Furthermore, the application of bacteria, biofilm, genetically altered microbes, algae, fungi, and microbial cells of immobilized nature has the capability to remove heavy metals through biosorption. For the sake of a sustainable environment, the heavy metal removal is preferred by the use of microbes resulting in the synergetic effects with many folds increasing (Yang et al. 2015). Heavy metals are not only the most important and long-lasting pollutant in the environment but economically very significant in industry. The current scenario of the ecosystem is combating pollution load due to heavy metals, which have been a toxic situation to the living organisms (Wu et al. 2010; Su 2014; Siddiquee et al. 2015; Okolo et al. 2016).

### 11.3.1 Microbial Remediation of Heavy Metals

Microorganisms follow different detoxification mechanisms to combat and survive amid of metal contamination. Microbes use various mechanisms to cope up metal toxicity such as the production of exopolysaccharide (EPS), biotransformation, extrusion, and use of enzymes (Dixit et al. 2015; Wu et al. 2010). The microbes respond to metals retained by the environment through many evolved ingenious metal detoxification and resistance mechanisms (Kumar et al. 2021). These

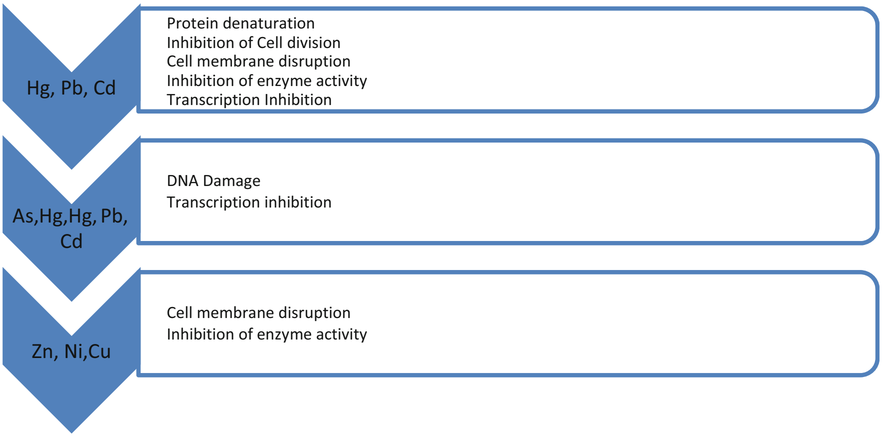

**Fig. 11.1** Heavy metal toxicity mechanism of microbes

mechanisms include electrostatic interaction, precipitation, ion exchange, surface complexation, on redox process alone or together (Yang et al. 2015).

Microorganisms decontaminate pollution by volatilization, valence conversion, and extracellular chemical precipitation (Tripathi and Hussain 2022). The microbes have negatively charged extracellular surface owing to the presence of anions that help microbes to increase the binding of cations (Gavrilescu 2004). The anionic sites on the microbial surface have the groups such as alcohol, phosphoryl, sulfonate, hydroxyl, sulfhydryl, ester, carboxyl, amine, thiol groups, and thioether, which facilitate the adsorption of metal (Gavrilescu 2004) (Fig. 11.1).

### 11.3.2 Toxicity of Heavy Metals to Microbes

Contamination by heavy metals is the potent source to cause toxic effects on microorganisms, and it is correlated with the preoccupied dose and accumulation of heavy metal (Rasmussen et al. 2000). Contamination of heavy metal involves several mechanisms, that is, destructing ion regulation, reacting as redox catalysts in the production of reactive oxygen species (ROS), breaking fatal enzymatic functions, and directly affecting the formation of DNA and protein (Hildebrandt et al. 2007). The biochemical and physiological properties of microbes are altered by the exposure of heavy metals. Metals such as cadmium (Cd) and chromium (Cr) have the capacity of calculating denaturation and oxidative injury of microbes and reducing the detoxification capacity of microbes.

Reactivity of metals such as Cr (III) can alter the activity and formation of enzymes due to the presence of thiol and carboxyl groups. The DNA with the negative charge on the phosphate group could interact with the complexes of cationic Cr (III) present intracellularly, and it might affect transcription and replication leading to the mutations.

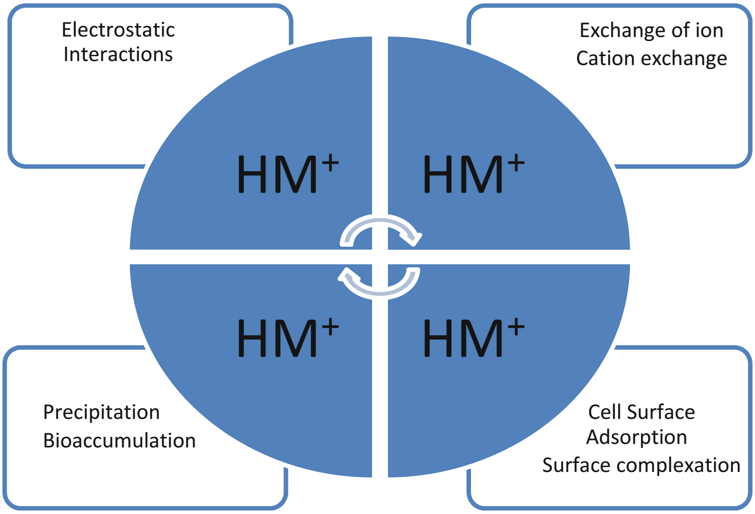

**Fig. 11.2** Heavy metal detoxification mechanism of microbes

Heavy metals cause severe injuries to DNA, lipids, proteins, and other cytoplasmic inclusions due to Fenton and Haber–Weis reactions, which catalyze the production of ROS. These ROS act as soluble electron carriers. Some metals are mainly responsible for DNA damage because they could enable the stabilizing superoxide radicals (Booth et al. 2015). Enzymatic interactions could be restricted through the configuration changes induced by competitive and noncompetitive inhibitors as heavy metals. The ionic imbalance is the result of the heavy metal adhesion on the cell surface and sometimes inflow through the transmembrane carrier or ion channels (Chen et al. 2014).

Lead (Pb) and cadmium (Cd) pose damaging effects on the cell membrane of microbes and cause DNA activity. This detrimental effect is responded due to the metal displacement from ligand interactions or native binding sites (Olaniran et al. 2013). The altered function of nucleic acid causes disrupted cell membrane, much functional disturbance, arrested enzyme activity, and oxidative phosphorylation (Fashola et al. 2016), which ultimately affect the growth, metabolism, and morphology of microbes (Fig. 11.2).

### 11.3.3 Heavy Metal Detoxification by Microbes

Microbes use different mechanisms to survive under metal exposure, which are extrusion, biotransformation, production of exopolysaccharide (EPS), use of enzymes, and synthesis of metallothioneins. The process involves procedures, electrostatic reactions, precipitation and exchange of ion, surface complexation, and redox interactions. Microbes withstand heavy metals by methylation, metal oxidation, organic–metal complex, enzymatic decrease, metal decrease along with efflux pumps, metal–ligand degradation, extracellular and intracellular sequestration of metals, removal by the permeable barrier, and presence of metal chelators such as metallothioneins and biosurfactants (Saghafi et al. 2019).

### 11.3.4 Biosorption Mechanism

The heavy metals uptake by microbes could be categorized into metabolically independent biosorption, that is prevalent on the cell surface, while metabolism-dependent bioaccumulation involves redox reaction, species transformation, and sequestration processes. A variety of metal ion compounds form complex for intracellular sequestration in the cytoplasm. The surface ligands interact with metals and increase their concentration within microbial cells, which are further transported to intracellular compartments. The metals are stored intracellularly by bacterial cells that have been efficiently utilized, especially in the effluent treatment methods. The rigidity of fungal cell wall is due to the presence of lipids, mineral, chitin, polyphosphates, protein, and polysaccharide with nitrogen constituents. The cell wall could be helpful in decontamination of metal ions by precipitation from the extracellular surface and intracellular components and uptake influence the valence conversion of some fungi aggravating metal accumulation to their spores and mycelium. The external surface of fungal cell wall can behave like a ligand and eliminate inorganic metals (Ayangbenro and Babalola 2017).

Microalgae are used as a bioindicator for the health of the aquatic ecosystem. The presence and absence of a particular type of species indicate the quality parameters or pollution level of any aquatic ecosystem. Many microalgae species, especially diatoms, have very unique structural cell structure, which makes them a perfect resource of pollutant degradation and accumulator of heavy metal (Tripathi and Hussain 2021; Hildebrand et al. 2012). On one hand, they absorb excess amounts of nutrients from wastewater and utilize it in increasing their population. On the other hand, microalgae use carbon dioxide for increasing their productivity like this they sequester excess amounts of greenhouse gas and contribute to the reduction in global warming (Janani and Kumar 2018). For example, *Thalassiosira* sp. is main diatom sp. having a dominant role in pollution reduction in water bodies. Heavy metals, cadmium, and copper are observed in almost every kind of aquatic habitats. Accumulation of these metals is observed in many microalgal species such as *Thalassiosira weissflogii.*

### 11.3.5 Extracellular Sequestration

The metal ions are extracellularly sequestered in cellular components, the periplasm, or complexation of metal ions as insoluble compounds. Copper-resistant *Pseudomonas syringae strains* result in copper-inducible proteins CopA, CopB (periplasmic proteins), and CopC (outer membrane protein), which attach with copper ions and microbial colonies. Recent studies have reported extraction of copper from contaminated soil by sulfur-oxidizing bacteria, followed by electrokinetic treatment using a DC current to further acidify the soil and collect the mobilized metals at the cathode. Iron-reducing bacteria such as *Geobacter* spp. and sulfur-reducing bacteria like *Desulfuromonas* spp. are able to reduce metals to less or nontoxic metals. Biological methods used for extraction of metals other than metal reduction include the organic acids production by heterotrophic organisms and the formation of sulfuric acid by oxidation of sulfur (e.g., by *Thiobacillus* spp.). *G. metallireducens*, an anaerobe, is able to reduce manganese (Mn), from lethal Mn. *G. sulfurreducens* and *G. metallireducens* are able to reduce chromium (Cr) from the lethal Cr (VI) to less toxic Cr (III). Sulfate-reducing bacteria produce hydrogen sulfide causing precipitation of metal cations (Frers 2009).

### 11.3.6 Extracellular Barrier of Preventing Metal Entry Into Microbial Cell

The plasma membrane, cell wall, or capsule fences metal ions from entering into the cell. Adsorption of metal ions is facilitated by ionizable groups of the bacterial cell wall. *Pseudomonas aeruginosa* biofilm cells show higher resistance toward copper, lead, and zinc ions than planktonic cells, whereas peripheral cells of the biofilm were killed. Accumulation of metal ions by extracellular polymers of biofilm takes place and then protects bacterial cells inside the biofilm.

### 11.3.7 Methylation of Metals

Metal toxicity is increased by methylation as a result increase in lipophilicity followed by increased permeability across cell membranes. Methylation plays a pivotal role in metal decontamination. Methylated compounds are usually explosive; for instance, Hg (II) can be biomethylated by bacteria such as *Bacillus* spp., *Escherichia* spp., *Clostridium* spp., *and Pseudomonas* spp. to gaseous methyl mercury (Ayangbenro and Babalola 2017).

### 11.3.8 Reduction in Heavy Metal Ions by Microbial Cell

Microbial cells convert the oxidation state of metal ions, hence reducing their toxicity. Bacteria for energy generation use metals and metalloids as electrons donors or acceptors. Metal ions are reduced by enzymatic activities, which result in the formation of the less toxic form of mercury and chromium inorganic metals.

## 11.4 Mechanism of Detoxification of Heavy Metals By Plants

The population has been increasing day by day, and the United Nations in 2018 estimated that the global births would be 74 million per year until 2027 through the Food and Agricultural Organization. This would be raising urbanization, industrialization, deforestation, agriculture, and energy supply to fulfill the global demands. The result is an increase in pollution and making the soil and water of poor quality. Human military activities, industrial mining, waste disposal, and farming practices are responsible for large-scale contamination with heavy metals and organic compounds in soil and water (Ayangbenro and Babalola 2017). The group of chemical elements of relatively high atomic number, atomic weight, and high densities are referred as heavy metals. The commonly found metalloids or heavy metals include lead (Pb), mercury (Hg), cadmium (Cd), zinc (Zn), arsenic (As), chromium (Cr), copper (Cu), and nickel (Ni). These are originated from natural and anthropogenic sources such as oil and gas industries (Neff et al. 2011), use of phosphate fertilizers in cropping (Hamzah et al. 2016; Rafique and Tariq 2016), sewage sludge (Farahat and Linderholm 2015), metal mining and smelting (Chen et al. 2016), and electroplating and burning fossil (Muradoglu et al. 2015). According to their roles in the biological system, heavy metals are grouped into essential and nonessential heavy metals. Zn, Cu, Ni, Fe, and Mn are referred to as essential heavy metals that require in physiological and biochemical activities of plants but become toxic when in large amount. Hg, Pb, As, and Cd are categorized as nonessential heavy metals that are highly toxic and decline agricultural productivity (Fasani et al. 2018).

The heavy metals are biodegradable-resistant and cannot be degraded by any physio- and biological processes, and they get accumulated into the human body through the food chain, persist in the environment, and increase in concentration eventually by biomagnification (Suman et al. 2018; Sarwar et al. 2010). Hence, heavy metals create a negative impact on the ecosystem and human health. Therefore, it is crucial to detoxify and remediate water and soil. Since from the decade, conventional methods of soil decontamination, which are pumping and treat, stabilization, soil washing, air stripping precipitation, thermal and adsorption, have been applied (Sheoron et al. 2011; Wuanna and Okieimen 2011; Rehman et al. 2017; Dal Carso et al. 2019). But these conventional techniques are expensive and furthermore do not really solve the problem. So, it is an urgent requirement for substitute practices, which are elegant, cost-effective, and capable to clean up heavily contaminated sites efficiently (Meagher 2000). A new alternative eco-friendly and cost-effective technology known as phytoremediation could be achieved. Phytoremediation is plant-based approach in which high-efficient metal-accumulated plants are used to make contaminated soil nontoxic. This method is also known as bioremediation, botanical bioremediation, or green remediation. In this technique, wild or genetically modified plants species are used to collect a large number of heavy metals from the soil so that it could lower the bioavailability and remove elemental pollutants from the soil.

### 11.4.1 Methods of Phytoremediation

Several methods of phytoremediation are as follows:

#### 11.4.1.1 Phytoextraction

This excludes organic compounds or metals from the soil due to their accumulation in the biomass. The roots absorb substances from the soil or water and concentrate it above the ground in the plant biomass (Ali et al. 2013). Plants that are hyperaccumulators can uptake higher amount of contaminants (Rascio and Navari-Izzo 2011).

#### 11.4.1.2 Phytodegradation

It is also called phytotransformation in which plants are utilized to store and degrade or mineralize inside the plant cells by specific enzymes that include nitroreductases, dehalogenases, and laccases (Rylott and Bruce 2008).

#### 11.4.1.3 Phytostabilization

The bioavailability of pollutants is reduced by binding or immobilizing them in the soil matrix. Plants make the substances limited to leach from the soil (Lone et al. 2008).

#### 11.4.1.4 Phytovolatilization

In this technique, plants use pollutants, and transform and release them into the atmosphere from growth matrix (Ruiz and Daniell 2009).

#### 11.4.1.5 Phytofiltration

The root system or other submerged parts of the plants absorb, precipitate, and concentrate the contaminants specifically heavy metals or sometimes radioactive elements (Javed et al. 2019).

#### 11.4.1.6 Rhizodegradation

It is also known as phytostimulation in which growing roots of plants stimulate the proliferation of microbes in degrading rhizosphere. They utilize metabolites and exudates of the plants as a source of energy and carbon (Ali et al. 2013; Jacob et al. 2018).

#### 11.4.1.7 Phytomining

Some metals can be restored from plant parts that have been applied by humans in potash. It may be profitable too. Besides accumulating toxic and unwanted minerals in their parts, plants also absorb contaminated organic compounds such as polychlorinated biphenyl (PCB).

### 11.4.2 Advantages and Limitations of Phytoremediation

It is in situ and passive technique, which has minimized environmental impact and contributed to landscape improvisation. This is useful to maintain the fertility of the soil (Othman Yahia and Daniel 2018). It is responsible to reduce dispersal of pollutants and dust by wind, leaching, surface run-off, and transportation of soil contaminants. Overall, it is low-cost and eco-friendly technique. Besides offering several advantages, phytoremediation has some limitations, which should be considered when seeking to apply it. Though it is of low cost but a time taking technology. Its treatment is slower than that of the traditional methods. The concentration of pollutants and other toxins could determine or exceed the tolerance limit of the plants. Usually, plants are selective in metal accumulation. If the concentration and toxicity of metals are more in the soil then it can be lethal for the plants too (Marques et al. 2009).

#### 11.4.2.1 Genetic Engineering in Phytoremediation

To overcome the limitations of phytoremediation, recombinant DNA technology has been proved an effective technique to improve the tolerance potential of plants toward heavy metals. To make the plant genetically modified, the foreign gene of interest is selected from a particular plant species or animal or bacteria, inserted, and transferred into the genome of the target. This process produces the inherited desired gene after DNA recombination and confers specific traits to that particular plant or animal or bacteria (Maurya et al. 2020; Chaurasia et al. 2021). This is comparatively unparalleled to the traditional method of breeding and advantageous to produce plants with desired traits such as phytoremediation (Marques et al. 2009). It is the most common strategy to produce GMPs for increasing the heavy metal efficiency in the plants to enhance phytoremediation.

## 11.5 New Innovative Approaches for Removal of Heavy Metals

Phytoremediation approach is not a new technique in terms of contaminant removal, but no doubt it has been overshadowed for a long time. Plants have been reported to remediate the wastewater even 300 years ago. This technique involves many different applications using plants, plant products, and microbes. The approaches include multiple processes such as degradation, filtration, extraction, stabilization, and volatilization.

### 11.5.1 Phytodegradation

Plant's metabolic processes convert the toxic organic pollutants into less toxic substances. Microbes associate the process of degradation (Marques et al. 2009). For example, *Populus* species and *Myriophyllum spicatum* have an enzymatic system to degrade the contaminants (Rylott and Bruce 2008).

### 11.5.2 Phytofiltration

This technique is also referred to as rhizofiltration. Plants absorb the contaminants through an aqueous medium, filtered by roots. Plants with high accumulation capacity or high root biomass and high tolerance potential are favorable for phytofiltration, for instance, *Phragmites australis*, *Helianthus annuus*, *Fontinalis antipyretica*, *Brassica juncea*, species of *Populus*, *Lemna*, *Salix*, and *Callitriche* (Prasad 2004; Pratas et al. 2012).

### 11.5.3 Phytoextraction

This technology is also referred to as phytosequestration or phyto-absorption or phytoaccumulation. The variety of studied plants are capable of storing the contaminants in their aerial parts. The major absorbing metals are Cd, Ni, Cu, Zn, Pb, and sometimes for Se and As, e.g., *Pteris vittata*, *Elsholtzia splendens*, *Thlaspi caerulescens*, and *Alyssum bertolonii* (Van der Ent et al. 2013).

### 11.5.4 Phytostabilization

It prevents the direct exposure of soil contaminants to wind, water, humans, and animals as they are covered by the plant community. Tolerant plant species are utilized to immobilize the translocation of metal and metalloids and restrict them in via dose zone (Nanthi et al. 2011). This technique is also effective in preventing secondary waste. But it needs the monitoring to check the stabilizing conditions.

### 11.5.5 Phytovolatilization

The process to absorb the contaminants, convert them into nontoxic simpler forms, and release them into the atmosphere by plants is referred to as phytovolatilization. Some rhizobia and their associates help in the degradation of organic contaminants (Jacob et al. 2018), e.g., *Astragalus bisulcatus*, *Brassica napus*, *Stanleya pinnata*, *Liriodendron tulipifera*, *Nicotiana tabacum*, *and Arabidopsis thaliana* (Ali et al. 2013).

### 11.5.6 Current Techniques

These abovementioned techniques have been improvised and categorized on the basis of contamination source. The techniques with some variations are stated mentioned:

#### 11.5.6.1 Hydraulic Barrier

The deep root system of large trees absorbs water in large quantities from the soil, resulting in major soaking of contaminants, which eventually evaporate through transpiration (Dushenkov 2003; Pratas et al. 2012), for example, *Populus* species.

#### 11.5.6.2 Vegetation Cover

It includes the trees, herbs, and shrubs to cover the landfills or tailings. This plant surface decreases the pollutant spread as the roots favor the aeration to microbial population, which increase the degradation of pollutants (Nanthi et al. 2011; Van der Ent et al. 2013; Sabir et al. 2015). The main limiting factor of this technique is difficulty in plant root development in tailings.

#### 11.5.6.3 Constructed Wetlands

This is the oldest process for wastewater treatment and is not considered as pure phytoremediation process due to the inclusion of multiple techniques, i.e., adsorption, filtration, precipitation, and ion exchange (Ali et al. 2013; Jacob et al. 2018). This is mainly present in the areas where water level remains at or near the surface for some seasons in the year. It consists of microbes, algae, vascular phytoplanktons, and organic soils.

#### 11.5.6.4 Phytodesalination

This is a recent approach to extract salt from saline soils through halophytes. This can be considered the extended part of phytoextraction owing to its peculiarities.

## 11.6 Conclusion and Future Prospective

Increasing metal pollution has become a menace for food, agricultural production, and health due to their persistence hence their exponential growth in the environment. To minimize and mitigate heavy metal contamination, multiple techniques have been explored. Genetic engineering approach has emerged as a futuristic potential aid to alter the target plant species with competent traits such as fast growth, good adaption, high biomass production and accumulation, and high heavy metal tolerance to various geological and climatic conditions. Furthermore, microbes and chelating agents can be used either to ameliorate soil health or to increase heavy metal bioaccessibility, which hastens heavy metal accumulation in plants. Phytoremediation is suitable for heavy metal remediation of water, sediments, and soil. The effectiveness of phytoremediation processes could be determined by many available monitoring tools. Despite a few limitations and disadvantages of the phytoremediation process, it is found an effective method for cleaning and recovery of the environment. In the current scenario, bioremediation of heavy metals is exhibiting a positive promising approach for detoxification and metal biosorption, especially from genetically modified microbes and biofilm-mediated techniques such as microbial fuel cell-based techniques and microbial gene transfer. The cell wall of biosorbents composed of polysaccharides and peptidoglycan favors the binding of metal uptake. It has multiple advantages in terms of cost-effectiveness, broad spectrum of pH for high metal affinity, temperature, faster kinetics, and safe technology. Furthermore, extended research intervention is required to be employed on biofilms for gene transfer in bioremediation. Contamination could be significantly decreased with the advances in the arena of environmental biotechnology using genomics in many fields of biology. Phytoremediation is mainly applied technology to decontaminate the site by using plants and related microbes. Purposefully, selected plant species could be efficiently used at metal-polluted sites and different strategies could be devised for future innovations such as chelate-assisted and microbe-assisted methods and genetic engineering, and methods. It would greatly enhance the future of phytoremediation.

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# Phytoremediation and Therapeutic Potential of Neglected Plants: An Invasive Aquatic Weeds and Ornamental Plant

# 12

Astha Gupta and Tulika Tyagi

## Abstract

Contamination of water and wastewater with heavy metals is emerging as a global environmental challenge. Increasing urbanization, industrialization, and overpopulation are leading to the degradation of the environment. The main hazardous contents of water pollution are heavy metals. Heavy metal pollution is considered a leading source of environmental contamination and poses serious hazards to human health and aquatic ecosystem. Water bodies are the main target for disposing of pollutants directly or indirectly. Conventional treatment technologies to remove contamination from wastewater are usually costly, time-consuming, and environmentally destructive. Therefore, the research is oriented toward cost-effective and eco-friendly technology for water purification, which will be beneficial for community. *E. crassipes* is an aquatic invasive plant with natural tendency to absorb pollutants from the water, especially heavy metals. This plant is suitable for the bio-cleaning of industrial wastewater contaminated with Cd, Cr, Co, Ni, Pb, and Hg. Furthermore, *E. crassipes* also has the ability to remove toxins other than heavy metals, such as cyanide, and is helpful in gold mining areas. Similarly, *P. stratiotes* (medicinal and aquatic plant) has also been extensively used for phytoremediation. It is helpful in the removal of some heavy metals from the industrial effluent and was found to be the best phytoremediator for Pb and Cu as it is successful in removing these metals. An

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ornamental plant includes Canna, which are being used for various purposes in addition to attractive ornamental plant in garden like noodle-making, animal fodder, jewelry, dye from seeds, paper from leaves, candies, and cakes. In addition to ornamental and industrial application, Canna is a potential source of vitamins, minerals, and nutraceutical components. Canna is also associated with environmental cleanliness and reported to degrade triazophos sulfide phosphate an organic phosphorous pesticide which is useful in pest control. *Canna indica* species can be used to increase water quality and for the phytoremediation of cadmium or many other toxic heavy metals. Despite various important roles of these underutilized plants, there are no any systematic efforts have been made to characterize and assess the extent of diversity available in aquatic and ornamental plants at phenotypic, biochemical, and molecular level. Moreover, this chapter further focused and elaborated on such plants and their bioactive compounds, which would be promising contributor of environment protection and pollution improvement programs.

Therefore, combination and application of *Eichhornia crassipes*, *Pistia stratiotes*, and *Canna* will make difference for detoxification of contaminants, heavy metals, and herbicides for sustainable environment and its improvement for human prosperity.

**Keywords**

Phytoremediation · Therapeutic agents · *Eichhornia crassipes* · *Pistia stratiotes* · *Canna* (ornamental plant)

## 12.1 Backdrop

Plants are one of the most fascinating creations on this earth. They capture the solar energy and transfer it to the living world. They are the source of our livelihood as they accomplish our basic needs and provide food, cloth, and shelter directly or indirectly in the form of cereals, pulses, fruits, vegetables, spices, fuel, fiber, wood, gums, resins, medicines, essential oils, etc. In addition, plants have also been associated with our esthetic, spiritual, recreational, and scientific aspects, and very importantly, they are also potential source to produce a wide variety of chemical compounds of great therapeutically and economical value.

Some aquatic and ornamental plants have the capacity to absorb heavy metals and pharmaceutical pollutants present in the wastewater. The wide application of these plants is due to their availability, bioaccumulation potentials, invasive mechanism, resilience in a toxic environment, and biomass potentials. Some wild ornamental plants and aquatic weed are found more tolerant, and they can act as a strong obstacle to stop the entry of contaminants into the food webs. This chapter covers various aspects of aquatic macrophytes and ornamental plant application in the phytoremediation of diverse aquatic inorganic and organic pollutants. Water hyacinth and water lettuce are aquatic plant species of ubiquitous nature, having great bioaccumulation potentials, resilience, invasive mechanism, and sporadic reproductive capacity used in developing phytoremediation systems (Ekperusi et al. 2019). Some ornamental plants have been evaluated as good candidate for metal-contaminated remediation includes *Chrysanthemum*, *Pontederia cordata*, *Mirabilis jalapa*, *Calendula calypso*, and *Tagetes patula* (Li et al. 2020; Xin et al. 2020; Ramírez et al. 2020; Farooq et al. 2020). Therefore, further investigation of most promising plant source would be needed for pollution control and metal detoxification.

## 12.2 Phytoremediation: Plant-Based Eco-Friendly Technology

Phytoremediation is a valuable, cost–effective, and environmentally friendly technology for the remediation of toxic metals from the environment. The plants used as a metal accumulator for the removal of heavy metals and other contaminants in phytoremediation were first introduced in 1983, but this idea is already 300 years old. In phytoremediation process, plants accumulate contaminants through their roots and then translocate pollutants to the other parts of their body (Ashraf et al. 2018; Sharma et al. 2015). Phytoremediation is also known as agroremediation, vegetative remediation, bioremediation, green remediation, green technology, and botano-remediation (Rahman and Hasegawa 2011; Sarwar et al. 2017; Kushwaha et al. 2018). The use of rich vegetation, soil, and microbiota along with other agrochemical practices makes the vegetative remediation a promising eco-friendly and green technology for the accumulation of different heavy metals (Mahar et al. 2016). There are myriad in situ and ex situ remediation applications in a phytoremediation mechanism. In situ applications are common because they decrease the multiplication of pollutant in water and airborne waste, which minimize the hazardous effect to the environment. Multiple pollutants can be treated on site by the phytoremediation, which reduces the spread of contamination by preventing soil erosion and leaching without the need for a disposal site. The utmost advantage of phytoremediation is its clean-up cost is quite reasonable than conventional techniques of remediation (Gerhardt et al. 2017). Phytoremediation is applicable for the remediation of large-scale area without any specific personnel and exclusive equipment over large-scale area where other conventional techniques are extremely inefficient and costly (Leguizamo et al. 2017). The widely distributed large number of toxic contaminants can be remediated by phytoremediation technologies such as insecticides, chlorinated solvents, petroleum hydrocarbons, radionucleosides, surfactants, explosive elements, heavy metals, polycyclic aromatic hydrocarbons (PAHs), and polychlorinated biphenyls (PCBs) (Leguizamo et al. 2017; Hussain et al. 2018). There are various plant species that accumulate higher concentrations of heavy metals in different parts of the body, such as a leaf, stems, and root, significantly without showing any sign of toxicity (Reeves et al. 2018).

Aquatic vascular plants (macrophytes) are important component of wetland habitats, and a diverse macrophyte assemblage can increase habitat heterogeneity in a wetland. Weeds are unwanted plants, which interfere with the consumption of land and water resources and thus adversely affect human welfare. They compete with the desired vegetation in croplands, forests, aquatic systems, etc. Scientists around the world are working hard to find out different uses of these weeds. Some weeds may have harmful effects on crops because of the chemical substances they release from their leaves and roots into the environment, but few substances may have beneficial effects on some plant species. Therefore, this chapter demonstrates underutilized plants with plant biotechnological tools and techniques, which have potential to treat environmental pollutants and have enormous pharmaceutical properties.

## 12.3 Neglected Plants for Phytoremediation

The aquatic ecosystem is an efficient and resourceful clean-up technique for phytoremediation over a large contaminated area. Aquatic plants act as a natural absorber for pollutants and heavy metals (Pratas et al. 2014). Removal of heavy metals and other contaminants through aquatic plants is the most effective and profitable method (Ali et al. 2013; Guittonny-Philippe et al. 2015). The selection of productive aquatic plant species for the accumulation of heavy metal is a crucial step for enhancing the phytoremediation (Sreelal and Jayanthi 2017). They have gained an overwhelming reputation over the years because of their capacity to clean up contaminated sites throughout the world (Gorito et al. 2017; Gopal 2003). Aquatic plants develop an extensive system of root system, which makes them the best option for the accumulation of contaminants in their roots and aerial parts.

### 12.3.1 Aquatic Plants

Aquatic plants with floating leaves and submerged roots are well known for their capability to eliminate the metals from the contaminated environment: water hyacinth (*Eichhornia crassipes*) (Gunathilakae et al. 2018), water lettuce (*Pistia stratiotes*), (Abbas et al. 2019), water ferns (*Salvinia minima*) (Tha and Bianchini 2015), duckweeds (*Lemna minor*, *Spirodela intermedia*) (da Silva et al. 2017; Daud et al. 2018), and watercress (*Nasturtium officinale*) (Shi et al. 2020). Passive transport is direct contact of the plant body with the pollution medium where heavy metals accumulate in aerial parts of the plant body. Water hyacinth, duckweed, and water lettuce are the most frequently used free-floating plants for the remediation of heavy metals from wastewater (Anaokar et al. 2018; Chen et al. 2018; Hua et al. 2012; Singh et al. 2012).

#### 12.3.1.1 Significance of Aquatic Plants

Phytoremediation of heavy metal contamination with aquatic plants has gained significant consideration due to its effectiveness, elegance, and eco-friendly nature (Sharma et al. 2015; Bokhari et al. 2016). Aquatic plants remove heavy metals via surface adsorption or absorption and then accumulate them in certain bounded forms. A wide variety of aquatic plants like water hyacinth, water lettuce, giant duckweed, *Salvinia* sp., and *Azolla* sp. have tremendous ability for the phytoremediation of wastewater (Soda et al. 2012; Rodríguez and Brisson 2015). The widespread availability, large biomass, high growth rate, and tolerance to toxic pollutants make them the best available phytoremediation plants. Aquatic plants possess sugar in the shape of starch, cellulose, and hemicelluloses. The fermentable sugar obtained after the hydrolysis of this carbohydrate results in the production of lactic acid, ethanol, and other important products. Therefore, sugar present in aquatic plants is a new potent feature supporting their role in the eco-sustainable environment. However, aquatic plants like *Pistia stratiotes* and *Eichhornia crassipes* have been reported to produce sugar during their enzymatic hydrolysis process.

### 12.3.2 Ornamental Plants

Plant flowers are very attractive and beautiful creation on earth, which has industrial and pharmaceutical application also. However, several colors of flowers exist worldwide that would be demanding agent in textile and food industry due to occurrence of natural herbal dye. Moreover, two most important ornamental plants are considered as potential hyperaccumulators like *Mirabilis jalapa* L. and *Tagetes patula* L., which were used for the remediation of alkaline soil co-contaminated with cadmium (Cd) and tetracycline antibiotics (TCs) (Li et al. 2020). However, surface water bodies may be polluted with various organic contaminants. Further, floating treatment wetlands (FTWs) established with *Canna flaccida* (ornamental plant) was helpful for eliminating the acetaminophen and carbamazepine (pharmaceuticals) and atrazine (herbicide) from contaminated water (Hwang et al. 2020). Recently, two different macrophytes, *Acorus calamus* and *Canna indica*, were explored with the vertical subsurface flow constructed wetlands for the treatment of domestic sewage (Barya et al. 2020). This strategy is required less resources like energy consumption and economic costs and environmental-friendly likewise.

#### 12.3.2.1 Application of Ornamental Plants

A number of woody and herbaceous ornamental plants were explored in urban areas for phytoremediation purpose and restoration of contaminated lands due to specific features of tolerance, absorbance, and translocate capability against heavy metals or dangerous organic compounds (Capuana 2020). Some important compounds have been explored from canna rhizomes, which include starch, vitamins, minerals, nutraceutical components, fiber, lipids, crude protein, and caloric value of 423 kcal/100 g dry weight (Ayusman et al. 2020). Additionally, HR LC-MS/MS analysis detected bioactive compounds from canna rhizome like psoromic acid, rosmarinic acid, usnic acid, coumaric acid, isoeugenitol, ellagic acid, and swietenine that is helpful for human health also (Ayusman et al. 2020). Four non-eatable floriculture plants were grown in heavy metal-contaminated soil and have significant phytoremedial potential such as antirrhinum, calendula, pansy, and marigold (Mahmood-ul-Hassan et al. 2020).

## 12.4 *Eichhornia crassipes*: An Aquatic Plant

*E. crassipes* (Water hyacinth) of Pontederiaceae family, a native of South America, is one of the free-floating macrophytes found in the aquatic environment such a ditch, ponds, and lakes. It is an herbal product rich in phytochemicals. The first description of water hyacinth was given by the German naturalist von Martius in 1823 while carrying out floral surveys in Brazil named it *Pontederia crassipes*. It was later transferred to the genus *Eichhornia* in 1843. It spreads to other tropical and subtropical regions by humans. The water hyacinth forms dense monocultures that can threaten local native species diversity and change the physical and chemical aquatic environment, altering the ecosystem. The rapid spread of the plant leads to the formation of mat-like coverage of fresh waterways. The effect of water hyacinth on phytoplankton, zooplankton, macro-invertebrates, fish, and birds is deleterious (Villamagna and Murphy 2010).

### 12.4.1 Taxonomical Classification

Kingdom: Plantae
Phylum: Spermatophyta
Subphylum: Angiospermae
Class: Monocotyledonae
Order: Pontederiales
Family: Pontederiaceae
Genus: *Eichhornia*
Species: *crassipes*

Habitat: Its habitat ranges from tropical desert to subtropical to rainforest zones. The temperature tolerance of the water hyacinth is minimum growth temperature is 12 °C (54 °F); its optimum growth temperature is 25–30 °C (77–86 °F).

Common names: Water hyacinth.

Distribution: It invaded about 62 countries in Africa, Asia, and North America and propagated extremely serious ecological, economical, and social problems in the region between 40° north and 45° south.

Morphological description: Water hyacinth is a free-floating perennial hydrophyte native to South America.

Roots: Roots are fibrous and featherlike.

Stem: There are two types of stems:

- Erect stems with flowers.
- Horizontal vegetative stems (stolon).

Leaves: Leaves are smooth, hairless, and glossy. There are two types of leaves.

- Leaves are arrow and erect with non-bulbous petioles.
- Leaves are thick stemmed with bulbous petioles.

Inflorescence: The inflorescence is a spike, which develops from the apical meristem, and each spike is subtended at the base by two bracts and has 8–15 sessile flowers (rarely 4–35).

Flowers: Flowers are funnel-shaped, light bluish-purple have six distinct petals.

#### 12.4.1.1 Application of *E. crassipes*: Green Cleaning Technology

*E. crassipes* and *P. stratiotes* were used for phytoremediation of nanoparticles at the rate of around 30–100% (Lazim et al. 2020). The analysis showed that root parts of both plants play a significant role in accumulating Ag nanoparticles compared to their aerial parts. This proves that aquatic plants are used in *green cleaning technology*. Thus, successful removal of heavy metal contamination using phytoremediation approaches with duckweeds, waterweeds, floating, and submerged macrophytes should be studied further for an environmental benign solution to the problem (Lazim et al. 2020).

Aquatic plants perform vibrant role in the remediation of heavy metals from the polluted sites. Heavy metal pollution is threat to human health and aquatic ecosystem. Aquatic plants *E. crassipes*, *P. stratiotes*, and *Lemna minor* are prominent metal accumulator plants for the remediation of heavy metal-polluted water (Ali et al. 2020). Aquaculture has large significant impacts on the environment. Water pollution caused by effluent from ponds is the main issues related to aquaculture. There are various conventional methods that help to remove pollutants from the aquatic environment but the most eco-friendly and low-cost-effective method is phytoremediation of removing pollutants from the environment. Therefore, five different plant species—*Centella asiatica*, *Ipomoea aquatica*, *Salvinia molesta*, *Eichhornia crassipes*, and *Pistia stratiotes*—are the best eliminators of total suspended solids from aquaculture wastewater and *E. crassipes* is the most effective for phosphate removal (Nizam et al. 2020).

##### Phytoremediation Potential of *E. crassipes*

Aquatic ecosystems are facing high level of stress and depletion due to the inputs of polluting materials. There are certain species of aquatic macrophytes that have ability to cope up with stressful conditions of various organic and inorganic pollutants present in water. These species are effective in polluted water treatment through phytoremediation. The various aquatic plant species are *Azolla*, *Eichhornia*, *Potamogeton*, *Spirodela*, *Lemna*, *Wolffia*, and *Wolfialla*. Among various plant species, *Eichhornia* is highly resistant and can tolerate the toxicity of heavy metals, phenols, formaldehyde, formic acid, acetic acid, and oxalic acid even in their high concentration (Ansari et al. 2020).

### Detoxification of Ni, Pb, Al, B, Cu, Mo, Zn, and Mn

The phytoremediation potential of algae, water hyacinth, and water lettuce was studied for the removal of Ni, Pb, and Mn and represented a very good accumulation potential into the root as compared to shoot. However, water hyacinth was able to accumulate Pb better, while water lettuce showed more preference for Ni and Mn (Alhaji et al. 2020). The highest metal accumulation properties shown by water hyacinth and water lettuce are attributed to their large body mass and their rooted nature, which support their rhizopheric activity and enhance metal uptake also (Alhaji et al. 2020). The waste of heavy metal lead (Pb) in water is removed through phytoremediation because it is highly toxic to the environment. The study determines the effect of *E. crassipes* ability in water to absorb heavy metal Pb and its effects to total dissolved solids (TDS) of groundwater during the bioremediation process. The results show that the *E. crassipes* was more effective for absorbing Pb up to 96% and TDS 62.55 ppm (Tanjung and Samawi 2020).

Water hyacinth is very efficient in taking up ferric, manganese, aluminum, boron, copper, molybdenum, and zinc favoring its growth over other aquatic species. It is one of the free-floating macrophytes found in the aquatic environment such a ditches, ponds, and lakes, and is mostly studied for the purposes of phytoremediation (Melignani et al. 2015). Further, it could produce high biomass in aquatic environment without much toxic symptoms under heavy metal stress condition (Malar et al. 2015). The beneficial aspects of water hyacinth as a potential fertilizer and utilization of several aquatic weeds in the preparation of vermin compost, which help the farmers to produce pollution-free manure, and it acts as an indirect method in controlling the aquatic weeds. However, *E. crassipes* is a common plant for the removal of Pb, Cu, Zn, Hg, Cd, Cr, and Mn in acid-mine water (Fawzy et al. 2012; Sasidharan et al. 2013; Mishra et al. 2013). A study to assess the growth of *E. crassipes* and its ability to accumulate Cu from polluted water results in high accumulation and translocation much lesser in leaves than in roots (Melignani et al. 2015).

### Phytoremediation of Ammoniacal Nitrogen (AN)

Ammoniacal nitrogen (AN) is present in both domestic wastewater and industrial wastewater, which is one of the main contributors of eutrophication. Phytoremediation is the potent solution to treat wastewater with high AN content due to its budget-friendly, ecological, and sustainable characteristics. Water hyacinth has been shown to be a feasible macrophyte for bioremediation of AN in wastewater (Tien et al. 2020).

### Phytoremediation of Fe (Iron)

Fe (iron) phytoremediation was performed using *E. crassipes*, *Lemna minor*, *P. stratiotes*, and *Salvinia cucullata* species. Efficiency of these four macrophytes was compared using different Fe concentrations of 1 mg $L^{-1}$, 3 mg $L^{-1}$, and 5 mg

$L^{-1}$ for 4 days, 8 days, and 12 days, respectively. Moreover, *E. crassipes* was the most efficient macrophyte, whereas *L. minor* was the least efficient. *E. crassipes* removed the highest percentage of Fe, i.e., 89% from 1 mg $L^{-1}$, 81.3% from 3 mg $L^{-1}$, and 73.2% from 5 mg $L^{-1}$ in 12-day experiment (Singh and Rai 2016). *E. crassipes* is very effective in the removal of nutrients from the aquatic bodies (Cristina et al. 2009). The plant can survive in extremely eutrophic water with anaerobically digested flushed dairy manure wastewater. Significant results were obtained in the physiological response of *E. crassipes* to the combined exposure of excess nutrients and Hg (Cristina et al. 2009). Moreover, *E. crassipes* has the capacity to remove heavy metals from landfill, which minimize pollution to an acceptable level (Akinbile and Yusoff 2012).

## 12.5 *Pistia stratiotes*/Jal Kumbhi: Medicinal and Aquatic Plant

*Pistia stratiotes*, also known as "Jal kumbhi" belongs to arum/Araceae family, is a free-floating aquatic plant that exists in streams, lakes, and ponds. It serves as a medicinal plant, utilized for the society benefits; however, it needs eradication and management where it has become a noxious weed.

Earliest descriptions of *P. stratiotes* L. (Araceae) were given by the ancient Egyptians and Greek philosophers Dioscorides and Theophrastus. *P. stratiotes* are the solitary member of the subfamily Pistioidea in Araceae. The synonyms and obsolete subspecific names (Plantatlas 2006) of this taxonomically isolated species are the only free-floating aroid (Araceae family).

### 12.5.1 Taxonomical Classification

Kingdom: Plantae
Division: Magnoliophyta
Class: Liliopsida
Order: Alismatales
Family: Araceae
Genus: *Pistia* L.
Species: *stratiotes*

Habitat: The *P. stratiotes* grows in a very wide variety of aquatic habitats, mainly stagnant water. It is usually a free-floating plant; it can survive for extended periods on moist soil.

Common name: Water lettuce, jal kumbhi, floating aroid, Nile cabbage, shell flower, tropical duckweed, water bonnet, water cabbage, water fern, water lily.

Morphological description: The morphology of *P. stratiotes* varies largely depending upon the environmental factors. The distinct hydrochemical characteristics of plants were regarding biomass, productivity allocation, pH of the cell saps, chlorophyll, nucleic acids, total free amino acid content of the leaves and total nitrogen, crude protein, and phosphorus in whole plants.

Roots: Roots are unbranched with many lateral rootlets. A clonal plant forms small colony through stolon.

Leaves: Leaves are pale-green, up to 20 cm long and 10 cm wide, mostly spathulate to broadly obovate with a rounded to truncate apex, surfaces, in particular the lower surface, covered by a dense mat of white woolly hairs.

Inflorescence: It is axillary, spathe 1.3–1.5 cm long, whitish; spadix with a single pistillate flower at base, and with 2–8 staminate flowers.

Flowers: Flowers are unisexual, staminate with two stamens, pistillate with unilocular ovary having numerous ovules, a slender style and penicillate stigma, the fruit with many thin seeds.

Fruits: Fruit thin-walled, many seeded.

Seeds: The seeds are housed in green berries. Mature seeds are hard, wrinkled, and golden brown in color.

#### 12.5.1.1 Application of *P. stratiotes*: Hyperaccumulator and Bioindicator

*P. stratiotes,* a perennial monocotyledonous aquatic plant present near all tropical and subtropical fresh waterways. Its growth habit makes it a weed in aquatic habitat, where it can kill native submerged plants and adversely affect the biodiversity. It is a common aquatic weed and may clog waterways in warmer states.

##### Phytoremediation of Crude Oil-Polluted Water

Bernard and Chukwuemeka (2020) explained that *Pistia stratiotes* is beneficial for the remediation of crude oil-polluted water. It showed potent ability to absorb heavy metals in crude oil-polluted water. The more the number of *P. stratiotes* in polluted water, the greater the ability of remediating it. The less concentration of crude oil spills can be easier achieved than heavily polluted water. *P. stratiotes* performed well in accumulating heavy metals and showed potentials for phytoremediation of polluted aquatic environment (Bernard and Chukwuemeka 2020).

##### Detoxification of Heavy Metals

Phytoremediation is a promising technique to remove excess nutrients from polluted water. *P. stratiotes* eligible to be used as a hyperaccumulators plant for the mitigation of organic contaminants and heavy metals from wastewater on a broad scale. Lower size of the plant for removal of heavy metals is a credit for this plant as compared to water hyacinth (Mufarrege et al. 2010; Singh and Sinha 2011). *P. stratiotes* can be used for Zn extraction from industrial wastes as it has strong affinity to Zn absorption in an eco-friendly manner (Nurhayati et al. 2012). The same is true about mercury too. The accumulation of heavy metals like Fe, Zn, Cu, Cr, and Cd does not cause any toxic effect on the plant and can be used for the phytoremediation of wastewater for heavy metals on large scale. It has been considered a promising plant for the remediation of contaminated waters. Water lettuce gave high antibiotic modification rates through root exudates tetracyclines and oxy-tetracyclines, the two frequently used antibiotics in veterinary feed and medicines.

Cadmium (Cd) detoxification for *P. stratiotes* L. and *E. crassipes* (Mart.) in two aquatic macrophytes is commonly used in phytoremediation in the open environment. Metal accumulation was much higher in the roots of *P. stratiotes*, whereas in *E. crassipes* a higher fraction was translocated to the leaves. Cd uptake capacity of floating macrophytes (*S. herzogii*, *P. stratiotes*, *Hydromistia stolonifera*, and *E. crassipes*) was determined in outdoors experiments. However, *P. stratiotes* efficiently removed Cd at the different concentrations. However, glutathione levels were remarkably higher and much more responsive to Cd exposure in *P. stratiotes* than in *E. crassipes*. Abundant phytochelatin synthesis occurred only in roots and leaves of *P. stratiotes*. On the other side, in *E. crassipes*, the constitutive levels of some antioxidant enzymes and ascorbate were higher and more responsive to Cd than in *P. stratiotes*. Thus, in these two aquatic plants grown in the open, different detoxification mechanisms might come into play to counterbalance Cd acute stress.

*P. stratiotes* L. can serve the mankind as the scavenger of Cr (VI) from wastewater (Dan et al. 2016). A research report assessed the effectiveness of three aquatic plants; *P. stratiotes* L. (water lettuce), *Spirodela polyrrhiza* (duckweed), and *E. crassipes* for the removal of five heavy metals (Fe, Zn, Cu, Cr, and Cd) in laboratory experiments showed *P. stratiotes* as the second to *E. crassipes* in efficiency in removing the selected heavy metals.

#### Phytoremediation of Dye Effluents

The efficiency of aquatic macrophytes *E. crassipes* and *P. stratiotes* is very efficient in reducing the pollution loads in dye effluents. Color reduction of effluent water was prominent in *E. crassipes* that proves to be an effective aquatic plant in reducing the pollution load of the effluent waters (Patel and Adhvary 2016). Therefore, incorporation of *E. crassipes* and *P. stratiotes* would be good strategy to control the environmental contamination. Consequently, *P. stratiotes* is an important medicinal aquatic plant used for phytoremediation or as bioindicator.

## 12.6 Canna: Ornamental Plant

Canna belong to the family Cannaceae with diploid, triploid, and tetraploid ($2n =$ 18, 27, and 36) nature of perennial herb. Different parts of canna includes seeds, shoot, flowers and root that plays potential application in numerous areas for sustainable development of environment. Cannas are being used for various purposes like attractive ornamental plant in garden, noodle-making, animal fodder, jewelry, dye from seeds, paper from leaves, candies, and cakes. Consequently, canna is very promising plant not only for ornamental purpose but also in industrial application (Gupta et al. 2013).

### 12.6.1 Taxonomic Classification

Kingdom: Plantae

Order: Zingiberales
Family: Cannaceae
Genus: Canna
Species: *C. indica*

#### 12.6.1.1 Importance of Canna

Rhizome extracts of *Canna indica* L. exhibited various properties including bioactive compounds, metabolites, antioxidant capacity, and α-glucosidase inhibitory metabolites, which would be used as functional food because it is also potential source of vitamins, minerals, and nutraceutical components (Ayusman et al. 2020). Further investigation of rhizomes explained availability of biologically and economically important compounds like starch (28.5%), fiber (25.1%), lipids (5.75%), crude protein (4.72%), and caloric value of 423 kcal/100 g dry weight (Ayusman et al. 2020).

Canna is also explored for environmental cleanliness and reported to degrade triazophos sulfide phosphate which is an organic phosphorous pesticide and useful for pest control. *C. indica* species can be used to increase water quality and for the phytoremediation of cadmium or many other toxic heavy metals. Despite various important role of this herb, there are no any systematic efforts have been made to characterize and assess the extent of diversity available in Canna at phenotypic, biochemical, and molecular level. Molecular diversity has been performed in 42 cultivars of canna through amplified fragment length polymorphism (AFLP) markers and established the genetic relationships (Gupta et al. 2013). There is need to identify novel and sequencing-based advance molecular markers simple sequence repeats and single nucleotide polymorphism (SSR and SNP), and its association with specific economically important cultivars is required for industrial use. Phytochemicals like alkaloids, glycosides, terpenoids, steroids, flavonoids, tannins, reducing sugars, and anthocyanin content may be one of the reasons, which create natural variation in plants. Due to mutation, when a pigment or phytochemical substances were lost in plant it creates a kind of polymorphism originated by pleiotropic effects of floral pigments. Primary source of diversity in flower color is might be due to variation in anthocyanin content. The anthocyanin is a pigment/co-pigment responsible for various colors of flowers, a quality indicator of flavor of berries, berry products, and fruits. Anthocyanin is water-soluble, nontoxic, phenolic plant metabolites belonging to the flavonoid family and biosynthesized by the general phenylpropanoid pathway. Anthocyanin pigment creates color differences so it may be a useful phenotypic marker for study the evolution of floral colors or morphological trait and indirect trait to select the cultivars that are adaptive to changing environment. Anthocyanin acts as antioxidant due to their free radical scavenging capacity and is believed that anthocyanin pigment in plants is responsible for natural variation in flower color (He et al. 2011) and may play role in adaptation and evolution because it provides endogenous protection from various environmental stresses. In humans, it protects cells from oxidative damage and reduces risk of some vital health conditions as neuroprotective, anti-inflammatory, and helpful in heart disease. According to Srivastava and Vankar (2010), the crude extract of red Canna contains four anthocyanin pigments showing very good antioxidant activity so well suited as nutraceuticals. Further, anthocyanin and carotenoid pigments have also been used as major component for preparation of natural dyes. The lutein, a carotenoid pigment of *Tagetes erecta* L., is used in perfume, leather, textile industry for staining cotton, woolen and silk fabrics, and food industry. The genetic relationship among anthocyanin coloration, soluble carbohydrate accumulation, and growth characteristics uses molecular marker in Leymus grasses. The assessment of wide range of genetic material for diversity is an integral part of any genetic improvement program as it helps in deciding the selection of parental genotypes for various cross combinations from large set of parental lines, which further helps in widening the genetic diversity through crossing and selection of desirable traits (nutraceutical, phytochemicals, and floricultural trait). However, there is not even single initiative has been taken. Therefore, there is need to focus for comparative study of diversity analysis and phytochemical characterization in Canna cultivars for extensive use.

## 12.7 Gas Chromatography-Mass Spectrometry (Gc-Ms): A Molecular Technique for Detection of Chemical Compound from Plant Extract

Molecular tools and techniques are very useful for detection and characterization of plant compounds. Gas chromatography-mass spectrometry (GC–MS) is one of the most extensively explored molecular techniques for study of endogenous soluble organic compounds involved in phytoremediation (Chang et al. 2020). The previous investigation was focused on two economically important plants, viz. *Eichhornia crassipes* and *Pistia stratiotes* belonging to the families Pontederiaceae and Araceae, respectively. The plant material was shade–dried, and different plant parts were collected and used as the experimental plant material for experimentation (Tyagi and Agarwal 2017).

### 12.7.1 Collection of Plant Material

Whole plant of *E. crassipes* (Mart.), Solms (Pontederiaceae), and *P. stratiotes* L. (Araceae) were collected from Kishore Sagar Lake, Kota City, Rajasthan, India.

### 12.7.2 Preparation of Plant Extract

The collected plant materials were ground into uniform powder. Dry powder of plant sample was extracted with ethanol using Soxhlet apparatus for 6 h. The extract was filtered, followed by concentrated using rotary evaporator. Finally, the extracted powder was suspended with the ethanol at the concentration of 100 mg/ml (w/v) followed by filtration through Varian Bond Elute C18 solid-phase extraction to remove impurities. Further, 1 μl of this solution was employed for GC-MS analysis. Extracts of all the plant parts of selected plant species were analyzed by GC-MS in Padmaja Aerobiological (P) Ltd., Turbhe, Navi Mumbai, Maharashtra (Tyagi and Agarwal 2017).

### 12.7.3 GC-MS Analysis

The GC-MS analysis was carried out using Agilent Technologies GC-MS (GC-7890A, MS 5975C) with fused silica 15 m × 0.2 mm ID × 1 μm of capillary column. At the end of this period, the oven temperature was 280 °C and maintained for 9 min. Injection port temperature was 250 °C, and helium flow rate is 1 ml/min. Mass spectral scan range was set at 30–450 (m/z).

### 12.7.4 Quantification of Phytocompounds

GC-MS is a valuable aid for identifying unknown peak for the identification of novel phytoconstituents. The structural type of the corresponding component was suggested on the basis of its mass spectral fragmentation and retention data. Composition was estimated on the basic calculation of the GC peak areas in percent by setting the total peak areas to 100%.

### 12.7.5 Statistical Analysis

Experimental results are expressed as mean value ± standard error mean (SEM). All measurements were replicated three times. IC50 values were also calculated by linear regression analysis.

## 12.8 Therapeutic Agents of Plant Extract

Plants used by folklore are valuable for modern medicine as they are direct sources of therapeutic agents. Herbal plants produce and contain a variety of chemical substances, of these substances and certain isolated compounds serve as models for new synthetic compounds and can be used as taxonomic markers for the discovery of new compounds.

A GC-MS analysis of the extracts from various plant parts of *Eichhornia crassipes* namely leaves, petiole, and root. Various constituents obtained from specific plant part are reported in Tables 12.1, 12.2, and 12.3. However, GC-MS spectra of *E. crassipes* from leaves, petiole, and root are shown in Figs. 12.1, 12.2, and 12.3. A GC-MS analysis was performed using leaves and root extract of *Pistia stratiotes* also. Various constituents obtained are reported in Tables 12.4 and 12.5.

**Table 12.1** Compound present in the ethanolic leaf extract of *Eichhornia crassipes* (Tyagi and Agarwal 2017)

| Retention time (RT) | Name of compound | Molecular formula | Molecular weight | Peak area (%) | Compound nature | Activity[a] |
|---|---|---|---|---|---|---|
| 2.59 | Ethane,1,1-diethoxy | $C_6H_{14}O_2$ | 118 | 9.77 | Ether | Flavoring agent |
| 7.09 | Propane,1,1-diethoxy-2-methyl | $C_8H_{18}O_2$ | 146 | 7.10 | Ether | No activity reported |
| 17.84 | n-Hexadecenoic acid | $C_{16}H_{32}O_2$ | 256 | 2.34 | Palmitic acid (saturated fatty acid) | Antioxidant, hypocholesterolemic, nematicide, lubricant, antiandrogenic, hmolytic, 5-alpha reductase inhibitor, antipsychotic |
| 18.20 | E-11-hexadecanoic acid, ethyl ester | $C_{18}H_{34}O_2$ | 282 | 1.04 | Stearic acid | Antimicrobial, antitumor |
| 18.30 | Palmitic acid, ethyl ester | $C_{18}H_{36}O_2$ | 284 | 12.09 | Stearic acid | Antifungal, antitumor, antibacterial |
| 20.66 | Phytol | $C_{20}H_{44}O$ | 296 | 2.12 | Diterpene | Antimicrobial, anti-inflammatory, anticancer, diuretic, antifungal against *S. typhi*, *resistant gonorrhoea*, *joint* dislocation, stimulant, and antimalarial |
| 21.27 | 9,12,15-Octadecatrienal | $C_{18}H_{30}O$ | 262 | 2.10 | Steroid | Antiarthritic, anticancer, hepatoprotective, antimicrobial, antiasthma |
| 21.53 | 9,12-Octadecadienoic acid, ethyl ester | $C_{20}H_{36}O_2$ | 308 | 3.79 | Polyenoic fatty acid | Hepatoprotective, antihistaminic, hypocholesterolemic, antieczemic |
| 21.65 | Linolenic acid, ethyl ester | $C_{20}H_{34}O_2$ | 306 | 26.26 | Linoleic acid ethyl ester | Hypocholesterolemic, nematicide, antiarthritic, hepatoprotective antiandrogenic, 5-alpha reductase inhibitor, antihistaminic, anticoronary, antieczemic, antiacne |
| 21.94 | Stearic acid, ethyl ester | $C_{20}H_{40}O_2$ | 312 | 0.98 | Fatty ester | No activity reported |
| 24.63 | Hexadecenoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester | $C_{19}H_{38}O_4$ | 330 | 0.87 | Amino compound | Antimicrobial |

(continued)

**Table 12.1** (continued)

| Retention time (RT) | Name of compound | Molecular formula | Molecular weight | Peak area (%) | Compound nature | Activity[a] |
|---|---|---|---|---|---|---|
| 25.64 | α-Glyceryl linoleate | $C_{21}H_{36}O_4$ | 352 | 1.35 | Fatty acid ester | Cosmetic, coloring agent |
| 26.43 | 1-Monolinoleoyl-glycerol trimethylsilyl ether | $C_{27}H_{54}O_4Si_2$ | 498 | 1.52 | Steroid | Anticancer, hepatoprotective, antimicrobial, antiasthma, diuretic |
| 27.68 | Linoleic acid, 2,3-bis-(O-TMS)-propyl ester | $C_{27}H_{54}O_4Si_2$ | 498 | 1.98 | Steroid | Antiarthritic, anticancer, hepatoprotective, antiasthma, diuretic |
| 28.59 | Stigmasterol | $C_{29}H_{48}O$ | 412 | 11.39 | Steroid | Antioxidant, hypoglycemic, and thyroid-inhibiting properties, precursor of progesterone, anticancer, antiarthritic, antiasthma, diuretic |

[a] Activity source: Dr. Duke's phytochemical and ethnobotanical databases (https://data.nal.usda.gov/dataset/dr-dukes-phytochemical-and-ethnobotanic-database)

**Table 12.2** Compound present in the ethanolic petiole extract of *Eichhornia crassipes* (Tyagi and Agarwal 2017)

| Retention time (RT) | Name of compound | Molecular formula | Mol wt. | Peak area (%) | Compound nature | Activity[a] |
|---|---|---|---|---|---|---|
| 2.14 | Isobutyl alcohol | $C_4H_{10}O$ | 74 | 28.82 | Alcohol | Biofuel |
| 2.58 | Ethane,1,1-diethoxy | $C_6H_{14}O_2$ | 118 | 4.15 | Ether | Flavoring agent |
| 3.48 | Formic acid,1-methyl ethyl ester | $C_4H_8O_2$ | 88 | 7.6 | Carboxylic acid | Preservative, antibacterial agent, treatment for warts |
| 4.24 | Ethanol,2-proxy | $C_5H_{12}O_2$ | 104 | 0.97 | Ether | Solvent, paints, and coating. |
| 7.09 | Propane,1,1-diethoxy-2-methyl | $C_8C_{18}O_2$ | 146 | 16.84 | Ether | No activity reported |
| 17.86 | n-Hexadecenoic acid | $C_{16}H_{32}O_2$ | 256 | 3.82 | Palmitic acid (saturated fatty acid) | Antioxidant, pesticide, hypocholesterolemic, nematicide, lubricant, antiandrogenic, hemolytic, 5-alpha reductase inhibitor, antipsychotic |
| 18.31 | Hexadecenoic acid, ethyl ester | $C_{18}H_{36}O_2$ | 284 | 23.7 | Palmitic acid ethyl ester | Antioxidant, hypocholesterolemic, pesticide, antiandrogenic, flavor, hemolytic, alphareductase inhibitor |
| 21.52 | Linolelaidic acid, methyl ester | $C_{19}H_{34}O_2$ | 294 | 1.62 | Fatty acid | No activity reported |
| 21.63 | 9,12,15-Octadecatrienoic acid, ethyl ester(Z,Z,Z) | $C_{20}H_{34}O_2$ | 306 | 5.50 | Linolenic acid ester compound | Anti-inflammatory, cancer preventive, hepatoprotective |
| 21.94 | Octadecanoic acid, ethyl ester | $C_{20}H_{40}O_2$ | 312 | 1.32 | Fatty ester | No activity reported |
| 24.63 | Hexadecenoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester | $C_{19}H_{38}O_4$ | 330 | 0.94 | Amino compound | Antimicrobial |
| 28.59 | Ethyl iso-allocholate | $C_{26}H_{44}O_5$ | 436 | 4.65 | Steroid | Antimicrobial, diuretic, anti-inflammatory, antiasthma |

[a] Activity source: Dr. Duke's phytochemical and ethnobotanical databases (https://data.nal.usda.gov/dataset/dr-dukes-phytochemical-and-ethnobotanic-database)

**Table 12.3** Compound present in the ethanolic root extract of *Eichhornia crassipes* (Tyagi and Agarwal 2017)

| Ret time (RT) | Name of compound | Mol formula | Mol wt. | Peak area (%) | Compound nature | Activity[a] |
|---|---|---|---|---|---|---|
| 1.94 | Propyl alcohol | $C_3H_8O$ | 60 | 5.53 | Alcohol | Engine fuel |
| 2.12 | 1-Propanol,2-methyl | $C_4H_{10}O$ | 74 | 4.85 | Alcohol | Industrial solvent |
| 2.57 | Ethane,1,1-diethoxy | $C_6H_{14}O_2$ | 118 | 5.14 | Ether | Flavoring agent |
| 3.46 | Formic acid,1-methyl ethyl ester | $C_4H_8O_2$ | 88 | 2.95 | carboxylic acid | Preservative, antibacterial agent, treatment for warts |
| 17.87 | n-Hexadecenoic acid | $C_{16}H_{32}O_2$ | 256 | 7.98 | Palmitic acid (saturated fatty acid) | Antioxidant, hypocholesterolemic, nematicide, pesticide, lubricant, antiandrogenic, 5-alpha reductase inhibitor, antipsychotic |
| 18.31 | Hexadecenoic acid, ethyl ester | $C_{18}H_{36}O_2$ | 284 | 28.33 | Palmitic acid ester | Antioxidant, hemolytic, hypocholesterolemic, nematicide, antiandrogenic |
| 21.53 | 9,12-Octadecadienoic acid, ethyl ester | $C_{20}H_{36}O_2$ | 308 | 9.99 | Unsaturated fatty acid ester | Anti-inflammatory, cancer preventive |
| 21.64 | Linolenic acid, ethyl ester | $C_{20}H_{34}O_2$ | 306 | 17.65 | Unsaturated fatty acid ester | Anti-inflammatory, anticancer |
| 21.94 | Octadecanoic acid, ethyl ester | $C_{20}H_{40}O_2$ | 312 | 2.27 | Fatty ester | No activity reported |
| 23.03 | Octadecane, 6-methyl | $C_{19}H_{40}$ | 268 | 1.66 | Alkane | Absorbent |
| 24.64 | Hexadecenoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester | $C_{19}H_{38}O_4$ | 330 | 2.51 | Amino compound | Antimicrobial |
| 28.59 | Cholesta-22,24-diene-5-ol,4,4-dimethyl | $C_{29}H_{48}O$ | 412 | 8.39 | Steroid | Antibacterial, trypanocidal activity |
| 28.98 | 1-Monolinoleoyl-glycerol trimethylsilyl ether | $C_{27}H_{54}O_4Si_2$ | 498 | 2.71 | Steroid | Antiarthritic, anticancer, hepatoprotective, antiasthma, diuretic |

[a] Activity source: Dr. Duke's Phytochemical and Ethnobotanical Databases (https://data.nal.usda.gov/dataset/dr-dukes-phytochemical-and-ethnobotanic-database

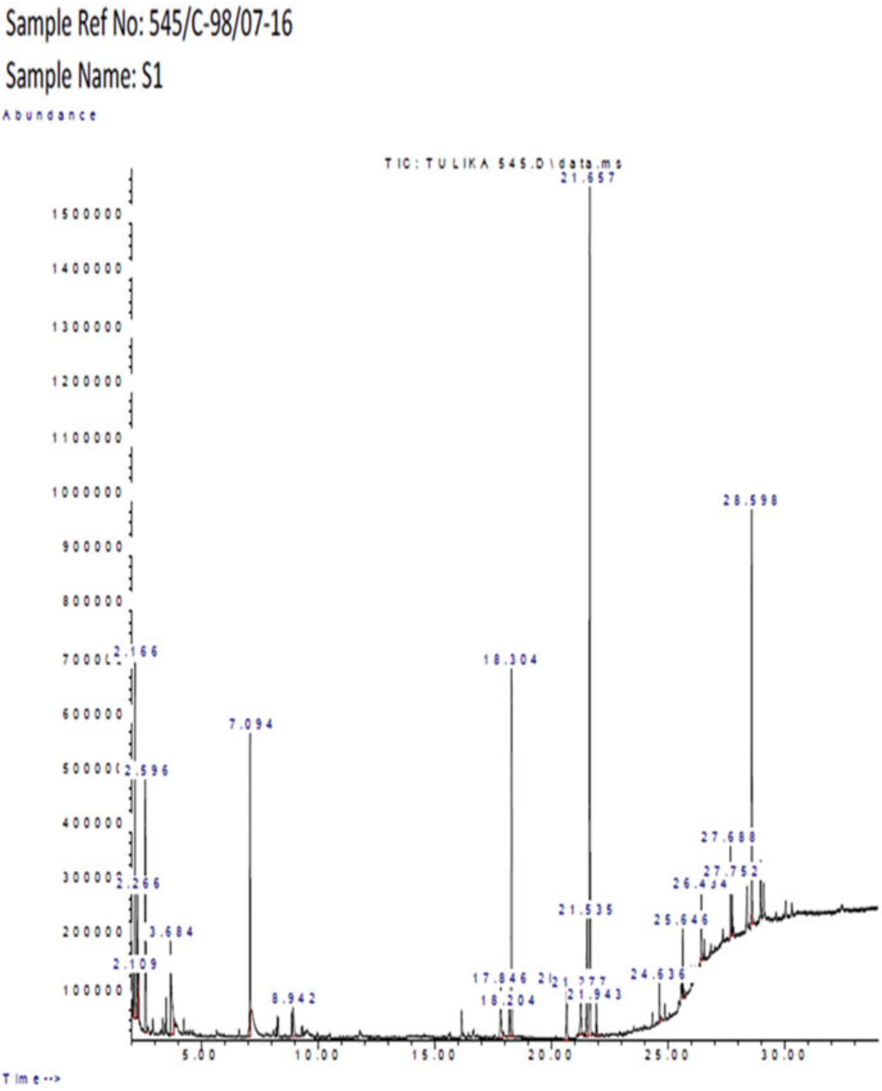

**Fig. 12.1** GC-MS spectra of ethanolic extract of *E. crassipes* leaf (Tyagi and Agarwal 2017)

GC-MS spectra of *P. stratiotes* from leaves and root are shown in Figs. 12.4 and 12.5.

Retention time (RT) is a measure of the time taken for a solute to pass through a chromatography column. It is calculated as the time from injection to detection. If the polarity of the stationary phase and compound are similar, the retention time increases because the compound interacts stronger with the stationary phase. As a result, polar compounds have long retention times on polar stationary phases and shorter retention times on nonpolar columns using the same temperature.

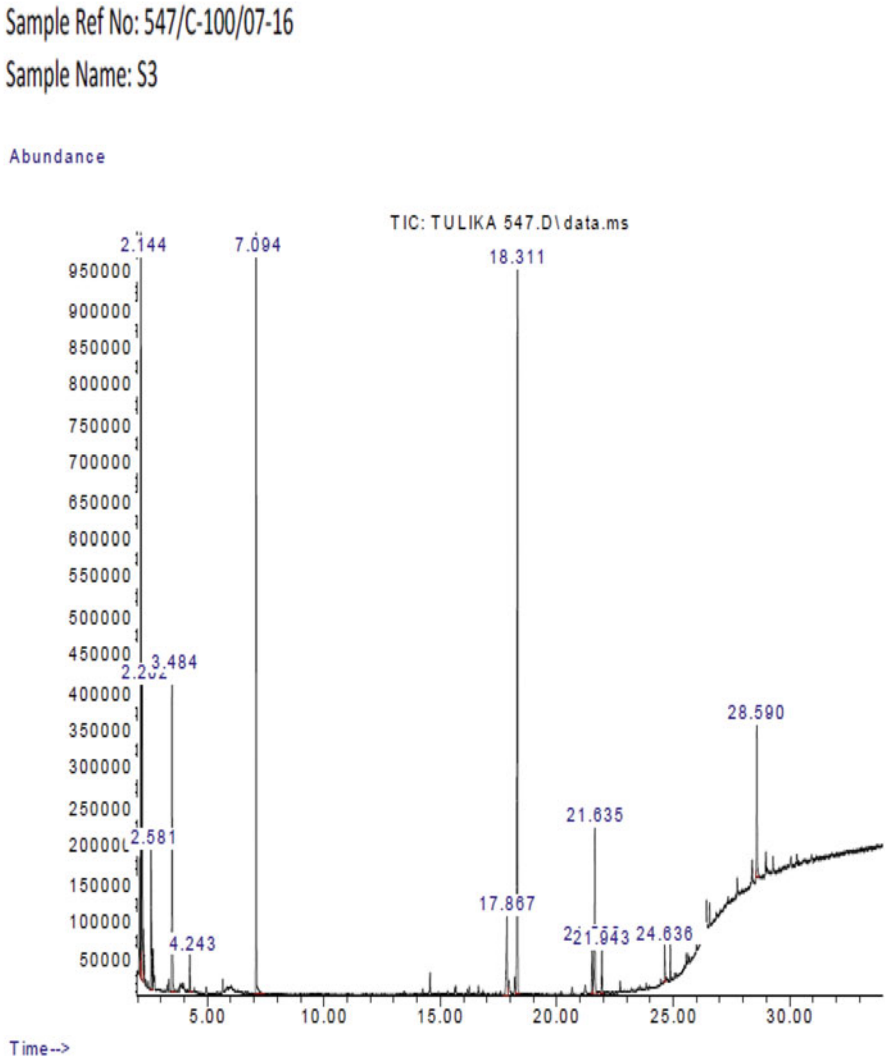

**Fig. 12.2** GC-MS spectra of ethanolic extract of *E. crassipes* petiole (Tyagi and Agarwal 2017)

## 12.9 Significance of Important Phytochemicals, Bioactive Compounds, and Its Properties for Sustainable Environment and Human Welfare

Among the identified phytochemicals in the study, there are some potentially active compounds that have been detected, which are the following:

Palmitic acid has the property of antioxidant, hypocholesterolemia, nematicide, pesticide, lubricant activities, and hemolytic 5-alpha is a reductase inhibitor (Jegadeeswari et al. 2012; Upgade and Anusha 2013).

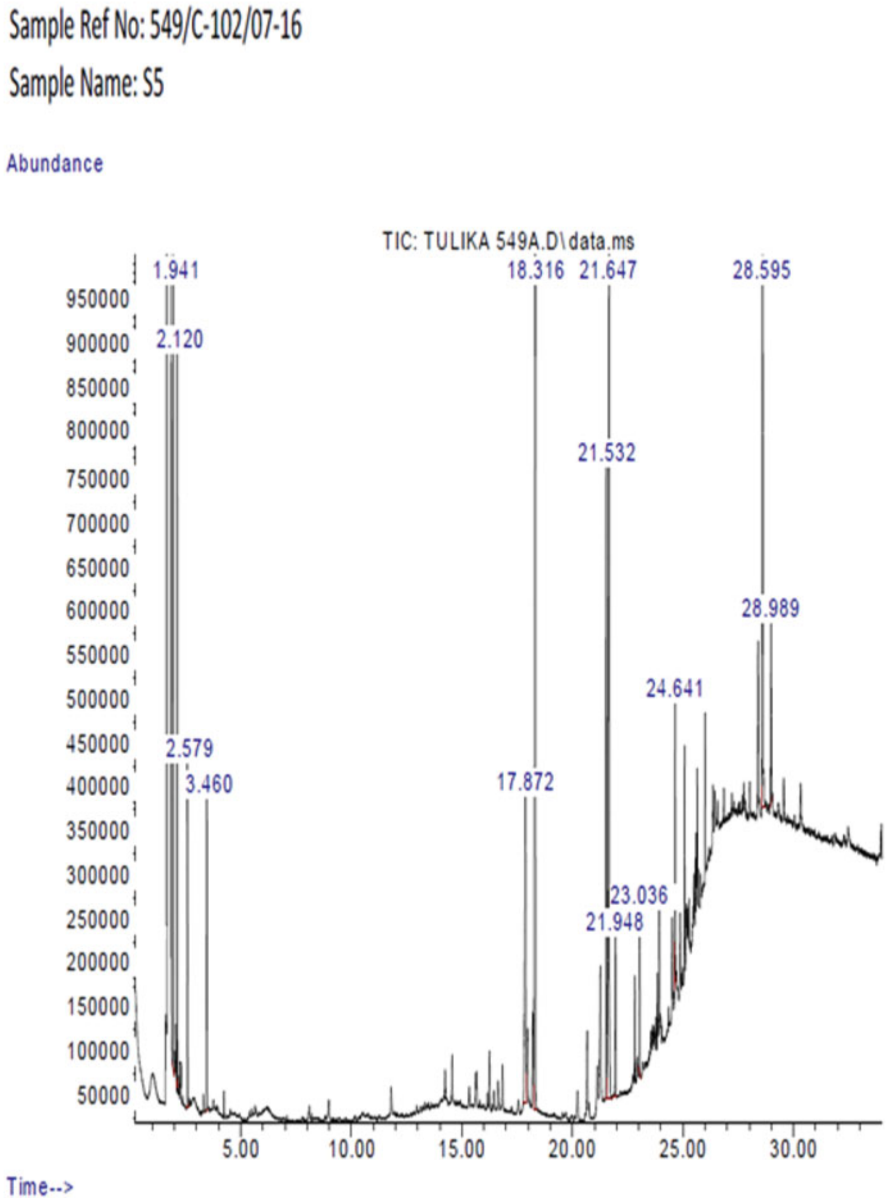

**Fig. 12.3** GC-MS spectra of ethanolic extract of *E. crassipes* root (Tyagi and Agarwal 2017)

n-Hexadecenoic acid is the common compound in the leaves of *P. stratiotes* and *E. crassipes*. It is used in the production of soap, cosmetics, and industrial mold release agents, anti-inflammatory, antibacterial, and antifungal (Praveen et al. 2010).

**Table 12.4** Compound present in the ethanolic leaf extract of *Pistia stratiotes* (Tyagi and Agarwal 2017)

| Ret time (RT) | Name of compound | Molecular formula | MW | Peak area (%) | Compound nature | Activity[a] |
|---|---|---|---|---|---|---|
| 2.14 | Isobutyl alcohol | $C_4H_{10}O$ | 74 | 5.42 | Alcohol | Biofuel |
| 3.48 | Formic acid,1-methyl ethyl ester | $C_4H_8O_2$ | 88 | 0.93 | Carboxylic acid | Preservative, antibacterial agent, treatment for warts |
| 7.08 | Propane,1,1-diethoxy-2-methyl | $C_8H_{18}O_2$ | 146 | 2.05 | Ether | No activity reported |
| 11.81 | L-Glutamine | $C_5H_{10}N_2O_3$ | 146 | 0.38 | Amino acid | Building block of protein |
| 17.93 | n-Hexadecenoic acid | $C_{16}H_{32}O_2$ | 256 | 7.18 | Palmitic acid (saturated fatty acid) | Antioxidant, hypocholesterolemic, nematicide, pesticide, antiandrogenic, hemolytic, 5-alpha reductase inhibitor, antipsychotic |
| 18.33 | Hexadecenoic acid, ethyl ester | $C_{18}H_{36}O_2$ | 284 | 13.29 | Palmitic acid ester | Antioxidant, hemolytic, hypocholesterolemic flavor, antiandrogenic |
| 21.53 | Linolelaidic acid, methyl ester | $C_{19}H_{34}O_2$ | 294 | 2.41 | Fatty acid | No activity reported |
| 21.64 | 9,12,15-Octadecatrienoic acid, methyl ester(Z,Z,Z) | $C_{19}H_{32}O_2$ | 292 | 2.7 | Steroid | Antiarthritic, anticancer, hepatoprotective, antimicrobial, antiasthma, diuretic |
| 23.92 | 12,15-Octadecadienoic acid, methyl ester | $C_{19}H_{30}O_2$ | 290 | 0.50 | Unsaturated fatty acid ester | No activity reported |
| 24.64 | Hexadecenoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester | $C_{19}H_{38}O_4$ | 330 | 0.96 | Amino compound | Antimicrobial |
| 24.90 | Diisooctyl phthalate | $C_8H_4(C_8H_{17}COO)_2$ | 390 | 53.84 | Plasticizer compound | Antimicrobial, Antifouling |
| 25.07 | Docosanoic acid, ethyl ester | $C_{24}H_{48}O_2$ | 368 | 0.69 | Fatty ester | No activity reported |
| 28.59 | Stigmasterol | $C_{29}H_{48}O$ | 412 | 2.57 | Steroid | Antioxidant, hypoglycemic, and thyroid-inhibiting properties, precursor of progesterone, anticancer, antiarthritic, antiasthma, anti-inflammatory, diuretic |

[a] Activity source: Dr. Duke's Phytochemical and Ethnobotanical Databases (https://data.nal.usda.gov/dataset/dr-dukes-phytochemical-and-ethnobotanic-database)

**Table 12.5** Compound present in the ethanolic root extract of *Pistia stratiotes* (Tyagi and Agarwal 2017)

| Ret time (RT) | Name of compound | Molecular formula | Mol wt. | Peak area (%) | Compound nature | Activity[a] |
|---|---|---|---|---|---|---|
| 2.13 | Isobutyl alcohol | $C_4H_{10}O$ | 74 | 1.70 | Alcohol | Biofuel |
| 2.59 | Ethane, 1,1-diethoxy | $C_6C_{14}O_2$ | 118 | 1.79 | Ether | Flavoring agent |
| 7.08 | Propane,1,1-diethoxy-2-methyl | $C_8H_{18}O_2$ | 146 | 1.61 | Ether | No activity reported |
| 15.66 | Octadecanoic acid, ethyl ester | $C_{20}H_{40}O_2$ | 312 | 0.07 | Fatty ester | No activity reported |
| 17.84 | n-Hexadecenoic acid | $C_{16}H_{32}O_2$ | 256 | 0.30 | Palmitic acid (saturated fatty acid) | Antioxidant, antipsychotic, hypocholesterolemic, nematicide, pesticide, lubricant, hemolytic, 5-alpha reductase inhibitor |
| 17.98 | E-11-Hexadecanoic acid, ethyl ester | $C_{18}H_{34}O_2$ | 282 | 0.24 | Stearic acid | Antifungal, antitumor, antibacterial |
| 18.29 | Hexadecenoic acid, ethyl ester | $C_{18}H_{36}O_2$ | 284 | 1.63 | Palmitic acid ester | Antioxidant, hemolytic, hypocholesterolemic, flavor, antiandrogenic |
| 24.92 | Bis(2-ethylhexyl) phthalate | $C_8H_4(C_8H_{17}COO)_2$ | 390 | 91.49 | Plasticizer compound | Solvent in glow stick |
| 27.27 | 1-Monolinoleoylglycerol trimethylsilyl ether | $C_{27}H_{54}O_4Si_2$ | 498 | 0.22 | Steroid | Antiarthritic, anticancer, hepatoprotective, antiasthma, diuretic |
| 28.59 | Ethyl iso-allocholate | $C_{26}H_{44}O_5$ | 436 | 0.92 | Steroid | Antimicrobial, diuretic, anti-inflammatory, antiasthma |

[a] Activity source: Dr. Duke's phytochemical and ethnobotanical databases (https://data.nal.usda.gov/dataset/dr-dukes-phytochemical-and-ethnobotanic-database)

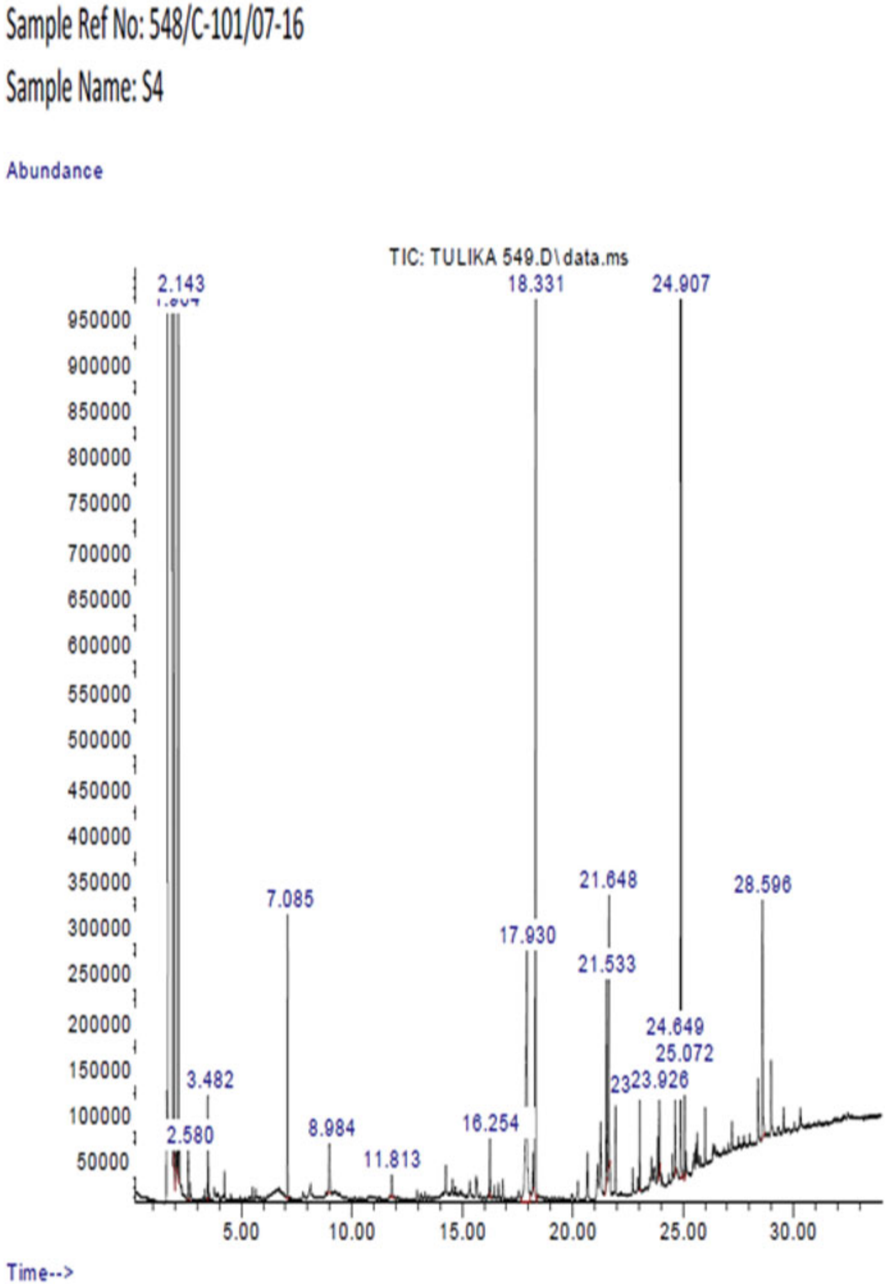

**Fig. 12.4** GC-MS spectra of ethanolic extract of *P. stratiotes* leaf (Tyagi and Agarwal 2017)

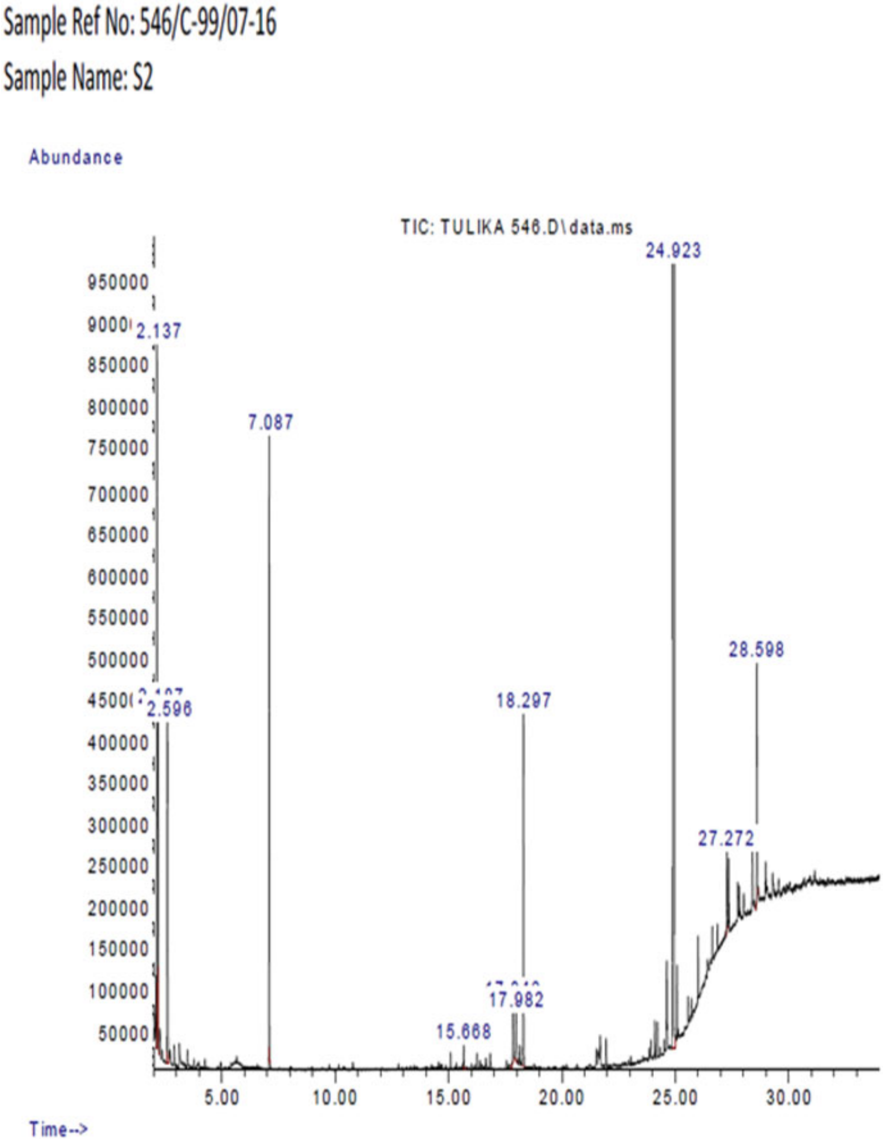

**Fig. 12.5** GC-MS spectra of ethanolic extract of *P. stratiotes* root (Tyagi and Agarwal 2017)

Hexadecenoic acid, ethyl ester found in the leaves of both plant extract, act as a flavoring agent used in foods including condiments and seasonings, hair and skin conditioning agent, nematicide, pesticide, lubricant, antiandrogenic anti-inflammatory, antioxidant, hypocholesterolemia, hemolytic, and 5-alpha reductase inhibitor (Shah et al. 2015).

Phytol is a diterpene compound that acts as an antimicrobial, anti-inflammatory, anticancer and diuretic. It acts as reactive oxygen species, which constitute potent novel class of pharmaceuticals for the treatment of chronic inflammatory diseases and rheumatoid arthritis.

9,12,15-Octadecatrienoic acid, methyl ester (Z,Z,Z), is a polyenoic fatty acid compound and it may act as an anti-inflammatory, hypocholesterolemia, cancer preventive, antidiabetic, hepatoprotective, nematicide, insectifuge, anti-histaminic, antiarthritic, anticoronary, antieczemic, antiacne, 5-alpha reductase inhibitor, and antiandrogenic (Vohra and Kaur 2011). It is used in biosynthesis of prostaglandins and cell membranes (O'Neil 2013; Adeoye-Isijola et al. 2018).

Cholesta-22, 24-dien-5-ol, 4,4-dimethyl is a steroid compound that possesses antimicrobial, anti-inflammatory, anticancer, diuretic, antiarthritic, antiasthma, and trypanocidal activity (Jenecius et al. 2012).

1-Monolinoleoylglycerol trimethylsilyl ether is the common compound in the roots of both the plant shows many biological activities such as antiarthritic, anticancer, hepatoprotective, antimicrobial, antiasthma, diuretic, antioxidant, anti-inflammatory, and antidiabetic (Senthil et al. 2016).

One of the interesting results from the present study was the separation and identification of phthalate compounds such as diisooctyl phthalate and bis (2-ethylhexyl) phthalate for the first time from the extract of *P. stratiotes* leaves and roots, respectively, using GC-MS technique.

Diisooctyl phthalate is a plasticizer compound; it acts as an antimicrobial and antifouling (Sangeetha and Vijayalakshmi 2011). Diisooctyl phthalate can also act as potent antidiabetic agents (Lawal et al. 2020).

Ethyl iso-allocholate is suggested to be a sterol compound, and it may use as an antibacterial, antitubercular, antioxidant, antitumor, cancer preventive, pesticide, and chemopreventive agent. To the best of our knowledge, phthalates showed diverse toxicity profiles according to their structures in the liver, kidneys, thyroid, and testes, which are involved in general toxicity.

## 12.10 Future Thrust

Phytoremediation is a cost-effective, environmentally friendly, esthetically pleasing environmental pollutant removal approach. The technologies based on the phytoremediation technique can be applied to both organic and inorganic pollutants present in soil, water, or air. Aquatic plants can be used as the natural catalysts to absorb and accumulate heavy metals in their tissues from wastewater. The selection of plant species is the most significant aspect as they hold steep efficiency for successful phytoremediation. Water hyacinth (*Eichhornia crassipes*), water lettuce (*Pistia stratiotes*), and duckweed (*Lemna minor*) along with some other aquatic (*Eichhornia crassipes* and *Pistia stratiotes*) and ornamental plants (Canna and marigold) are prominent metal accumulator plants for the remediation of heavy metal-polluted water. In future, integrated approach would be helpful for pollution control in sustainable manner. Furthermore, some economically and medicinally important plants are underutilized due to lack of knowledge.

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# Phytoremediation of Coastal Saline Vertisols of Gujarat Through Biosaline Agriculture

# 13

G. Gururaja Rao and Jitendra Chikara

## Abstract

Sustainability of an ecosystem rests on the scientific management based on a sound database. Managing the coastal agricultural ecosystem is more complex when it is affected by natural phenomena like seawater ingress and the introduction of large-scale human interventions. Such a situation is prevailing in the coastal districts of Gujarat state, India, where salinity of the agricultural lands is constantly on the increasing trend. The problems of environmental degradation in Gujarat are as diverse and complex as the ecological fabric of the state. About 6.73 million hectares (mha) of land is salt-affected in India, of which 2.22 mha is present in Gujarat State. Of this, about 60% area is affected by coastal salinity problems with highly saline groundwater. Three approaches, changes in natural resource management, changes in technology used, and development of salt-tolerant varieties of the crops can be used to overcome the constraints of salinity of soil and groundwater. The use of salt-tolerant varieties and economic halophytes through saline agriculture is an effective, economic, and eco-friendly approach in the management of salt-affected vertisols. Soil salinity either before sowing or that occurs during the growing period is detrimental to crop growth and productivity. Unlike salt-loving plants, the halophytes, salt-sensitive ones, and the glycophytes, which comprise the crop plants, are greatly affected by salinity of soil and irrigation waters. Thus, identifying salt-tolerant crop plants/varieties and their cultivation on coastal saline vertisols holds promise for maximizing the production. Research efforts made resulted in identifying and evolving agrotechnologies, viz. (1) cultivation of *Salvadora persica* on highly

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saline vertisols (for nonedible seed oil); (2) cultivation of *Salicornia brachiata* (edible oil and herbal salt); (3) forage production using halophytic grasses (fodder); (4) cultivation of seed spice, *Anethum graveolens*, on moderately saline vertisols (spice and pharmaceutical derivatives), (5) salt-tolerant herbaceum/desi cotton (*Gossypium herbaceum*) (commercial) and wheat accessions (food) on costal saline vertisols, (6) farming system model for maximizing on-farm production, and (7) saline water use in conjunctive mode in different arable crops (wheat, dill, safflower, mustard) and some fruit species. While cultivation of *Salvadora* resulted in reduction in soil salinity over the years, desi cotton was found ideal and profitable crop to the farmers in saline and rainfed coastal areas. Desi cotton accession, G. Cot 23, gave yield in the range of 1.6–1.9 t ha$^{-1}$ under the average salinity of 7.6 dS m$^{-1}$. In saline vertisols of southern, central, and Saurashtra areas of Gujarat (EC range 5.9–7.2 dS m$^{-1}$), salt-tolerant wheat varieties KRL 210 and KRL 19 gave yield in the range of 36–39.5q ha$^{-1}$. The details of technologies, economics of their cultivation, and their role in remediation of salinity along with future prospects are discussed in the chapter.

**Keywords**

Coastal salinity · Conjunctive use · Halophytes · Phytoremediation · Saline vertisols

## 13.1 Introduction

Agricultural salinity management of soils and irrigation waters has become major research and developmental issue in the arid and semi-arid regions of the world. Global demand for food, fiber, and bioenergy is growing at a rapid rate primarily due to the burgeoning population of people. In the most developing countries, growth rate in agriculture has failed to catch up with the increase in population growth. Soil salinization is considered a major factor contributing to the human-induced land degradation. Soil salinity has been a major concern to global agriculture throughout human history. In India, about 6.727 million hectares (mha) of land is affected by salinity and scarcity issues that have become limiting factors in agricultural crop production.

Vertisols are churning heavy clay soils with predominantly smectitic mineralogy and possess a high proportion of swelling clays; i.e., montmorillonite-rich clays are often also called heavy cracking clay soils or swell-shrink soils (Fig. 13.1). Vertisols are not a uniform soil entity, and they occur under different agroclimatic environments, which is a limitation to the transfer of technology for managing them. Proper understanding of their characteristics in relation to their potential use for food production is a justifiable aim of research on vertisols, which should lead to the effective management and viable use of these soils. The development of salinity and sodicity in black soil region is generally associated with poor drainage and waterlogging. In low-technology societies, the major obstacles to agricultural land

**Fig. 13.1** Typical vertisols with distinct cracks

use of vertisols include the adverse physical properties and their poor workability. These soils have a considerable potential for agricultural production, but special management practices like tillage and water management are required to secure sustained production. Though vertisols have a great potential for productive cropping because of their stored water that can sustain crops through drought/stress periods, the current level of crop production in harsh environments, viz. saline and waterlogging conditions, is inadequate to meet the needs of rapid increase in human population. Saline and sodic vertisols may develop under irrigation, but they are rare under natural conditions. In India, the saline and sodic vertisols *by and large* remain fallow during the rainy season, which results in soil erosion. Vertisol is one of the important soil orders of soil taxonomy. Vertisols, the churning heavy clay soils with a high proportion of swelling clays, soils form deep wide cracks from the surface downward when they dry, which happens in most years. These soils have a vertic horizon within 100 cm from the soil surface; possess 30% or more clay in all horizons to a depth of 100 cm or more; and possess cracks that open periodically.

## 13.2 Distribution of Vertisols

These soils are distributed mainly in the states of Maharashtra, Madhya Pradesh, Gujarat, Andhra Pradesh, Karnataka, Tamil Nadu, and Rajasthan and to some extent in Orissa and Bihar (Fig. 13.2), and are cultivated mainly with cotton, sorghum, pearl millet, pigeon pea, groundnut, chickpea, and at places with wheat and soybean under rainfed conditions. The major area under these soils is cultivated with Kharif (July–September) crops, while some area receiving winter rains (October–December) is cultivated with Rabi crops. Rice and sugarcane cropping is also practiced where irrigation facility is available. The yields of crops grown on these soils under the rainfed condition are comparatively low, but there is a great potential to increase the same. The unreliability of rainfall has been considered one of the reasons for the low agricultural productivity in the black soils. Whenever irrigation is provided, cultivators have reaped rich harvest, but in the long run, problems of salinity and sodicity do crop up, and these soils have become saline and/or sodic. The problem of water stagnation and moisture stress are common features of the vertisols of the

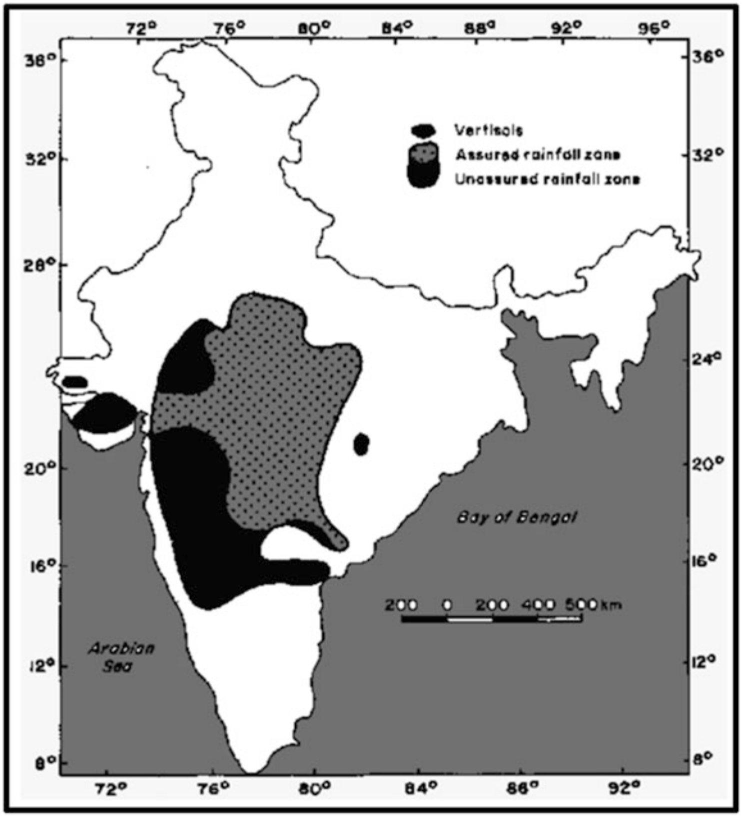

**Fig. 13.2** Vertisol distribution in India

semi-arid regions, which further intensify with sodification. Thus, crops suffer badly due to oxygen stress in the initial stage and moisture stress after the termination of the monsoon or delay in irrigation.

## 13.3 Main Production Constraints

Vertisols are imperfect to poorly drained, with limited leaching of soluble weathering products, and contain high available calcium and magnesium, and the pH is above 7. This is due to the very low hydraulic conductivity of a vertisol: Once the soil has reached its field capacity, practically no water movement occurs. Flooding can be a major problem in areas with higher rainfall. Surface water may be drained by open drains. Mole drainage is virtually impossible.

Vertisols are of very hard consistency when dry and very plastic and sticky when wet and thus are difficult to work. Thus, the workability of these soils is often limited to very short periods with medium (optimal) water status. However, tillage operations can be performed in the dry season with heavy machinery. Mechanical tillage in the wet season causes serious soil compaction. Really wetland is impassable. The adverse physical properties and poor workability of vertisols are a major obstacle to agricultural land use, especially in low-technology societies. Vertisols have a considerable potential for agricultural production, but special management practices (tillage and water management) are required to secure sustained production. The main parts of the vertisol regions are used for low-intensity grazing.

Vertisols are base-rich soils and are capable of sustaining continuous cropping. They do not necessarily require a rest period for recovery because the pedoturbation brings subsoil to the surface. However, the overall productivity normally remains low, especially where no irrigation water is available. Nitrogen is normally deficient and phosphorus. Phosphate fixation (as tricalcium phosphate) may occur, but it is not a major problem. Potassium levels are variable. Secondary elements and micronutrients are often deficient. In semi-arid areas, free carbonate and gypsum accumulations are common. Saline and sodic vertisols may develop under irrigation, but they are rare under natural conditions.

The major production constraints prevailing in vertisols comprise the following:

- Reduced permeability in a swollen state resulting in low infiltration and internal drainage
- Poor aeration of wet soils and related poor root development
- Narrow optimum moisture range for tillage and seeding operations
- Rapid drying of granular surfaces and scaling and crusting resulting in germination problems
- Salinity hazards associated with rising groundwater table and use of poor quality irrigation water
- Irrigation-induced salinity development under canal command areas

## 13.4 Soil Salinity Problems in India

Agriculture is the principal lever of economic and social development. The ever-increasing human population needs a massive amount of food, and to meet this, intensive agricultural practices that are followed have been resulting in the degradation of arable lands occurring due to waterlogging, salinity, chemical pollution, etc. The type of situation is more alarming in the arid and semi-arid regions with low rainfall and high evapotranspiration. The agricultural land is decreasing constantly due to population pressure, adverse environmental conditions, continuously increasing natural calamities, and global climate change (Hasanuzzaman et al. 2013a, b). More than 45 mha of irrigated land is affected by salinity that accounts for 20% of total land, and about 1.5 mha of land is taken out of production every year (Munns and Tester 2008); if it continues in such a way, it is likely that about 50% of cultivable lands will be lost by the middle of the twenty-first century. Land degradation due to salinity-related problems is becoming a serious challenge for food and nutritional security in any region. Inadequate attention paid in the planning stage of the irrigation projects led to the alarming increase in the waterlogging and salinity. The increasing problem of salinity of soils and groundwater in the irrigation commands and coastal area is of great concern that needs a holistic approach for its management.

It is reported that about 6.727 mha of land is salt-affected in India (Singh et al. 2010; Fig. 13.3), which is inclusive of coastal areas along the 8129 km long sea coast. In India, the environmental degradation issues are as diverse and complex as their ecological fabric in many states. While some of the problems are widespread

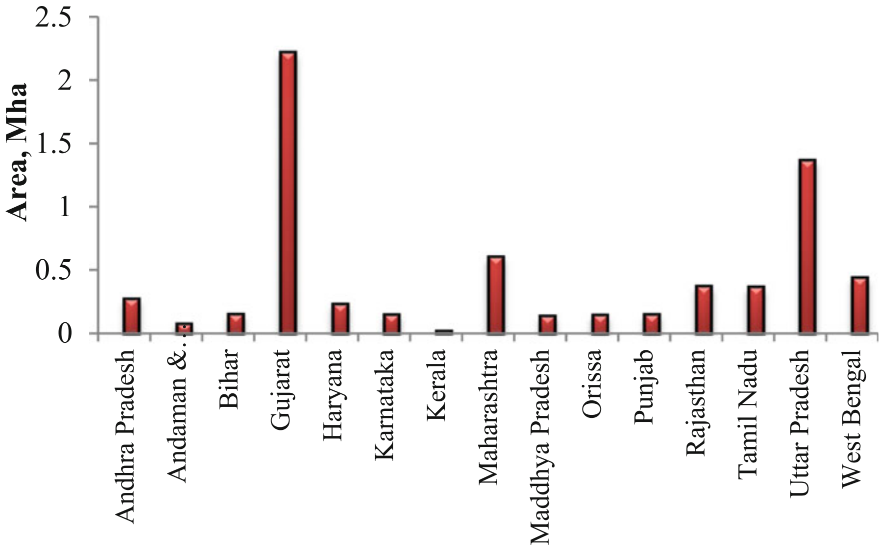

**Fig. 13.3** State-wise distribution of salt-affected soils in India (total area—6.727 mha)

and operate over a long term, the others are mainly localized and more intense in their impacts. Soil and water salinity problems are essentially multisectoral and are complex in nature. Vast areas are in imminent danger of turning barren, and production and productivity have simply declined due to secondary salinization. Soil salinity problems of any area are further compounded by highly saline groundwater, and such areas *by and large* remain barren for want of economically feasible technological interventions. The adverse effects of salinity have put the food and nutritional security at stake while creating environmental pollution and affecting health.

### 13.4.1 Coastal Saline Soils

The coastal ecosystem in India poses a delicate equilibrium between land and water masses among its different components but with a high degree of vulnerability in spite of having bountiful natural resources. India with 8129 km coastline spreads over 9 states, 2 union territories, and 2 island ecosystems and is subjected to many spells of sea-level changes (Fig. 13.4). Though natural calamities like cyclones, storms, tsunami, sea ingress, and tornado are frequent visitors causing monumental losses to the lives and properties of the people living in the coastal regions, this region is endowed with rich diverse natural resources. Thus, the management of natural resources and sustenance of ecology in the coastal region is of paramount importance. The major problems encountered in the coastal region are as follows:

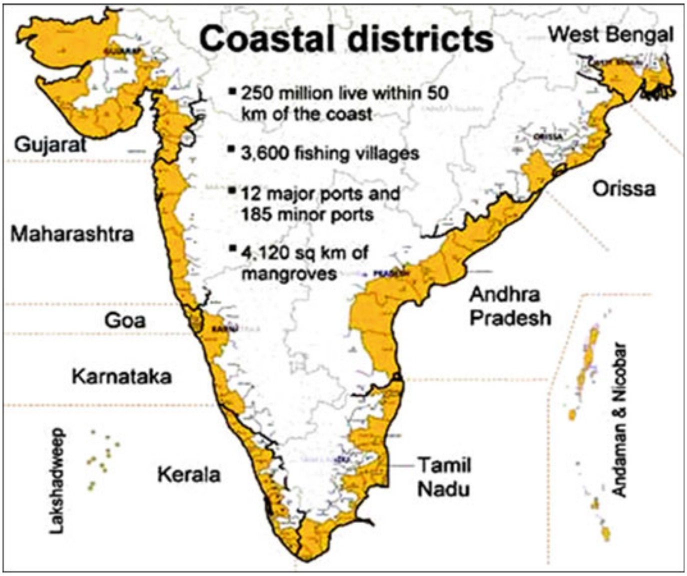

**Fig. 13.4** Coastal belt of Indian subcontinent

- Lands are subjected to the influence of tidal waves and periodical inundation by tidal water;
- Shallow water table enriched with salt contributes to increase in soil salinity during winter and summer months;
- Heavy rainfall resulting in excess water during the *kharif* season;
- Poor surface and subsurface drainage conditions;
- Lack of good-quality irrigation water; and
- Poor socioeconomic conditions of the farmers limiting introduction of high-investment technologies.

### 13.4.2 Salinity Build-up in Soil and Soil Quality

Saline ecosystems are a familiar feature in many continents. The seas and oceans of the world, which contain on average about 3.5% (w/v) salt, cover some 72% of the surface of the globe, and so, it is inevitable that salt affects some of the land. Salinity ingress of groundwater occurs due to (1) excessive and continued abstraction of groundwater from the coastal plain aquifers; (2) seawater and tidal water ingress; (3) relatively less recharge, and (4) poor land and water management resulting in salinity build-up.

### 13.4.3 Seawater Intrusion

Overexploitation of groundwater resulting in sea intrusion in different pockets renders danger to the sensitive freshwater aquifers. Saltwater intrusion takes several forms. Horizontal intrusion occurs as the saline water slowly pushes the fresh inland groundwater landward and upward. The cause can be both natural (due to rising sea levels) and anthropogenic (abstraction of freshwater from the coastal wells). Pumping from coastal wells can also draw saltwater downward from surface sources such as tidal creeks and canals. Options for control of seawater ingress into aquifers include (1) modification in groundwater pumping and extraction patterns; (2) artificial groundwater recharge; (3) injection barriers; (4) subsurface barriers, tidal regulators, check dams, and reservoirs (Sen et al. 2012; Gururaja Rao 2012; Gururaja Rao et al. 2012, 2013c, 2014). However, for an effective and long-term solution to the problem of seawater intrusion into the groundwater in the coastal plains, it is vital to develop location-specific optimization methods and models to identify and earmark ideal and suitable locations of the pumping wells and rates of withdrawal of the groundwater. Studies carried out by Central Soil Salinity Research Institute (CSSRI) through artificial recharge of groundwater (Gururaja Rao et al. 2014) and the efforts in this direction (Gururaja Rao et al. 2012, 2013c) have paid dividends.

Sustainable development of this ecosystem requires an integrated approach to the soil and water management, and, through this, to make necessary measures to conserve the ecology. To maximize crop productivity, these areas should be brought under utilization where there are options for removing salinity or using the salt-tolerant crops. Due to the rapid climate change, the saline area in the world is increasing day by day, and currently, there is an ample need to develop highly salt-tolerant crops to cope with the adverse situation. Since these crops do not remove the salts, cultivating with halophytes having capacity to accumulate and exclude the salt forms an effective strategy. Methods for salt removal include agronomic practices or phytoremediation. The former is cost and labor-intensive and needs some developmental strategies for implication; on the contrary, the phytoremediation by halophytes is more suitable as it can be executed very easily without those problems (Hasanuzzaman et al. 2013b).

Salt stress severely limits the plant growth and yield; in fact, no toxic substance restricts the plant growth more than salt on a world scale. Salinity is a perpetual problem in coastal areas, which are much vulnerable to this problem caused by natural and anthropogenic forces affecting the agricultural regions and degrading the landscape. Consequently, it has become imperative to search for salt-tolerant and high-yielding crops, to develop ecologically and economically viable technologies to produce plants of economic importance. Keeping in view the increasing scarcity of freshwater, there is a dire need to focus on nature-based solutions to help tackle the challenges posed by food insecurity. Halophytes, for instance, offer a great opportunity to utilize unused salty water and coastal saline lands and that augment food and fodder production. In the present chapter, while some important halophytes that are used for regreening the coastal saline areas are covered in detail, some other potential candidate species are further suggested in coastal salinity management through biosaline agricultural approach.

## 13.4.4 Coastal Saline Vertisols

### 13.4.4.1 Impact of Salinity

Salinity problems in vertisols may be inherited from the parent materials or may arise due to aridity in climate, the topographical situation, unscientific and faulty irrigation practices, and rise in groundwater level resulting from the canal seepage and associated drainage issues. The leaching of excess salts is hardly possible. Surface leaching of slats from the paddy fields can be achieved by draining the standing water on regular basis. Saline vertisols contain excess neutral soluble chlorides and sulfate of sodium, calcium, and magnesium with electrical conductivity of the saturation extract of soil (ECe) $>4$ dS $m^{-1}$, pH $<8.2$, and exchangeable sodium percentage (ESP) $<15$. The hard consistency of vertisools, when dry and very plastic and sticky when wet, the workability of vertisols is often limited to very short periods (Gururaja Rao 2015). Soil texture plays a key role in determining water and solute movement within the soil profile. The sum of silt and sand fraction was higher (43.27%) in saline vertisols compared to nonsaline nonsodic vertisols (39.28%) as reported by Worku and Bedadi (2016). This indicates that the formation of salt-affected vertisols is associated with high silt and sand fractions, which could be due to larger pore sizes that favor more permeability for solute transport and easy for evaporation. As a result, there is higher accumulation of salt crust in the soil surface.

### 13.4.4.2 Water Logging-Related Problems

Poor drainage is one of the problems of saline vertisols. Deep vertisols have impeded drainage in the rainy season with consequent loss of trafficability and poor air–water relations. The poor trafficability of the soil when wet seriously interferes with the planting operations. The initial effect of waterlogging results in wilting of plants due to the lack of oxygen to respire, while other gases such as carbon dioxide ($CO_2$) and ethylene ($C_2H_4$), which are detrimental to root growth, also accumulate in the root zone. Waterlogging occurs when three basic events take place such as (1) presence of sufficient amount of water in the root zone to produce saturation, (2) mechanism and physical processes by which water is supplied to and retained within the root zone, and (3) sufficient time for saturation of the plant roots to produce anaerobic conditions and associated changes in the biological and chemical activity, which is harmful to the plant health. Subsequently, root tissues decompose and decay. The plant growth and development are severely affected, and if the waterlogging prolongs, the crop stand may become poor (Fig. 13.5) or even completely whither-off. The adverse impact of waterlogging on crop performance depends on factors like duration of waterlogging, the proportion of the crop root zone affected, the oxygen depletion rate, extent of the effect on nutrient uptake, and the amount of toxins accumulated in the active root zone. The depletion of oxygen in the root zone

**Fig. 13.5** Poor crop stand of cotton on water-logged saline vertisols

is governed by soil factors such as temperature, organic matter status, and extent of salinity/acidity.

#### 13.4.4.3 Opportunities with Coastal Saline Vertisols

Annual run-off and soil loss are significantly less in vertisols compared to alfisols. The mean annual run-off was 199.7 mm and 117.8 mm for alfisols and vertisols, respectively. The mean annual soil loss was 4.78 and 1.68 t $ha^{-1}$, respectively, for alfisols and vertisols (Pathak et al. 2013). The smaller run-off from the vertisols was attributed to preferential infiltration of local run-off into the soil cracks. The water-holding capacity of these soils is good due to the high clay content. Water is adsorbed at the clay surfaces and retained between crystal lattice layers. Most of these soils have high cation exchange capacity (CEC) and a high base saturation percentage. The dominant cations are $Ca^{2+}$ and $Mg^{2+}$, while $Na^+$ plays an important role (Gururaja Rao et al. 2013c). The presence of high $Mg^{2+}$ results in dispersion and probably leads to restricted drainage and limited use for paddy followed by wheat cultivation. The soil reaction varies from weakly acid to weakly alkaline (pH 6.0–8.0). The CEC varies between 30 and 80 Cmol (+) $kg^{-1}$ dry soil. The CEC of the clay varies between 50 and 100 Cmol (+) $kg^{-1}$ clay. The base saturation percentage is greater than 50 and often close to 100 with $Ca^{2+}$ and $Mg^{2+}$ occupying more than 90% of the exchange sites; the Ca/Mg ratio is normally between 3 and 1. In saline vertisols, the relation between the salinity measured as a 1:2 soil–water extract ($EC_2$) and electrical conductivity (EC) in soil saturation extract (ECe) is given below.

$$\text{EC in soil saturation extract (ECe)} = -2.191 + (4.012 \times \text{EC in 1 : 2 soil} - \text{water extract}), r^2 = 0.985.$$

The level of salinity experienced by the plants in the alfisol was more severe than in the vertisols with the same level of salt application. An increased EC in the soil solution in the alfisol compared to the vertisol is likely due to the low cation exchange capacity and the relatively low level of organic matter of the alfisol (Krishnamurthy et al. 2011).

### 13.4.5 Coastal Salt-Affected Vertisols in Gujarat State, a Western Province of India

Salt-affected vertisols of Gujarat State are formed from the interaction of the sea and various drainage basins resulting in varied and complex salinity problems. This area comprises Bhal area, Bara tract, and Ghed tract in Saurashtra coast, which are formed due to the interaction of sea and inland areas and have varied and complex salinity problems. The area falls under arid and semi-arid climate with 650 mm of mean annual rainfall. Salinity in the region is mainly due to (1) weathering of

**Fig. 13.6** Salt-affected black soil in Gujarat

minerals either in situ or elsewhere and subsequent accumulation and transportation; (2) inherent salinity—as this area remained under the sea for long period and high saline water table has made the agricultural land saline; (3) the accelerated exploitation of saline groundwater in the past has made the land saline, which in the later period transported and deposited in the subsurface horizon of the soil profile; and (4) lateral seawater intrusion in the lower aquifer due to below sea-level water table caused by accelerated groundwater exploitation. The problems, coastal and inland salinity encountered in these regions *by and large*, have resemblances with the problems of the other areas of the state.

These soils are generally very deep (150–200 cm), fine-textured with clay content ranging from 45 to 68% (Fig. 13.6). These soils are calcareous in nature with calcium carbonate (2–12% $CaCO_3$) occurring in the form of nodules, kankar, and powdery form and exhibit alkaline reaction. While soils of the Bhal area are highly saline, soils of the Bara tract have a significant amount of soluble salts in sub-soils, with the surface layer remaining with relatively low concentration. In the Bara track, while only 39.6% of surface soils are free from salinity ($<2$ dS $m^{-1}$), 49.3% soils are saline with salinity ranging from 2 to 4 dS $m^{-1}$ and only 11.1% soils have salinity greater than 4.0 dS $m^{-1}$, whereas 10% of the sub-soil are having salinity less than 2 dS $m^{-1}$, 15% between 2 and 4 dS $m^{-1}$, and 75% greater than 4 dS $m^{-1}$ (Gururaja Rao et al. 2001, 2013c).

## 13.5 Management Options

The major strategies for reclamation and management of vertisols include the following:

- Salinity control through ameliorative measures;
- Resource conservation comprising rainwater harvesting, groundwater recharging, nutrient, and water budgeting and recycling of residues;
- Identifying ideal crops/plant species and economic halophytes;
- Conjunctive use of saline groundwater with conserved rainwater for crop production; and
- Evolving multi-cropping and integrated farming systems comprising crops/fruit species/vegetables/biomass species and aquaculture

### 13.5.1 Phytodesalinization of Coastal Saline Vertisols

Phytodesalinization or phyto/bioremediation involves growing of salt-tolerant plants, crops, and trees for removal of excess salts and/or water for the management of saline and waterlogged vertisols. The selection of plant species or crops depends on the extent of salinity, agroclimatic condition, and the economic potential of the plants. The following strategies may be employed depending upon the location-specific problems.

#### 13.5.1.1 Phytoremediation by Wild Edible Species and Fodder Crops

There are several wild plant species, which are highly tolerant to salinity. The species such as *Reichardia picroides*, *Sonchus oleraceus*, and *Urospermum picroides* are tolerant to salinity level of 8 dS $m^{-1}$. They possess salt-accumulating mechanisms as with increasing salinity their yield was not affected, whereas the Na content in leaf tissues increased (Salonikioti et al. 2015). In coastal areas, these plant species could be cultivated for removal of salts where poor quality irrigation water is used. Coastal saline vertisols can also be phytodesalinized by the cultivation of fodder crops, *Echinochloa stagnina* Retz. Beauv. In two cropping seasons (8 months' time), the salt stock in the top soil (0–10 cm) decreased by 81% and in the 0–40 cm soil layer decreased by 40% due to the cultivation of *E. stagnina*. In addition to salt removal, the production of forage (24–27 t $ha^{-1}$) was sufficiently high to have an economic interest for farmers (Ado et al. 2016).

### 13.5.2 Saline Agriculture: A Potential Tool for Phytoremediation

Biosaline agriculture is a prospective area of research where the genetic resources of halophytes and salt-tolerant plants could be utilized for producing human and animal diet and a variety of other raw materials on saline soaks using saline irrigation water (Dagar et al. 2006). The sustainable cultivation of halophytes and other salt-tolerant crops can serve commercial purposes without the degradation associated with large-scale annual monocultures and modern industrial agriculture in general. Many halophytes combine high biomass and high protein or mineral levels with outstanding ability to withstand a wide range of environmental stresses (Arora et al. 2013; Arora and Mehta 2018). Salt-tolerant plants with potential to produce food for humans and forage/fodder for livestock have been identified for coastal saline lands (Fig. 13.7). Though high salt concentration of the vegetative tissues is noticed, systems can be developed using feed mixes for livestock ruminants (Norman et al. 2013).

Although halophytes represent less than 2% of the plant population, they are of significant economic potential, albeit contribute tremendously toward the ecorestoration. World over, these plants are used as sources of food, fodder, chemicals, protective fence, besides phytoremediation, etc. (Ventura et al. 2015). Nikalje et al. (2019) described that halophytes are the source of food, feed, fiber, medicine, bioenergy crops, and a number of products. Looking at an array of benefits, cultivation of these plants on highly saline soils has been suggested by a number of workers (Ventura and Sagi 2013; Loconsole et al. 2019; Hongbo et al. 2018).

Halophytes are salt-loving plants that survive and reproduce in environments where the salt concentration exceeds 200 mM of NaCl (~20 dS $m^{-1}$; Flowers and Colmer 2008). Thus, they are often described as salt-tolerant, salt-loving, or saltwater plants, whereas practically all of our domesticated crops are considered glycophytes having been selected and bred from freshwater ancestor (Arora et al. 2013). Halophytes are also called euhalophytes because they have increased productivity with increasing salt levels and actually grow better under salinity conditions than under freshwater conditions. Halophytes have been divided into two groups, obligative halophytes, which invariably need salt for their growth and metabolism, and facultative halophytes, which grow and adapt to saline and nonsaline conditions. They show sufficient growth and development under high saline conditions, *i.e.*, halophytes belonging to the Chenopodiaceae. Facultative halophytes are able to establish themselves on salty soils, but their optimum tolerance lies in a salt-free or at least low-salt condition. The members that can tolerate salt are rom the families, Poaceae, Cyperaceae, and Brassicaceae and dicots like *Aster trifolium*, *Glaux maritima*, and *Plantago maritima*. Habitat indifferent halophytes are *Chenopodium glaucum*, *Myosurus minimus*, and *Potentilla anserina* (Hasanuzzaman et al. 2014). The mechanism of salt tolerance of halophytes is salt exclusion, excretion, and accumulation with salt excretion as the most efficient mechanism.

Halophytes occupy niches from the marine to the arid areas, from salt deserts to salt marshes, and this range of habitats is reflected in a variety of recognized "physiotypes." Halophytic plants provide options for livestock feeding in both arid and saline landscapes. These plants are variable in both biomass production and nutritive value, and they are characterized by slow growth, low digestibility (therefore, low metabolizable energy), and high content of antinutritional factors. The major hallmark of all halophytes is their ability to use inorganic ions such as $Na^+$ and

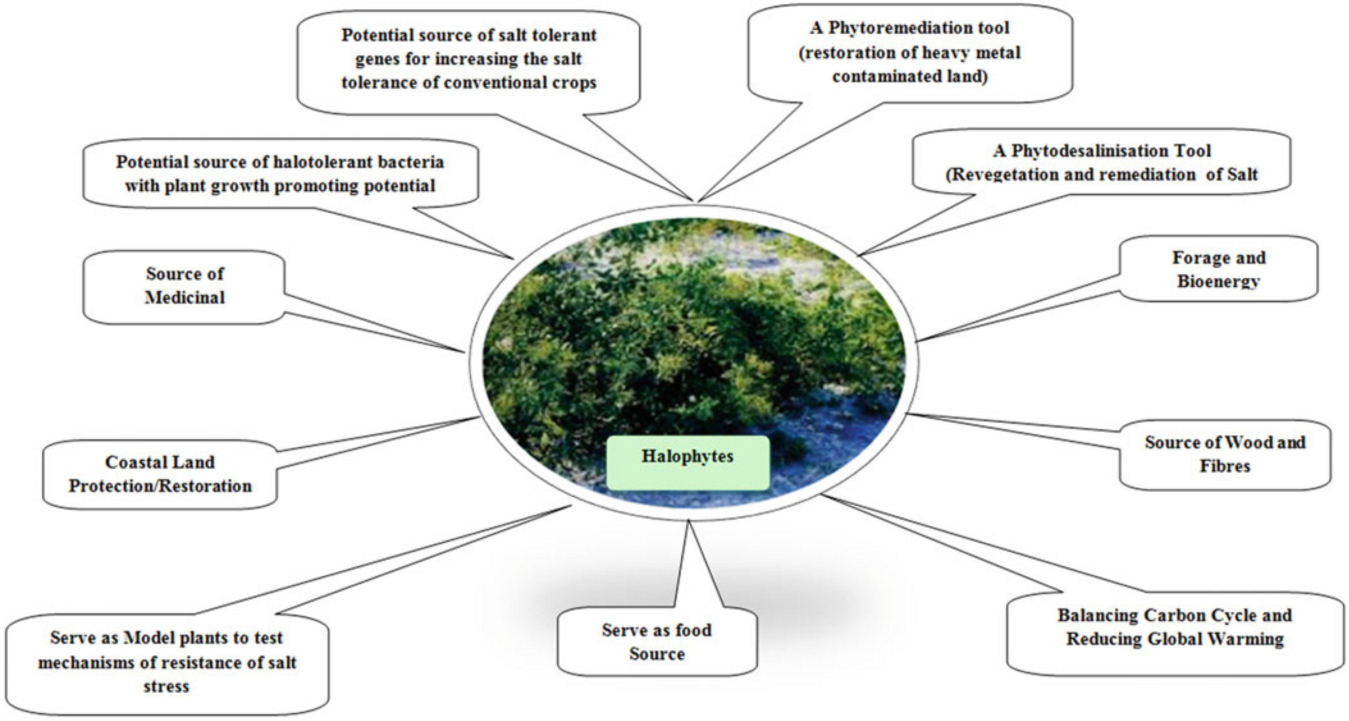

**Fig. 13.7** Halophytes and their uses

$Cl^-$ (available in the external media "for free"—i.e., ions capable of being taken passively along the electrochemical gradient without spending any energy (ATP) to drive this process) for osmotic adjustment in their tissues when grown under saline conditions. Halophytes tolerate these ions because of their superior ability to sequester cytotoxic $Na^+$ in root and leaf cell vacuoles—a trait conferred by the constitutive expression of tonoplast $Na^+/H^+$ NHX exchangers (Apse and Blumwald 2007) complemented by the efficient control of slow (SV) and fast (FV) vacuolar ion channels (Bonales-Alatorre et al. 2013) to prevent $Na^+$ from leaking back into the cytosol. In contrast, glycophytic crops possess only a limited ability to use $Na^+$ for osmotic adjustment and rely heavily on de novo synthesis of organic osmolytes.

Halophytes are equipped with well-defined adaptive mechanisms that enable them not only to withstand periodical high salinity, but also to complete their entire life cycles at high salinities. The tolerance of halophytes to salinity relies mainly on the controlled uptake of ions and the vacuolar compartmentalization of $Na^+$, $K^+$, and $Cl^-$ with the achievement of an osmotic balance between vacuoles and cytoplasm by synthesis of osmotically active metabolites (Flowers and Colmer 2008).

The most important feature of halophytes is that the seeds remain relatively salt-free, enhancing their potential for immediate use without any additional treatment. The relatively small size of their seeds, perceived as a disadvantage in harvesting and processing, can often be compensated for by relatively high yields per hectare. Although they do not constitute good food staples, amaranth is an example with extremely small grains that have met commercial expectations while providing superior nutritive qualities. Similarly, a number of salt-tolerant cereal grasses (*Distichlis*, *Pennisetum*, *and Sporobolus*) and *Chenopodium* produce large amounts of small high-protein seeds that contain a good balance of amino acids and essential fatty acids, essential vitamins and minerals, and important starches/carbohydrates. The use of saline and brackish water resources has been recommended for growing cash crops as food, fuel, fiber, fodder, and medicine for the ever-increasing human population (Flowers and Colmer 2008).

### 13.5.3 Halophytes as Alternate Food/Feed Crops

The agricultural use of saline water or soils is an important approach in the management of saline wastelands. Nonconventional crops (including halophytes) are seen as an alternative for farming in regions where only saline waters and saline soils are available and freshwater is considered a scarce resource. Identification of economic halophytes and salt-tolerant plants with potential to grow under saline water for irrigation would form an ideal management strategy in biosaline agricultural programs.

There has been a shift from producing halophytes as animal feed or salty vegetables for human consumption to the mass production of nonfood crops. Halophytes (e.g., *Crithmum maritimum*, *Portulaca oleracea*, *Salicornia* spp., and *Aster tripolium*) are well known for their ability to synthesize secondary metabolites, which have several functions, such as osmolytes and scavengers of reactive oxygen species. The secondary metabolites comprise simple and complex sugars, amino acids, quaternary ammonium compounds, polyols and antioxidants (e.g., polyphenols, β-carotene, ascorbic acid, and ureides) (Ventura and Sagi 2013). Osmolytes can be utilized in functional food, which is defined as having disease-preventing and/or health-promoting benefits (Buhmann et al. 2010; Buhmann and Papenbrock 2013a, b). Several halophytes such as *Salicornia* spp. and *Aster tripolium* are already being sold as sea vegetables and salad crops in the European markets at comparatively high prices (Böer 2006). The nonseasonality and year-round availability were an important step in the dissemination of the *Salicornia* crop and should be realized for any further halophyte vegetables (Böer 2006).

Halophytes have been tested as vegetable, forage, and oilseed crops in agronomic field trials. The most productive species yield 10–20 t $ha^{-1}$ of biomass on seawater irrigation, equivalent to conventional crops. *Salicornia bigelovii*, an oilseed halophyte, for example, yields 2 t $ha^{-1}$ of seed containing 28% oil and 31% protein, which is similar to soybean yield and seed quality (Pandya et al. 2006). Many plants have been used traditionally as herbs and vegetables (Ventura and Sagi 2013). Some of the halophytes are good fodder and hence can be used for animal feeding in saline-prone areas. However, it is to be taken into consideration that some halophytes may cause nutritional barrier due to partially high salt content and antinutritional compounds. As the reclamation of salt-affected soils is not completely feasible and is not always cost-effective, the researchers are searching for biosaline agriculture, and thus, it is obvious to explore better understanding of how naturally adapted plants (halophytes) handle salts (Koyro et al. 2011).

Manousaki and Nicolas (2011), Hasanuzzaman et al. (2014), and Jesus et al. (2015) summarized the use of halophytes for phytoremediation purpose because these plants follow different type of pathways (like phytoextraction or accumulation) to sustain in the environment. Owing to salt accumulation in plant tissues, halophytes are also proposed for soil desalination. There are success stories of the cultivation of a few halophytes like *Atriplex nummularia*, *Suaeda fruticosa*, *Suaeda salsa*, and *Sesuvium portulacastrum* (Panta et al. 2014). Jesus et al. (2015) analyzed the viability of phytoremediation of saline soils and explored the mechanism involved in the process (direct uptake or through cation exchange enhancement). The progress made on utilization of halophytes for phytoremediation of heavy metal-contaminated soils world over is summarized by Liang et al. (2017).

### 13.5.4 Halophytes in Bioremediation Programs

Bioremediation is an approach wherein plants/halophytes are used for reducing soil and water contamination (by organic and inorganic pollutants) that results from the improper disposal of aquaculture, agriculture, and industrial effluents. On salt-affected soils, phytoremediation is often the only effective and economical method employed for removing or reducing the contaminates, particularly where physical/chemical treatments and leaching are not economically feasible. Halophytes have also been shown to have the potential to accumulate enormous salt quantities, which depends on their above-ground biomass (hyperaccumulating plants). This could be of great importance, particularly in arid and semi-arid regions, where insufficient precipitation and inappropriate systems fail to reduce the salt burden in the rhizosphere of plants. Environmentally safe and clean techniques to address the salinity problems comprise the introduction of salt (ion)-removing species to control and maintain the sustainability of agricultural fields. Large-scale decontamination of soils and underground water using phytoremediation techniques requires plants with high salt uptake rate, large biomass, and tolerance to a wide array of environmental conditions and constraints. Furthermore, salt marshes, especially salt-accumulating halophytes, are the dominant species in the coastal region and the introduction of salt-removing halophytic species could potentially create both environmental and economic solutions to remediate saline soils. After reclamation studies are over, these cultivated halophytes can be utilized as animal fodder or for making organic composts.

Cultivation of *Salicornia* may also provide economic with selenium-rich animal feed. A number of halophytic grasses have been found effective in revegetating brine-contaminated soils that typically result from gas and oil mining. Eid (2011) showed the removal of Zn, Cu, and Ni by *Sporobolus virginicus* and *Spartina patens*. *Sesuvium portulacastrum* is a salt hyperaccumulating halophyte that compartmentalizes the toxic $Na^+$ in the vacuoles, and, therefore, membrane-bound transport system-regulating cytosolic ($Na^+$, $K^+$, and $Ca^{++}$) and ion accumulation by increasing the vacuolar volume. This high salt-accumulating trait in its tissues may be exploited for reducing salt levels in the potential coastal saline soils by its continued cultivation. Studies by Ramaswamy et al. (2017) in coastal Tamil Nadu, India, indicated the efficacy of this species to remove salt to a tune of 7.37 dS $m^{-1}$ from the original of 13.7 dS $m^{-1}$ in a span of 30 days after planting and 5.34 dS $m^{-1}$ in 60 days after planting.

## 13.6 Bioremediation of Coastal Saline Vertisols: Some Interventions

### 13.6.1 Intervention 1: Cultivation of *Salvadora persica* on Highly Saline Black Soils (ECe >45 dS $m^{-1}$)

Coastal and inland saline vertisols in India pose a serious threat to the economy. Agrotechnology for cultivating economically potential salt-tolerant halophyte, *Salvadora persica* L. (Meswak, Fig. 13.8), a good source for seed oil, has been evolved for the management of moderate to highly saline vertisols. This is a medicinal plant of great value with its bark containing resins and an alkaloid called salvadoricine. The seeds are a good source of nonedible oil rich in C-12 and C-14 fatty acids having immense applications in soap and detergent industry (Gururaja Rao 2015; Gururaja Rao et al. 2003, 2004; Reddy et al. 2008). *Salvadora persica*, being a large, well-branched evergreen shrub or small tree having soft whitish-yellow wood with numerous branches, drooping, glabrous, and shining, has the

**Fig. 13.8** *Salvadora persica* on highly saline black soils: immature (**a**, left) and mature (**b**, right) plants

**Table 13.1** Cost of cultivation of *Salvadora persica* on highly saline black soil

| Field operations (input costs) | Cost (₹ $ha^{-1}$) |
|---|---|
| Field preparation (by tractor) | 500.00 |
| Pitting (625 pits of $1' \times 1' \times 1'$) | 625.00 |
| Cost of saplings at Rs. 0.90 $plant^{-1}$ | 565.00 |
| Planting | 50.00 |
| Irrigation during the first year (saline water) | 150.00 |
| Digging of pit of $2.5 \times 2.0 \times 1$ m (for saline water) | 300.00 |
| Fertilizer (at 50 g DAP/plant) and FYM | 300.00 |
| Plant basin making at Rs. 0.35 $plant^{-1}$ | 220.00 |
| Miscellaneous (gap filling at 5%) | 50.00 |
| Total | 2760.00 |

Source: Gururaja Rao et al. (2003)

potential of regreening the highly saline soils that cannot be put under arable farming.

The saplings could be raised using saline water of 15 dS $m^{-1}$, an advantageous feature in areas with limited freshwater resources. The cost of cultivation comes to ₹2760/hectare in the first year (Table 13.1). By the fifth year, the plants would yield about 1800 kg $ha^{-1}$, with net returns of Rs. 8400/hectare. This species, thus, while giving economic returns on costal saline black soils with salinity up to 50 dS $m^{-1}$,

**Table 13.2** Seed production and returns from Salvadora cultivation on highly saline vertisols (ECe >55 dS $m^{-1}$)

| Year | Seed yield (t $ha^{-1}$) | Returns (₹ $ha^{-1}$) | | Cost/benefit ratio |
|---|---|---|---|---|
| | | Gross | Net | |
| I Year | Nil | Nil | Nil | Nil |
| II Year | 0.725 | 3625.00 | 365.00 | 10.03 |
| III Year | 0.978 | 4890.00 | 4340.00 | 0.13 |
| IV Year | 1.58 | 7900.00 | 7250.00 | 0.09 |
| V Year | 1.838 | 9190.00 | 8440.00 | 0.09 |

Source: Gururaja Rao et al. (2003)

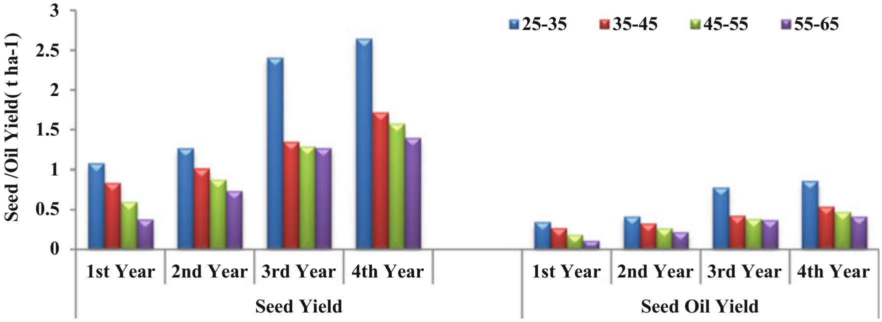

**Fig. 13.9** Seed and oil yield in *Salvadora persica* grown on saline vertisols at different salinities

also provides ecorestoration through environmental greening and forms a niche for highly saline black soils (Table 13.2 and Fig. 13.9). A spacing of 4 × 4 m has been found ideal for planting on saline black soils (Gururaja Rao et al. 2017).

Field studies on *S. persica* clearly elucidated this as a potential species for highly saline vertisols with salinity of 45–55 dS $m^{-1}$. National Bank for Agriculture and Rural Development (NABARD) in association with CSSRI came out with a bankable model for cultivation of *S. persica* (Fig. 13.10) under the refinancing module.

#### 13.6.1.1 Salt Compartmentation

Sodium and chloride ion distribution in different plant parts of *S. persica* grown at different salinities indicated bark and senescing leaves as the potential sinks for such toxic ions, thus enabling immature leaves, partially mature and physiologically mature leaves to perform their normal physiological activity (Fig. 13.11). Further, the senescing leaves act as potential sinks for toxic ions, thereby reducing the load on other photosynthesizing tissues, which remain by and large salt-free (Gururaja Rao et al. 2004).

#### 13.6.1.2 $Na^+$ and $Cl^-$ Concentration and Flux

The rate and ion transport (flux) from root to shoot and to the whole plant was calculated using the formula, $Js = (Ms_2 - Ms_1) \ln (WR_2/WR_1/(t_2 - t_1)(WR_2 -$

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TECHNICAL ASPECTS AND AGRONOMIC PRACTICES OF *SALVADORA*

With R&D grant assistance from NABARD Central Soil Salinity Research Institute, Regional Research Station, Anand (now in Baruch) have conducted a field experiments and standardized package of practices for growing Salvadora. Based on a detailed study conducted by NABARD in Gujarat and also discussions had with Scientists of CSSRI, Regional Research Station, Bharuch, the techno economic aspects of *Salva-dora* has been finalized.

*References:*

1. Technical Bulletin No.1/2003 "Salvadora persica: A life support species for salt affected black soils" by G Gururaja Rao, A K Nayak and Anil Chinchmalatpure, published by Central Soil Salinity Research Institute (ICAR), Regional Research Station, Bharuch, Gujarat

2. R&D Project Report "Management of salt affected black soils using Salvadora – forage grass based land use system" submitted to NABARD by CSSRI, Regional Research Station, Bharuchin 2002.

**Fig. 13.10** Bankable model scheme developed by NABARD Mumbai in association with CSSRI RRS, Bharuch, for cultivating *Salvadora* on highly saline black soils

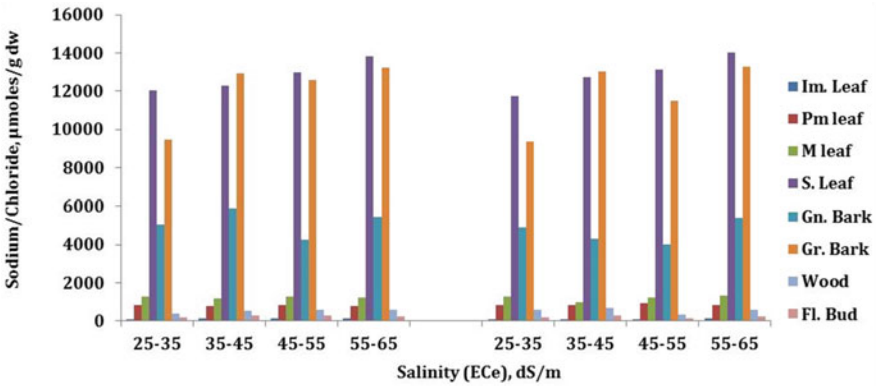


**Fig. 13.11** Compartmentation of sodium and chloride in *Salvadora persica* grown at different salinities on highly saline black soil

$WR_1$), where *Js* is the rate of transport (flux), Ms1 and Ms2 are the amounts of ion in the shoot/whole plant, and $WR_1$ and $WR_2$ are the fresh weights of the roots at the harvest times *t*2 and *t*1 (Pitman 1975).

Sodium and chloride concentration in all the plant parts was found to increase with increase in salinity of the soil. While maximum amount of $Na^+$ and $Cl^-$ ions was seen in the bark, root, and senescing leaves, the immature (expanding) and mature (fully expanded) leaves remained relatively with lower concentration (Table 13.3). These tissues act as a potential sink for excess $Na^+$ and $Cl^-$ ions. The capacity of the sink increased with age of the plant and increase in salinity that indicates the presence of a very well-developed salt compartmentation mechanism in *S. persica* (Gururaja Rao et al. 2004). Though $Na^+$ concentration increases with increase in salinity, the total Na uptake showed a decreasing trend, which may be obviously due to decrease in the biomass yield with increase in salinity. Similarly, chloride uptake in the root is much higher than that of the shoot. The rate of flux of $Na^+$ and $Cl^-$ ions to the whole plant while increasing with increase in salinity showed a decreasing trend with age (Table 13.4). The flux of these ions from the root to the shoot was a fraction of that to the whole plant, indicating that roots accumulate more ions than shoots. In this species, roots act as both $Na^+$ and $Cl^-$ accumulator.

#### 13.6.1.3 Soil Salinity Under Plantations

The soils showed high degree of spatial and temporal variation in soil salinity ranging from 65 to 70 dS $m^{-1}$ in the top layer in the initial years of planting. Salinity of the soil decreased with depth, from the surface to 90 cm depth (Fig. 13.12). Cultivation of *S. persica* up to 5 years resulted in a slight decline in soil salinity as compared to the pre-planting salinity. Changes in surface salinity are partly attributed to the ability of plants to extract the salt and partly due to root activity, which improves the physical properties of the soil. However, the magnitude of fluctuation in salinity was not much at the lower layers. The groundwater table might be contributing to salinity causing only minor changes at lower depths. The spatial variability of surface salinity under a 5-year-old plantation (Fig. 13.13) showed significant difference from the initial salinity prior to planting.

### 13.6.2 Intervention 2: Phytoremediation by *Salicornia*

Among halophytes, the genus *Salicornia*, a succulent, occurs naturally in abundance on mud flats. It is the most valuable and commercially exploited one for its seed oil and nutritional value and for its succulent tips used as green salad. The glasswort (*Salicornia bigelovii*) is a leafless salt marsh annual plant (Fig. 13.14) with green jointed and succulent stems indigenous to the Arabian Sea coasts of Pakistan and India. It is extremely salt-tolerant and used as salad (greens), while the seed forms a good source of high-quality edible oil; the residual meal provides superior feed for livestock and shrimp/fish. It produces seeds with 30% oil and 35% protein and the oil similar to soybean oil. In addition, the stem and straw can be utilized as hay in mixed feeding regimes, manufactured into pressed board for construction purposes or in

**Table 13.3** Concentration of $Na^+$ and $Cl^-$ (%) ions at organ level in *Salvadora persica* grown on highly saline black soil

| | Salinity range (dS $m^{-1}$) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 25–35 | | | 35–45 | | | 45–55 | | | 55–65 | | |
| Plant part | 2nd year | 3rd year | 4th year | 2nd year | 3rd year | 4th year | 2nd year | 3rd year | 4th year | 2nd year | 3rd year | 4th year |
| $Na^+$ | | | | | | | | | | | | |
| Root | 1.13 | 1.62 | 1.86 | 1.61 | 2.11 | 2.30 | 1.91 | 2.37 | 2.57 | 1.93 | 2.39 | 2.60 |
| Wood | 0.02 | 0.03 | 0.03 | 0.03 | 0.04 | 0.04 | 0.03 | 0.04 | 0.05 | 0.04 | 0.06 | 0.06 |
| Bark | 1.59 | 1.72 | 1.95 | 2.12 | 2.29 | 2.60 | 2.33 | 2.52 | 2.86 | 2.73 | 2.95 | 3.34 |
| Im. leaf | 0.02 | 0.02 | 0.02 | 0.02 | 0.02 | 0.03 | 0.02 | 0.02 | 0.03 | 0.03 | 0.03 | 0.03 |
| M. leaf | 0.18 | 0.18 | 0.21 | 0.19 | 0.20 | 0.23 | 0.21 | 0.22 | 0.25 | 0.22 | 0.24 | 0.27 |
| S. leaf | 1.66 | 1.81 | 2.06 | 2.11 | 2.30 | 2.61 | 2.30 | 2.38 | 2.71 | 2.39 | 2.51 | 2.83 |
| CD 0.05 | 0.36 | 0.13 | 0.18 | 0.46 | 0.21 | 0.22 | 0.89 | 0.16 | 0.30 | 0.69 | 0.46 | 0.71 |
| $Cl^-$ | | | | | | | | | | | | |
| Root | 2.13 | 2.65 | 2.70 | 2.65 | 3.36 | 3.60 | 2.88 | 3.00 | 3.90 | 2.94 | 4.00 | 4.01 |
| Wood | 0.04 | 0.05 | 0.06 | 0.05 | 0.06 | 0.06 | 0.05 | 0.07 | 0.07 | 0.07 | 1.00 | 0.10 |
| Bark | 2.49 | 2.69 | 3.05 | 3.43 | 3.70 | 4.01 | 3.74 | 4.04 | 4.41 | 4.26 | 4.62 | 5.14 |
| Im. leaf | 0.03 | 0.04 | 0.04 | 0.04 | 0.04 | 0.05 | 0.04 | 0.04 | 0.04 | 0.5 | 0.05 | 0.06 |
| M. leaf | 0.28 | 0.29 | 0.32 | 0.31 | 0.31 | 0.38 | 0.33 | 0.34 | 0.39 | 0.35 | 0.40 | 0.42 |
| S. leaf | 2.63 | 2.82 | 3.19 | 3.17 | 3.67 | 2.71 | 3.58 | 3.76 | 4.23 | 3.58 | 4.04 | 4.59 |
| CD 0.05 | 0.40 | 0.16 | 0.39 | 1.05 | 0.28 | 0.30 | 0.58 | 0.22 | 0.40 | 0.95 | 0.98 | 0.83 |

**Table 13.4** Uptake and flux of $Na^+$ and $Cl^-$ ions in *S. persica* on saline black soils

|  | Uptake (g) |  |  |  | Flux ($\mu g\ g^{-1}\ day^{-1}$) |  |  |  |
|---|---|---|---|---|---|---|---|---|
|  | Shoot |  | Root |  | Shoot |  | Root |  |
| Salinity class ($dS\ m^{-1}$) | $Na^+$ | $Cl^-$ | $Na^+$ | $Cl^-$ | $Na^+$ | $Cl^-$ | $Na^+$ | $Cl^-$ |
|  | 2nd year |  |  |  |  |  |  |  |
| 25–35 | 6.44 | 10.18 | 8.40 | 15.86 |  |  |  |  |
| 35–45 | 5.12 | 8.53 | 9.31 | 15.29 |  |  |  |  |
| 45–55 | 4.10 | 6.57 | 6.58 | 9.91 |  |  |  |  |
| 55–65 | 3.68 | 5.64 | 4.97 | 7.56 |  |  |  |  |
| CD 0.05 | 1.21 | 1.88 | 1.93 | 2.12 |  |  |  |  |
|  | 3rd year |  |  |  | Between 3rd and 2nd year |  |  |  |
| 25–35 | 16.01 | 25.90 | 27.36 | 44.93 | 29.9 | 46.1 | 9.8 | 16.2 |
| 35–45 | 14.21 | 22.95 | 27.69 | 44.08 | 39.0 | 61.3 | 12.9 | 20.4 |
| 45–55 | 10.13 | 16.21 | 18.56 | 29.43 | 50.2 | 81.3 | 16.8 | 26.9 |
| 55–65 | 9.82 | 15.59 | 13.62 | 22.84 | 78.8 | 131.4 | 19.5 | 52.6 |
| CD 0.05 | 2.11 | 2.88 | 3.58 | 5.35 | 10.5 | 13.8 | 4.3 | 5.8 |
|  | 4th year |  |  |  | Between 4th and 3rd year |  |  |  |
| 25–35 | 22.31 | 34.71 | 38.33 | 56.66 | 10.8 | 12.9 | 3.9 | 5.5 |
| 35–45 | 18.42 | 28.69 | 37.64 | 58.73 | 12.3 | 17.8 | 3.7 | 5.0 |
| 45–55 | 14.43 | 22.30 | 37.23 | 37.23 | 17.8 | 23.5 | 7.3 | 9.7 |
| 55–65 | 13.51 | 20.84 | 29.35 | 29.35 | 29.7 | 410.2 | 11.9 | 16.9 |
| LSD $p \leq 0.05$ | 3.95 | 4.23 | 0.53 | 1.88 | 1.88 | 3.50 | 1.20 | 1.70 |

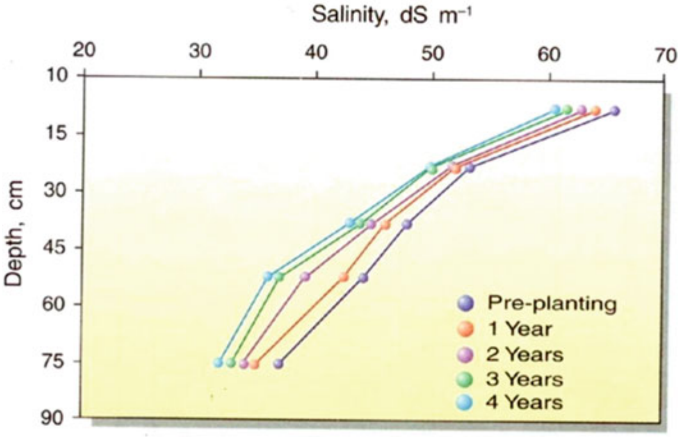


**Fig. 13.12** Soil salinity variations over the years under *S. persica* grown on highly saline black soil (55–65 $dS\ m^{-1}$ range)

paper making. Though a number of halophytes are available for phytoremediation, *Salicornia* looks to be an ideal plant due to multifaceted economic uses. Its potential

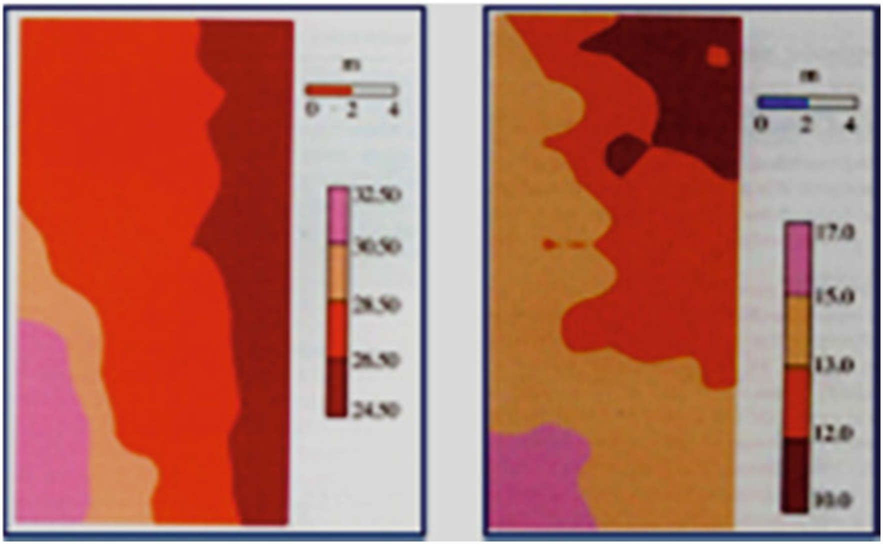


**Fig. 13.13** Spatial variability in soil salinity prior and after planting of *S. persica*

**Fig. 13.14** *Salicornia brachiata* on highly saline soil

as a source of vegetable salt may be exploited besides medicinal values to bring in entrepreneurs, private players for coastal land development.

Earlier studies by Pandya et al. (2006) showed that application of nitrogen (N) up to 100 kg $ha^{-1}$ had significantly increased the seed yield (29 and 87%) and plant biomass (29 and 51%), over 75 and 0 kg N $ha^{-1}$, respectively. Plant attributes like canopy, spike length, number of segments, and harvest index were also found to be increased with the increase in N application. Application of 75 kg $P_2O_5$ $ha^{-1}$ was also found significant, resulting in higher seed yield (48%) and number of spike segments (43%) over the control. The interaction study between the applied doses of N × P was found significant at the highest fertilizer levels (N-100 kg $ha^{-1}$ × P-75 kg $ha^{-1}$) and produced maximum seed yield over the control but remained and at par in case of plant biomass. The plant nitrogen content in biomass (spike+seed) though found increased with N application but has remained at par in case of P application (Table 13.6). Nitrogen and potassium content and their uptake were found to increase significantly with N application. The plant density had a significant effect on yield, biomass, and other important yield attributes. Plant canopy increased significantly during different phases of growth. The findings here indicate potential benefit of *Salicornia brachiata* if resorted and integrated to the crop production system in saline soils intruded with sea water for enhancement of scope of sustainable marine ecosystem and as remedial measure of coastal saline agriculture of the world inclusive of salt-affected waste lands. Optimum plant density of 278 plants 10 $m^{-2}$ and 100 kg N $ha^{-1}$ and 75 kg $P_2O_5$ $ha^{-1}$ improved the vegetable biomass and oil of *Salicornia brachiata* (Fig. 13.15; Table 13.5), which is of immense help to the producers with a new alternative cropping system having high industrial potential for its valued nutritional salt and linoleic-rich oil and plant bioactive derivatives.

Commercial plantations of *Salicornia bigelovii* and *S. europaea* have also been raised in Dubai with seawater for high culinary value. Since the seeds are a source of high-quality unsaturated oil (30%) and proteins (40%), its potential use in biofuel and animal feed is advocated. The seed weight in *S. bigelovii* ranged from 6.39 to 9.17 g $plant^{-1}$ (Shahid et al. 2013; Shahi 2017). This seed weight is quite high as compared to its Indian counterpart that showed 4.5 g $plant^{-1}$ (Gohil et al. 2008). Seed and dry biomass production in the species is very low and does not sustain as a commercial crop. Therefore, it was emphasized to develop useful products to make the cultivation more sustainable. Attention was given to extract the absorbed salt in tissues (30–40%). The quality of the salt was estimated, and later extraction was scaled up to get salt from the vegetative dry biomass after the plants completed its life cycle. Processes were developed to produce vegetable salt, and its variations from *Salicornia* contained several important nutrients not normally found in sea salt and named as Saloni. Low sodium salt was considered to be beneficial for cardiovascular patients.

Recently, the potential of *Salicornia* was assessed for phytoremediation of saline soils (Sharma et al. 2010). Attempts were made to cultivate *S. brachiata* with reject RO water. The optimum plant growth was observed when irrigated with RO reject water having TDS of 27,000 ppm (approx.). The utilization of RO reject brackish water would be a sustainable and environment-friendly approach for the

**Fig. 13.15** High-density crop of S*alicornia brachiata*

management of RO reject water technologies world over (Singh et al. 2018). *Salicornia* grown in tannery waste water-treated lands indicated that industry can use this plant to solve salt accumulation problems arising from tannery (Ramesh et al. 2009).

Reddy et al. (2008) studied the effect of bromide on growth, ion content, and metabolic changes. Enzymatic activities decreased significantly with increase in fluoride treatment. Their studies demonstrated that bromide was not significantly toxic to *Salicornia* up to 600 mM indicating its suitability only for moderately Br-contaminated soils and also summarized the bromide tolerance of a number of halophytes along the Indian coast like *Salicornia brachiata*, *Suaeda nudiflora*, and *Salvadora persica.* An easy, simple, and eco-friendly method was developed to determine bromide content in plant parts. The bromide content ranged from 0.086–0.2 g in roots, 0.175–0.443 g in stems, and 0.287–0.432 g in leaves per g dry plant material. These plants were suggested for remediation of bromide affected soils.

Sharma et al. (2010) showed the accumulation of heavy metals like Cd, Ni, and arsenic in *Salicornia brachiata*, making it suitable for phytoremediation coastal saline soils. Catalase activity significantly increased with the exposure to heavy metals. Recently, Kaviyani et al. (2017a, b) demonstrated that Ni- and Pb-contaminated soils can be remediated with *Salicornia iranica*. Padro et al. (2013) also demonstrated the influence of cadmium contamination on *Salicornia ramosissima* and phytoremediation capacity of the species as it was observed that with increase in salinity, cadmium, and salinity affected the growth and bioaccumulation of cadmium.

**Table 13.5** Effect of phosphorus on yield and yield attributes in *Salicornia brachiata*

| Phosphorus levels | Seed yield (kg $ha^{-1}$) | Dry biomass (kg $ha^{-1}$) | Plant height (cm) | Canopy spread (cm) | Main branches (Number) | Spikes/branch (Number) | Spike length (cm) | Segments (Number) | HI (%) |
|---|---|---|---|---|---|---|---|---|---|
| $P_0$ | 571.6 | 5192 | 30.10 | 123.9 | 33.2 | 424.2 | 9.80 | 5.7 | 24.9 |
| $P_1$ | 609.0 | 6016 | 31.70 | 125.9 | 33.3 | 423.8 | 11.00 | 5.9 | 28.9 |
| $P_2$ | 846.2 | 7144 | 30.20 | 122.3 | 34.1 | 564.2 | 11.40 | 8.2 | 41.8 |
| S. Em | 88.10 | 759.2 | 0.51 | 4.51 | 2.91 | 52.06 | 0.56 | 0.49 | 13.1 |
| LSD ($p \leq 0.05$) | 215.6 | NS | NS | NS | NS | NS | NS | 1.72 | NS |

$P_1$ and $P_2$ indicate phosphorus application at 50 and 75 kg $ha^{-1}$, respectively; *HI* harvest index
Source: Pandya et al. (2006)

### 13.6.3 Halophytes in Biosaline Agroforestry

The main focus of this intervention is the remediation of saline wastelands through cultivation of biomass species for energy production, biomaterials, and fodder and focusing on the tree component of agroforestry systems. For example, in saline areas trees and salt-tolerant plants can be an alternative to conventional agriculture. Trees on saline wastelands produce timber for construction or for energy, i.e., charcoal for cooking or electricity production through gasifiers. They also function as windscreens, protect the soil against erosion, add organic matter and nitrogen in soil, help in breaking hard pans in alkali soils, and above all sequester carbon helping in mitigating climate change (Sharma et al. 2010; Gururaja Rao and Dagar 2020).

The desert halophyte plants, *Tamarix aphylla* and *Euphorbia tirucalli*, are a good source of biomass under extreme desert conditions (Eshel et al. 2011). *Tamarix aphylla* (erect type) trees produced 52 and 26 t ha$^{-1}$ organic biomass when irrigated with reclaimed sewage (EC approx. 3 dS m$^{-1}$) or brine (EC approx. 7–10 dS m$^{-1}$), respectively. *Euphorbia tirucalii*, a desert succulent, suggested as a potential biofuel crop, was found to exhibit 60-fold weight increase 18 months after transplanting when irrigated with saline sewage (EC 8–10 dS m$^{-1}$), generating a crop rich in carbon and hydrogen that has potential for being directly converted into biofuel (Eshel et al. 2010).

### 13.6.4 Intervention 3: Cultivation of Forage Grasses

Agriculture and animal husbandry in India are interwoven as farming and livestock rearing form an integral part of rural living. India supports nearly 20% of the world livestock. Most often and especially in arid and resource poor regions, livestock is the only source of cash income for subsistence farms. It insures some livelihood in the event of crop failure. At present, India faces a net deficit of 61.1% green fodder, 21.9% dry crop residues, and 64% feeds. Due to ever-increasing population pressure, arable land will mainly be used for food and cash crops, leaving little chance of having good-quality arable lands for fodder production. Therefore, poor quality saline/sodic lands provide a good avenue to increase the availability of fodder if some grasses can be identified to suit the saline/sodic environment. Forages like *Leptochloa fusca*, *Cynodon dactylon*, *Dactyloctenium sindicum*, *Paspalum vaginatum*, *Chloris gayana*, *Echinochloa turnerana*, *E. colonum*, *Eragrostis tenella*, *Dichanthium annulatum*, *D. caricosum*, *and Brachiaria mutica* are the predominant ones in coastal saline soils (Khan and Ansari 2008). Forage grasses, kallar grass (*Leptochloa fusca*), Rhodes grass (*Chloris gayana*), and Para grass (*Brachiaria mutica)* are highly salt-tolerant and high biomass yielders. While Prosopis and kallar grass are highly promising for firewood and forage production on sodic soils, *Salvadora persica* with kallar grass and/or *Dichanthium annulatum* constitute a viable option for saline vertisols (Gururaja Rao et al. 2017). Singh et al. (2014) reported a silvipastoral system comprising *Prosopis* with kallar grass has been found ideal and highly remunerative in the early four years. Growing *Acacia nilotica*,

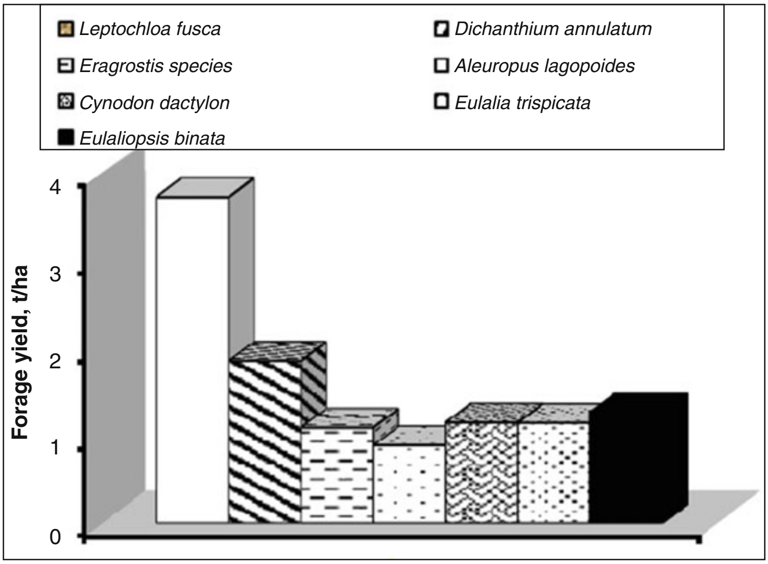


**Fig. 13.16** Forage yield of grasses on saline black soils (ECe, 14.6 dS/m). Source: Gururaja Rao et al. (2012)

*Eucalyptus tereticornis*, and *Parkinsonia aculeata* on ridges and kallar grass in trenches has been found ideal. Cultivation of kallar grass in furrows and *Dichanthium* on ridges has been found beneficial on saline Vertisols (Gururaja Rao et al. 2011). Agrotechnology for the cultivation of forage grasses, *Dichanthium annulatum* and *Leptochloa fusca* (8–10 dS $m^{-1}$) and *Eragrostis* and *Aeluropus lagopoides* on saline black soils having salinity up to 14–16 dS $m^{-1}$, have been evolved. Application of nitrogen at 46 kg $ha^{-1}$ as urea increased the forage yield by about 70% in *Dichanthium annulatum* (Gururaja Rao 2015).

Other grasses like *Aeluropus lagopoides* and *Eragrostis* spp. are ideal for cultivation on highly saline black soils. *Dichanthium annulatum* is another grass with better forage qualities and high slat tolerance (Fig. 13.16; Gururaja Rao 2015). This species possess a well-defined salt compartmentation mechanism wherein the roots act as potential sinks for toxic $Na^+$ and $Cl^-$ ions making the shoots relatively salt-free.

Kallar grass actually thrives in sodic and waterlogged soil conditions and has been used in their reclamation programs. Studies on moderately saline black soils with salinity of 14–18 dS $m^{-1}$, in a ridge and furrow system of planting, indicated that this grass yields well when planted in furrows (Gururaja Rao 2015; Gururaja Rao et al. 2017).

*Dichanthium annulatum* and *Leptochloa fusca* in a ridge-furrow planting system with 50 cm high ridge and 1 m between midpoints of two successive ridges were found ideal in saline vertisols having salinity up to 8–10 dS $m^{-1}$ (Table 13.6). For maximizing forage production, *Dichanthium* on ridges and *Leptochloa* in furrows form an ideal proposition on saline black soils. Nitrogen given at the rate of 45 kg

**Table 13.6** Growth and yield of forage grasses under ridge and furrow planting system

| | Height (m) | | Tillers (plant$^{-1}$) | | Green forage yield (t ha$^{-1}$) | |
|---|---|---|---|---|---|---|
| Grass species | Ridge | Furrow | Ridge | Furrow | Ridge | Furrow |
| *Leptochloa fusca* | 1.18 | 1.02 | 10.62 | 9351 | 3.17 | 3.73 |
| *Dichanthium annulatum* | 0.91 | 0.74 | 6.41 | 5.32 | 1.85 | 1.76 |
| $CD_{0.05}$ | | Height | | Tillers | | Yield |
| Planting method | | 0.12 | | 0.91 | | NS |
| Grass species | | 0.16 | | 1.53 | | 0.82 |
| Planting method × grass species | | NS | | 2.24 | | NS |

Salinity of the saturation extract (0–30 cm): 15.4 dS m$^{-1}$
Source: Gururaja Rao et al. (2013c)

**Table 13.7** Effect of nitrogen on growth and forage yield (t ha$^{-1}$) of forage grasses

| | Height (m) | | Tillers (plant$^{-1}$) | | Green forage yield (t ha$^{-1}$) | |
|---|---|---|---|---|---|---|
| Grass species | +N | −N | +N | −N | +N | −N |
| *Leptochloa fusca* | 1.39 | 0.99 | 12.54 | 4.46 | 3.21 | 2.13 |
| *Dichanthium annulatum* | 1.01 | 0.87 | 10.24 | 7.38 | 2.24 | 1.32 |
| $CD_{0.05}$ | | Height | | Tillers | | Yield |
| Planting method | | 0.13 | | 3.11 | | 0.88 |
| Grass species | | 0.22 | | 2.32 | | 0.55 |
| Planting method × grass species | | NS | | NS | | NS |

Source: Gururaja Rao et al. (2013c)

ha$^{-1}$ (in the form of urea) at the time of rooted slip planting, boosts forage production, and improves forage quality traits. *Dichanthium annulatum* has been found most suitable for saline black soils as it possessed well-defined salt compartmentation, wherein the roots act as potential sinks for toxic ions like sodium and chloride, making the shoot portions relatively salt-free (Table 13.7, Gururaja Rao et al. 2017).

Studies on forage grasses on saline vertisols further revealed the presence of a well-defined salt exclusion mechanism in *Dichanthium annulatum*. Application of nitrogen at 46 kg ha$^{-1}$ (Table 13.7) as urea increased the forage yield by about 70% in *Dichanthium annulatum*. The cattle and camel populace form the important livestock of the region. Cultivation of salt-tolerant grasses like *Dichanthium annulatum* and *Leptochloa fusca* on moderate saline soils result in 1.9 t ha$^{-1}$ and 3.2 t ha$^{-1}$, respectively.

**Physical Impact** The technology has been widely adapted in saline vertisols of Bhal. These grasses being perennial in nature, while providing fodder continuously, also add to the environmental stability in the area.

**Unit Cost** ₹3000 $ha^{-1}$ in the first year for planting. The grasses used to give 3–4 cuts and together gave economic returns of about ₹10,000 $ha^{-1}$.

Halophytic forage grasses, viz. *Aeluropus lagopoides* and *Eragrostis*, have been found ideal for saline agriculture on saline black soils. Of these two, *Aeluropus* was found to possess better forage qualities and salt removal ability from the soils. These grasses responded well to saline water up to 30 dS $m^{-1}$ and thus form suitable for cultivation on coastal saline Vertisol with plenty of saline groundwater resources (Ahmed et al. 2011; Gururaja Rao et al. 2011).

#### 13.6.4.1 Salt Uptake and Ion Flux

The $Na^+$ and $Cl^-$ ions in leaf and stem increased with the increase in salinity in these grasses. The shoot (leaf and stem) sodium content after two irrigations increased from 2000 μmoles to 5900 μmoles in the leaf of *Eragrostis* spp. and 3500 μmoles to 5100 μmoles in *Aeluropus lagopoides*. The $Na^+$ and $Cl^-$ contents were higher in the stem compared to the leaves, indicating the stem a potential sink. Among the grasses, $Na^+$ and $Cl^-$ contents were found to be more in *Aeluropus lagopoides* than in *Eragrostis* spp. (Ahmed et al. 2011; Gururaja Rao 2015; Gururaja Rao et al. 2011, 2013c). The uptake and flux of $Na^+$ and $Cl^-$ and the total $Na^+$ uptake showed a decreasing trend with increase in salinity of irrigation water in both the grasses. *Aeluropus lagopoides* showed higher uptake than that of *Eragrostis* spp. though the increase was only marginal (Tables 13.8 and 13.9). The total $Na^+$ content is less in the shoot than in the root in both the grasses irrespective of salinity and age of the plant. Chloride uptake, however, is relatively more in the root than in the shoot. The rate of flux of $Na^+$ and $Cl^-$ to the whole plant though increased with salinity and the age of the plant.

#### 13.6.4.2 Salt Compartmentation and Sodium and Potassium Budget

Ion uptake, partition, and ion flux indicated that roots act as potential sinks for toxic ions like $Na^+$ and $Cl^-$ (Table 13.10). *Aeluropus* showed higher ion uptake than *Eragrostis*. While roots of *Aeluropus* showed 65.4% uptake of total salt, the shoots showed only 34.6% and in *Eragrostis* roots retained 70.6% and the shoots only 29.4%. The lower ion content in the shoots reduces the salt toxicity to the photosynthesizing tissue and also helps in the forage quality. Ion partitioning ($Na^+$ and $Cl^-$) in the shoot and roots of the two grasses indicated that roots act as sinks for these toxic ions. The grasses have been found very effective in salt removal from the soil layers. *Aeluropus* was found to remove more salt than *Eragrostis*.

Ion compartmentation at the organ level indicated a higher amount of sodium in roots followed by stem and old leaves and the least in inflorescence in both the grasses. Similar trend was observed in potassium in that foliage and roots had higher potassium than inflorescence (Table 13.10). Higher accumulation of sodium in roots, old leaves, and stems indicate the physiologically mature foliage had relatively low tissue sodium. Of the two forage grasses, *Aeluropus* had higher potassium in foliage, while *Eragrostis* had higher potassium in roots. Contrary to this, sodium was found to be more in the foliage of *Eragrostis*, while roots of *Aeluropus* had marginally

**Table 13.8** Uptake and flux of $Na^+$ and $Cl^-$ ions in *Aeluropus lagopoides* under saline water irrigation

| | Uptake (g) | | | | Flux (ug $g^{-1}$ $day^{-1}$) | | | |
|---|---|---|---|---|---|---|---|---|
| | Shoot | | Root | | To whole plant | | To shoot | |
| Salinity (dS $m^{-1}$) | $Na^+$ | $Cl^-$ | $Na^+$ | $Cl^-$ | $Na^+$ | $Cl^-$ | $Na^+$ | $Cl^-$ |
| | 1st week | | | | | | | |
| 10 | 3.91 | 4.37 | 5.42 | 5.14 | | | | |
| 20 | 3.64 | 4.12 | 4.86 | 4.58 | | | | |
| 30 | 3.42 | 3.90 | 4.39 | 4.32 | | | | |
| 40 | 3.08 | 3.76 | 4.14 | 4.24 | | | | |
| | 2nd week | | | | Between 1st and 2nd weeks | | | |
| 10 | 4.78 | 4.36 | 5.96 | 4.35 | 7.90 | 9.92 | 2.18 | 3.42 |
| 20 | 4.32 | 4.04 | 5.78 | 4.14 | 8.62 | 10.42 | 3.62 | 3.75 |
| 30 | 4.02 | 3.92 | 4.14 | 3.62 | 9.36 | 12.62 | 3.92 | 4.62 |
| 40 | 3.64 | 3.51 | 4.04 | 3.44 | 10.42 | 18.80 | 4.14 | 5.96 |
| | 3rd week | | | | Between 2nd and 3rd weeks | | | |
| 10 | 4.92 | 4.88 | 5.84 | 4.98 | 10.39 | 12.82 | 3.14 | 4.36 |
| 20 | 4.81 | 4.64 | 5.72 | 4.86 | 13.86 | 16.01 | 3.36 | 5.62 |
| 30 | 4.32 | 4.38 | 4.92 | 4.64 | 15.14 | 22.41 | 3.92 | 6.98 |
| 40 | 4.02 | 4.14 | 4.44 | 4.32 | 19.52 | 24.62 | 4.79 | 9.39 |
| | 4th week | | | | Between 3rd and 4th weeks | | | |
| 10 | 5.64 | 5.84 | 6.10 | 5.14 | 15.76 | 19.72 | 3.79 | 4.72 |
| 20 | 4.92 | 4.92 | 5.72 | 5.02 | 16.80 | 25.6 | 5.16 | 6.72 |
| 30 | 4.84 | 4.63 | 5.32 | 4.84 | 18.44 | 26.8 | 6.13 | 8.42 |
| 40 | 4.12 | 4.24 | 4.79 | 4.36 | 26.12 | 28.42 | 7.14 | 8.92 |

higher sodium. Once the flowering occurs, higher sodium is found to be more in older leaves in *Eragrostis*, when compared to *Aeluropus* while older leaves showed lesser sodium when compared to shoot (Gururaja Rao et al. 2011).

#### 13.6.4.3 Salt Removal

Salt budgeting including contributions of saline water, subsurface salinity, and the salt uptake by the halophytic grasses indicated that *Aeluropus* had better salt removal, i.e., 43.9% when compared to *Eragrostis* with 39.7% (Fig. 13.17). This feature is highly useful in using these grasses under saline agriculture programs for lowering soil salinity in coastal saline soils, which over the years will help cultivation of lesser tolerant and more economically potential species.

#### 13.6.4.4 Forage Production

The forage yield of these grasses is given in Fig. 13.18. The data indicated higher forage yield in *Eragrostis* when compared to *Aeluropus lagopoides* at a salinity of 14.6 dS $m^{-1}$. *Eragrostis* spp. showed higher fresh and dry plant biomass than *Aeluropus lagopoides*. Among the treatments, *Eragrostis* did not show much variation in the fresh and dry shoot biomass, whereas *Aeluropus lagopoides* showed

**Table 13.9** Uptake and flux of $Na^+$ and $Cl^-$ ions in *Eragrostis species* under saline water irrigation

| | Uptake (g) | | | | Flux (ug $g^{-1}$ $day^{-1}$) | | | |
|---|---|---|---|---|---|---|---|---|
| | Shoot | | Root | | To whole plant | | To shoot | |
| Salinity (dS $m^{-1}$) | $Na^+$ | $Cl^-$ | $Na^+$ | $Cl^-$ | $Na^+$ | $Cl^-$ | $Na^+$ | $Cl^-$ |
| | 1st week | | | | | | | |
| 10 | 4.20 | 4.85 | 5.82 | 5.13 | | | | |
| 20 | 3.92 | 4.60 | 5.20 | 4.93 | | | | |
| 30 | 3.61 | 4.05 | 4.85 | 4.14 | | | | |
| 40 | 3.40 | 3.70 | 3.85 | 3.96 | | | | |
| | 2nd week | | | | Between 1st and 2nd weeks | | | |
| 10 | 5.15 | 4.74 | 6.40 | 4.68 | 8.78 | 1.88 | 2.42 | 3.62 |
| 20 | 4.95 | 4.44 | 6.00 | 4.53 | 9.92 | 13.84 | 3.03 | 4.81 |
| 30 | 4.45 | 4.10 | 5.28 | 3.96 | 12.62 | 14.09 | 3.84 | 4.99 |
| 40 | 3.62 | 3.78 | 4.62 | 3.65 | 13.12 | 26.32 | 4.12 | 8.92 |
| | 3rd week | | | | Between 2nd and 3rd weeks | | | |
| 10 | 5.60 | 5.66 | 6.78 | 5.25 | 13.42 | 16.24 | 3.62 | 5.14 |
| 20 | 5.05 | 5.05 | 6.18 | 4.94 | 17.32 | 20.33 | 5.14 | 6.24 |
| 30 | 4.62 | 4.60 | 5.85 | 4.60 | 18.92 | 28.75 | 5.92 | 8.36 |
| 40 | 4.28 | 4.20 | 4.90 | 4.28 | 24.32 | 32.48 | 7.96 | 10.62 |
| | 4th week | | | | Between 3rd and 4th weeks | | | |
| 10 | 6.05 | 6.20 | 6.90 | 6.18 | 19.70 | 26.78 | 6.32 | 8.32 |
| 20 | 5.25 | 5.00 | 6.23 | 5.54 | 26.30 | 32.14 | 8.44 | 10.64 |
| 30 | 5.05 | 5.05 | 5.63 | 4.84 | 28.15 | 39.36 | 9.63 | 14.20 |
| 40 | 4.60 | 4.60 | 5.00 | 4.12 | 30.10 | 42.74 | 10.40 | 16.32 |

**Table 13.10** Ion partitioning in halophytic grasses grown on saline vertisols

| | Aeluropus lagopoides | | | Eragrostis species | | |
|---|---|---|---|---|---|---|
| Part of the plant | $Na^+$ | $K^+$ | Na/K | $Na^+$ | $K^+$ | Na/K |
| Inflorescence | 2.6 | 4.4 | 0.590 | 4.3 | 4.9 | 0.876 |
| Mature foliage | 12.4 | 8.8 | 1.409 | 11.6 | 7.6 | 1.526 |
| Stem | 16.1 | 10.4 | 1.548 | 12.4 | 7.9 | 1.570 |
| Old foliage | 13.6 | 7.9 | 1.722 | 14.2 | 7.4 | 1.972 |
| Root | 30.2 | 8.8 | 3.432 | 29.4 | 9.1 | 3.231 |

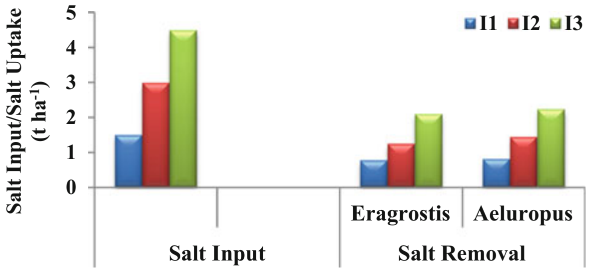


**Fig. 13.17** Salt input and salt uptake by forage grasses under saline water irrigation on saline vertisols (I1, I2, I3 indicate numbers of irrigations)

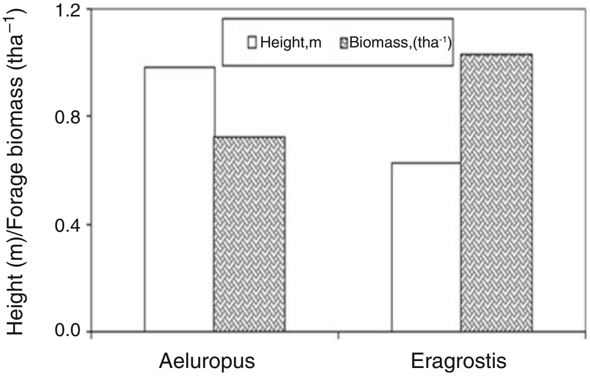


**Fig. 13.18** Growth and forage yield of grasses grown on saline black soil (ECe 12.8 dS m$^{-1}$; CD 0.05: height: 0.26; biomass: 0.418)

**Table 13.11** Effect of nitrogen on forage biomass of halophytic grasses irrigated with saline water

| | *Aeluropus lagopoides* | | | *Eragrostis species* | | |
|---|---|---|---|---|---|---|
| Irrigation | N0 | N30 | N60 | N0 | N30 | N60 |
| I1 | 1.01 | 1.24 | 1.29 | 1.12 | 1.25 | 1.34 |
| I2 | 1.10 | 1.28 | 1.36 | 1.19 | 1.28 | 1.41 |
| I3 | 1.15 | 1.31 | 1.41 | 1.22 | 1.31 | 1.44 |
| $CD_{0.05}$ | | | | | | |
| Nitrogen | 0.18 | | | 0.08 | | |
| Irrigation | 0.09 | | | 0.12 | | |
| N × I | 0.11 | | | 0.11 | | |

Source: Gururaja Rao et al. (2013c)

distinguishing variation in these parameters indicating the higher tolerance of *Eragrostis* spp. The root biomass, however, was found to be more at higher salinity in both the grasses, indicating that the roots of these grasses had better salt tolerance than shoots. This is also clearly evident from the shoot/root biomass ratios of both the grasses. The increase in salinity resulted in a decrease in shoot biomass indicating an inverse relation between salinity and biomass production as reported earlier by Gururaja Rao et al. (2011).

#### 13.6.4.5 Effect of Nitrogen on Growth and Forage Yield

Nitrogen given at 60 kg ha$^{-1}$ has boosted the growth and biomass (Table 13.11). However, no significant differences were noticed when the grasses were irrigated at 15 and 30 days interval, indicating that by giving irrigation once in 30 days, saline water can be saved by 50%. In water-scarce regions like coastal saline vertisols, this becomes handy for taking up other halophytes/salt-tolerant plants for saline agriculture programs. Nitrogen given at 60 kg ha$^{-1}$ though slightly enhanced forage yield with three irrigations when compared to two irrigations, and thus, by compromising slight forage loss, saline water can be saved up to 33%.

Nitrogen application has been found to enhance forage biomass (Fig. 13.19). Moreover, nitrogen being the most limiting nutrient in saline vertisols that are being low in N and organic matter, when given with saline water it resulted in significant

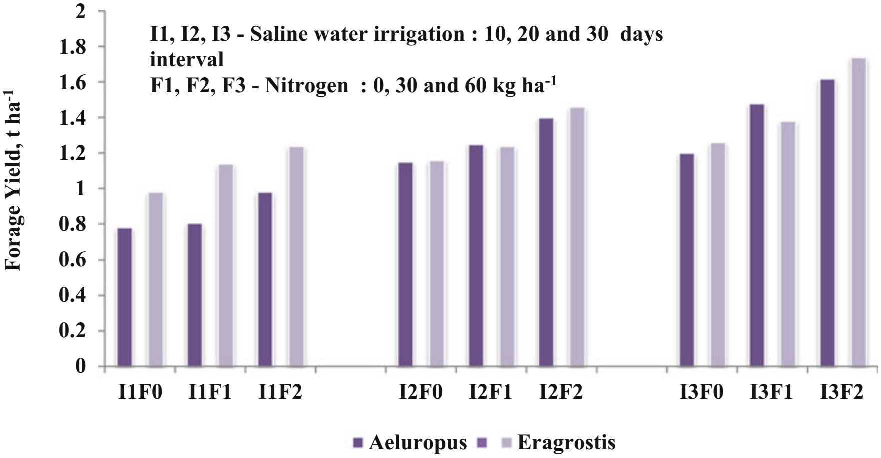


**Fig. 13.19** Forage yield of halophytic grasses as influenced by nitrogen

**Table 13.12** Effect of nitrogen on biomass (t ha$^{-1}$) of halophytic grasses with saline water (source: Gururaja Rao et al. 2013c)

| Irrigation | *Aeluropus lagopoides* | | | *Eragrostis* species | | |
|---|---|---|---|---|---|---|
| | Nitrogen (kg ha$^{-1}$) | | | | | |
| | 0 | 30 | 60 | 0 | 30 | 60 |
| $I_1$ | 1.01 | 1.24 | 1.29 | 1.12 | 1.25 | 1.34 |
| $I_2$ | 1.10 | 1.28 | 1.36 | 1.19 | 1.28 | 1.41 |
| $I_3$ | 1.15 | 1.31 | 1.41 | 1.22 | 1.31 | 1.44 |
| $CD_{0.05}$ | | | | | | |
| Nitrogen (N) | 0.18 | | | 0.08 | | |
| Irrigation (I) | 0.09 | | | 0.12 | | |
| N × I | 0.11 | | | 0.11 | | |

increase in forage yield of both the grasses. Of the two, *Eragrostis was* found to have higher growth, tillers, and forage yield with 60 kg ha$^{-1}$ N application when saline water was applied at 15 days of interval (Table 13.12). The grasses were found very effective in salt removal from the soil layers, *Aeluropus* removing more salt than *Eragrostis*. Analysis of tissue sodium and chloride indicated their content per se decreased when compared to those given no nitrogen. This low tissue sodium and chloride, however, improved the forage quality parameters. Nitrogen given at 60 kg ha$^{-1}$ resulted in lowered tissue ion content, resulting mainly from the increased biomass, which resulted in lowered salt distribution per unit weight of the tissue.

### 13.6.5 Intervention 4: Cultivation of Seed Spice, Dill (*Anethum graveolens*), for Remediation of Moderately Saline Vertisols

Dill, *Anethum graveolens*, a nonconventional seed spice crop, has been identified as a potential crop for cultivation on saline black soils having salinity up to 6 dS m$^{-1}$ in

**Table 13.13** Seed yield of dill (q ha$^{-1}$) as influenced by different salinity levels under different farm sites

| Salinity (dS m$^{-1}$) | Khanpur | Warsada | Bamangam |
|---|---|---|---|
| 2–4 | 3.74 | 4.09 | 3.16 |
| 4–6 | 3.03 | 2.25 | 2.37 |
| 6–8 | 2.34 | 1.76 | 1.64 |
| 8–10 | 1.95 | 1.36 | 1.56 |
| CD (0.05)<br>Farm<br>Salinity<br>Farm × salinity | NS<br>0.13<br>NS | | |

Source: Gururaja Rao et al. (2009a)

**Table 13.14** Effect of quality and number of irrigation waters on yield of dill (t ha$^{-1}$) on saline black soils

| | One irrigation | | Two irrigations | | Three irrigation | |
|---|---|---|---|---|---|---|
| Salinity (dS$^{-1}$) | Seed | Seed | Seed | Stover | Seed | Stover |
| BAW | 0.784 | 2.352 | 0.834 | 2.500 | 0.914 | 2.651 |
| 4 | 0.650 | 1.958 | 0.815 | 2.526 | 0.906 | 2.808 |
| 8 | 0.354 | 1.200 | 0.417 | 1.334 | 0.567 | 1.814 |
| 12 | 0.209 | 0.689 | 0.292 | 0.992 | 0.367 | 1.212 |

CD (0.05)
No. of irrigations (*I*) 0.030
Quality of water (*Q*) 0.033
$I \times Q$ 0.081

Source: Gururaja Rao et al. (2009a, b)

the rabi season with the residual soil moisture. It has multiple uses viz. pot herb, leaves as vegetable, seeds used as condiments, and seed oil for aromatic and medicinal purposes. The herb contains vitamin C as high as 121.4 mg g$^{100}$. The oil of dill seeds and its emulsion in water (Dill water) are considered to be aromatic, carminative, and effective in colic pains and possess anti-pyretic and anthelmintic properties. The crop gives fairly good yield on saline black soils having salinity of 4–6 dS m$^{-1}$ (Table 13.13). The crop responds well to saline water irrigation. Three critical stages for saline water irrigation, vegetative, flowering, and seed formation stages have been noticed. A substantial increase in yield can be obtained by using saline groundwater in conjunction with the best available surface water (Table 13.14). The cost of cultivation comes to ₹6000 ha$^{-1}$, and the crop would yield net returns of ₹16,500 ha$^{-1}$. The benefit:cost ratio noticed was 2.75 (Table 13.15). This crop thus would help farmers of the region to go for the second crop in the rabi season on lands, which remained fallow lands due to water and salinity constraints (Gururaja Rao et al. 2009a, 2013b).

#### Impact

Nonconventional crop like dill can be grown using residual moisture resulting in 2.6q/ha seed yield with net returns of ₹8000. This crop forms an ideal option for the state in general and the region in particular, which by and large faces water scarcity

**Table 13.15** Cost of cultivation Dill on saline black soils and economic returns

| Items of expenditure | Cost (Rs.) |
|---|---|
| Field preparation *n* | 1200 |
| Seed material | 150 |
| Seed treatment and sowing | 300 |
| Fertilizers | 800 |
| Application of fertilizer | 200 |
| Interculture and weeding | 350 |
| Irrigation[a] | 1500 |
| Harvesting and threshing | 1000 |
| Miscellaneous | 500 |
| Total | 6000 |
| Returns | |
| Yield of Dill (0.75 t $ha^{-1}$) | |
| Gross returns at Rs. 30,000 $t^{-1}$ | 22,500 |
| Net returns | 16,500 |

Source: Gururaja Rao et al. (2009a, b)

[a] Labour cost of six irrigations

problems. Under saline water irrigation, crop would yield net returns of ₹16,500 $ha^{-1}$ with ₹6000/hectare as cost of cultivation. The benefit: cost ratio works out to be 2.75. This crop thus would help farmers of the region to go for the second crop in the rabi season on lands with water and salinity constraints. Dill crop thus can be taken up using residual moisture and/or with saline groundwater.

### 13.6.6 Intervention 5: Cotton Pulse Intercropping for Moderately Saline Vertisols

In the Bara tract and parts of Saurashtra region in Gujarat, farmers growing cotton as a rainfed monocrop, invariably face crop losses due to occurrence of salinity at later stages of crop growth. Under such situations, intercropping with pulses provides remuneration in the event of the failure of the cotton crop. On-farm trials have indicated that cotton cluster bean intercrops (Fig. 13.20) proved to be beneficial on moderately saline black soils having salinity of 4–6 dS $m^{-1}$. Cotton intercropped with cluster bean produced cotton seed yield at par with that of sole cotton. Nitrogen application at 80 kg N $ha^{-1}$ significantly increased the seed cotton yield under saline conditions (Table 13.16). Cluster bean while improving the fertility of the soil provides an insurance against the failure of the cotton crop.

In the Bara tract particularly in Vagra and Amod talukas, the farmers have been adopting the cotton intercropped with pulse technology in about 165 ha area. This system would fetch about ₹16,000 per hectare from cotton and further the pulses due to their nitrogen-fixing ability to enrich the soils with nitrogen. Cotton and pulses can be taken as rainfed crops, providing saline water irrigation, if available further boost the crop yields. The use of saline water in cotton has been proved beneficial on saline black soils (Gururaja Rao et al. 2013a, b, c; Meena et al. 2019).

**Fig. 13.20** Cotton cluster bean intercrop on saline vertisols of Bara tract (Gujarat)

**Table 13.16** Performance of cotton intercropped with pulses under different levels of fertilizers on moderately saline Vertisols of Bara tract

| Treatments | Seed cotton yield (kg $ha^{-1}$) | Treatments | Seed cotton yield (kg $ha^{-1}$) |
|---|---|---|---|
| Main-plot treatments | | Subplot treatments | |
| Inter crops (pulses) | | Fertilizer levels (kg $ha^{-1}$)<br>N + 40 $kgP_2O_5$ $ha^{-1}$ | |
| Sole cotton | 572.0 | Control | 355.1 |
| Cotton + black gram | 532.7 | 20 | 440.3 |
| Cotton + cluster bean | 559.3 | 40 | 537.6 |
| Cotton + soybean | 556.9 | 60 | 626.1 |
| S Em (±) | 9.3 | 80 | 676.3 |
| LSD ($p \leq 0.05$) | 22.7 | 100 | 685.8 |
| | | S Em (±) | 15.2 |
| | | LSD ($p \leq 0.05$) | 30.7 |
| Coefficient of variation | | 6.7% | |

Source: CSSRI Annual Report, 2003–2004

### 13.6.7 Intervention 6: Cultivation of Salt-Tolerant Crops, Cotton and Wheat, as Ideal Interventions for Coastal Saline Vertisols

#### 13.6.7.1 Desi Cotton on Coastal Saline Vertisols

Sustainability of an ecosystem depends on the scientific management based on a sound database. For the management of saline vertisols of the Southern and Saurashtra regions, the use of salt-tolerant crop varieties is considered an ecologically and economically viable option. To overcome the constraints of saline soil and saline groundwater, the use of salt-tolerant varieties is an effective, economic, and eco-friendly approach in the management of saline vertisols. Although cotton is classified as a fairly salt-tolerant crop, its tolerance to salinity is far from that of halophytes (Dong 2012). Therefore, growth and yield reduction are inevitable under high salinity, which often reduces vegetative growth to a greater degree with more reduction noticed in shoots than roots (Dong 2012). Research conducted in this direction resulted in identification of ideal *herbaceum* cotton cultivars like G. Cot 23, G Cot DH 7, GBav 109, and GBav 120 (Gururaja Rao et al. 2013a, b, c). Studies by Gururaja Rao et al. (2013a, b) with diverse cotton accessions clearly indicated that on saline soils of Bara tract and Bhal area, *herbaceum* cottons showed better adaptability and seed cotton yield under rainfed conditions. Seed cotton yield obtained from the farmers' fields is found at par with that of experimental yield (Table 13.17).

In vertisols with high subsurface salinity (Gururaja Rao et al. 2013a), *herbaceum* and *arboreum* showed seed cotton yields comparable to those obtained under well-managed experimental sites, suggesting the possibility of their expansion in other saline areas. In view of their low water requirements, minimum or no-pest problems (Gururaja Rao et al. 2013a, b, c; Meena et al. 2019), and satisfactory yields, *desi* (local) cotton is preferred across different farm units in coastal Gujarat where the salinity at harvest ranged from 7.4 to 8.8 dS m$^{-1}$ (Fig. 13.21). A strong negative relation ($r^2 = 0.8821$) between soil salinity and seed cotton yield was noticed. Seed cotton yield dropped below 1.7 Mg ha$^{-1}$ only at 9 dS m$^{-1}$ (Fig. 13.22). As compared to hybrids and Bt lines, *desi* cotton possessed higher salt tolerance and showed lower

**Table 13.17** Performance of desi cottons in Bara tract area

| District/Taluka | Village | Crop | Mean yield (Mg ha$^{-1}$) | Soil salinity (dS m$^{-1}$) |
|---|---|---|---|---|
| Bharuch/ Jambusar | Kalak | G. Cot 23 | 1.78 | 9.20 |
| | Magnad | G Cot 23 (herbaceum)<br>GBav 120 (arboreum) | 1.89<br>1.68 | 8.40<br>8.45 |
| | Bojadara | G Cot 23<br>GBav 120 | 1.84<br>1.76 | 8.82<br>8.75 |
| CSSRI farm | Samni | G Cot 23<br>GBav 120 | 1.91<br>1.80 | 7.86<br>8.02 |
| | | Total average | 1.81 | 8.50 |

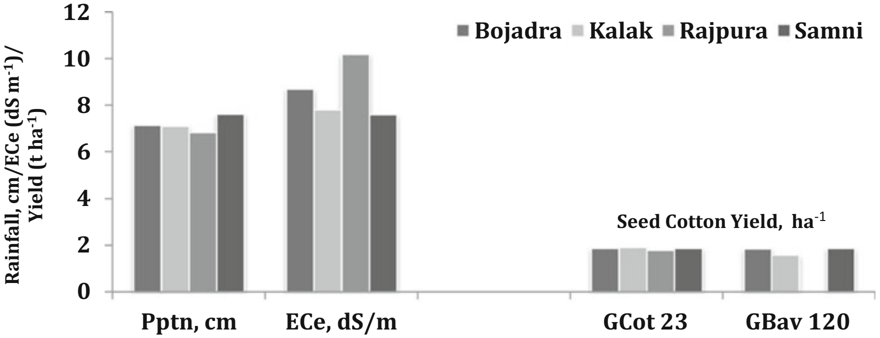


**Fig. 13.21** Rain fall (Pptn), soil salinity ($EC_e$) and seed cotton yield in South Gujarat (Source: Gururaja Rao et al. 2016a, b)

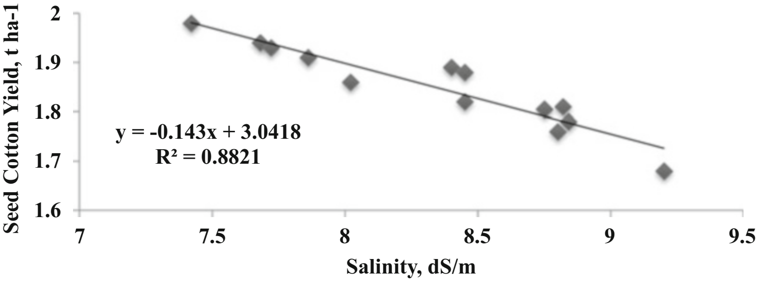


**Fig. 13.22** Relation between soil salinity and seed cotton yield (Source: Gururaja Rao et al. 2016a, b)

yield drop at salinity more than 6 dS $m^{-1}$ (Gururaja Rao et al. 2013a). Unlike *hirsutum*/hybrids, which have a shallow root system and shortened lifespan (July to October) along with their high water requirement, *desi* cottons have a deep root system and longer life cycle (July to March). The low water requirement by *desi* cottons rendered them ideal ones for rainfed saline vertisols (Gururaja Rao et al. 2013a, b, 2016b).

Cultivation of *desi* cotton (G. Cot 23) on saline vertisols of Saurashtra region showed seed cotton yield in the range of 1.62–1.78 Mg $ha^{-1}$ at salinity of 6.2–7.2 dS $m^{-1}$ (Fig. 13.23). When compared to the soils of South Gujarat or Bhal area, the soils of Saurashtra are of clay loam type and needed more irrigations, which, however, has not resulted in higher root zone salinity.

Saline lands having salinity up to 8–10 dS $m^{-1}$ had been profitably brought under cultivation with herbaceum cotton, G. Cot 23, in all the three coastal regions of the state. This had resulted in gross income in the range of ₹70,000–75,000/hectare and net income of ₹45,000–50,000/hectare, particularly in South Gujarat, i.e., in Bojadra and Kalak with B/C ratio of 1.8–2.0. The lower salt tolerance coupled with lower seed cotton yield of hybrids and Bt lines compared to herbaceum and arboreum (Gururaja Rao et al. 2013b) formed a basis for the farmers going for desi cottons in

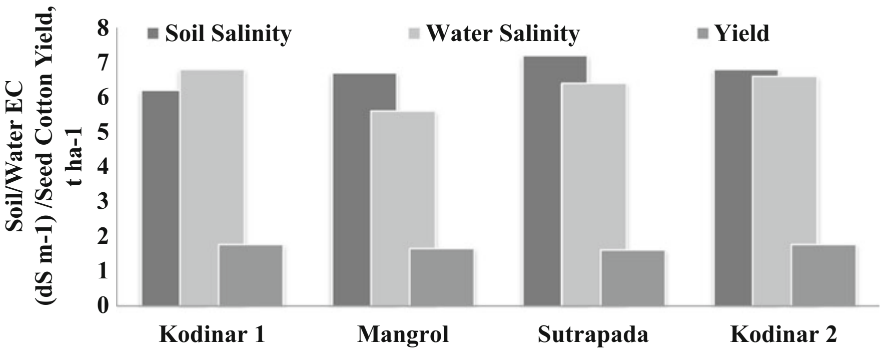


**Fig. 13.23** Performance of cotton (G. Cot 23) in Junagadh district (Source: Gururaja Rao et al. 2016a, b)

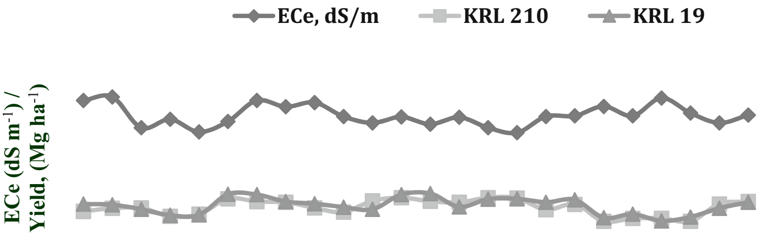


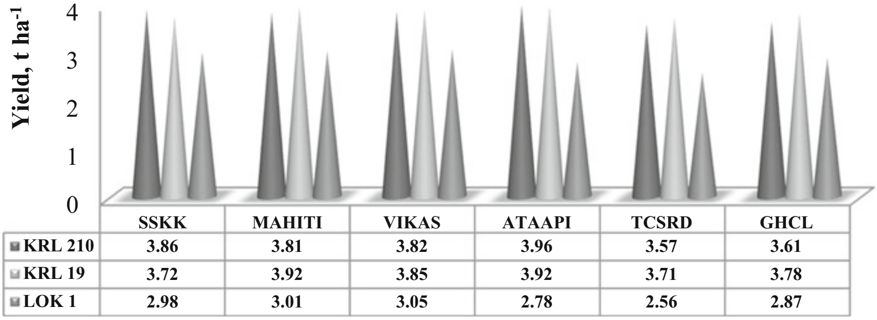
**Fig. 13.24** Wheat yield in different clusters taken up by NGOs (Source: Gururaja Rao et al. 2016a, b)

coastal saline areas. Further, earlier studies by Gururaja Rao et al. (2013a, b) also indicated lower input costs primarily due to reduced disease incidence in desi cotton coupled with application of less number of irrigations.

#### 13.6.7.2 Wheat on Coastal Saline Vertisols

Central Soil Salinity Research Institute, Karnal, has developed salt-tolerant wheat varieties (KRL 1-4, KRL 210, KRL 213, and KRL 19), which have become popular in parts of northern India. Farmers' field trials conducted by ICAR-CSSRI, RRS, Bharuch, in different farm units in collaboration with Non-Government Organization (NGO) like SSKK and GHCL (Saurashtra), MAHITI (Bhal area), VIKAS and ATAAPI (Bara tract), and TCSRD (Jamnagar district) indicated almost consistent yield performance (3.6–3.95 Mg ha$^{-1}$) of both wheat accessions, KRL 210 and KRL 19 under salinity of 5.9 to 7.2 dS m$^{-1}$ (Fig. 13.24).

Similarly, under field trials conducted with KRL 210 in Junagadh District, the seed yields ranged between 3.4 and 3.95 Mg ha$^{-1}$ (Fig. 13.25). In Una and Veraval, wheat yield of 3.8 t ha$^{-1}$ was noticed at salinity ranging from 6.4 to 6.9 dS m$^{-1}$, whereas at similar salinity, the seed yields reduced by 20 kg ha$^{-1}$ in Jafrabad due to high clay content of the soil. The seed yield of the salt-tolerant wheat lines were much higher than local variety Lok-1, which yielded about 2.5–3.0 Mg ha$^{-1}$



| | SSKK | MAHITI | VIKAS | ATAAPI | TCSRD | GHCL |
|---|---|---|---|---|---|---|
| ▨ KRL 210 | 3.86 | 3.81 | 3.82 | 3.96 | 3.57 | 3.61 |
| ▨ KRL 19 | 3.72 | 3.92 | 3.85 | 3.92 | 3.71 | 3.78 |
| ▨ LOK 1 | 2.98 | 3.01 | 3.05 | 2.78 | 2.56 | 2.87 |

**Fig. 13.25** Comparative performance of CSSRI wheat varieties over local variety (Lok 1) under saline soils of coastal Gujarat (Source: Gururaja Rao et al. 2016a, b)

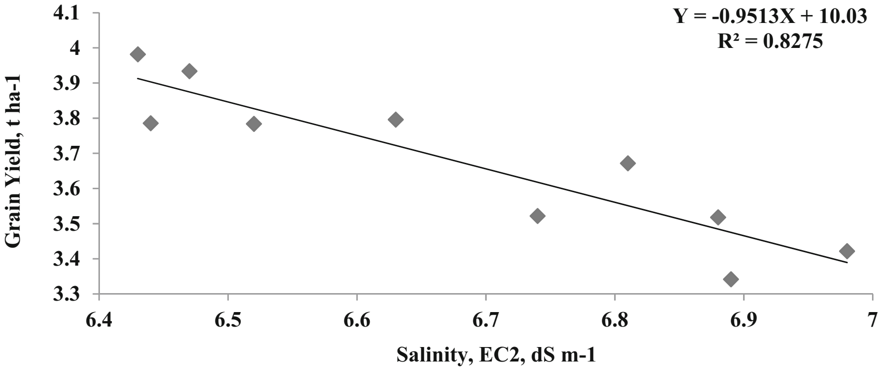


**Fig. 13.26** Relation between soil salinity and wheat yield (Source: Gururaja Rao et al. 2016a, b)

indicating the superiority of salt-tolerant lines and thus their suitability for saline vertisols of coastal Gujarat (Gururaja Rao et al. 2016a, b). However, significant negative relation was noticed between wheat yield and salinity ($Y = -0.9513X + 10.03$; $R^2 = 0.8275$, Fig. 13.26).

Studies on salt-tolerant wheat and desi cotton in coastal saline vertisols indicated while desi cotton (G. Cot 23) cultivation increased in Bara tract, salt-tolerant wheat varieties (KRL 210 and KRL 19) have been found getting wider acceptability in Central Gujarat and Saurashtra regions (Gururaja Rao et al. 2016a, b).

### 13.6.8 Intervention 7: Conjunctive Use of Saline Water with Surface Water for Crop Production—A Tool to Mitigate Salinity on Saline Black Soils

Sustained irrigated agriculture is critical for food and fiber production to support the growing human population. Sustainability and success of saline water use depend on sound implementation and management. The three major approaches that involve the use of saline and/or sodic waters for crop production in a conjunctive mode include cyclic, blending, and sequential use and reuse of such waters through surface irrigation. A number of studies have been carried out during the last two decades that evaluated these approaches on a field scale (Gururaja Rao et al. 2013b, 2016a, b).

Proper utilization of land and groundwater resources is of paramount importance in agricultural production in the Bara tract. The nonuse of saline groundwater in saline vertisols of the Bara tract is not only making the crop production stagnant but also contributing to an increase in the groundwater table and salinity. The salinity of groundwater is too high in the saline tracts of vertisols and cannot be used for irrigation purposes as such, and thus, it needs to be mixed with limited surface water available. In the absence of inadequate irrigation water supplies in the region, technologies are evolved for the conjunctive use of saline groundwater in mixing and cyclic modes for growing oilseed crops like dill, mustard, and safflower. It proved to be remunerative due to its long-term potential impacts on economic development, employment generation, and environmental improvement (Gururaja Rao et al. 2013b, 2016a, b).

Studies by Gururaja Rao et al. (2013b) (Table 13.20) in diverse crops like dill, mustard, safflower, and wheat using saline water in cyclic mode showed the following results. Dill, a seed spice crop, could be grown during *rabi* season on rainfed saline black soils with salinity of 4–5 dS $m^{-1}$ with a seed yield of 3 Mg $ha^{-1}$, which otherwise remained fallow. However, the conjunctive use of saline groundwater with surface water can improve the productivity manifold. In dill, if surface water is available for one irrigation, it should be applied at the seed formation stage and saline water at the vegetative and flowering stages. If surface water is available for two irrigations, the same should be applied at the time of flowering and seed formation stage and saline water at the vegetative stage. In areas with high groundwater table and lack of sufficient surface water, surface water up to 66% can be saved by application of saline groundwater (4 dS $m^{-1}$) at branching and flowering stage and surface water at seed formation stage without further increase in soil salinity (Gururaja Rao et al. 2013b). This method can increase seed yield by 150% over the yield obtained under unirrigated condition.

Branching and flowering stages were found to be sensitive stages for saline water irrigation in safflower. If surface water is available for one irrigation, it should be applied at the branching stage and saline water at the vegetation and the flowering stages. If surface water is available for two irrigations, it should be applied at the branching and flowering stages and saline water at the vegetative stage. In safflower by applying saline groundwater (4 dS $m^{-1}$) at flowering and grain filling stages and surface water at branching stage, 86% increase in yields over the yield obtained under unirrigated conditions (370 kg $ha^{-1}$) can be obtained (Gururaja Rao et al. 2013b).

Likewise, Indian mustard (*Brassica juncea*) can be grown on saline black soils with saline groundwater having EC of 4 dS $m^{-1}$ in conjunction with the limited surface water. Flowering and pod formation stages are relatively more sensitive to saline water irrigation. Application of two saline water irrigations (of ECIW 4 dS

**Table 13.18** Effect of conjunctive use saline water and surface water in cyclic mode on the yield (kg ha$^{-1}$) of arable crops on saline black soils

| Treatments | Dill | Safflower | Mustard | Wheat |
|---|---|---|---|---|
| T1—All BAW | 832 | 825 | 735 | 2910 |
| T2—SW at branching stage/crown root initiation stage in wheat + rest BAW | 793 | 745 | 665 | 2720 |
| T3—SW at flowering stage/maximum tillering stage in wheat + rest BAW | 784 | 765 | 629 | 2810 |
| T4—SW at seed formation stage/flower initiation stage in wheat + rest BAW | 768 | 775 | 645 | 2740 |
| T5 (SW—branching/tillering and flowering) rest BAW | 752 | 635 | 559 | 2220 |
| T6 (SW—branching/tillering and seed) + rest BAW | 744 | 685 | 519 | 2300 |
| T7 (SW—flowering and seed) + rest BAW | 736 | 688 | 535 | 2230 |
| T8 (all saline) | 682 | 499 | 326 | 1680 |
| LSD ($p \leq 0.05$) | 06 | 21 | 75 | 500 |

The interventions evolved for the conjunctive use of saline water and groundwater for four important crops of the region would improve dill yield by 446 kg ha$^{-1}$, mustard by 237 kg ha$^{-1}$, safflower by 518 kg ha$^{-1}$, and wheat by 1200 kg ha$^{-1}$ over the yield under unirrigated condition. The surface water saved per hectare would create 0.8 ha additional command under irrigation. This technology as well can be extrapolated to other saline vertisols

*BAW* best available water

Source: Gururaja Rao et al. (2013b)

m$^{-1}$) at branching and pod formation stages and surface water flowering stage resulted in a yield of 559 kg ha$^{-1}$ (Gururaja Rao et al. 2013b). Flowering and pod formation stages are relatively sensitive to saline water irrigations. This method while saving 66% surface irrigation water increases the yield by 123% over the yield obtained under unirrigated conditions (220 kg ha$^{-1}$).

Good-quality water, if available, should be applied at crown initiation stage and saline water at maximum tillering and flower initiation stages in wheat. If surface water for two irrigations is available, it should be applied at crown root initiation and flower initiation stages. Under high saline water table prevailing in saline black soils, in wheat, when saline water is applied, flowering and maximum tillering stages are equally sensitive as that of crown root initiation stage for salinity because of their exposure to increase in groundwater salinity (Gururaja Rao et al. 2013b). Application of saline water (4 dS m$^{-1}$) at these stages and good-quality water at crown root initiation stage resulted in an increase of 180% seed yield when compared to the yield obtained under unirrigated condition (Table 13.18).

### 13.6.9 Other Interventions: Agroforestry for Coastal Saline Vertisols

Since the afforestation and agroforestry add new dimensions to saline vertisols in terms of resource generation and upliftment of the populace of the area, its prospects are manifold in the overall development. Reclamation of sodic/saline vertisols require additional inputs such as amendments (gypsum, pressmud, and pyrite in case of alkali lands) or installation of drainage (for saline or saline water-logged soils), use of fertilizers (including green manure), and infrastructure for farm operations. Therefore, afforestation or agroforestry involving trees, grasses, and low-water-requiring crops (when using saline water for irrigation) is considered an ideal land use for reclamation and management of salty lands and utilization of degraded lands using saline water (Singh 2009; Garg et al. 2017; Gururaja Rao and Camus 2021). Besides providing fuel, fodder, and timber, afforestation will also lead to bioamelioration of salty lands. Afforestation of these lands will not only help in ecological and environmental considerations, but also be useful in relieving pressure on traditionally cultivated lands and forests.

The salty black soils are generally either contemporary or of secondary origin. The contemporary ones exist in the topographic situation under poor drainage conditions. However, the soils that have become sodic due to injudicious use of irrigation water can be encountered in the irrigation command area. Restoring the productivity of these lands once they become salinized is much more difficult as compared to alluvial sandy loam soils of the Indo-Gangetic Plain. Researchers made consistent efforts to study the basic, strategic, and applied aspects of salt-affected Vertisols (Gururaja Rao 2015; Gururaja Rao et al. 2003, 2004). Technologies like cultivation of *Salvadora* (tolerates salinity up to 50 dS $m^{-1}$) for the restoration of highly saline soils (>40 dS $m^{-1}$, Gururaja Rao et al. 2003; Gururaja Rao et al. 2009a, 2017) and cultivation of dill (tolerates salinity of 4–6 dS $m^{-1}$) have been adopted by the farmers (Gururaja Rao et al. 2009a).

Long-term experiments indicated that *P. juliflora* and *Azadirachta indica* were the most successful species for sodic soils (Singh et al. 2014) and saline Vertisols (Patil et al. 2005). The data on properties of the soil surface (0–15 cm) showed that after 7 years of plantation under *P. juliflora*, *pH*, ECe, and ESP reduced from 8.8, 4 dS $m^{-1}$, and 35 to 8.5, 1.29 dS $m^{-1}$, and 10, respectively, and under *A. indica* these values reduced to 8.5, 1.3 dS $m^{-1}$, and 14, respectively. Among grasses, *Aeluropus lagopoides*, *Leptochloa fusca*, *Brachiaria mutica*, *Chloris gayana*, *Dichanthium annulatum*, *Bothriochloa pertusa* and species of *Eragrostis*, *Sporobolus*, and *Panicum* are most successfully.

Aromatic grasses such as *Vetiveria zizanioides* and *Cymbopogon martini* can be grown easily. *Matricaria chamomile* can withstand both high pH and ESP. In a separate fruit trial on the soil of ESP 25, 40, and 60, it was found that gooseberry (*Emblica officinalis*) and *ber* (*Ziziphus mauritiana*) are the most successful plantations for these soils. In one trial, an oil-yielding bush *Salvadora persica* was grown in combination with *Leptochloa fusca*, *Eragrostis* spp., and *Dichanthium annulatum* forage grasses on saline vertisol in Gujarat. The soil was clay loam (clay 40%, silt 31%, sand 29%) with pH ranging from 7.2 to 8.9 and ECe from 25 to 70 dS $m^{-1}$. The underground water was 0.5–2 m from the surface with EC ranging from 55 to 60 dS $m^{-1}$. These grasses could produce on an average 3.72, 1.0, and 1.8 Mg $ha^{-1}$ of green forage, respectively. During the fourth year, the seed yield of *Salvadora persica* ranged from 1.84 to 2.65 Mg $ha^{-1}$ with oil contents ranging from 576–868 kg $ha^{-1}$ at different salinity levels (Gururaja Rao et al. 2003, 2013b, 2017). The experiments conducted in sodic vertisols with ESP 40, growing *Leptochloa fusca*, *Brachiaria mutica*, and *Vetiveria zizanioides*, showed increased forage biomass during the second year. The uptake of sodium by *L. fusca* with the highest followed by *B. mutica* at every stage of cutting. During 3 years, these grasses could remove 144.8, 200.0, and 63.5 kg ha$^{-1}$ sodium from the soil, respectively. Thus, besides producing biomass, silvipastoral system helped in amelioration of soil in terms of reducing soil pH, EC, and ESP and increasing organic matter.

### 13.6.10 Biomass Species for Remediation of Saline Vertisols

Experiments with *Casuarina equisetifolia*, *Acacia nilotica*, *Dalbergia sissoo*, *Azadirachta indica*, *Sesbania grandiflora*, *and Hardwickia binata* on saline vertisols under Tungabhadra command showed reduction in soil salinity, calcium carbonate, and soil soluble sodium, bicarbonate; chloride, sulfate, and sodium adsorption ratio was observed under all the tree species apart from improvement in organic carbon (Patil et al. 2005). Tree species, *A. nilotica* and *C. equisetifolia* with higher biomass production, were found to exhibit greater bioamelioration potential in saline vertisols.

Multipurpose trees evaluated under saline and high water table conditions of vertisols of Tungabhadra command showed better performance of *Casuarina equisetifolia*, *Acacia auriculiformis Dalbergia sissoo*, *Syzygium cuminii*, *Pongamia pinnata*, and *Gliricidia maculata* based on height and collar diameter. The moderate-tolerant species identified were *Albizia amara* and *Pongamia pinnata*. All tree species enriched the soil nutrient pool (N, P and K) and organic carbon and decreased dispersibility of clays. Further studies also indicated *Azadirachta indica* and *Acacia nilotica*, the tree species along with native check plant *Prosopis juliflora*, are helpful in reclamation of sodic vertisols. A successful crop production can be taken up after the removal of tree species (10–12 years) under rainfed situations (Patil et al. 2005).

Reduction in soil salinity, calcium carbonate and soil soluble sodium, bicarbonate, chloride, sulfate, and sodium adsorption ratio was observed under tree species *Casuarina equisetifolia*, *Acacia nilotica*, *Dalbergia sissoo*, *Azadirachta indica*, *Sesbania grandiflora*, *and Hardwickia binata* apart from improvement in organic carbon. Tree species such as *A. nilotica* and *C. equisetifolia* with higher biomass production exhibited greater bioamelioration potential in saline vertisols (Patil et al. 2005). Some important tree species adapted to the saline vertisols are *Casuarina equisetifolia*, *Terminalia arjuna*, *Acacia nilotica*, *A. auriculiformis*, *Eucalyptus robusta*, *Salvadora persica*, *Pongamia pinnata*, *Ziziphus species*, *Tamarindus indica*, *Prosopis juliflora*, etc. *Cenchrus ciliaris* and fodder sorghum along with *Acacia nilotica* are a widely adapted silvipastoral system.

### 13.6.11 Horticultural Plants for Remediation of Saline Vertisols

Under horticultural plants, guava, aonla, pomegranate, tamarind, dates, karonda, ber, and jamun are found performing well on alkali soils (Singh et al. 2014). The planting costs of these fruit species were compared both in pit planting and in auger-hole methods and found that the latter is significantly less cost-intensive, i.e., ₹20,396 ha$^{-1}$ when compared to the one by pit planting (₹42,214 ha$^{-1}$). These species provide staggered income to the farmers and thus fit into the economically unwell coastal ecosystem.

Other fruit crops tested beneficial on saline vertisols include sapota, guava, pomegranate, aonla, and *jamun*. Field studies conducted on saline vertisols using saline water under the drip system and conventional basin irrigation showed the former was much more beneficial in terms of income generation (Gururaja Rao et al. 2013b). Effect of saline water irrigation through surface and drip methods in guava on saline vertisols indicated higher fruit yield, net income, and better benefit/cost ratio under drip irrigation system than surface/basin irrigation (Table 13.19, Gururaja Rao et al. 2013b).

### 13.6.12 Farming System Model: A Tool to Use Coastal Saline Vertisols

Studies on farming systems approach for the small farmer in the rainfed vertisols of the Western zone of Tamil Nadu by Shekinah et al. (2005) indicated a model comprising (crop + pigeon + goat + buffalo + agroforestry + farm pond) as the profitable enterprise that generated higher employment year-round. This system also facilitated the maximum recycling of resources and residues generated on the farm among the enterprises. The output and the waste of one enterprise served as input to another. The nutritive value of the system *from the* carbohydrates, proteins, and fats was the highest with this combination. Farming systems with a combination of cropping (fertilized with composted buffalo manure), with pigeon (10 pairs), goat (5:1 female:male), buffaloes (2 milking buffaloes + 1 calf), agroforestry, and farm pond. The system comprised crop component of maize (F) + cowpea (F) + chickpea + coriander (0.25 ha); sorghum (F) + cowpea (F) + chickpea + coriander (0.25 ha); sorghum (G) + cowpea (G) (0.20 ha); and sunflower + coriander (0.10 ha); agroforestry (*Acacia nilotica* + *Cenchrus ciliaris*) (sorghum (F) + cowpea (F) of 0.10 ha); animal component (0.6 ha) and farm pond of 0.4 ha.

Energy budgeting in the above farming system suggested that crops like chickpea with high contribution to the output and high milk production from buffalo have resulted in the highest energy output of 175,689 MJ year$^{-1}$ from the FS4 system, indicating the supremacy of the enterprise. Manure from the goat enterprise adds to the system total energy output because crops in the FS4 system are grown with recycled and composted buffalo manure. The energy output of the goat and the buffalo enterprises are low as the energy value of milk and meat is only 4.90 and 4.94 MJ kg$^{-1}$, respectively, as against 18 MJ kg$^{-1}$ for grains produced. Crop-livestock integration, therefore, increases the overall productivity of ecosystems.

**Table 13.19** Performance of guava under drip and surface irrigation with saline water in saline vertisols

| | Fruit yield/block[a] | | Fruit yield (kg $ha^{-1}$) | | | Net income (₹ $ha^{-1}$) | | B/C ratio | |
|---|---|---|---|---|---|---|---|---|---|
| ECIW (dS $m^{-1}$) | Surface | Drip | Surface | Drip | % Gain under drip | Surface | Drip | Surface | Drip |
| BAW (0.4) | 98.75 | 96.3 | 1312 | 1283 | −2.2 | 6560 | 6415 | 1.64 | 1.60 |
| 2 | 74.40 | 81.2 | 992 | 1082 | +9.1 | 4960 | 5410 | 1.24 | 1.35 |
| 4 | 66.10 | 75.9 | 881 | 1011 | +11.4 | 4405 | 5055 | 1.10 | 1.26 |

Cost of cultivation taken at ₹4000/ha (30 plants/block)
Cost of plants: ₹2000; planting costs: ₹1200; on-farm expenses/year: ₹800
During the first year, we had obtained ₹3800 from 180 plants. Spacing: 5 × 5 m
[a] Block of 25 plants

| Component | Area, m$^2$ | Crop |
|---|---|---|
| Pond | 2000 | |
| Dykes | 900 | Banana and papaya, Vegetables |
| Fruit species | 4000 | Banana, Jamun and Aonla |
| Biomass species | 3500 | Eucalyptus and Pongamia |
| Compost pit (m$^{3)}$ | 48 | |
| Total | 11200 | |

**Fig. 13.27** Components of farming system use on saline vertisols of Gujarat

Livestock in small-holder farming systems not only increases food production and nutritional supply but also produces residues/wastes that can be recycled and utilized for maximum benefits. Cropping provides an avenue for recycling of animal waste as manure, thereby helping in creating a closed system.

Farming studies comprising components, viz. rainwater harvesting structure (farm pond), fruit, vegetable, biomass species, and field crops (spices) covering an area of 1.12 ha (Fig. 13.27) on saline Vertisols showed low-water-requiring crops like papaya, dill, and coriander had higher water productivity than banana, which is high water-requiring species (Gururaja Rao et al. 2009b). Data on crop components, crop production, water productivity, and benefit/cost ratio are given in Table 13.20

**Table 13.20** Water productivity and benefit/cost ratios of components in the farming system

| Component | Banana | Papaya | Dill | Coriander | Brinjal | Tomato | Bottle gourd | Cabbage |
|---|---|---|---|---|---|---|---|---|
| Plot area ($m^2$) | 190 | 225 | 1600 | 1600 | 150 | 160 | 150 | 250 |
| Water applied (mm) | 953 | 180 | 120 | 122 | 94 | 104 | 112 | 122 |
| Economic yield (kg/plot) | 887 | 652 | 122 | 116 | 208 | 220 | 324 | 364 |
| Water productivity (kg $m^{-3}$) | 0.931 | 3.62 | 1.017 | 0.951 | 2.21 | 2.12 | 2.89 | 2.98 |
| Water productivity (Rs. $m^{-3}$) | 3.58 | 19.25 | 19.85 | 33.28 | 15.21 | 10.00 | 13.88 | 17.90 |
| Cost of cultivation (Rs.) | 1590 | 1450 | 960 | 1120 | 400 | 280 | 490 | 440 |
| Gross income (Rs.) | 5002 | 4916 | 3342 | 5180 | 1830 | 1320 | 1944 | 2184 |
| Net income (Rs.) | 3412 | 3466 | 2382 | 4060 | 1430 | 1040 | 1554 | 1744 |
| B/C ratio | 2.15 | 2.39 | 2.48 | 3.63 | 3.8 | 3.71 | 3.17 | 3.96 |
| Total net income from the system (under yielding stage (Rs/ha)): 43,840 | | | | | | | | |

Source: Gururaja Rao et al. (2009a, b)

indicated that spice crops like dill and coriander had higher water productivity (in terms of monetary gains) when compared to vegetables. Vegetables, however, had higher water productivity when yield is taken into account. High water-requiring crops like banana had the lowest water productivity (both in terms of economic yield and monetary gains) and also the benefit/cost ratio when compared to low and moderate water-requiring crops like species and vegetables. Among the fruit species, papaya was found to be beneficial both in terms of water productivity and benefit/cost ratio and found suitable for Bara tract areas.

The vegetable crops like tomato, brinjal, bottle gourd, and cabbage are moderate in water consumption and due to their continued yielding provide subsistence income to the farmers. The spices, however, because of very low water requirement and high water productivity, have been found ideal for water-scare areas like Bara tract. The B/C ratio of papaya, dill, and coriander was higher than banana. Vegetables, brinjal, tomato, and bottle gourd showed higher water productivity and also higher B/C ratio than banana. These crops, with low crop duration and low water needs, form ideal components that provide regular income to the farming community. The crops like papaya, dill, and coriander along with vegetables because of their higher water productivity and B/C ratio are found to be suitable for the saline vertisols of Bara tract.

For small and marginal farmers (with a landholding of 2 ha), an integrated multi-enterprise model comprising diverse components (field and horticultural crops, fishery, cattle, poultry, and beekeeping) was developed for ensuring sustainable resource use efficiency, high and regular incomes, and employment generation. This model was found to reduce the production costs substantially by synergistic recycling of resources among different components (Sharma and Chaudhari 2012).

Woody biomass species, Eucalyptus and *Pongamia*, and fruit crop, *Syzygium cumini*, were introduced as components of the farming system on saline vertisols (Gururaja Rao et al. 2009b; Meena et al. 2019), and the litter yield was studied. *Eucalyptus* and *Pongamia*, while showing good growth, yielded significant amounts of litter (Fig. 13.27). The litterfall was found to be more in *Syzygium* when compared to *Eucalyptus* and *Pongamia*. The compost generated from the litter (Fig. 13.28) of different components was analyzed, and the data indicated that it had dry matter of 54.9% and 15% organic matter. The composition of the compost indicated that it was rich in nitrogen and calcium and thus forms a good nutrient source and thus provides a scope for minimizing the use of inorganic fertilizers.

### 13.6.13 Biodiesel Species for Mitigating Salinity

Biodiesel from nonfood feedstock is gaining attention around the world. Great emphasis is being given to the production of biodiesel in view of its enormous economic, social, and environmental benefits. Biodiesel is a fast-developing alternative fuel in many developed and developing countries of the world. Shortage of edible oil for human consumption in developing countries does not favor its use for biodiesel production. Hence, in India, the focus on tree-borne oilseeds as the source

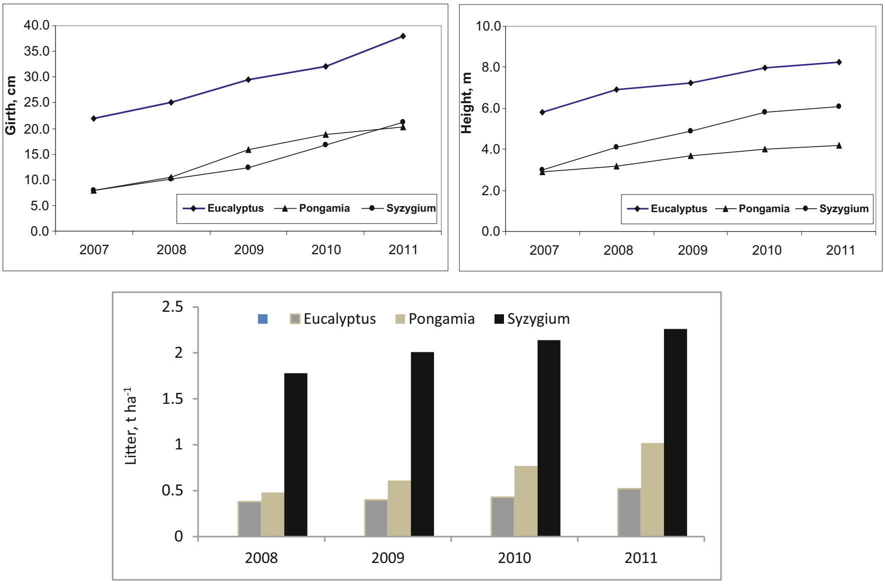


**Fig. 13.28** Growth and litterfall in biomass species on saline vertisols

of feedstock for biodiesel production has highlighted the role of *Pongamia pinnata* (L.) and *Jatropha curcas*. *Pongamia* is valued for shade, ornamental value, seed oil, fodder, and green manure. In recent times, the interest in this tree is mainly focused on the use of its seed oil as biodiesel, which is environmentally safe, nontoxic, and biodegradable.

#### 13.6.13.1 Jatropha

*Jatropha curcas* is considered the most suitable biofuel crop since it uses lands, which largely remain unproductive and are located in poverty-stricken and watershed areas and degraded forests. Besides, this crop may not replace other important food crops and in turn will not have a major impact on cropping pattern. Studies showed that *Jatropha* grows and performs well in soils having salinity up to 10 dS $m^{-1}$. Similarly, *Jatropha* irrigated with saline groundwater (11.6 dS $m^{-1}$) on vertisols with subsurface salinity also indicated good growth, flowering, and seed production. Plants irrigated with saline water at intervals of 10, 20, and 30 days indicated no significant difference between 20 and 30 days of irrigated plants in terms of growth and seed yield (Table 13.21; Sharma et al. 2008; Singh et al. 2015). This suggests that marginal quality of saline groundwater can be saved if the crop is irrigated during hot summer once a month. Though only a marginal decline in seed yield occurred in plants irrigated by saline water at 20 or 30 days, by foregoing this seed yield loss, there can be a saving of marginal saline water by 50–66%. Application of lesser quantities of saline water also reduces salt build-up in the soil.

**Table 13.21** Effect of saline water (11.6 dS $m^{-1}$) irrigation on seed and oil yield of Jatropha on saline vertisols second-year plantation (1111 plants $ha^{-1}$)

| Irrigation frequency (days) | Irrigation water applied (l/plant) | Plant height (m) | Seed yield (g/plant) | Seed yield (kg/ha) | Seed oil content (%) | Seed oil yield (kg/ha) |
|---|---|---|---|---|---|---|
| 10 (6) | 90 | 1.36 | 268 | 2977 | 35.2 | 1047.9 |
| 20 (3) | 45 | 1.22 | 184 | 2044 | 36.2 | 739.9 |
| 30 (2) | 30 | 1.14 | 173 | 1911 | 36.3 | 693.7 |
| CD at 5% | | 0.06 | 8.54 | 15.57 | NS | 10.53 |

Figures in the parenthesis indicate number of irrigations. Source: Sharma et al. (2010)

#### 13.6.13.2 Intercropping of Dill with Jatropha curcas

Salt-affected vertisols with moderate salinity can also be brought under *Jatropha* cultivation. Experiments conducted indicate response of *Jatropha* to saline water up to 11.6 dS $m^{-1}$. Since dill also grows and yields well under saline water irrigation, this can be taken up as an intercrop in the initial years since *Jatropha* starts giving economic yield from the third year onwards. This approach would provide the farmer with returns even during initial years of *Jatropha* cultivation.

### 13.6.14 Medicinal Trees in the Bioremediation Program

Many plants in traditional agricultural systems in the tropics possess medicinal value. These can be found (either planted or carefully tended natural regenerations) in home gardens, as scattered trees in croplands and grazing lands, and on field bunds. *Acacia* species such *as Acacia nilotica* and *A. polyacantha* have medicinal value (Gururaja Rao 2021). Similarly, “arjun” (*Terminalia arjuna*) has been found to possess medicinal uses. Holy basil or “tulsi” (*Ocimum sanctum*), drumstick (*Moringa oleifera*), and curry leaf (*Murraya koenigii*) are routinely used for common ailments or in food preparations. Earlier studies by Singh et al. (2014) indicated that medicinal and aromatic plants such as isabgol (*Plantago ovata*), aloe (*Aloe barbadensis*), and kalmegh (*Andrographis paniculata*) produce high biomass under saline (ECIW 8.5 dS $m^{-1}$) irrigation.

#### 13.6.14.1 Medicinal Plants as Intercrops with Woody Species

Many tropical medicinal and aromatic plants (MAPs) are well-adapted to partial shading, moist soil, high relative humidity, and mild temperatures allowing them to be intercropped with timber and fuel wood plantations, fruit trees, and plantation crops. Medicinal plants, safed musli (*Chlorophytum borivilianum*), sarpagandha (Rauwolfia serpentina), turmeric (*Curcuma longa*), and ginger (*Zingiber officinale*) have been successfully intercropped with fuel wood trees (e.g., *Acacia auriculiformis*, *Albizia lebbeck*, *Eucalyptus tereticornis*, *Gmelina arborea*, and *Leucaena leucocephala*) (Prajapati et al. 2003) and quinoa (Ramesh et al. 2019). Only 10 out of 64 herbaceous medicinal plants tried in intercropping with 2-year-old poplar (*Populus deltoides*) spaced 5 m apart gave poor performance (Gupta and

Dagar 2016), indicating that many medicinal plants can be grown in agroforestry systems.

#### 13.6.14.2 Aromatic Plants as Intercrops with Woody Species

Studies have been conducted in India since the mid-1980s on the feasibility and economic aspects of intercropping aromatic plants with timber trees. Various studies in India demonstrated that aromatic *Mentha* spp. (*M. arvensis*, *M. piperita*, *M. citrata var. citrata*, *M. spicata*, *M. cardiaca*, *and M. gracilis*) and *Cymbopogon* spp. [lemon grass (*C. flexuosus*); Java citronella (*C. winterianus*); and palmarosa (*C. martinii*)] can be grown intercropped with *Populus deltoides* or *Eucalyptus* spp. Some of the above aromatic species and *Pelargonium* sp. (Geranium) can also be intercropped with another essential oil-yielding tree *Eucalyptus citriodora (*lemon- or citron-scented gum). The essential oil profiles of citronella and palmarosa grown in open and partial shade were not significantly different, but the oil of menthol mint (*M. arvensis*) produced in the open was richer in menthone, while that produced under shade was richer in menthol (Gururaja Rao 2021). These intercropping systems gave two to four times greater economic returns than sole cropping of the components. These studies indicate that aromatic plants can be profitably grown in association with linearly growing fuel and timber trees such as *L. leucocephala*, *Casuarina* spp., and *Grevillea robusta*.

## 13.7 Summary

Saline and sodic vertisols in coastal areas with their inherent physical constraints like high clay content, low infiltration rate, swelling and shrinking behavior, and low moisture range pose severe challenges for agricultural crop production. In order to bring such soils under production system, evolving economically viable interventions with salt-tolerant crops comprising food crops, horticultural crops, forages, medicinal and aromatic plants, woody biomass species, and halophytes, which would form ideal ones in phytoremediation of coastal saline vertisols. This is very challenging as these soils provide many constraints even at low salinity. Farming system modules with low-water-requiring salt-tolerant crops, fruit species, biomass species, and vegetable crops along with medicinal and aromatic crops have been found as ideal options. Silvipastoral system has been advocated as a promising sustainable option for phytoremediation of these soils. *Salvadora persica* has been proved quite remunerative even on highly saline vertisols with salinity up to 45 dS $m^{-1}$. The interventions comprising halophytes, field crops, biomass species, forages, plants of industrial importance, and their role in mitigating salinity in coastal saline vertisols have been detailed. Aromatic herb, *Anethum graveolens*, proved to be beneficial on moderately saline vertisols. Cultivation of medicinal plants along with woody tree species on saline vertisols has also been suggested. Among other halophytes, *Salicornia* and *Arthrocnemum* have been found ideal for saline vertisols. Coastal saline vertisols, thus, provide a challenging area and the interventions evolved have been field demonstrated and can be used as ideal management options for addressing salinity issues.

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**Dr. G. Gururaja Rao**, born on July 1, 1953, in Andhra Pradesh of India, completed M.Sc., in Botany with Applied Plant Physiology specialization from Sri Venkateswara University, Tirupati, completed Ph.D., in Physiology of Salt Tolerance in 1981, subsequently joined Rubber Research Institute of India, Kottayam, as Research Assistant, subsequently got elevated to Environmental Physiologist, worked on drought tolerance of Hevea, and came out with markers for drought tolerance in Hevea. After joining ICAR-Central Soil Salinity Research Institute, Regional Research Station, Bharuch, Gujarat, in 1989 as Senior Scientist, he subsequently got appointed as Joint Director/Head of the Station in 2003 and continued in the same position till 2014 end before superannuation in June 2015. During his association with CSSRI, he came out with several agro-interventions for the management of saline vertisols, i.e., cultivation of *Salvadora persica*, cultivation of Dill, a seed spice crop, halophytic grasses for saline vertisols, cotton pulse intercrop for saline vertisols, conjunctive use of saline water for crop production, groundwater recharging for augmenting groundwater levels, cultivation of *Desi* cotton and salt-tolerant wheat on coastal saline soils, use of treated industrial effluent in diverse crop interventions, and developed farming system model for maximizing production on saline vertisols. A total of 220 research papers were published in peer-reviewed national and international journals, symposia presentations, book chapters, and popular articles. He guided 28 students for their PG dissertations in Plant Physiology and Biochemistry. He received groundwater augmentation award, Dr. JSP Yadav Memorial Award, ISCAR Fellowship during service period, and Korni Venkatagiri and Smt. Ganjam Venkataramanaiah Memorial Prize during postgraduation.

**Dr. Jitendra Chikara** was born in November 1952 and completed his Ph.D. in Botany (cytogenetics) from CCS University, Meerut (erstwhile Meerut University), in 1982. During 1979–1981, he served as Research Associate in Vivekananda Parvatiya Krishi Anusandhan Shala (ICAR), Almora, for working on genetic improvement of millets. Later, in 1981 he joined CSIR-CSMCRI, Bhavnagar, as Scientist to work on seawater agriculture. Since 1981, he worked on salinity tolerance of crops and screened a number of varieties of various crop plants like wheat, millets, and barley and identified salt-tolerant and susceptible species and varieties. Later, selection and assessment of yield potential of Jojoba plants were undertaken on nonsaline wastelands in Gujarat and other states. Cultivation of Jojoba on farmers and entrepreneur's fields was promoted with cultivation package of practice. Genetic improvement and multiplication through tissue culture of these nontraditional plants like Jojoba, Salvadora, and Simarouba was undertaken. Micropropagation of halophytes like *Salicornia brachiata* and *Suaeda nudiflora* was also attempted, and protocol through callus cultures was developed. Later, he worked on developing sustainable agronomic practices for Jatropha cultivation on wasteland/marginal lands. Survey of wildly growing Jatropha was undertaken from different parts of the country to collect the germplasm of plus trees for assessment. Genetic variability at the molecular level among Jatropha population had been studied for identification of molecular markers related to yield attributes. Basic data were generated to study the full life cycle analysis. Also, he participated in the technology development for application of seaweed saps as a foliar spray to increase the yields in different crops at Pan India level. Survey of coastal halophytes along the Gulf of Cambay was undertaken for one of the sponsored project. A total of 26 research papers were published in peer-reviewed journals, while 22 papers were presented in national and international seminars/ symposiums. Besides above, seven book chapters and one book were also written. Two students completed their Ph.D. degree under my supervision. About 25 graduate and postgraduate students completed their dissertations under my supervision. Later, he superannuated as Chief Scientist in 2012.

# Emerging Biotechnologies in Agriculture for Efficient Farming and Global Food Production

## 14

Bablesh Ranawat, Freny Shah, Sonam Dubey, Aneesha Singh, and Sandhya Mishra

## Abstract

If the global population continues to grow, it will be difficult for agricultural productivity to keep pace without overtaxing essential environmental resources. To address global food security and place agriculture on a path to sustainability, a diverse and integrated approach will be needed. Modern biotechnology focuses on the enhanced application of genetically modified (GM) crops which represent an important range of resources that can promote sustainable agriculture and increased food security. This chapter deals with innovative research in the field of agricultural biotechnology by emphasizing novel and evolving techniques for efficient farming and global food production. This topic illustrates the importance of agriculture biotechnology for sustainable agronomy and improved food security which can further be used to encourage the production and adoption of beneficial GM crops.

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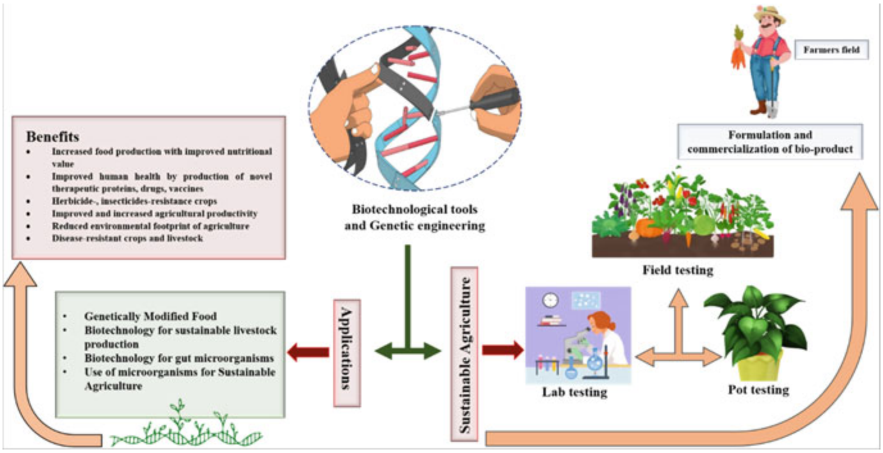
## Graphical Abstract



**Keywords**

Food security · Sustainability · Agriculture biotechnology · Genetically modified crops · Plant growth regulators · Plant development

## 14.1 Introduction

The major goal of agricultural husbandary has been to produce sufficient feed, biofuel, fiber, and food to meet the requirements of the population and society. Sustainable agriculture aims to satisfy the aforementioned rudimentary common stipulations with a focus on reducing agricultural inputs, and quality perseverance of the soil, reducing the impact on the environment, and ensuring economic viability (Gilbert et al. 2010). It has been predicted that the world population can be reached to 9 billion within the next 30 years resulting in the escalating requirement for fiber, feed, biofuels, and food. This could be the exigent demand for the agronomy sector to run with the need (Gilbert et al. 2010). Agricultural production would be affected by pressure of growing population, and change in the climate and related severe conditions of weather along with the availability of the arable land and water availability for agricultural purpose. There is a requirement for more land to be converted to cultivated acres deprived of the crop production that can outperform current varieties, and more inputs will be required to meet growing societal demands. For example, the researcher estimated that if current agricultural patterns continue, an additional ~1 billion Ha (hectares) of cultivable land would be required by 2050 (Tilman et al. 2011). Without raising the carbon impact or carbon footprint, improving food safety in the face of these challenges would take a unified and varied perspective.

Food protection happens when “all individuals have physical and economical access to adequate, safe, and nutritious food at all times to satisfy their nutritional requirement and food preferences for an active and balanced life”. Even though agricultural production is complex and depends on several political factors, infrastructure, and socioeconomic in a global context, the capacity of the agricultural sector optimizes the yield of crop output without abandonment of environmental resources. Agronomy has delivered tremendous prospect to boost sustainability in agricultural sector and safeguarding food security, but the functional and significant applications of agronomy are only beginning to be realized in the sense of food security and sustainability. The convention on biological diversity (CBD) describes biotechnology as “any technical application that creates or modifies processes or products for particular use using living organisms, biological systems, or derivatives thereof” (Secretariat of the Convention on Biological Diversity 2005). Within this comprehensive definition, for thousands of years, agriculture has been using biotechnology to improve germplasm, improve crop genetics, and select desired traits by selective breeding. Such biotechnology techniques help to promote the creation of pure genetic lines, compared to conventional breeding, and have improved the efficiency and speed of selecting desirable characteristics. In addition, transgenic plants are better known as genetically modified plants/crops that are fundamental to the area of contemporary agronomy developed using genetic and molecular tools. The espousal rate of GM plants/crops with biotechnological traits is expected to enhance continuously, especially in the developing countries. Much of the developments in GM crops to date are based on a variety of main row crops (such as corn, soybean, and cotton). Alternative approaches like promising resistance to various herbicides, and a range of stacked features offering tolerance to herbicides in combination with multiple insect resistance modes of action are also becoming readily available to farmers. In addition to the diversity of plants/crops being modified (like banana, wheat, cowpea, rice, and vegetable crops), further developments in the agriculture field are growing, but also the range of advanced genetic modifications (such as fungal, viral, and bacterial resistance, tolerance to abiotic stresses, improved nutrition, improved digestibility, and modified composition) (USDA-ERS, 2015).

A major global concern has been the effect of industrial agriculture on natural resources. Growth in the increased demand and population for agricultural products is increased day by day which leads to enhance pressure on land and water supplies. Low efficacy in the usage of resources, especially nitrogen, needs a major attention for many intensively controlled system related to agricultural having greater external inputs. Eventually, elevated usage leads to low performance outcomes in environmental issues such as eutrophication, ammonia and GHGs emissions, groundwater contamination, and soil erosion. Hence, the need is to turn existing agriculture toward highly productive, resource-efficient, ecologically sustainable, and socially acceptable. In order to improve the quality of a crop under suboptimal circumstances, the steady increase in global temperatures and the decline in freshwater supply pose crucial challenges for agricultural researchers. In the current scenario of developed agricultural practices, the key sources of contaminants that are highly harmful to animals and humans are the inefficient use of fertilizers and other chemicals in water, soil, and air. The twenty-first century's need is to move toward new farming techniques increasing the availability of feed and food, while at the same time mitigating the negative effects on agricultural productivity, such as the global warming.

Food availability in simple term can be explained as the physical presence of food. Food supply is a mix of domestic food stocks, commercial food exports and imports, and supplies of food along with the production of food at a national level. It is a fact that food could be grown and purchased at the household level from the local markets.

The growing necessities for nourishing, safe, and balanced food due to a growing population and the dedication to preserving biological diversity, climate change, and other sources are of a major concern for agriculture. Healthy and diverse foods can reduce diet-related diseases, largely based on plant-derived foods. Plant science investments will be required to develop a variety of cropping systems that balance efficiency, nutritional quality, and sustainability. Diversity of cultivars and nutrient composition are essential. To reduce degradation of soil, we need more diversed and nutritious cultivars by eliminating the application of hazardous chemical fertilizers and pesticides. To enhance the adaptability of crops toward the change in climate and resisting power to evolving pests, we need to collaborate with medical scientists and food materials, the food sector industries, breeders, and formers. The current global concerns of today are reduction in biodiversity, emission of greenhouse gases (GHGEs), climatic change, poverty, water scarcity, hunger, and malnutrition. Additional global issues are diet-related disorders like those associated with being overweight, obese, and diabetes. The provision of agricultural technologies such as genetic engineering and molecular technology often needs long-term plant science funding (Dwivedi et al. 2017).

A specially commission to direct sustainable food choices has shown that modest reduction in GHGE (<30%) is consistent with nutritional affordability and adequacy without adding substantial changes in the group of food in accordance with the guideline of the nutrition.

The execution of food system approach will help to evaluate, address, and recognize trade-off between social, nutritional, economic, and environmental priorities and limitation (Kumar et al. 2015). Agriculture practices, however, should fulfill the requirement of consumer food protection and quality, build successful value-chain linkages, and decrease ecological tension while increasing their sustainability. As shown in Fig. 14.1, three main features of a sustainable agriculture present in the food systems are depicted (Sukhdev et al. 2016).

Current agricultural strategies are mostly powered through features like predictability, high yield, quality, low cost, and nutritional values and not by taste (Kemp 2016). Instead of nutritional and functional characteristics, current agriculture programs have concentrated primarily on host plant resistance, low labor input, and edible yield (like driving herbicide-resistant cultivars). Therefore, the choice of cultivars according to nutritional value has received little attention (Leoncini et al. 2012).

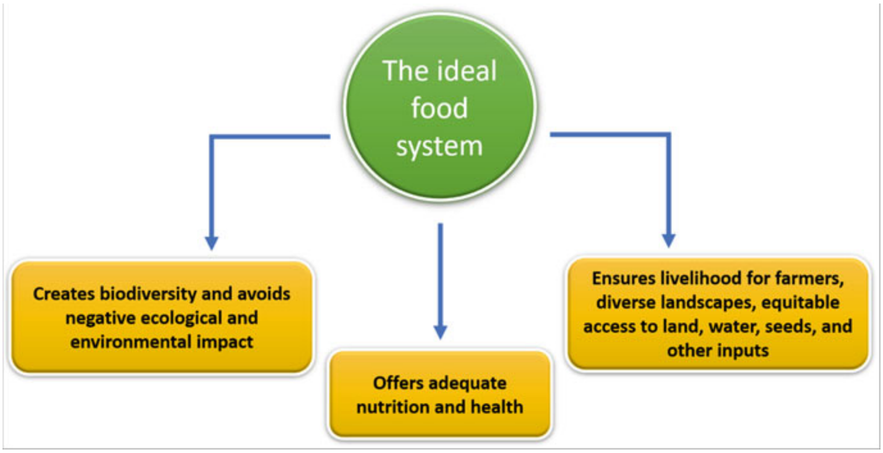


**Fig. 14.1** Key attributes of a sustainable food system (adapted from Sukhdev et al. 2016)

Nowadays, 95% of the nation's energy comes through 30 crop species, however, as mentioned above, nearly half of the world's demand for calories, comes from three significant crops, notably wheat, maize, and rice. The study suggested that among the 30,000 only about 150 edible crop species were capable of growing. According to World Health Organization (2015) report, this lack of diversity alone was responsible for creating adverse effects on the health. The potential of biotechnology is to encourage and foster sustainable agriculture and rural development. Such innovations are beneficial for the environment, particularly given that renewable genetic outputs. In order to further promote rural and sustainable agriculture development, the potential of genotypes and genes, for example, species or varieties to replace renewable resources, is of great importance. It is critical to underline that biotechnology should not be viewed as a replacement for traditional crop improvement tools but rather as a means of integrating recombinant techniques in to traditional crop methods to boost agricultural research and development output (Singh and Mondal 2017). Biotechnology advances have typically led to the following accomplishments:

a. To get better understanding of the plant's roles and responses toward environment,
b. Molecular techniques for genetic resources elucidation, safety, and implementation,
c. Selection of objective that aim to improve the efficacy and yield of plants, crops, fish, farm animals, and food storage quality,
d. Make farm animals and fish immune to disease that is life-threatening,
e. Enhanced production of crops by rising disease resistance capacity,
f. Improved food quality by using high-proteinic GM crops with more suitable amino acid, nutrients, and starch levels,

g. Efficient molecular diagnostics for the identification and treatment of pathogens, parasites, and pests,
h. Healthier products—the longevity of products can be improved by genetic modification. Consumers can have more access to full foods of greater nutritional value through easier movement of fresher items. In addition, it will promote the prevention of injury, spoilage, and decreased nutritional value,
i. As genetic engineering lowers insecticides can remain in the diet, it can be a natural advantage. Therefore, the leaching of pesticides into groundwater will be decreased and the interaction of people working in the farm with toxic and lethal composites can be reduced,
j. Usage of molecular or genetic techniques (DNA) to provide understanding toward crop production.

The aim of this chapter is to highlight advances in agronomy for sustainable agriculture and food security. The emphasis is on genetically modified plants/crops and, particularly, on biotechnology tools in sustainable agriculture, but it should be emphasized that GM crops are unable to replace other kinds of technologies needed to make system of production sustainable. In the future alternative, tactics need to be distinct. Yield enhances will remain key point, but it is essential to look for the ways of loosening the interdependence between yield and external use of input and making production systems more responsive to ecological stresses.

## 14.2 Potential Benefits and Effects of GM Crops

Genetically modified (GM) plants/crops have considerable potential to endanger the survival of sustainable agriculture and biodiversity. Centers of origins and megadiverse nations and/or crop species diversity are especially vulnerable areas of the world. Sustainable agriculture's future could be irreversibly jeopardized by the contamination of genetic resources retained in situ, threatening a strategic resource for global food protection. Since genetically modified plants/crops are genuinely scientific novelties, their deployment into the ecosystem expressed concern about both the uncertain evolutionary and ecological outcomes which may be expressed in the medium and long term by GM species/varieties itself and the associated fauna and flora. The widespread pollution of natural flora by GM traits and the depletion and deterioration of the widely owned genetic resources available today for agricultural production may be one of the implications of such processes. When release into atmosphere, GM crops/plants bearing industrial and pharmaceuticals characteristics can pose even more dangerous risks (Table 14.1).

The effect of commercialized GM crops on the benefits, use, and yield of chemical pesticides by farmers using micro-level data collected in different countries has been analyzed in a large number of studies (Qaim 2009; Carpenter 2010; Finger et al. 2011; Areal et al. 2013).

**Table 14.1** Possible risks and benefits of genetically modified organisms (adapted from Lucht 2015)

| Risks | Benefits |
|---|---|
| Religious, cultural, and ethical issues<br>Issues related to health<br>• Toxins and allergens<br>• Stability of antibiotic<br>Ecological and environmental issues<br>• Insecticide resistance<br>• Threat to biodiversity and to genetic diversity<br>• Impacts on nontarget species | • Enhanced production of food with better-quality nutritious value<br>• Human health improvement by production of innovative therapeutic drugs, proteins, and vaccines<br>• Herbicide-resistant and insecticide-resistant crops<br>• Improved and increased agricultural productivity<br>• Reduced environmental footprint of agriculture<br>• Resistance toward diseases |

## 14.3 Application of Agricultural Biotechnology

### 14.3.1 Genetically Modified Food

Current revelations in molecular genetics and functional biology have shown that in the near future, the associated biotechnology products would be very practical. Improved crop diversities could therefore be tailored using GM technology for marginal agro-ecological regions, which were largely neglected by the green revolution. These all techniques offer the possibility of adding a beneficial trait from strongly related crops/plants without associated detrimental genes, or from related species that do not intersect promptly with crops of interest, or from entirely unrelated species, or from other taxonomic phylae. It creates the opportunity also for containment of pests/insects/diseases, food fortification in cereals with vital vitamins for example vitamin A, micronutrient like zinc, iron, and crucial amino acids such as lysine, and for the development of crops/plants that are resistant to drought or generally capable of growing well in harsh environments. The farmer approachability of GM techniques is a chief characteristic, as it is assembled in an efficient seed form. The key benefit of approaching same technique in agronomy is that it is possible to improve productivity by using new varieties with pest- and disease-resistant properties. The injury to the plants/crops is caused mostly by fungus and insect larvae, to some point by adult insects. There has been rapid progress in using GE for plant/crop enhancement in terms of pest-resistant, male sterility systems, and herbicide resistance due to improvements in gene expression and genetic transformation over the last decade. In 1987, the first transgenic plants were produced with *Bacillus thuringiensis* (Bt) genes. Bt cotton is a GM crop containing *B. thuringiensis*-isolated foreign gene. A novel technique based on the RNA interference mechanism (RNAi) has been introduced to avoid such problems. Using *Agrobacterium* as the carrier, the particular genes from the parasite are transmitted into the plant. The genes are inserted in a manner that generates both sense RNA (in insects) and antisense RNA (in plants). They form a double-stranded RNA, as these two RNAs are complementary. As a consequence, in the transgenic

**Table 14.2** Listed technologies for GM crops at the field trial level (adapted from Qaim 2016)

| Crop | Trait | Countries |
|---|---|---|
| Apple | Reduced bruising/browning | Canada |
| Banana | Provitamin A content<br>Bacterial resistance<br>Insect/nematode resistance | Uganda |
| Bean | Virus resistance | Brazil |
| Cabbage | Insect resistance | India, China |
| Canola | Herbicide tolerance<br>Omega-3 content | Australia, USA, Canada<br>USA |
| Cassava | Virus resistance<br>Provitamin A content | Kenya, Indonesia, Uganda<br>Kenya, Nigeria, Uganda |
| Chickpea | Insect resistance | India |
| Cotton | Insect and herbicide resistance | India, USA, Pakistan, Ghana, Burkina Faso, Kenya, Cameroon |
| Cowpea | Insect resistance | Burkina Faso, Ghana, Nigeria |
| Eggplant | Insect resistance | India, Philippines |
| Groundnut | Virus/fungal resistance | India |
| Maize | High phytase (quality)<br>Stacked insect and herbicide resistance | China<br>India, Indonesia, Pakistan, South Africa, USA |
| Mustard | Male sterility | India |
| Orange | Bacterial resistance | USA |
| Pigeonpea | Insect resistance | India |
| Potato | Fungal resistance | Bangladesh, India, Indonesia |
| Rice | Insect resistance<br>Iron content, provitamin A content | China, India<br>India, Philippines, Indonesia |
| Sunflower | High oleic acid | Australia, Argentina |
| Sorghum | Stacked provitamin A, iron, zinc | Kenya, Nigeria |
| Soybean | Multiple pest resistance, yield enhancement, and modified fatty acid | USA |
| Sugarcane | Insect resistance and herbicide tolerance, and drought tolerance | Australia, USA<br>Brazil, Indonesia |
| Tomato | Fungal resistance and insect resistance | Argentina, India, Chile |
| Wheat | Drought tolerance<br>Insect resistance<br>Fungal resistance<br>Virus resistance<br>Herbicide tolerance<br>Improved grain quality | Australia, Egypt<br>UK<br>China<br>China<br>USA<br>Australia |

host, the parasite does not line up and the transgenic plant is protected from the pest. In both developing and developed countries, transgenic crops/plants with desired genes will play a pivotal role in agriculture field. This will play a notable role in improving crop production, reducing insect-related losses, and improving the situation of poor people of rural areas. The production and deployment of transgenic crops/plants with pest management insecticidal characteristics would result in reduction of sprays for insecticides (Table 14.2) (Singh and Mondal 2017).

## 14.3.2 Biotechnological Approach for Sustainable Livestock Production

### 14.3.2.1 Embryonic Transfer and Superovulation

With the advent of artificial insemination (AI), the manipulation of reproduction in the domestic animals began in the 1930s. During the 1960s, AI organization stated to make regular use of freeze semen to make its application widespread, making better use of the cells of the egg by superovulation of the genetically superior animals. The transfer of embryos is a very effective technology to increase the productivity of an animal (Madan 2005). This technique has also made it possible to greatly promote imports and exports of valuable genetic materials, to establish new breeding principles, to conserve genes through freezing techniques, to produce twins, to introduce new genes into closely related herds, and to manipulate embryos and transgenic animals. The implementation of AI and the transfer of embryos has fully changed the bovine breeding scheme. A new dimension has been created by recent advances in the field of in vitro fertilization and oocyte maturation in farm animals.

### 14.3.2.2 Gene Transfer and Transgenic

The advancement of gene transfer techniques has created innovative new opportunities for modifying animal outputs (Hammer et al. 1985). Transfer of gene provides a strong perspective toward the development of animal growth and reproductive efficiency manipulation techniques.

### 14.3.2.3 Gene Knockout

A molecular technique that precisely silences a target gene is gene knockout or gene disruption. This technique can be useful to silence genes that have local or general (tissue-specific) function, including a feature that causes the entire body or particular tissue to grow slowly.

### 14.3.2.4 Gene Therapy

Another approach to bringing exogenous genes into animals is gene therapy. Originally, gene therapy was developed as a way of correcting human disorders based on genetics. This approach can also be used, however, to provide genes that can change the composition of the body and development.

### 14.3.2.5 Somatotrophin in Milk Production

A possible technological method for increasing the production of milk in animals that are important for dairy industries is to inject growth hormones (GH) or bovine somatotrophin (BST). BST is a hormonal protein released by the anterior pituitary gland that occurs naturally and is widely used to increase milk production which has been made available by recent developments in recombinant DNA technology.

### 14.3.2.6 Vaccines and Diagnostics

Biotechnology has transformed vaccine production science, leading to new understandings in the field of biochemical processes of pathogenesis and infectious disease, and has unlocked the new approaches for recently developed vaccines such as recombinant DNA vaccines and peptide vaccines that have been chemically synthesized. Live and killed microorganisms are vaccines produced using traditional ways. There is a replicated agent in live vaccines, while the killed vaccine includes a non-infectious agent and an adjuvant.

### 14.3.3 Biotechnology for Gut Microorganisms

Biotechnology, which is very important for animal health and development, can be used to enhance the metabolism and behavior of gut microorganisms. Three main approaches are as follows:

1. Use of products that are produced biotechnologically for improvement of the gut ecosystem.
2. To promote the growth of advantageous bacteria for the preparation of prebiotics and probiotics.
3. Implementation of new strains or species of microorganisms into the gut.
4. Genetic manipulation of microorganism that is present naturally in the gut in order to boost their ability to perform defined functions or introduce new functions. Implementations of different genes have been investigated in gut microorganisms. GM microorganisms are capable of either digesting fibrous and forage lignin products, degrading contaminants, synthesizing essential amino acids, reducing the formation of methane, or tolerating acids.

Owing to technological challenges and public concerns, biotechnology for intestinal microorganisms is far from commercially applicable.

### 14.3.4 Use of Microorganisms for Sustainable Agriculture

Microorganisms play a crucial role in functioning of ecosystems and conservation of sustainable agriculture. This sustainable agriculture provides the ability to meet our present time agricultural requirements. The microorganisms associated with plants display a wide range of living habits whose saprophytic or symbiotic relationships with the plant may be beneficial. Most of these microorganisms reside in the rhizospheric zone, but some of them successfully penetrate and live within plant tissues, known as endophytes. For the purpose of sustainable and balanced crop production, many soil bacterial groups are utilized protecting the biosphere via improving plant nutrition, soil quality, and health, and these soil microorganisms play a significant role in agriculture (Lugtenberg 2015). In agriculture practices, the benefits of applying microbes include not only sustainable agriculture, but also other ecosystem-associated benefits. These include the regeneration of habitats, improving the resilience of plant species, the recovery of endangered flora, and adaptive management strategies for diversity (Barea et al. 2013). For this cause, many techniques are proposed to use the beneficial microbial community more effectively to assist sustainable environmental-friendly agrotechnological practices. For a continuous supply of essential nutrients for growth and plant defense, the main objective of using microbes is required. Because several biotic and abiotic factors and agronomic management influence the interactions between the microbial populations and plants, the impact of environmental stress factors must be overcome, mainly in the current global climate change scenario, as they severely harm the interactions between plants and microbes.

## 14.4 Food Security and Sustainable Agriculture

Food sustainability is one of the main problems with regard to climate change in the modern era. Unfavorable climate changes through heat stress and water affect cereal production, but are also correlated with frost logging and waterlogging, pest dynamics, and disease. As changes in unusual and uncertain weather conditions impact populations worldwide, climate change is a recurring force of change in livelihoods. Rising populations, shifting lifestyles, losing groundwater source, urbanization, and the additional demand for cereals like maize for fuel and fodder pose major challenges for production of cereal in the foreseeable future, even without any climate change (Hubert et al. 2010). Global demand for cereals is estimated to exceed 3 billion tons by 2050 (Alexandratos and Bruinsma 2012).

Reduced seed set, decreased grain size, pollen sterility, quality, shriveled seed, and stigma drying (in wheat), poor anther dehiscence, nonviable pollen, poor pollen development, early embryo abortion (in rice), pollen deposition on stigma, tassel blast, leaf firing, pollen sterility, accelerated senescence, barren plants, and decreased seed set are various qualitative indicators of heat stress. Wheat early flowering and poor grain filling are some of the qualitative indicators for drought stress, poor panicle emergence (in rice), decrease in leaf expansion, early senescence, inferior grain set, and decreased grain weight, leaf senescence (in maize), increase in anthesis-silking interval, barren plants, decreased gran set, and poor ear tip filling (in pearl millet).

Food security was described according to the UN (1975), as the “availability at all times of adequate world food supplies of basic foodstuffs to support a steady expansion of food consumption and to offset production and price fluctuations.”

## 14.5 Current and Future Trends

Growing the worldwide population, shifts in the nature and size of per capita demand, improving photosynthesis, defeating exotic diseases, climate change, reducing environmental change, leveraging genome advances, competition for key resources and changes, understanding diet and health in consumer values, and ethical roles are important factors affecting the food system. Maintaining future agriculture consumption, increasing effciency with existing/new science and technology tools and processes, resolving unknown risks, and minimizing waste are priorities. It is also necessary to resolve the danger of future instability in the food system, to end hunger and reducing GHGEs. It is important to enforce strategic policies to conserve the environment and biodiversity for sustainable food production. Promoting sustainable agriculture is also important to safeguard the economic viability of farmers. Sustainable agriculture is indeed a collection of agriculture practices which can, in the long-term, wish to maintain efficiency, profitability, and farm productivity without degrading natural resources and the environment. It may be of significant response to promote the implementation of native skills, less dependency on external inputs, major prominence on the diversity of crop, rotation of the crops and development based on needs, and easy marketability to ensure the survival of small farmers.

Global food prices have decreased dramatically over the twentieth century due to the massive upsurges in the production of agricultural products due to the significant advancement in the various technologies. The long-term adverse inflation rate made many assume that with economic growth, food prices will always decline. The price spike in year 2007–2008 and subsequent years has shown that food shortage is not just a problem (FAO 2011). Farmers who sell crops/plants are content with increasing wages and earnings, while customers who buy food cannot afford high expenses. Many poor and food-insecure households in the world purchase greater amount of food than they sell, that is why suffering from increased costs. Therefore, price rises aim to worsen global poverty and food in security (Ecker and Qaim 2011).

Although rate of population increasing is no longer as high as they used to be in the past, with over 70 million new people added to the planet each year, the global population continues to rise more. Furthermore, the agronomy-based products are used more precisely for non-food uses, like biofuels, which have just augmented dramatically in value. According to the report, the total need for agronomical products like feed and food has gained by an average of 1.8–2.0% per year over the past 15 years (Qaim and Klümper 2013). Other developments will have to produce more productivity gains, particularly improvement in agronomy. However, advancement in breeding is constrained by the genetic variation that is or can be created in the species of interest. Traditional methods alone would not be enough to maintain past rates of breeding success on a sustainable basis. GE provides tremendous potential to increase the genetic diversity that breeders will use to cope with the challenges ahead (Tester and Langridge 2010).

Over the next few decades, growth in agricultural production will continue to be substantial leading to a further rise in the global population, rising profits, and the increasing use of plant-based goods and substances for energy and other industrial processes (Godfray et al. 2010; Hertel 2015). Rising natural resources scarcity, including water and land, environmental concerns like soil depletion and extinction of biodiversity, and changing weather patterns pose major encounters which need to be tackled by improved technology. It is often suggested that current priority should be given to by decreasing post-harvesting losses, surplus, and restricting food intake rather than rising production. Undoubtedly, decreasing waste and losses are concern objective and reduction in food consumption would also be beneficial, particularly in rich nations. Such improvements, however, are not easy to achieve and alone will not be sufficient to alter global supply and demand patterns (Qaim and Klümper 2013). Therefore, different policies need to be followed concurrently, and one of them needs to be sustainable increase in agricultural production. Despite forecasts and concerns to the breeders, farmers, contrary, and agronomists/scientists have developed and implemented agricultural technologies that maintain that growth in food production is sufficient to meet the growing demand for food. There is no reason to assume, from a mere technical viewpoint, that this will not be feasible in the future as well. Combined with other promising innovations, genetic engineering and modern biotechnology may play a significant part in boosting production and product quality, while decreasing the use of limited resources and agricultural chemicals. But nowadays, the prices and direction of farming techniques are importantly dependent on public tendencies. Emerging innovations need to be used cautiously, but we cannot afford to rule out promising fields of research solely on the basis of bias, given the challenges ahead.

## 14.6 Strategies and Approaches of Sustainable Agriculture

Social justice, environmental security, and economic development are the golden triangle of sustainable agriculture. The techniques are aimed at minimizing degradation of soil and erosion, preserving the quality of the soil, saving water, use of chemical fertilizers, generating large amount of nutritious food at low cost, fewer pollutant-causing pesticides, addressing food protection, and maintaining biodiversity, ensuring customer access to quality and quantity of food. Strategies should be systematic in their existence, including social and policy decisions, agricultural input control, creative farming methods, and genetic modification.

## 14.7 Discussion

The major global concern has been effect of industrial agriculture on natural resources. Growth in the population and increased demand for agricultural products are continuously increasing the pressure on land and water supplies. The overall amount of arable land available for agriculture is small and may be of low quality for intensive agricultural production in some regions of the world. Sustainable agriculture is fundamental to responsible management of mental resources in the environment, such as renewable and non-renewable energy, soil, water, and nutrient inputs. For crop varieties with improved characteristics (like improved nutrition, increased yield, and improved stress tolerance), conventional breeding has been used in the past to pick. The advent of modern biotechnology and the production of GM crops, however, have enhanced the toolbox of the breeder and enabled agriculture to move much faster on a path to sustainability. Such innovations have a wide and established track record of improving yield and prosperity, benefiting more from smallholder farmers in developing regions than in developed regions (Klümper and Qaim 2014).

The technologies addressed here are only a few examples of what can be done to improve food protection and environmental stewardship. While these new developments are highly promising, it is important to note that agricultural biotechnology represents only one set of instruments to improve the sustainability of agriculture and food security. In order to improve sustainability and manage the environmental burden of agriculture, an integrated approach including precision farming for input applications, cover cropping conservation tillage, increased crop diversity, and other best management practices would all be necessary (National Research Council 2010). Every concept seems to have a diverse focus and a separate collection of analytical questions. It remains a theoretical and conceptual hurdle to overcome incompatibilities between these approaches and among the objectives of sustainable agriculture in general. The analysis and understanding of sustainable agriculture presented here, however, offer a basis for evaluating agriculture's sustainability.

## 14.8 Conclusion

Agriculture productivity can also be understood via an economic, ecological, and social viewpoint and should be analyzed in relation to all three dimensions of agriculture. Sustainable agriculture can also be presented on various time scales as a set of interacting structures. In thinking about and practicing sustainable agriculture, conceptual diversity occurs. Limiting the effect on the environment of agriculture and enhancing the quality of food nutrition would involve innovative food production approaches and, thus, innovative plant breeding practices. To address nutritional and food protection and achieve environmental sustainability, there is no single solution. Nutrition, agricultural productivity, and health are multidimensional, interconnected, and complex. An assessment is adopted where together with enhanced nutritional value, management of integrated natural resources, and resource-efficient plants/crops will mitigate the adverse impact of climate change on agriculture. Dietary trends and crop diversity that reduce the effects on the environment and improve health need to be recognized and adopted. Based on the use of heterogeneous cultivars, processing systems contribute to the diversity of diets and thus to better well-being and human health. Since nourishment is a complex function, we recommend a comprehensive perspective to seizure nutrition's complexity in relation to human health. In addition to recognizing dietary trends with lower environmental effects, coupled with the promotion of more active lifestyles, policies that promote the adoption of healthier diets are a positive strategy to improve public health.

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# Role of Beneficial Microbes in Alleviating Stresses in Plants

# 15

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## Abstract

Global climatic change has resulted in the increased activity of biotic and abiotic stresses on the agricultural crops and forest ecosystem. To cope with stresses has now

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become crucial for nature's survival. Biotic stress is referred to as the phytopathogens, while abiotic stress includes temperature, salinity, drought, and heavy metals in soil due to excessive use of sprays and chemical detergents. Apart from affecting the crop yield, it also affects the bioremediation efficiency and ecosystems of forests. In this case, agronomists and agriculturalists are looking toward the use of plant growth-promoting microbes (plant growth-promoting rhizobacteria (PGPRs), plant growth-promoting bacteria (PGPB), and plant growth-promoting fungi (PGPF)) for better production and health of plants. This way is environmentally friendly, effective, and sustainable as compared to the conventional system, i.e., pesticides and chemical fertilizers. Plant–microbe interaction increases the survival of plants by minimizing the negative effects of biotic and abiotic stresses. The use of "Omics and bio-formulations" is the recent achievements made in this sector by considering future concerns against biotic and abiotic stress-causing factors.

**Keywords**

Bioremediation · Biotic and abiotic stresses · Drought · Plant growth-promoting hormones · Salinity

## 15.1 Introduction

The key determinant affecting the productivity, quality, and yield of agricultural products around the globe is due to stress caused by both biotic and abiotic factors. Global warming will have resulted in the average rise of overall earth temperature by 2.5 °C by the mid of twenty-first century, besides the tornados, salinity in the soil, increased carbon dioxide proportion, drought, unexpected monsoon showers, and floods are responsible for massive economic losses. The moisture level within the soil almost affected 2/3 of the overall arid and semi-arid regions, and one of the main examples is in India with 11 million hectares of cultivatable land affected by salinity. To overcome this issue, scientists are trying to introduce the colonies of advantageous microbes and rhizosphere to enhance the crop yield by either direct or indirect techniques. The induction of phyto-growth promoter rhizobacteria is an eminent technique, and by addressing their basic physiology, their adaptability in field conditions will help to resolve any issues. Food security is the priority for every nation on this planet due to the escalation in urbanization and industrialization, but the climatic changes along with poor management strategies made it difficult to achieve as the deforestation inclined in recent years. The highest annual deforestation rate in 2015–2020 was in Africa (4.41 million ha), followed by South America (2.96 million ha) and Asia (2.24 million ha). In Africa, most of the deforestation was in Eastern and Southern Africa (2.20 million ha per year) and Western and Central Africa (1.90 million ha per year). In Asia, the deforestation occurred mostly in South and Southeast Asia (1.96 million ha per year) (FAO 2020).

The stability of agroecosystems has now become crucial as the world population is exceeding the benchmark of about 9.5 billion by the year 2050, so it will be impossible to feed the additional 2 billion people with the same limit of resources (Meyers and Kalaitzandonakes 2012). Indirectly, the side effects of the organophosphates are harming humanity via their consumption in the form of fruits and vegetables grown traditionally all over the world. The use of plant growth-promoting microbes PGPM has become popular within a few years due to their remarkable results in the sustainability of agroecosystems and ecological problems. The wastes excreted as an outcome of anthropogenic activities like industrialization and urbanization are also treated by bioremediation using the microbes. The multi-role PGPM in the current scenario has got a prime reputation along with endophytes. The induction of bio-inoculants for a safe agri-food system considering human health has gained vital importance after the legislative changes adopted by several countries to abate the escalating use of chemicals in soil in the form of fertilizers and pesticides. The natural equilibrium of soil nutrients disturbed by chemical inoculants can be reversed to its natural state for a healthier lifestyle through microbial inoculants (Woo and Pepe 2018). Although several microbial strains (fungal and bacterial) had been successfully inoculated in laboratory trials for optimized productivity of crops and vegetables, the PGPM is commonly applied at the field level due to its versatile mechanism of action. PGPM acts directly and indirectly for enhancing the production, in terms of direct action they generate specified agents that regulate the plant growth rate including nitrogen fixation, indole 3 acetic acid, phosphate solubility, and indirectly via overthrowing the phytopathogens (Khan et al. 2020). Apart from the PGPM, the role of PGPR cannot be neglected as they put forth a favorable effect on plant’s health during drought, salinity, and other abiotic stress-causing factors by restoring the contaminated soils (van Oosten et al. 2018). The interaction of plants with arbuscular mycorrhizal fungi is also helpful in enhancing the surface area of roots for better absorption of nutrients. The effectiveness of any microbial inoculant can be judged by its capability to incorporate effectively externally and internally in host plant to build a link with other microbes localized in the surrounding environment through its rhizocompetence chattels. In an overall scenario, the rhizosphere adherence within the plant's root happens through many processes including survival in contrast to other microbes, the movement of bacteria, and synergistic association with the host plant (Bhattacharyya et al. 2017).

The microbes residing in the plant's tissues like roots, stems, and leaves without affecting their natural physiology are termed as endophytes. Under ambient conditions, the role of endophytes is to elevate the growth either by secondary metabolism or by nutrient acclimatization besides provoking the disease attack (Zhao et al. 2011). Apart from the exact mechanism involved in the linkage between the microbial disintegration and ecosystems, the existence of microbial diversity is very important. The bacteria localized in the rhizosphere (the junction between roots and soil) exert a positive impact on plant growth and termed as plant growth promoters.

The alterations caused by abiotic stress on plants involved their anatomy, normal physiology, molecular level, and biochemical changes that adversely affect the growth, health, and production of crops and plants. Water shortage, elevated salinity level, and higher temperature often interconnectively caused cellular mutilation ultimately resulted in decreased production. For an instance, the osmotic pressure stress is acquired by drought and saline conditions of soil deliberately disturbing the natural homeostasis and ion concentration in contrast to optimum cell membrane potential. Heat shock proteins failed to respond under severe temperature variations, if not resolved on time leading to agglutination of cellular proteins and ultimately cell death. The imbalance in thermotolerance, especially the elevated temperature, stops the transcription and translation mechanisms, halting the plant growth at a microlevel. Similarly, the intolerance of heat shock proteins HSP during the cold stress cannot overcome the disturbed metabolic physiology, which ultimately causes alterations in cellular structures and stopping of enzymatic activities within the cell.

Specified transport mechanisms are involved in the uptake of heavy metals by plants, reaching the storage sites, targeting the functioning enzymes like Cu/Zn SOD. The plant-associated bacteria assisted plant growth and show tolerance to heavy metals and reduce their uptake or translocation to aerial parts of plants by decreasing the metal bioavailability in the soils. The presence of free copper (Cu) is dangerous as it declined the oxygen level within plant tissues and generates free radicals like $H_2O_2$ and $OH^-$ radicals that lead to apoptosis (Khan and Singh 2021). The fundamental structural proteins got damaged by oxidative stress that is the outcome of increased temperature, high saline level, and drought. The response of plants against the abiotic stress is a complex mechanism, starting from the signal receiving and then transduction followed by genetically and physiologically retortion besides the production of ROS, altered water turgor, and osmolytes. Figure 15.1 illustrates the abiotic and biotic stresses on a plant.

The negative impact of abiotic factors on the forest ecosystem cannot be neglected, and this effect varies with the severity, type, and duration of the natural disasters like tornados, volcano eruptions, fires, thunderstorms that hit the ecological system badly. This overwhelming situation on the forest ecosystem can be reversed by promoting the natural microbes within the earth's soil. Natural disasters not only disturb the habitat of animals living there but also exerts massive pressure on the microbial community (fungi and bacteria) residing over plants by completely vanishing them from the region. A favorable point here is that forest managers having a keen interest in the productivity and sustainability of forests are taking serious measures to overcome the short-term consequences resulted from natural disasters with the help of international corporation and advanced research protocols to conserve the natural habitat and making this planet a healthy place (Dinerstein et al. 2019).

## 15.2 Microbial Role in Combating Salinity and Temperature Stress

Higher temperatures provoke microbial colonization and affect the productivity of crops. The immediate response by microbes against the temperature rise is the producing heat shock proteins (HSP), polypeptides in nature.

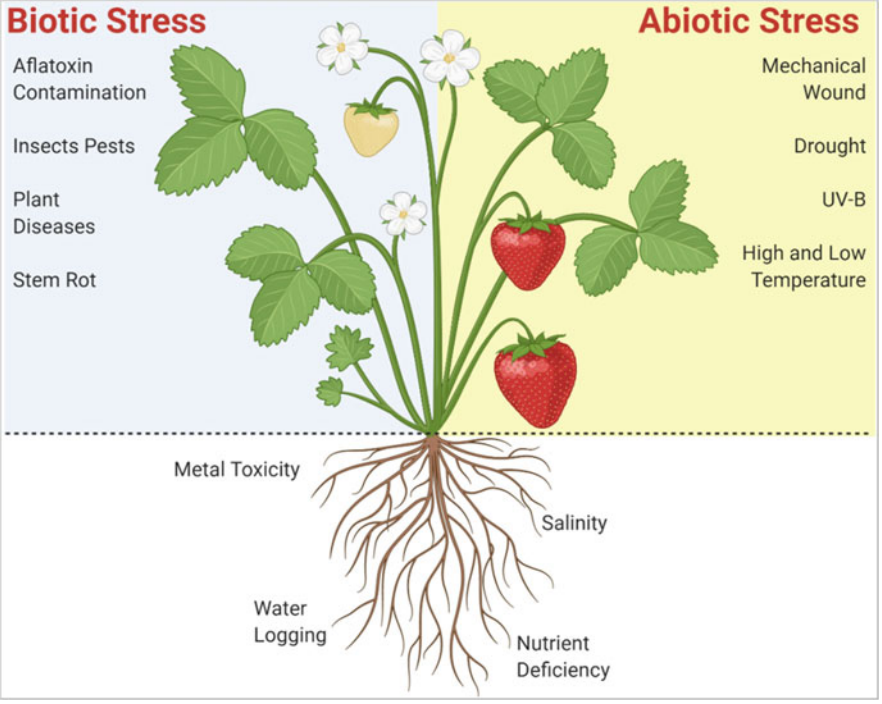

**Fig. 15.1** Biotic and abiotic stress on a plant

The addition of thermotolerant *P. putida* strain AKMP7 in wheat substantially enhanced the root (27.7 cm, 1.19-fold increase) and shoot length (52.93 cm, 1.12-fold increase) over uninoculated (root, 23.13 cm, and shoot, 47.12 cm) plants. Likewise, considerable growth in dry biomass of inoculated plants was recorded (16.8 g/plant, 1.6-fold) as compared to uninoculated control (10.5 g/plant). During the advancement phase of any plant, the inoculant bacteria must effectively colonize in the roots and by creating a beneficial association trigger the adaptive mechanism to tolerate abrupt to extensive changes in the temperature (Mitra et al. 2021). *Burkholderia phytofirmans*, a bacterial strain, after colonizing increases the phenols, starch, and proline levels and helps in protecting the plant from cold stress like frostbite and higher temperatures (Wang et al. 2015). Uptake of the food at the seedlings stage of wheat has been recorded by inserting the strain SRM and *Pantoea dispersa*, apart from food uptake an increase in the IAA levels, phosphate solubility, and HCN availability at a temperature range of 4–15 °C noted (Khan and Singh 2021).

About 900 million hectors of arable land are affected by salinity that is equivalent to 6% of the total landmass, and if the globalization after meth's is not controlled on an emergency basis this 6% will reach up to 50% by the mid of twenty-first century (Shrivastava and Kumar 2015). Salinity results in the imbalanced ionic ratio $Na^+$ and $Cl^-$, reduced osmotic turgor pressure, reactive oxygen species in the chloroplast, oxidative degeneration, plasma lysis, and closed stomata, thus affecting the plants' growth and yield. The highly reactive nature of ROS made them cause massive level damage to the plant's cell membranes and its electrolytes including nucleic acid too. In general, the salinity results in cellular necrosis, loss of turgor, impaired photosynthesis, reduced height, weight, and yield. The salinity stress can be resolved by considering the measures essential for maintaining the water homeostasis and effective photosynthesis in plants thus advances the plant's health and yield. Soil microbe's causes a change in the ion concentration of salinized soil and reported to enhance the productivity of crops grown over the saline-affected region. Moreover, a change in the plant hormone level also proved to be helpful in salinity issues. Several mechanisms cordially work to tackle the salinity problem in crops. A restriction of $Na^+$ ions takes place through exo-polysaccharide stimulation during high salinity, besides the AM fungus regulates the production of osmoprotectants in corn and beans. The availability of free auxin in plants is also helpful in tolerating the salinity stress. In canola, the insertion of 14 halotolerant microbial strains reduced the ethylene production through the ACC deaminase working. Similarly, the inoculation of *Pseudomonas fluorescens* in maize crop grown over saline soil showed a gain in live weight and root elongation via the ACC deaminase activity. Figure 15.2 summarizes the role of PGPB.

Plant growth-promoting rhizobacteria alter the plant's hormonal profile and control the salinity stress by actively regulating the ion concentration, especially the $K^+$: $Na^+$ ratio in contrast to toxic ($Na^+$ and $Cl^-$), ultimately improving the stomata conductivity rate, reducing the toxic influences within plant cells, and promoting the growth. The role of silicon is important in salinity as it counters the $Na^+$ uptake via an unknown pathway, increasing the integrity of the cell membrane, enhancing photosynthesis and antioxidant enzyme processes, and lowering the water loss. Many PGPRs like *Azospirillum*, *Pseudomonas*, *Bacillus*, and *Rhizobium* sustain their growth promoter capability even at higher saline concentrations by generating osmo-protective agents. The salt stress is mitigated by PGP's by regulating EPS production via a vital pathway, this EPS also retains the water capacity in plant cells, and anchoring chattels leads to better soil moisture level for efficient holding of the stem (Table 15.1). PGPR lowered the ethylene production that is a stress hormone produced under saline conditions (Tewari and Arora 2015).

## 15.3 Microbial Role in Combating Drought Stresses

An estimate showed that by the mid of twenty-first century the ratio of the land affected by drought will be doubled due to depletion of natural water resources by 30%, which will be resulted in creating a food security risk as the production of the crops will be restricted all over the globe and especially in the arid and semi-arid areas. Different PGPRs when inoculated successfully mitigate the drought problem by synthesizing exo-polysaccharides, ACC deaminase, indole acetic acid, and proline, and by improving the water transportation in cells. EPS formation by generating macro-aggregates improves plants' resistance and advances the soil configuration to

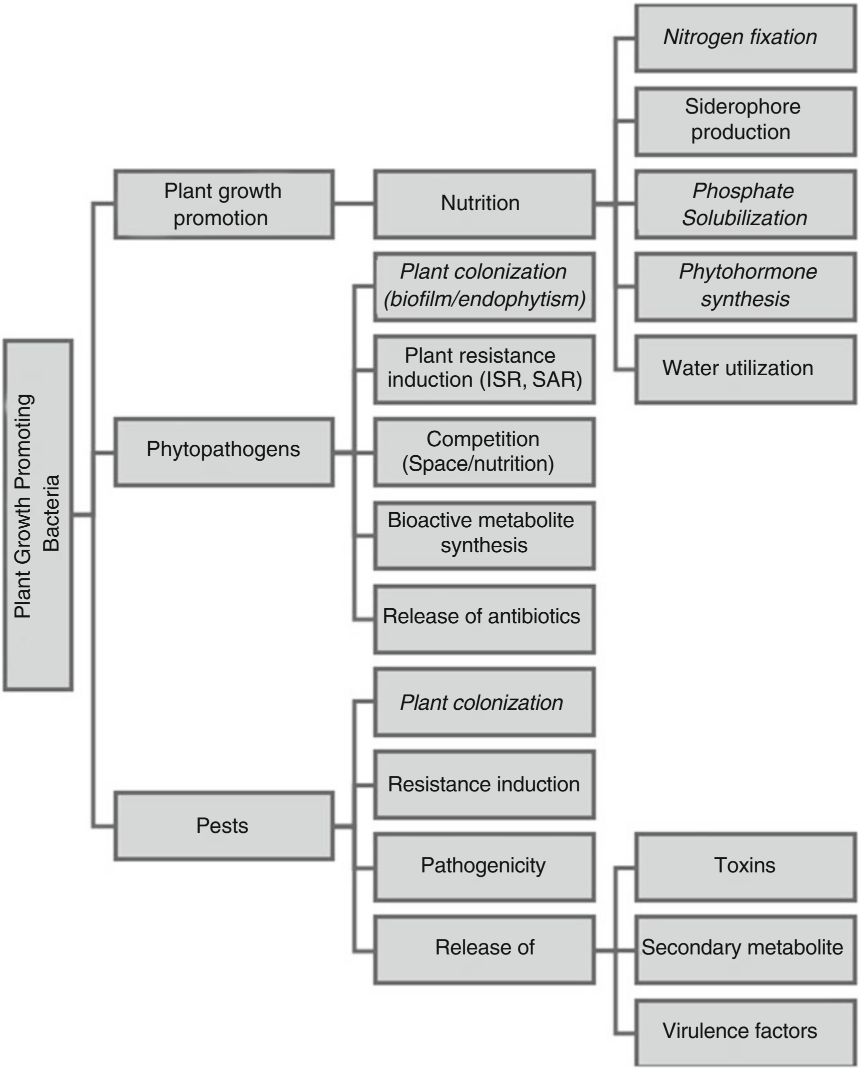

**Fig. 15.2** A flow chart explaining PGPB's different mechanisms

cope with the drought by increasing the mineral uptake by affecting the root adhering soil-to-root tissue ratio (Tewari and Arora 2015). PGPRs modulate the plant's response and assist in development stages during water scarcity by mediating the delay in reproductive stages and altered transpiration rate. *Arabidopsis thaliana,* a plant, exhibited an enhanced tolerance against drought stress when treated with *Phyllobacterium brassicacearum* 196 strain by persuading reproductive suspension and functional changes (Bresson et al. 2013). Drought stress is conferred through PGPR by generating tolerance in metabolism resulting in the formation of cytokines,

**Table 15.1** List of crops upon inoculation of PGPB showing positive effect against salinity

| Crops | Microbial species | Effect | Reference |
|---|---|---|---|
| *Zea mays* | *Azospirillum* | Osmo-protection | (Hamdia et al. 2004) |
| *Lactuca sativa* | *Azospirillum* | Uplift seed germination | (Barassi et al. 2006) |
| *Brassica napus* | *Pseudomonas putida* | Promote plant growth | (Cheng et al. 2007) |
| *Arachis hypogaea* | *P. fluorescens* | ↑ACC deaminase activity | (Saravanakumar and Samiyappan 2007) |
| *Gossypium* sp. | *P. putida* Rs-198 | ↑ Absorption of the $Mg^{2+}$, $K^{2+}$, and $Ca^{2+}$. ↓$Na^{2+}$ from the soil | (Yao et al. 2010) |

**Table 15.2** PGPB for countering the drought, salinity, and temperature stress

| Stress type | Microbe | Crop | Reference |
|---|---|---|---|
| Drought | *Pantoea agglomerans* | Wheat | (Grover et al. 2011) |
| Drought | *Rhizobium* sp. | Sunflower | (Grover et al. 2011) |
| Salinity | *A. piechaudii* | Tomato | (Grover et al. 2011) |
| Salinity | *B. amyloliquefaciens* | Wheat | (Grover et al. 2011) |
| Temperature | *P. fluorescens* | Wheat | (Egamberdieva et al. 2009) |

antioxidants, ethylene degradation, and assimilation of abscisic acid, which ultimately force the stomata to close and avoid water loss from leaves. Table 15.2 reveals the role of different PGPB in abiotic stress factors.

The mycotic role in alleviating drought stress can never be neglected as the mycorrhizal in soil improves the soil exploitation via hyphal association to promote the water uptake and deepens the root penetration in dry areas. The native alterations resulted from mycorrhiza helps soil microbes to give maximum output against abiotic factors. It is hypothesized that the production of ABA is the symptom that sequentially regulates the role of aquaporins in both AMF crops and non-AMF crops during drought stress. Under the drastic effects of abiotic factors, the arbuscular fungi help in the advancement of growth by directing the transpiration ratio, absorption, and photosynthesis index by taming the texture of soil, making it a better substitute under drought. A significant difference was recorded between two parameters including the transpiration rate and stomatal activity in plants colonized with arbuscular mycotic fungi and non-AMF plants. Similarly, a comparative study was conducted between arbuscular mycotic fungi-inoculated plants and non-arbuscular-treated plants. Results showed a constant growth pattern for AMF-treated plants with restricted water supply in contrast to non-AMF plants with full-time water supply. The seedlings of an evergreen oak plant with hard structure with AMF inoculation mitigate the drought stress effectively and showed an advanced development, nutrition, osmotic potential, and antioxidant enzyme activity and proposed the logical use of AMF in the refurbishment of vegetation and grasses in the Karst region that occupies 12% of overall landform (Zhang et al. 2013).

## 15.4 Plant Growth-Promoting Bacteria, Rhizobacteria, and Fungi [PGPB, PGPR, and PGPF]

### 15.4.1 PGPB

Several microbial species have been reported to provide enough support during the abiotic stress to maintain the plant growth at optimum level by generating the cytokines, antioxidants, and ABA production degrading the toxic reactive oxygen species. PGPRs produce different substances like IAA and gibberellins to tolerate the stress via improving nutritional uptake through enhanced root volume against oxidative stress. The agricultural plant species under abiotic stress performed very efficiently when treated with ACC containing PGPR by elongating the length of the roots to uptake water effectively from deeper soil. The PGPR can make up to 107 CFUs/g of root and constructively affect the plant's development, these growth-promoting microbes not only increase the yield but also made the plants healthy with a better fresh look. The hot spot for colonizing the microbes is rhizosphere, as the microbes are divided into two main categories as PGPR and PGPF. The solubility of inorganic materials, availability of organic substances, secretion of plant hormones, and chelators are the key roles done by PGPR and

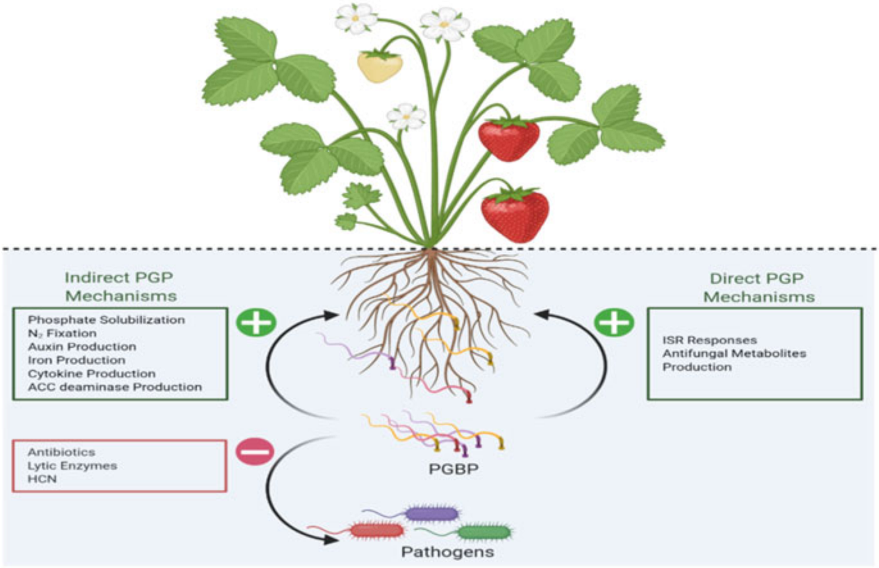

**Fig. 15.3** Illustrates the direct and indirect mechanism of PGPB in plants

PGPF to enhance the yield and health of plants (Spaepen et al. 2009). Figure 15.3 illustrates the direct and indirect mechanism of PGPB in plants.

### 15.4.2 PGPR

The term PGPR is referred to those growth-promoting soil microbes, especially the bacteria that reside on plants' roots, leaves, or within the tissues by making a positive association. A wide range of PGPR has been reported including the *Pseudomonas*, *Rhizobium*, *Azospirillum*, *Azotobacter*, *Klebsiella*, *Enterobacter*, *Alcaligenes*, *Arthrobacter*, *Burkholderia*, *Bacillus*, and *Serratia* (Ahemad and Kibret 2014). Their key function is to perform the vital processes needed for plant growth like nitrogen fixation, phosphate solubility, regulation of plant hormones, provocation of iron Fe radicals, and indirect support by precluding from toxic plant pathogens (Sayyed et al. 2013). The indirect pathway adopted by PGPR for the advancement of host plant growth is the provision of an environmental niche and generation of systematic resistance against a wide array of pathogens and abiotic stress factors.

The transformation of the nitrogen N present in the atmosphere into ammonia via associative or symbiotic relation between microbes of plant and soil for the betterment of plant health is termed as the biological nitrogen transformation or fixation process BNF. From all the nitrogen fixation microbes, only the rhizobia (bacteria-forming root nodules) have keen importance due to their capability of making a positive association with legumes. After localizing in the root system, these microbes made nodules that act as the storage house of fixed nitrogen, and this whole process is so intricate that only specified rhizobia affect a few selected plants genera from a species. Several scientists reported the enhancement of N content of legumes, especially in the pulses after the use of rhizobia biofertilizers by creating a healthy symbiosis between plant and N-fixing PGPR. For the enhancement of phosphorous uptake by plants, the use of phosphate-solubilizing bacteria (PSB) as inoculant is a very common practice at the commercial level. The frequently used PSB includes *Pseudomonas*, *Bacillus*, and *Rhizobium*. The generation of siderophores by PGPR helps plants to uptake the Fe iron content by reversibly chelating it as labile complex. The siderophores have a lower molecular weight and act as extracellular components for $Fe^{+3}$. A strong complex is formed between the siderophores and the $Fe^{+3}$ that passes through the cell membrane via receptors. Till now after the detection of 500 different siderophores, only 270 are well-characterized (Ali and Vidhale 2013). PGPR not only generates the siderophores but also eliminates the unwanted microbes from the root ecological niche, while the secretion of hydrogen cyanide (HCN) and an antimicrobial substance to alleviate the root diseases resulted from the Phyto-associated pathogens.

The activation of ISR with the help of PGPR against a wide array of plant pathogens is another important mechanism, and in general, it closely resembles the classic pathogen-induced resistance, where the noninfected areas of the plant from the previous microbial attack become stronger. Figure 15.4 shows the comparison between plants treated with and without PGPR. The lysis of pathogenic fungal

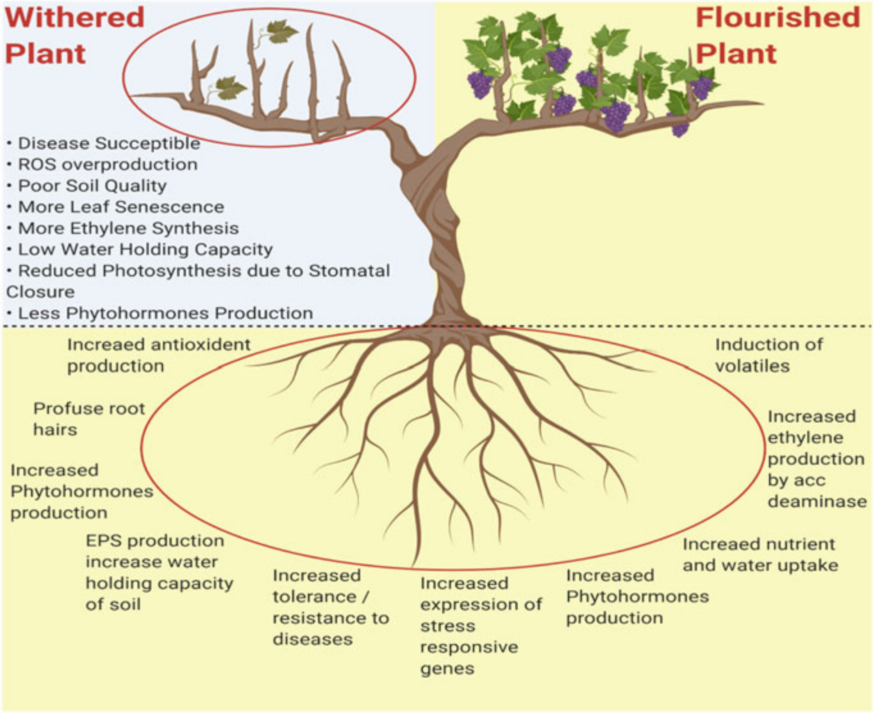

**Fig. 15.4** PGPR effects on the plants

hyphae occurs by the production of hydrolytic enzymes including the chitinases, proteases, and lipases due to anti-agonistic activities of PGPR. The bacillus species are taken as one of the best in the agriculture sector for more than a century as microbial inoculating agents in the form of pesticides, fertilizers, and fungicidal for growth promotion (Sivasakthi et al. 2014).

Likewise the *Bacillus*, another astounding PGPR belongs to the genus *Pseudomonas* and several research studies have confirmed its significance in phytocommunity due to its effective biocontrol chattels and growth development (Tewari and Arora 2015).

### 15.4.3 Plant Growth-Promoting Fungi (PGPF)

The plant growth-promoting chattels of the fungi resident of the plants rhizosphere have been discussed by several scientists. Of these PGPF, *Phoma*, *Penicillium*, *Aspergillus*, *Fusarium*, *Trichoderma*, and arbuscular mycorrhizal fungi (AMF) are the species considered specifically due to their effective role in disease subdual and growth enhancement. The pathway by which the PGPF helps plants is via the phytohormone production, organic matter degradation, assurance of the unavailable

**Table 15.3** Effect of PGPF on plants against abiotic and biotic stress

| PGPF | Effect | Reference |
|---|---|---|
| *Penicillium* spp. GP15-1 | Growth enhancement and ISR against leaf infection by the anthracnose pathogen *Colletotrichum orbiculare* | (Hossain et al. 2014) |
| *Penicillium* sp. (UOM-PGPF 27) | Seed quality enhancement of pearl millet and induce resistance to downy mildew disease | (Murali et al. 2012) |
| *Penicillium oxalicum* | Plant growth and induces resistance in pearl millet against Downy mildew disease | (Murali and Amruthesh 2015) |

soil nutrients by effective solubilization, and give safety from the biotic and abiotic stress-causing factors (Khan et al. 2012). Apart from the direct effect on plant growth, some other processes are also involved in the indirect growth enhancement by excluding the niche, activation of antibiosis, fungal parasitism, ISR, and predator avoidance. AMF's importance in the plant's growth has been acknowledged for decades ago, and the main character of AMF involves the uplift of nutrients typically the phosphorous P. Crops and the plants inoculated with AMF exhibited an enhanced growth and yield ratio. The multifunctional property including both the nutritional and nonnutritional aspects of AMF has now been successfully acknowledged by scientists. The AMF belonging to the family *Gigasporaceae* are the efficient phosphorous P uptakers, whereas the AMF from the *Glomeraceae* is best known for possessing root pathogen protective abilities. Table 15.3 explains the role of PGPF on plants, while Fig. 15.5 explains the role of PGPF in enhancing the roots and shoots growth, and protection from the airborne and soil-borne pathogens by triggering the defense mechanism.

### 15.4.4 Roles of PGPM in Agriculture Sustainability and Improving Soil Fertility

The term agricultural sustainability can be explained as the adoption or implementation of practices to cope up with the present and future demands of edible products, human health, and ecological safety to fully benefit the human race. In other words, agriculture sustainability must ensure social, economic, and environmental stability. To make the agriculture sector sustainable by the implementation of microbial use is a burning issue. It is now experimentally proved that manipulation of rhizosphere by root and bacterial associations can effectively enhance the production quality and sustainability of agriculture. For any sustainable agroecosystem, the application of plant growth-promoting microbes PGPM as a biofertilizing agent in contrast to the synthetically prepared nitrogen N, potassium K, and phosphorus P fertilizers has been acknowledged as the best practice for ecological safety (Mishra et al. 2015).

A soil is said to fertile if it is capable of providing the basic physiochemical and biological prerequisite necessary for the growth of crops and plants. The advancement of technology in agriculture and irrigation systems and extended usage of

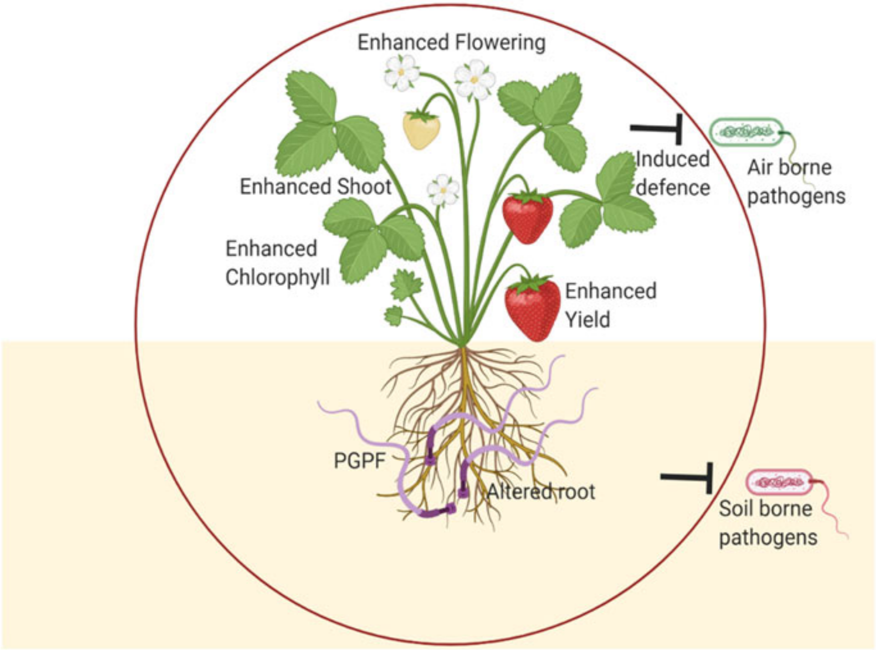

**Fig. 15.5** Role of PGPF in plants

synthetically prepared fertilizers have led to the degradation and imbalancing of the naturally available nutrients of the soil, hence making it nonviable for effective yield. A land that is under repeated cultivation or is cultivated with the same crops, again and again, mislays its natural organic matter and as a result, the crumbling structure, bulk density, porosity index, and biological activity of soil are disturbed. Soil organic matter is one of the crucial components indicating soil fertility or quality. Several different mechanisms are carried out by PGPM for sustaining the fertility of the soil, of which one of the direct benefits by PGPM to the soil is their vibrant role in maintaining the SOM. Soil conservation and restoration are the main attribute carried by SOM. Just like the soil organic matter (SOM), the presence of dissolved organic components (DOM) in the soil retrieves the ammonium $NH^{+4}$, adjust the microbe triggered N conversions, and maintain carbon-to-nitrogen ratio (C: N) of soil. The soils affected with lower SOM levels when treated with PGPM exhibited an enhancement of yield, production, and quality of crops. For efficient water movement and retention in the soil, the aggregation of soil is the chief physical factor that improves the fertility index.

Out of the 25,000 known fungal soil species that contribute to 70% of whole microbes, AMF holds a share of about 50% of that microbial biomass and by adopting several mechanisms impart a significant influence on the carbon C, nitrogen N, and phosphorous P soil contents. The mycelium of AMF penetrates deeper into the soil for the carbon recycling process of soil to improve the soil texture. The advantage of fungal hyphae is that they can penetrate up to 8 cm beyond the root extent with an increased surface area to regulate the C content of soil (Corrêa et al. 2015).

## 15.4.5 Endophytes as Source for Bioactive and Novel Compounds in Plant Health

The emergence of two new techniques the bioremediation and phytoremediation for the endophytes has gained much impetus from the last few years. The biological control or biocontrol, stimulation of plants or the phytostimulation, and the use of biofertilizers are the three main mechanisms in which endophytes play a crucial role. The specialty of endophytes is the release of specified bioactive metabolites or agents without causing any damage to the host plant (Liarzi et al. 2016). The resistance of the plants has been increased by the synthesis of different biologically active agents from endophytes. These metabolites possessing anticancerous, antibacterial, antidiabetic, and antiviral chattels are now successfully used in the pharmaceutical industry for human welfare. The separation of the alkaloids, terpenoids, some steroids, peptides, flavonoids, and quinolones from the *Azadirachta Indica* also probed for further industrial and pharmaceutical purposes. Several biologically active metabolites possess the antioxidative, antitumor, anti-leishmanial, cytotoxic, and fuel-generating chattels (Wang and Dai 2011). Cryptocandin, pestaloside, cryptocin, ecomycins, pestalopyrone, and pseudomycins are some of the examples of the antifungal agents produced by the endophytes for the sake of growth advancement. The release of biological metabolites from endophytes can be affected in two ways either genetically or physiochemically. One of the innate sources for the collection of flavonoids, saponins, and carbohydrate tannins is the mycotic endophytic species (Liu et al. 2016). Contrary to this, some beneficial mechanisms regulated by endophytes include extracellular enzyme production, nitrogen fixation, antioxidation, phytohormone production, ACC deaminase activity, heavy metal stress control, biological control, and nutrition deficiency stress.

### 15.4.5.1 Extracellular Enzyme Production

The regulation of the extracellular enzyme from the endophytes has a crucial role in maintaining the plant's health. These extracellular enzymes are also required for the colonization of endophytes in the plants. Wide range use of the extracellular and exo-enzymes has been documented by several researchers in the biotechnology field, for example, in the fermentation process. The cell wall lysis capability of several enzymes, especially the β-1,3-glucanase, protease, lipase, and chitinases aligned with endophytes, can be used as a biological control against pathogenic microbes (Fouda et al. 2015). Xylanases, cellulases, pectinases, lipases, proteases, phosphatases, amylase, and glucosidases are some of the enzymes that are successfully used in host plant during endophytic microbial progression but reported to reduce the pathogenic load when acknowledged as phytoprotective agents (Pereira et al. 2016).

#### 15.4.5.2 Biological Control Agents

The borer insect of the sorghum crop was biologically controlled by an endophytic fungus named *Beauveria bassiana.* Similarly different endophytic bacteria have antagonist chattels in vitro against other endophytic bacteria, like the effect of *Bacillus subtilis* is antagonistic against *Botrytis cinerea* (rotting causative agent) during tomato storage. The emergence of new endophytes like *Burkholderia pyrrocinia* JK-SH007 and *Bacillus cepacia* has been successfully tested as a biocontrol agent against poplar canker (Ren et al. 2011).

The latest approach in the category of the biocontrol strategy is the induction of mutation at the genetic level, by expressing that mutation the endophytes secrete an anti-pest substance of protein nature like lectins to control the insects. The *Chaetomium globosum* YY-11, *Enterobacter* sp., and *Bacillus subtilis* were isolated from the rape seedlings and rice, respectively, and were used for the expression of *Pinellia ternata* agglutinin (*PtA*) gene. Previously, sap-sucking pests were controlled by expressing the *PtA* gene by using different endophytic mycotic and bacterial strains. Another example is the biocontrol of the black planthopper *Sogatella furcifera,* by expressing the *PtA* gene using the *Enterobacter cloacae* bacteria (Zhang et al. 2011).

#### 15.4.5.3 ACC Deaminase and Phytohormone Production

The production of ACC deaminase from the endophytes benefits the plants in their growth and improves stress tolerance capacity by degrading the ethylene that is the predecessor for the ACC production. The reduction in the plant's ethylene levels done by degrading the predecessor ACC deaminase can reduce plant ethylene levels by cleaving its precursor (1 aminocyclopropane-1-carboxylate) to 2-oxobutanoate and ammonia ultimately triggering the ethylene production (Glick 2014). Ethylene, being the crucial phytohormone actively, takes part in bacterial localization in roots, seed germination, and capacity building of plant against several stress factors. The assimilation of ethylene against stress factors is proved to be fatal for the health of plants. Besides the provision of relief against the stress factors, the ACC deaminase enzyme evokes the bacterial endophyte settlement. A case study documented the loss of root elongation capacity in canola plants upon inactivation of the ACC deaminase gene.

Indole-3-acetic acid (IAA) is one of the chief auxins resultant from the growth-promoting endophytes. Auxins by degrading the ethylene enhance the development of the root and can be considered as an efficient tool for managing the salt stress in halophytes (Witzel et al. 2012). IAA by interfering with the host's defense mechanism enhances the capability of endophytes for colonization into roots; thus, the production of such phytohormones by endophytes that triggers the colonization character must be considered significant given in Fig. 15.6. Abscisic acid (ABA), indole-3-acetic acid (IAA), and the gibberellins are the phytohormones produced by a halophyte *Prosopis strombulifera.* ABA is an important hormone that regulates the plant's growth, seed and bud dormancy, stomatal closure, controls the hydal balance, and tolerates the osmotic stress. An experimental trial confirms the improved health

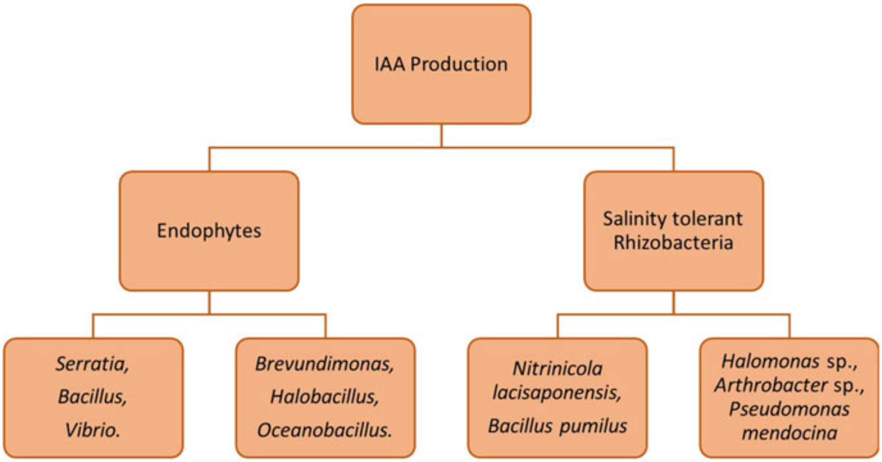

**Fig. 15.6** Phytohormone IAA production against salinity

of wheat plants sown over saline soil but treated with IAA-bearing rhizobacteria (Tiwari et al. 2011).

#### 15.4.5.4 Nitrogen Fixation

Besides other qualitative traits of endophytes in plants, nitrogen fixation is one of the main components adopted by plants against unfavorable environmental circumstances. Several root endophyte species (*Azoarcus* spp., *Acetobacter diazotrophicus, and Herbaspirillum* spp.) take part in the nitrogen fixation (Ruppel et al. 2013). The fixation of nitrogen by endophytes under harsh environments improves the overall health status of the plant even if present in lower amounts. This mechanism is so vivid that it explains the nitrogen content status either for the microbial demand or available for the host's plant. *Paenibacillus* P22 an endophyte taken from the poplar trees convened the fixed nitrogen toward the total N content and also tempted alterations in the metabolism (Hardoim et al. 2015).

#### 15.4.5.5 Heavy Metal and Nutrient Stress

Almost 25–80% of the cultivated crops had got deteriorated by heavy metals toxicity, which is one of the major abiotic stress-causing factors. The main reason for lower production and abridged soil fertility index of the acidic soils is the presence of higher contents of manganese (Mn) and aluminum (Al) along with minerals deficiency of potassium (K), magnesium (Mg), phosphorous (P), and calcium (Ca). The presence of traces of heavy metals in the soil leads to roots toxicity and affects overall productivity. The ability of bacterial endophytes regarding the mobility and immobility of metallic cations has to be acknowledged, as they interfere with the possible availability of cations to the plants (Pandey et al. 2016). Cadmium (Cd) is another metal found in the soil, and the (Cd) affected soil when treated with dark septate endophyte (DSE) *Exophiala pisciphila* in the *Zea mays* trial. The advanced antioxidative enzymes working was recorded (Friesen et al. 2013). The availability of micro- and macronutrients or minerals by the endophytes to the plants takes place in several ways. Microbes that are responsible for the fixation of nitrogen N can also process the root exudates and in reverse provide the N for amino acid fusion. The growth promotion by endophytes is dependent on the production of phytohormones gibberellins (GAs), IAA, phosphate solubility, siderophore generation, and the transfer of vital vitamins to the plants. Many studies have confirmed the better nutrient uptake upon endophyte treatment, phosphorous P availability, and solubility was improved in wheat and rice by *Pseudomonas* sp. via regulation of GAs. Similarly, the zinc (Zn) endorsement also improved in the wheat crop by using the *Azotobacter chroococcum* (Abadi and Sepehri 2016).

## 15.5 Development of Microbial Inoculum for Small-Level Farming

For enhancing the crop production, there is an inclining demand for microbial inoculums availability in commercial markets all over the world (Lobo et al. 2019). A standard microbes inocula must be packed with such material that is able to provide optimum environment like carbon level, required pH, and water besides maintain a longer shelf life and viability of microbes. Some other requisites for microbial inocula includes its availability, environment friendly nature, nutritional supplementation, and cost-effectiveness (Soumare et al. 2020). A study conducted by Gitonga et al. (2021) stated how traditional and organic small-scale farming styles and soybean cultivars affect native *Bradyrhizobium* presence in soil. Another method " On Demand Seed Inoculation" has been adopted by seed manufactures, which involves the farmers will of treating the seeds with microbial inoculums (PGPB, PGPF, PGPR) before packaging and delivering (Deaker et al. 2012). This approach can be implemented easily on small-scale level along with some awareness campaigns and national-level conferences, especially in the developing countries, but still more research and partnership are required for bioprospecting the efficient PGPMs in local environment.

## 15.6 Abiotic Stress Factors Concerning Forest Ecosystem and Global Economy

The abiotic stresses or disturbances are referred to as the constraints resulted from nonliving elements, imparting an essential effect either positively or negatively on the forest's ecology. Forests' configuration, structure, and working are crucial for the maintenance of biotic diversity and facilitating the restoration as shown in Fig. 15.7. About 4000 different abiotic disturbances cause events to take place in the last decade, resulting in the loss of 1 million precious human lives, affecting around 2.5 billion inhabitants around the globe, and economic losses of a trillion US dollars

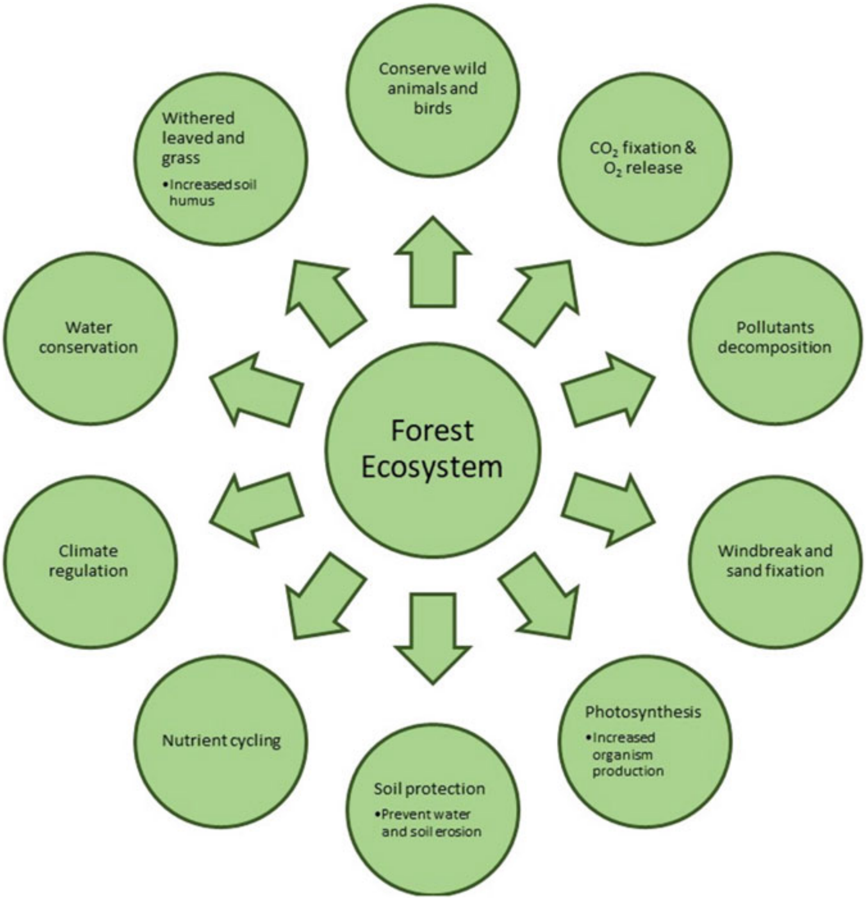

**Fig. 15.7** Forest ecosystem's role in maintaining biodiversity

(RCS 2010). Such devastating losses indicate the importance, extent, and sternness of abiotic conflicts.

- In 2005, the southern part of Sweden was hit by a severe wind storm (abiotic stress) and damaged around 1.2 million hectares of forests including the natural ecological niche (FAO 2020).
- More than 32,000 events of the fires were recorded by Russia in 2010 burning up to 2.3 million hectares of conifers along with mixed trees forests. 62 people lost their lives, while hundreds of houses were damaged (Williams et al. 2011).
- In 2010, floods killed about 2000 people and mutilated about 1.7 million homes in Pakistan. Millions of livestock individuals, forests, wildlife, and millions of hectares of ripe crops were destroyed (UNEP 2011).

To counter the escalating frequency and intensity of the abiotic disturbances to affect the natural habitat of forests, an adaptive forest management approach is required to conserve the worldwide natural forests resources. To moderate or avert the prospective influences, it is necessary to promote the cultivation of varying plant species, amalgamated cropping confirmation for resistance, and avoidance of growing trees more disposed to abiotic stress. A collaborative approach by all the countries to counter the deforestation and assurance of the reforestation will reduce the atmospheric carbon dioxide level, ultimately improving the status of global warming, and ozone depletion.

## 15.7 Future Challenges and Conclusion

To boost the agricultural yield without making damage to natural ecosystems is a vital challenge for scientists. Farmers generally made efforts to increase the soil fertility by adopting various physiochemical methods, like the use of expensive fertilizers, pesticides, insecticides, and fungicides to uplift the production of crops, but unluckily they neglect the essential role frolicked by the soil microbes for the enhanced yield (East 2013). Thus, it is necessary to encourage farmers regarding the use of PGPM as biofertilizers, to prevent the crops and plants from lethal pathogens, and helps to positively combat against abiotic and biotic stress factors. Still another drawback on field conditions is the strangeness regarding the role of PGPM for pollutant degradation, spoiled cultivated land organization, and mitigation of stresses. Recently, an extensive evolution has been done for assuring the role of PGPM in the optimum health, yield, and growth of plants. Through advanced technologies like the "Omics," scientists become able to provide data on the physiological and anatomical aspects of genes and proteins in the plant's growth process and through metabolomics cellular metabolites and exudates got recognized (Swarupa et al. 2016). The adoption of such highly advanced technologies will help sort out the fabricated bioformulations having a complete pathway from inoculation to regulation of hormones and several other applications. Currently used bioformulations lacking in reliability and quality, this scenario can be altered by fabricating such bioformulations that completely coordinate with PGPM accompanied by other additives and metabolites (Arora and Mishra 2016).

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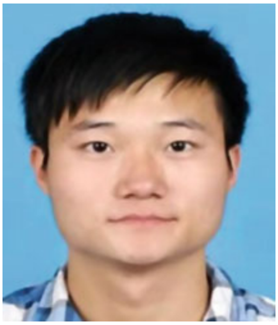

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# 16

# Mainstreaming of Underutilized Oilseed Safflower Crop Through Biotechnological Approaches for Improving Economic and Environmental Sustainability

Astha Gupta

## Abstract

Oilseed crops are the backbone of agriculture for vegetable oil production. Safflower is one of the considerable oilseed crops because of high-quality nutritious seed oil and biofuel production. A range of species (16 species: wild and cultivated) are well-known internationally, of which few are *C. tinctorius*, *C. palaestinus*, *C. oxyacanthus*, *C. lanatus*, and *C. creticus*. However, *Carthamus tinctorius* L. is domesticated species and is extensively cultivated across world including India for seed oil under rainfed condition. Different parts of plant including seed, straw, flower, root, and shoot, are profitable, and are exploited for herbal dye production, agrochemicals, phytoremediative ability (due to presence of antioxidant compound: phytochelatin and α-tocopherol), as well as medicinal and pharmaceutical purpose. Moreover, safflower exhibit deep root system and xerophytic spines (adaptive traits) that represent its ability to tolerate drought and heat, and therefore would be competent climate-resilient crop. Nutritionally safflower oil is very important for medicinal purpose and economical level also which is due to presence of low amount of saturated fatty acids (palmitic and stearic acids) and high amount of unsaturated fatty acids (oleic and linoleic acids) that minimize the risk of cardiovascular and other severe diseases. However, promising safflower crop has not been utilized up to its potential by reason of low yield, spines, and disease susceptibility. Flowers of safflower are extensively harnessed for religious ceremonies, and their floral extract is a rich source of herbal dye (safflomins and carthamin), which can be further utilized as natural coloring agent for textile and food industry. Plant extracts, secondary metabolites, and bioactive compounds of safflower possess antioxidant activity,

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anti-inflammatory effects, weed control, pain relief properties and is a traditional herb. Therefore, there is a need to focus on their improvement due to agricultural, environmental, and economic importance of safflower for human consumption and associated industries. Application of genome-wide molecular markers, quantitative trait loci (QTLs), and biotechnological approaches would be beneficial for the improvement of yield, oil, and stress resistance (abiotic and biotic)-related traits. Most adaptive traits may be explored and introgressed from wild relatives and/or appropriate germplasm to cultivated individual of safflower under adverse environmental conditions. In addition, development of high yielding spineless varieties with good quality oil content (desirable traits) may be developed through molecular breeding, gene pyramiding, and marker-assisted selection that will be applicable for safflower cultivation at small farmer's field also.

**Keywords**

Safflower · Oilseed crop · Molecular biology · Biotechnology · Environment and biofuel

## 16.1 Backdrop

Oil and fuel are incredibly essential components and alarming issue at global level due to continuous consumption and limited resource availability. However, oilseed crops are second most important sector after cereals, which need to focus in agriculture area because of high demand and speedy exploitation (Laxmi and Sahu 2020). India has cultivated nine different oilseed crops (255 lakh hectares) in 2018–2019 and has been major oilseed producer about 323 lakh tons (average yield of 1265 kg/hectare). Furthermore, India has been second largest importer and third largest consumer of edible oil (Joseph 2020). In the past, group of agriculture scientists and ministries have established the "Technology Mission on Oilseeds" (TMO) in 1986 to enhance the edible oil productivity and quality, which was also known as "*Yellow Revolution*," but later in 1998, import duty was reduced from 65 to 15% (Shukla and Chaudhary 2020; Joseph 2020). Therefore, oilseed crop improvement and its yield should be noticed further to fulfill the need of vegetable oil and related economical and agricultural products.

Out of nine oilseed crops exist, seven are known to produce edible oil (soybean, rapeseed–mustard, groundnut, sunflower, sesame, niger, and safflower) and remaining two produce non-edible oil (castor and linseed), which may be cultivated in diverse agroclimatic conditions (Laxmi and Sahu 2020). However, oilseed crops are primary source of vegetable oil that are grown mainly under rainfed conditions and some secondary sources of vegetable oil are also reported like rice bran, coconut cottonseed, tree-borne oilseeds (TBOs), and oil palm (Laxmi and Sahu 2020). Vegetable oil has considerable amount of fat-soluble vitamins, and essential fatty acid and nutrition value therefore are extensively valuable for cooking purpose and human health (Osunrinade et al. 2020). However, peanut oil and refined blended plant oil have adverse impact on health due to association with type 2 diabetes, but sesame oil, soybean oil, and canola oil were not correlated with diabetes risk (Zhuang et al. 2020).

Moreover, sustainable management of waste vegetable oil is achievable through transformation into biodiesel via transesterification process that improve the energy conservation and environmental impact (Di Fraia et al. 2020). In addition, waste vegetable oil is a good resource of renewable energy and alternative fuel that may contribute to smart and resilient society in world energy crisis situation (Dey and Ray 2020; Di Fraia et al. 2020). Further, fossil-based fuels would be a demand of future generations (climate mitigation and energy efficiency) to control pollution and bioresource accumulation worldwide. Therefore, substitute fuels like biodiesel would be a upcoming energy requirement (Hosseinzadeh Samani et al. 2020; Yesilyurt et al. 2020). Biodiesel is non-hazardous, biodegradable, recyclable, and renewable fuel and is obtained from animal fats, vegetable oils, and their wastes using different techniques but the only drawback is dependency on feedstock (Yesilyurt et al. 2020). The biodiesel industry has been growing internationally, particularly in India with optimized kinetic and thermodynamic factors for sustainable biodiesel production using waste vegetable oil (Pugazhendhi et al. 2020). Safflower (*Carthamus tinctorius* L.) is considered as imperative oilseed crop, which has been widely cultivated in Asia, Europe, Australia, and Americas for its high-quality nutritious vegetable oil and industrial utilization. Safflower is a potential cash crop and dried flower is a significant raw material for cosmetics, dyes, herbs, bioactive compounds, and again pigment associated with flavonoids (primarily quinone chalcones) (Chen et al. 2020). It is also exploited for traditional medicines as a purgative, analgesic, antipyretic, an antidote to poisoning, menstrual problems, postpartum hemorrhage, osteoporosis, medicinal and pharmaceutical applications (Turgumbayeva et al. 2018).

Safflower exhibit deep root system, xerophytic spines, and low water requirement and represent an ability to with stand drought and heat (Bhattarai et al. 2020). Crop rotation of safflower would be beneficial for local production of healthy vegetable oil for human consumption and protein-rich meal for the animal also. Safflower oil is economically very important due to the presence of low amount of saturated fatty acids (palmitic and stearic acids) and high amount of unsaturated fatty acids (oleic and linoleic acids) (Kutsenkova et al. 2020: Chakradhari et al. 2020). Unsaturated fatty acids of safflower seed oil make it a high-quality nutritional vegetable oil with minimized risk of cardiovascular diseases, obesity, and metabolic disorders (Zhang et al. 2020; Nimrouzi et al. 2020). Although safflower is a promising crop for oil production, it still has not been utilized in agriculture due to various shortcomings like low yield, spines on mature flowers, insect–pest susceptibility, and limited information about germplasm's genetic diversity (Ali et al. 2020). Flowers of safflower are extensively used in religious ceremonies, and their floral extract is a rich source of pigment or herbal dye (safflomins and carthamin), which can be further utilized as coloring agent in textile and in food industry (Patanè et al. 2020; Adeel et al. 2020).

In summing up, most of the safflower plant parts, its bioproducts, and commodities are fruitful for environment but has not been explored at large scale due to few undesirable characteristics. However, spines present on head and leaf of safflower plant are one of the considerable feature that pose difficulty in harvesting manually, low yield, disease sensitivity, and farmer's limited knowledge. Therefore, owing to agricultural and economic importance, there is a need to spotlight their improvement for better human consumption and current allied industry.

## 16.2 Safflower: Neglected Oilseed Crop

### 16.2.1 General Narrative as False Saffron

Safflower is well-known like false saffron, belongs to the Compositae or Asteraceae or Daisy family that is branched, thistle-like annual plant, and herbaceous species normally grown in an arid climate condition of Southern Asia, Egypt, China, Iran, and India (Shirwaikar and Khan 2010; Delshad et al. 2018). However, saffron authentication and adulteration was examined using safflower plant-derived adulterants of saffron with high-performance thin-layer chromatography (HPTLC) coupled with multivariate image analysis (MIA) (Amirvaresi et al. 2020). Recently, fraudulent admixture with allied plants "safflower and calendula" was explored and *Safranal* was detected as a marker to discriminate saffron from safflower (Farag et al. 2020).

### 16.2.2 Distribution of Species and Specific Characteristics

Generally, it is a dicot, largely grown in the arid and semi-arid regions of the wide geographical zones across world and with considerable production in Kazakhstan and India (Turgumbayeva et al. 2018; Ali et al. 2020). The Kazakhstan species of safflower- Ak-Mai is exploited for 20 biologically active compounds from volatile oil of flower (Turgumbayeva et al. 2018). In a recent study, 89 safflower accessions were grouped in same cluster associated from Iran, India, Pakistan, and Turkey because of same genetic makeup, whereas accessions related to Near East (China and Afghanistan) were found in different clusters (Hassani et al. 2020). Further, this was based on hypothesis that safflower was domesticated in west of Fertile Crescent and then extended through Europe and Africa.

Moreover, 16 species of the genus Carthamus from 34 populations were examined using nuclear DNA samples and flow cytometry (Garnatje et al. 2006). They described species and origin, which are as follows: (1) *Carthamus alexandrines:* Egypt, (2) *Carthamus anatolicus* (Boiss.): Isreal, (3) *Carthamus boissieri:* Greece, (4) *Carthamus creticus*: Morocco, (5) *Carthamus dentatus* Vahl ssp.: Greece, (6) *Carthamus glaucus*: Turkey, (7) *Carthamus lanatus*: Greece and *Carthamus lanatus* L. ssp. *Montanus*: Tunisia, (8) *Carthamus leucocaulos* Sibth. & Sm: Greece, (9) *Carthamus turkestanicus* Popov: Armenia, (10) *Carthamus gypsicola* Iljin:

**Fig. 16.1** Safflower (*Carthamus tinctorius L.*) growing at Agricultural Research Station, University of Delhi, Bawana Road, New Delhi, India (latitude: 28°38′N, longitude: 77°12′E and altitude: 252 m)

Armenia, (11) *Carthamus palaestinus* Eig: Isreal, (12) *Carthamus persicus* Desf. exWilld: Lebanon, (13) *Carthamus oxyacantha* M. Bieb: Iran, (14) *Carthamus tenuis*: Isreal, (15) *Carthamus nitidus* Boiss: Isreal, and (16) *Carthamus tinctorius* L.: Kazakhstan. Genus *Carthamus* represented three basic chromosome numbers ($x = 10$, 11, and 12) and further explained putative hybrid origin includes allopolyploid species *C. creticus* and *C. turkestanicus* ($2n = 64$) through combination of *C. lanatus* ($2n = 44$) with *C. leucocaulos* ($2n = 20$) and *C. glaucus* subsp. glaucus ($2n = 20$), respectively (Garnatje et al. 2006; Khidir and Knowles 1970).

Lipophilic and hydrophilic components (bioactive compound) were explored in seed oils of *C. tinctorius* (cultivated; Fig. 16.1) and *C. oxyacantha* (wild) and found predominated form of carotenoids (over 37% which is hydrophilic in nature), while elevated levels of lutein (15%) and β-carotene (25%) were detected primarily in *C. Oxyacantha* (Chakradhari et al. 2020). Considerable amount of fatty acid was investigated in *C. tinctorius* and *C. Oxyacantha* includes stearic (~2%), palmitic (~5%), oleic (~15%), and linoleic (~78%) in addition to α-tocopherol and β-sitosterol (phytosterols) (Chakradhari et al. 2020). Shafiei-Koij et al. (2020) constructed a phylogenetic tree using *fad2-1* gene sequences and concluded that *C. tinctorius* was closely related to two wild species, i.e., *C. palaestinus* and *C. oxyacanthus* due to grouped in same cluster and *C. lanatus* was the probable progenitor of *C. Creticus* (synonym *C. boeticus* and *C. turkestanicus*). Approximately, 90% loss in yield of safflower reported due to Alternaria leaf spot (ALS) diseases. (Kammili et al. 2020). There are some wild species, which would be good source of biotic and abiotic stress including *C. palaestinus*, *C. Lanatus*, and *C. oxyacanthus*. However, cultivated species of safflower were not exhibited ALS resistance but some wild species like *C. palaestinus* and *C. lanatus* were tolerant, therefore promising source to recover yield from disease (Kammili et al. 2020).

### 16.2.3 Safflower (*Carthamus tinctorius* L.): A Cultivated Species

Safflower (*Carthamus tinctorius* L.) is a domesticated species, and its flowers and seeds are valuable component in traditional Chinese medicine due to recovery of cerebral blood flow and nutritional vegetable oil (Ren et al. 2020; Hosseinzadeh Samani et al. 2020; Kutsenkova et al. 2020). *Carthamus tinctorius* L. is a diploid ($2n = 24$), self-pollinating sps. with approximately 1.4 GB genome size and one of the essential underutilized oilseed crop as compared to other oilseed crops, which is resilience to dry condition (Fig. 16.1).

### 16.2.4 Effect of Climatic Conditions on Growth and Development

Safflower cultivars behave differently in diverse environmental conditions like temperature and photoperiod. For example, Padideh cultivar is a late flowering safflower variety, which is sensitive to photoperiod and most suitable for growing in cold regions as compared to warm regions when compared with other cultivars (Torabi et al. 2020). Further cooperative effects of sowing time and plant density (25 and 50 plants/$m^2$) were investigated for different traits in safflower (cv. Catima) like floret and pigment production (safflomins and carthamin) in semi-arid Mediterranean environments (Patanè et al. 2020). They also found high plant density and delayed sowing that significantly increased heads and decreased florets' yields (Patanè et al. 2020). In contrast, early sowing elevated amount of pigment content in florets approximately 54.6 g/100 g safflomins and 2.97 g/100 g carthamin accordingly (Patanè et al. 2020). However, pre-treatment of high temperature is helpful in producing high ethanol yield from safflower straw (Hashemi et al. 2020).

Safflower oil yield and water use efficiency (WUE) were considerably improved under pre-season irrigation, but extreme in-season irrigation did not benefit these traits (Bhattarai et al. 2020). However, key adaptive traits of safflower (*Carthamus tinctorius* L.) i.e. deep root structure, was examined under semi-arid agriculture and rainfed conditions (Bhattarai et al. 2020). Other very promising and potential characters are low water requirements and capability to abide to abiotic stresses that make safflower an attractive alternative crop for the Southern High Plains that supports high water-demanding crops (Bhattarai et al. 2020).

A prediction model was constructed to understand the plant performance in terms of days to flowering, plant growth, and yield (Torabi et al. 2020). Some parameters were selected for creation of prediction model related to flowering days in safflower that includes (1) a field experiment (12 sowing dates); (2) safflower cultivars (Esfahan, Sofeh, Goldasht, and Padideh); (3) three temperature and two photoperiod-related functions; etc. (Torabi et al. 2020). Further, this model may be integrated into the simulation model for interpreting yield and growth of safflower.

### 16.2.5 Potential Relevance of Safflower Crop

Safflower is largely cultivated for its commercial importance which includes oil, biofuel, biological active compounds, animal feed, secondary metabolites, pharmacological and medicinal properties (Mani et al. 2020). Safflower has been exploited in traditional and modern medicine and plays key role in central nervous, vascular, cardiac, anticoagulant, gastrointestinal, reproductive, antioxidant, metabolic, hypolipidemic actions, rheumatism, psoriasis, paralysis, mouth ulcers, vitiligo, phlegm humor, numb limbs, black spots, poisoning, and melancholy humor (Delshad et al. 2018; Mani et al. 2020). Secondary metabolites (sesquiterpene lactone dehydrocostuslactone) of safflower roots (*Carthamus tinctorius*) exhibit phytochemical activity against stimulation of parasitic plant germination (*Orobanche cumana* and *Phelipanche ramosa*) and weed control (*Lolium perenne*, *Echinochloa crus-galli*, and *Lolium rigidum*). Consequently, safflower root extract would serve as potential component for ecological balance and creation of agrochemicals (natural products) against weed control (Rial Tomé et al. 2020).

## 16.3 The Agricultural, Environmental, Industrial, Medicinal, and Economic Importance of Safflower

Safflower plant as individual and its products are directly or indirectly involved in numerous sectors and complement multiple fields like agriculture, medicine, and pharmaceuticals. Further, the application of safflower components contributes to for sustainable environment and economic development.

### 16.3.1 Agricultural and Economical Aspect

Economic enhancement is possible through revolution of oil, cereals, and milk production in crisis. Different parts of safflower plant (straw, seed, flower, fodder, and root) are involved in agricultural development and can be improved through molecular breeding. Two fundamental parts of safflower includes (1) straw is abundant, low-priced, and renewable lignocellulosic waste; (2) seeds are directly accountable for vegetable oil, both are promising plant component to contribute in agriculture in an environmental-friendly manner. However, safflower straw (79.6 wt %) and seed oil (20.4 wt%) are very potential resource for bioethanol and biodiesel production and further residual seed cake is useful for biogas production through anaerobic digestion mechanism again (Hashemi et al. 2020). Further, they explained that oilseeds and seed cake contribute considerably to production of biodiesel and methane that were 90.3% and 185.8 mL/g of volatile solids, respectively (Hashemi et al. 2020). However, the Turkey has been cultivating safflower crop at commercial scale due to its economic and agricultural importance that includes high oil ratio, quality, physicochemical features, and potential biodiesel feedstock (Al-Samaraae et al. 2020). In safflower, 24-epibrassinosteroids (24-EBR) regulates plant growth that would be useful to enhance seed yield, chlorophyll content, oil percentage, biochemical properties, proline, carotenoid amount, and oil content even in drought stress condition (Zafari et al. 2020). Biomass-related traits (weight and length of root and shoot) and chlorophyll content of safflower cultivars were reduced, whereas enzymatic activity (activities of catalase, hydrogen peroxide content, peroxidase enzymes, and superoxide dismutase) was enhanced under water stress condition

(60% field capacity) (Farooq et al. 2020). Some phenotypic traits like leaf area and number, dry and fresh weight of shoot and shoot length decrease while other traits like fresh weight, root length and secondary metabolites increase, under drought stress (Chavoushi et al. 2020).

### 16.3.2 Textile and Food Industries

Microwave irradiated extract of safflower (*Carthamus tinctorius* L.) has application in dyeing of silk fabric and this treatment enhanced the carthamin dyeing behavior and color characteristics (Adeel et al. 2020). Two economically and environment-friendly valuable pigments of safflower namely (1) safflomins and (2) carthamin, were explored which are promising candidates for natural colorants and showed negative correlation with air temperature (Patanè et al. 2020). In addition to food and textile industries, extracted natural plant pigment would be helpful as coloring agent for staining the various products without side effects on human health and environment. These coloring agents may be used for preparation of cake, pastries, candies, gem, paint, cosmetics (lipstick), and herbal color.

### 16.3.3 Environment-Friendly Biofuel and Biodiesel

Biofuel has the biological origin from plant and emits limited pollution and greenhouse gas when compared to conventional fossil fuel and is generally produced from renewable resources. Types of biofuel include bioethanol, biodiesel, and biogas, which are essential and appealling to replace for clean environment. Whole plant is important source for biofuel production and hydrothermally pretreated safflower straw increases the bioethanol production at high temperature 120–180 °C for 1–5 h (Hashemi et al. 2020). Biodiesel from safflower oil (B100) was used as fuel to substitute the diesel and prepared through the transesterification method (Thiyagarajan et al. 2020). Further studies discuss the power of methanol (10%) and n-pentanol (30%) pre-injection to evaluate the performance, emission, and combustion parameters, and which result in lower smoke emissions. (Thiyagarajan et al. 2020). Such kind of preliminary initiative for low level of smoke emissions would be an initial step towards pollution control and environment safety. Safflower oil biodiesel blend fuels of EP10 (90% biodiesel–10% ethyl proxitol) and MP10 (90% biodiesel–10% methyl proxitol) significantly reduced the emissions of hydrocarbons (HC) and carbon monoxide (CO) (Aydın 2020). Ultrasonic system was explored to examine the likelihood of biodiesel and fuel production from safflower oil for diesel power generator (Hosseinzadeh Samani et al. 2020). As recent study on biorefinery (plant to biofuel) depicts that each kg of safflower produced several economically beneficial components like ethanol (97.2 g), methane (22.4 L), and biodiesel (46.6 g) that was comparable to 0.168 L gasoline per kg plant (Hashemi et al. 2020).

Safflower residues (SRs) acquired from oil extraction were studied for pyrolysis kinetics and thermodynamic parameters through *py*-GC/MS. Results indicated the existence of high energy-containing compounds, which would be potential resource for pyrolysis to generate biofuel (Tahir et al. 2020). However, operating parameters (engine load, biodiesel ratio, and injection pressure) of diesel engine were determined via biodiesel combination of safflower, canola, and waste vegetable oil mixture (made by trans-esterification) with the help of response surface methodology (RSM) to estimate the usability of biodiesel (Simsek and Uslu 2020). Appropriate amount of dietary oils (safflower oil and fish oil) and yeast would be a wonderful feeding strategy for livestock to establish cleaner ecosystem and GHG mitigation and decreases $CO_2$, $CH_4$, and $H_2$ emission (Velázquez et al. 2020).

### 16.3.4 Medicinal and Pharmaceutical: Bioactive Compounds

A potential compound of safflower could be used as an alternative drug for treatment of bone disease (steroid-induced avascular necrosis of the femoral head: SANFH) in a rabbit model, named SPAW that is a water-soluble polysaccharide and has (1→3)-linked β-d-glucan (Cui et al. 2020). Similarly, antioxidant activity of safflower extracts (hydroxysafflor yellow A and anhydrosafflor yellow B) was also increased with rising concentration as reported, which would be helpful for cardiovascular and cerebrovascular diseases (Zhang et al. 2020). Three active compounds of safflower explored in traditional chinese herbal prescription Xuebijing: XBJ, include safflor yellow A (SYA), hydroxysafflor yellow A (HSYA), and anhydrosafflor yellow B (AHSYB), which plays important role in pain relief and decreases inflammation (Wang et al. 2020). Safflower seed extracts and oil contribute to control fructose-induced metabolic syndrome through antioxidant and anti-inflammatory property and homeostasis of trace elements and improve beta-oxidation ability of the liver with the help of CD36, FAS, and CPT-1beta gene high expression (Nimrouzi et al. 2020). Additionally, some pharmaceutical products of modern plant biotechnology approaches or transgenic safflower were also explored for human welfare like gamma-linolenic acid (GLA) safflower oil or insulin and apo-lipoprotein (Flider 2013).

### 16.3.5 Secondary Metabolites and Regulating Molecule

Recent study reported strong relation between light intensities and flavonoid biosynthesis in safflower; furthermore, flavonoids from flowers of safflower have pharmacological effects (Ren et al. 2020). Recently, advanced plant biotechnological approaches have been applied to explore molecular mechanism of flavonoid biosynthesis and 209 flavonoid metabolites were detected through metabolomic and transcriptome study (Chen et al. 2020).Therefore, drought stress can be useful for improved production of secondary metabolites, salicylic acid (SA), and sodium nitroprusside that would be a great mitigation approach for normal plant function and development (Chavoushi et al. 2020). These two potential regulating molecules of safflower, i.e., salicylic acid (SA) and sodium nitroprusside, were involved in improving anthocyanin content, rate of photosynthesis and phenylalanine ammonia lyase activity, growth of aerial parts, and hindered the root elongation due to their free radical scavenging capability (Chavoushi et al. 2020). The signaling molecule melatonin (MEL), enhanced the phytoremediative ability (phytochelatin content) of safflower plants (Namdjoyan et al. 2020). Moreover, MEL molecule reduced Pb uptake and its translocation (root to shoot) and enhanced the survival and biomass production (roots, stems, and leaves) in Pb-contaminated soils by regulating antioxidant defense process (Namdjoyan et al. 2020). Additionally, exogenously treatment of ascorbic acid (AsA) represented encouraging result on the safflower plants growth under water-deficit conditions, which was associated with AsA-induced improved osmoprotection and antioxidant defense system (Farooq et al. 2020). A number of photosynthetic pigments and secondary metabolites (flavonoids, phenol, anthocyanin, and phenylalanine ammonia lyase activity) were also reported in safflower (Chavoushi et al. 2020).

### 16.3.6 Nutritional Importance

Chemical composition, calorie content, and rheological properties of ground safflower seeds and dough were analyzed accordingly and developed the technology for shortbread using safflower seeds (Kutsenkova et al. 2020). However, safflower seeds are good source of high-grade vegetable protein (approximately 16%), linoleic and linolenic acids (polyunsaturated fatty acids), vitamins, dietary fiber, and minerals therefore considered as protein fortifier for shortbread (Kutsenkova et al. 2020). Furthermore, GLA safflower oil is good dietary supplements source of omega-6 fatty acid and includes more than 60–70% gamma-linolenic acid (GLA) that was considered as first genetically modified (GM) GLA plant oil (Flider 2013).

## 16.4 Crop Improvement Approach: Molecular Markers and QTL Mapping

Due to agricultural and economic importance, safflower crop should be extensively explored and utilized in experimental and farmers field, respectively. Some undesirable traits or characteristics may exist in cultivated varieties of safflower (Chakradhari et al. 2020). Hence, crop improvement is needed for a number of specific quantitative agricultural traits like seed yield, oil quality, and disease resistance, later which would be helpful for good quality vegetable oil production at commercial level. Therefore, desired trait for safflower improvement can be morphological, physiological, or metabolic traits that can be tagged by quantitative trait loci (QTL) mapping, association mapping, or marker-assisted selection with the help of molecular markers. QTL analyses usually start with the construction of linkage map using a segregating population of genetically distinct parents and polymorphic markers.

"Identification of genomic regions or DNA sequences linked with a quantitative trait may be mapped using molecular markers is called QTL mapping. In other terms QTL analysis is a statistical study to detect an association between phenotype and molecular markers." Basic components required for QTL mapping are:

- Diverse parents for desired trait/s
- Molecular markers
- Segregating population
- QTL mapping software

### 16.4.1 Molecular Markers: DNA-Based Markers

Application of genome-wide molecular markers would be beneficial for the improvement of yield and oil-related traits in safflower. However, phenotypic markers are observable descriptor and sensitive to environment which has been explored in conventional breeding. Therefore, observable descriptors are not useful in crop improvement programs that includes color and shape of flower, leaf and seed etc. Detection of phenotypic and genetic variability correlated with plants is essential to investigate most adaptive trait and unique loci or novel allele for improvement (Gupta 2020). Molecular markers are more accurate and authentic than phenotypic markers due to insensitivity towards environment and early stage detection in plant life cycle. Moreover, molecular markers are DNA-based markers used extensively in molecular breeding to identify the genetic diversity. Molecular markers are categorized as following: hybridization-based markers (RFLP), PCR-based markers (RAPD, AFLP, SCAR, ISSR, and InDel), and sequencing-based markers (SSR and SNP). However, restriction fragment length polymorphism (RFLP), random amplification of polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), insertion or deletion (InDel), and inter simple sequence repeat (ISSR) markers were explored in safflower and other crops for diversity analysis, population structure, and germplasm characterization (Hamdan et al. 2012; Gupta et al. 2013; Maurya et al. 2013, 2015; Mirzahashemi et al. 2015). Sequence characterized amplified region (SCAR) markers were linked to very high linoleic acid content and male sterility in safflower (Hamdan et al. 2008). Further, simple sequence repeats (SSR) and single nucleotide polymorphism (SNP) markers were also explored in Arabidopsis, Jatropha, and safflower for desired traits (Hamdan et al. 2012; Pearl et al. 2014; Maurya et al. 2013, 2015; Gupta et al. 2020).

### 16.4.2 Quantitative Trait Loci (QTL) Mapping in Safflower

In general, plant shows two type of characters/traits that include qualitative trait (effect of one gene with major effect) and quantitative trait (governed by more than one gene with minor effect) which may change phenotypes, characteristics, and productivity of plants depending upon their gene interaction (dominance, additive and epistatic). Natural allelic variation may be governed either by quantitative traits or by qualitative traits. Moreover, quantitative trait loci (QTLs) refers to a gene or chromosomal region that affects a quantitative trait. In other terms, QTL is region or DNA sequence in the genome, which may be present in coding or noncoding region and regulates agronomic traits. For their detection, QTLs should have allelic variation and must exhibit association or linkage with polymorphic marker allele. Identification of genomic regions associated with particular traits in safflower is a prerequisite for elaborate systematic breeding programmes. A number of marker system and mapping populations have been developed using diverse parents for several agronomical and economical traits to perform QTL mapping in safflower (listed in Table 16.1). Diversity analysis and linkage map of safflower have been constructed using different types of markers that include RAPD, AFLP, SCAR, ISSR, InDel, RFLP, SSR, and SNP (Hamdan et al. 2008, 2012; Mayerhofer et al. 2010; Pearl et al. 2014; Mirzahashemi et al. 2015). Pearl et al. (2014) found 12 linkage group using 244 SNP markers and identified total 61 QTLs for 21 traits in contrast to other study (Table 16.1). Comparative analysis of prior studies indicates that selection of less number of markers (119 markers) and small population size (66 individuals) lead to development of 24 linkage groups in safflower (Mirzahashemi et al. 2015). However, previous studies have been performed to map quantitative trait loci (QTL) for a number of traits (average leaf size, internodes number and length, achene traits, flower color, oil content and quality, etc.) using $F_2$ mapping populations (range 66–276 individuals) in safflower through QTL study (Hamdan et al. 2008, 2012; Mayerhofer et al. 2010; Pearl et al. 2014; Mirzahashemi et al. 2015). Additionally, Mayerhofer et al. (2010) developed inter-specific $BC_1$ population (120 individuals) and constructed the linkage map using SSR and RFLP markers for seed oil content and quality traits of safflower. Further, they selected three different parents for their study, and initially, they crossed Centennial (yellow flower color: USDA PI 538779) with *C. oxyacanthus* (noxious weed in the USA and carries a number of undomesticated traits: USDA PI 426185) and backcrossed their $F_1$ with S-317 (USDA PI 599253). Different parents of safflower have been selected for development of mapping population(s) to detect the quantitative trait loci for preharvest (plant height, number of heads per plant, spininess, and growth habit) and postharvest traits (seed weight and number, seed oil content, oleic and linoleic acid content). For example, AC Sunset × *C. Palaestinus*, CL-1 (nuclear male-sterile line) × CR-9 (high oleic acid lines, >84%), CL-1 (nuclear male-sterile line) × CR-6 (>75% oleic acid), MS line CL1 (64–79% linoleic acid) × CR-142 (84–90% linoleic acid), Centennial (yellow flower color) × NP-12 (USDA PI 401577: female white flower color), Centennial (yellow flower color) × *C. oxyacanthus* (noxious weed: undomesticated traits), and IL.111 (Iranian semi drought-sensitive

**Table 16.1** List of identified QTLs for several traits in safflower using different molecular markers

| Traits | Chromosome/linkage group/ number of QTLs | Type of marker | Mapping populations (type and size) | Genotype/cultivars | Reference |
|---|---|---|---|---|---|
| *Preharvest traits* | | | | | |
| Average leaf size | 2,8 | SNP | $F_2$ (276) | AC sunset, (PI 592391) × *C. palaestinus* (PI 235663) | Pearl et al. (2014) |
| Average leaf roundness | 4,7,8,12 | | | | |
| Spininess | 5,8,12 | | | | |
| Days to flower | 4,8,9 | | | | |
| Primary capitulum height | 1(2QTL),4(2QTL),8,9,12 | | | | |
| Primary disk diameter | 1,8,9,12 | | | | |
| Number of heads | 8 | | | | |
| Stem height | 5,8,9 | | | | |
| Number of internodes | 3,12 | | | | |
| Internode length | 1,5,12 (2 QTL) | | | | |
| Lowest branch height | 7 | | | | |
| *Postharvest traits* | | | | | |
| Number of selfed seed | 3,8,9 | SNP | $F_2$ (276) | AC sunset, (PI592391) × *C. palaestinus* (PI235663) | Pearl et al. (2014) |
| Achene weight | 3,8,9,11 | | | | |
| Achene length | 3,4,9,11 | | | | |
| Achene width | 3,9,10,11 | | | | |
| Seed dormancy | 5 | | | | |
| *Oil traits* | | | | | |

(continued)

**Table 16.1** (continued)

| Traits | Chromosome/linkage group/ number of QTLs | Type of marker | Mapping populations (type and size) | Genotype/cultivars | Reference |
|---|---|---|---|---|---|
| Seed oil | 9(2QTL),10,12 | SNP | $F_2$ (276) | AC Sunset, (PI 592391) × *C. palaestinus* (PI 235663) | Pearl et al. (2014) |
| Palmitic acid | 5 | | | | |
| Oleic acid | 3,7,8 | | | | |
| Linoleic acid | 7,8 | | | | |
| Oleic acid | *3* (*Ol*gene) | SCAR, RAPD, SSR, InDel | $F_2$ (116) | CL-1 (nuclear male-sterile line) × CR-9 (high oleic acid lines, >84%) | Hamdan et al. (2012) |
| Oleic acid | *3* (*Ol*gene) | SCAR, RAPD, SSR | $F_2$ (119) | CL-1(nuclear male-sterile line) × CR-6 (>75% oleic acid) | Hamdan et al. (2012) |
| Very high linoleic acid content | 3 (*Li* gene) | SCAR, RAPD | $F_{2:3}$ (162) | MS line CL1 (64–79% linoleic acid) × CR-142 (84–90%) | Hamdan et al. (2008) |
| *Flower color traits* | | | | | |
| Flower color | 4 | SNP | $F_2$ (276) | AC Sunset, (PI 592391) × *C. palaestinus* (PI 235663) | Pearl et al. (2014) |
| Flower color | 9 (gene *ctfc1*) | SSR, RFLP | $F_2$ (138) | *C. tinctorius*: centennial (yellow flower color: USDA PI 538779) × NP-12 (white: USDA PI 401577) | Mayerhofer et al. (2010) |
| *Other traits* | | | | | |
| Nuclear male sterility (NMS) | 3 (*Ms* gene) | SCAR, RAPD, SSR, InDel | $F_2$ (116) | CL-1 (nuclear male-sterile line) × CR-9 (high oleic acid lines, >84%) | Hamdan et al. (2012) |
| Nuclear male sterility (NMS) | 3 (*Ms* gene) | | $F_2$ (119) | CL-1 (nuclear male-sterile line) × CR-6 (>75% oleic acid) | Hamdan et al. (2012) |

| | | | | | |
|---|---|---|---|---|---|
| | | SCAR, RAPD, SSR | | | |
| Nuclear male sterility (NMS) | 3 (*Ms* gene) | SCAR, RAPD | $F_{2:3}$ (162) | MS line CL1 (64–79% linoleic acid) × CR-142 (84–90%) | Hamdan et al. (2008) |
| Enzyme (oleoyl-phosphatidylcholine desaturase) | 3 (FAD2-1 locus: candidate gene for high oleic acid content underlies the *Ol* gene) | SSR, SCAR | $F_2$ (145) | CL-1 (nuclear male-sterile line) × CR-50 (palmitic acid content) | Hamdan et al. (2012) |
| *Morphological traits under drought stress* | | | | | |
| Drought tolerance | 2,4,6 (major QTL) | SSR, ISSR | $F_{2:3}$ (66) | IL.111 (Iranian semi drought-sensitive line) × Mex.22-191 (drought tolerance line) | Mirzahashemi et al. (2015) |
| Plant height | 6 (2 QTL) | | | | |
| Branches/plant | 4(2QTL),6 | | | | |
| Capsules/plant | 2 | | | | |
| Dry weight/plant | 2,4,6 | | | | |
| Seeds/plant | 2,3,4,7,9,18 | | | | |
| Seed yield/plant | 2,9 | | | | |
| 1000-seed weight | 5 | | | | |

line) × Mex.22-191 (drought tolerance line). On the basis of prior study, oleic acid and flower color-related QTLs have been found on linkage groups 3 and 4, and 9, respectively (Mayerhofer et al. 2010; Hamdan et al. 2012; Pearl et al. 2014). However, further analysis of genomic region of oleic acid QTL indicated that *Ol* gene was found under this QTL locus, which controlled the high oleic content in safflower (Hamdan et al. 2009, 2012). Candidate gene mapping approach of *FAD2-1*gene showed that *FAD2-1*(oleoyl-phosphatidylcholine desaturase) locus was linked to *Ol* gene on linkage map 3, which was responsible for high oleic acid in safflower (Hamdan et al. 2012). Thus, QTL mapping of agronomical traits would be useful for improvement of safflower through marker-assisted breeding programs. Further, fine mapping and map-based cloning of identified QTLs would be useful to detect genes associated with respective traits. Recently, diversity array technology was used to identify 12,232 silicoDArT markers for 100-seed weight trait using 94 safflower accessions (26 countries) to evaluate diversity analysis (phenotypic and genetic), population structure study, and marker–trait association (Ali et al. 2020). DArTseq technology is a kind of competent genotyping-by-sequencing (GBS- a Next Generation Sequence, NGS technology), which was used by other researchers to explore selected safflower accessions (89 individuals) (Hassani et al. 2020). In safflower, 19,639 DArTseq markers have been developed through DArTseq genotyping analysis, which includes 1136 silicoDArTs and 2295 SNP polymorphic markers out of 10,130 silicoDArTs and 9509 SNPs, respectively, and applied for investigation like genetic diversity analysis, population structure study, and linkage disequilibrium (Hassani et al. 2020).

## 16.5 Application of Biotechnology and Modern Approaches for Sustainable Development: Toward Climate-Resilient Oilseed Crop

Oil is a necessary product worldwide; therefore, biotechnological techniques would be useful for development of novel lines and varieties with desired adaptive agronomic traits. As compared to the traditional breeding methods, double haploid production can save years of the breeding process through shortening the several generations of selfing. Plant breeders are putting in tremendous efforts to improve quality and quantity of plants to increase nutrition and availability of plant products. Although researchers have made great efforts to facilitate safflower improvement through conventional breeding, it is not stable due to adverse climatic conditions and environmental interactions. Therefore, there is a need to explore molecular breeding and marker-assisted selection in safflower.

The anther cultures have attracted considerable attention as supplementary tools for rapid production of haploid and inbred lines for obtaining hybrid cultivars. Haploids are plants with gametophytic chromosome number and double haploids undergo chromosome duplication to yield homozygous plants. In a single step, development of complete homozygous lines can be achieved from heterozygous parents. In vitro culture of safflower anther/microspore will be one of the promising biotechnological strategies for speed breeding. Haploid production along with molecular breeding would be a considerable approach for safflower improvement within short duration of time.

Flavonoids are the major dynamic and active compounds in safflower; transcriptome analysis was performed using flowers of safflower associated with diverse light intensities for flavonoid biosynthesis (Ren et al. 2020). Later, results indicated that 78,179 unigenes (total of 99.16 Gb data) were annotated and 13 genes were correlated with flavonoid biosynthesis and seven genes were validated through RT-PCR (Ren et al. 2020). They also found that expressions of HCT genes were significantly associated with flavonoid accumulation and plays imperative function in safflower flowers, which is important source for several commercial products. Furthermore, depth analysis of secondary metabolites and biologically active compound must be explored for enhance the economic value of safflower crop.

Generally, oil quality and quantity is regulated by specific genes and regulating molecules in plant system. Eleven fatty acid desaturase (*fad2*) genes have been examined using 17 *Carthamus tinctorius* accessions and 28 other Carthamus accessions and concluded that *fad2-1* gene plays major function in oleic acid to linoleic acid conversion (Shafiei-Koij et al. 2020). Interestingly, one base pair deletion of *fad2-1* gene-coding region at position 604 was positively related to high levels of oleic acid content in five mutants of *C. tinctorius* accessions. Moreover, two genes *fad2-1* and *fad2-8* regulate oleate desaturase activity in safflower (Shafiei-Koij et al. 2020). In addition, oleate desaturases were associated with endoplasmic reticulum and responsible for variation of oleic and linoleic acid content (Tonnis et al. 2020). Furthermore, oleic acid content is a key parameter to evaluate oil stability and seed nutritional quality and FAD2 enzyme regulates oleic acid content in seeds of peanut also (Tonnis et al. 2020). Consequently, exploration of diverse germplasm should be performed through molecular markers for development of new mapping population to tag novel QTLs/candidate genes.

Plant biotechnological approaches may enhance safflower vegetable oil content and quality to identify potential genes or locus that would be possible through marker-assisted breeding. Introgression of desirable traits of wild species into cultivated individuals will be a great success to enhance safflower yield and tolerance. Further pyramiding of multiple genes for high yield, oil content, and quality, stress tolerance is a future demand for agricultural, economic, and environmental demand, which is achievable through molecular breeding in underutilized oilseed crop of safflower.

Uncertainties of climate change have harmful impact on plant growth and development by alteration of metabolism, carbon sequestration, soil fertility, microbial activity, plant yield, and diversity that may create food security crisis (Dhankher and Foyer 2018). Therefore, climate change-resilient crops are need of future for food protection and agriculture security, which can provide tolerance against broad spectrum of stresses like drought, cold, heat, salinity, flood, and insect pests. Genomic-assisted breeding has application to develop climate-resilient crops and improve global food safety and security (Dhankher and Foyer 2018). Further, evaluation of suitable individual (germplasms or accession or wild varieties) is necessary for commercial manufacturing of safflower seed oil and environment-friendly biofuel production.

## 16.6 Future Thrusts

Safflower is not only an important oilseed crop but also a promising source of biofuel and may fulfill human requirement with balanced life and environment security. Optimum production of vegetable oil, biodiesel, herbal coloring dye, and bioactive medicinal and nutritional compounds will make a difference for gradual development of society and countries universally. Germplasm characterization for attractive agricultural traits will definitely contribute for economically important products due to its global distribution. Molecular breeding of safflower for high oil content, enhanced level of oleic acid, stable herbal dye, seed yield, and bioactive and medicinal compound are some remarkable traits, which need to be focussed in improvement programs. However, novel genetic variations may be created through crossing of two individuals for detection of active alleles/loci in safflower. In safflower, there are limited number of quantitative trait loci (QTLs) that have been identified using different types of molecular markers (AFLP, SSR, and SNP) and parent-specific traits, but their number should be increased. Characterization of germplasm and validation of QTLs for one or multiple traits may be helpful for the identification of candidate genes/true QTL using map-based cloning and meta-QTL analysis. Further gene tagging, pyramiding, and marker-assisted selection will be appropriate strategy for development of "supervariety" with desired traits in region-specific and/or global condition as per need. Moreover, deep root system and drought tolerance trait of safflower will be a good opportunity for biofuel and oil production under high temperature and adverse environmental conditions as compared to other oilseed crops for sustainable development.

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# Clean Energy for Environmental Protection: An Outlook Toward Phytoremediation

# 17

P. P. Sameena and Jos T. Puthur

## Abstract

The contamination of arable lands with toxic heavy metals is a serious problem. The reduction in the arable land area affects the food security of the increasing population. Likewise, heavy use of fossil fuels releases a surplus quantity of carbon dioxide into the atmosphere, which leads to an alarming rise in atmospheric temperature and global warming. In this scenario, the cultivation of energy plants in polluted lands for phytoremediation gets the double benefits of bioenergy production and decontamination of land. With the use and production of bioenergy, the effective utilization of a country's biomass is enhanced, which results in the considerable reduction of the use of fossil fuels and associated energy sources. Therefore, the use of bioenergy as a renewable energy source plays a significant role in reducing the environmental impact in relation to $CO_2$ emissions. The implementation of a bioenergy system accelerates the use of regional energy access, and consequently, it reduces the dependence on fossil fuels for sustainable development.

## Keywords

Bioenergy · Biomass · Environmental safety · Heavy metals · Renewable energy · Sustainable development

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## 17.1 Introduction

The contamination of cultivated lands due to the presence of toxic heavy metals has become a serious environmental issue, especially after the large-scale development of industrialization and urbanization (Hong et al. 2020). The presence of heavy metals negatively affects environmental stability, and it also threatens the living system on earth. Heavy metals are originally derived through natural processes taking place on the earth's crust, but the anthropogenic activities like mining and smelting of ores, agricultural activities like application of chemical pesticides and fertilizers, sewage sludge, and industrial effluents are largely contributing toward the heavy metal pollution of soil and water bodies (Abdelsalam et al. 2019). Because of their persistence, non-degradability, penetration into the food chain, and biological toxicity, heavy metal pollution has become the most serious threat to the ecosystem (Sall et al. 2020). Most of the plants and aquatic organisms can accumulate and bioconcentrate these heavy metals in small quantities, but when the concentration exceeds the standard toxicity levels, it will ultimately lead to severe toxicity symptoms. Once entered into the food chain, the heavy metals pose severe negative effects in the organisms, such as disturbances in the metabolic processes, developmental disorders, and even damage the genetic makeup (Ali et al. 2019).

It has been reported that one-third of the arable land is lost in the past 40 years because of soil erosion and/or environmental pollution, which adversely affects food security (Milman 2015). Therefore, it is necessary to restore these lands for the effective cultivation of food crops, which requires the characterization and remediation of the pollutants (Javaid et al. 2020). Different heavy metal remediation technologies have been employed in the field, which includes physicochemical and biological remediation methods (Liu et al. 2018). The physicochemical remediation technologies such as chemical precipitation, flotation, adsorption and soil washing, flushing, landfilling, and ion exchange are less cost-effective and can lead to irreversible alterations in the soil characteristics (Akhtar et al. 2020). But among the available remediation technologies, phytoremediation is plant-based, eco-friendly, economically viable, cost-effective, and widely accepted method, which is used to immobilize or deactivate the contaminants, including heavy metals and metalloids from soil and water.

Nowadays, the dependence on fossil fuels for energy purposes is increasing due to the rise of population and industrialization. These non-renewable fuels supply around 88% of the world's energy demand, which includes coal, oil, and natural gas for providing electricity and transportation energy. The burning of fossil fuels results in increased $CO_2$ and other greenhouse gas emissions at alarming rates, causing global temperature rise and climate change. Therefore, the renewable energy sources, especially bioenergy, appear to be one of the most effective and proficient forms of energy (Scarlat et al. 2015). Approximately 14% of global energy usage comes from bioenergy, and photosynthesis is the primary source of creating bioenergy, which can be extracted by using advanced technologies (Ale et al. 2019).

In this regard, the two crucial environmental problems, i.e., soil pollution with heavy metals and temperature elevation due to burning of fossil fuels, can be solved by the coupling of bioenergy production with heavy metal phytoremediation using heavy metal hyper-accumulating non-edible bioenergy plants (Lima et al. 2019). Cultivating the energy plants in the heavy metal-contaminated lands for the phytoremediation purpose is getting the benefits of the production of different forms of bioenergy such as biogas and bioethanol (Abdelsalam et al. 2019). In the present literature, heavy metal pollution and its impacts on the ecosystem, mechanisms of phytoremediation, bioenergy production from the phytoremediation biomass, the environmental and socioeconomical benefits of bioenergy, strategies to enhance the metal accumulation potential in bioenergy plants, and sustainable development are taken into consideration, and an extensive analysis has been made for the production of clean energy for environmental protection.

## 17.2 Heavy Metal Pollution and Environmental Impacts

Among the various pollutants in soil, heavy metals are the most detrimental pollutant, and it is primarily because of the considerable variation in the activity of soil enzymes and functional diversity of soil microorganisms (Fazekašová and Fazekaš 2020). Heavy metals include both essential and non-essential elements. The essential heavy metals such as cobalt (Co), copper (Cu), iron (Fe), manganese (Mn), molybdenum (Mo), selenium (Se), and zinc (Zn) have diverse roles in the physiological and metabolic activities in living organisms on minimal concentrations; however, higher concentrations lead to toxicity symptoms. In contrast, some metals like arsenic (As), cadmium (Cd), chromium (Cr), lead (Pb), and mercury (Hg) are non-essential, causing detrimental effects even if in minute concentrations. They adversely affect the growth, development, and metabolism of plants, microbes, and animals (Ali et al. 2019).

Plants uptake these heavy metals and bioconcentrate in their tissues and subsequently get transferred through the food chain, which becomes a potential threat to the health of animals and human beings. Considering the higher level of metal concentration on cultivable lands due to bore-well irrigation, mining, and other industrial activities, effective remediation of these lands is important for sustainable development (Hou et al. 2020). Heavy metals exert toxicity effects in the soil biota by altering the soil microbial activities and inhibit the metabolism of plants even at low concentrations. The toxicity symptoms produced by plants, animals, and humans due to heavy metal exposure include imbalances in the nutrient uptake, production of reactive oxygen species (ROS), and associated membrane damage (Tarekegn et al. 2020).

## 17.3 Coupled Phytoremediation and Bioenergy Production

Plants having the ability to accumulate exceedingly higher concentrations of the heavy metals in their biomass with the bioenergy potential have been commonly used for the coupled phytoremediation and bioenergy production (Barbosa et al.

2015). The energy plants such as *Ricinus communis*, *Jatropha curcas*, *Arundo donax*, *Helianthus annuus*, *Panicum virgatum*, *Salix alba*, *Glycine max*, *Populus deltoides*, and *Miscanthus fuscus* are the heavy metal hyperaccumulating plants and are also used for the efficient production of bioenergy (Bauddh et al. 2017).

## 17.3.1 Mechanisms of Phytoremediation

Phytoremediation is an in situ remediation technology, utilizing the innate heavy metal accumulation potential of plants and their mechanisms, and the efficiency depends on the nature of the pollutant, bioavailability, and properties of the soil (Rai et al. 2019). There are different methods adopted by plants to cope with the heavy metal toxicity, of which phytostabilization and phytoextraction are the most important ones in relation to heavy metal absorption and the associated processing of plant biomass.

### 17.3.1.1 Phytostabilization

Phytostabilization is a method of the immobilization of heavy metals by precipitation or absorption in the root system and thereby prevents leaching of the metals to the groundwater and also reduces the shoot translocation of the metal. Because of the root immobilization and reduced translocation, phytostabilization turns to be significant in the cultivation of food crops in the contaminated lands (Janeeshma and Puthur 2020). Unlike other phytoremediation mechanisms, in phytostabilization, the metals are not removed from the soil permanently, but get stabilized in the root system or soil and thereby reduce the mobility and bioavailability of the metal into the food chain (Lan et al. 2020). For a plant to achieve phytostabilization potential, it must have a fast growth rate, deep root system, and reduced translocation factor.

### 17.3.1.2 Phytoextraction

Phytoextraction refers to the uptake and translocation of the metals from soil to the shoot biomass of the plants without affecting the normal metabolic activities. When sufficient biomass has been attained, they are harvested and removed from the field. Hence, the metal concentrations in the soil are reduced to acceptable levels (Lu et al. 2019). The characteristics of the plants used for phytoextraction purpose includes well-developed root system, higher rate of biomass production, and enhanced level of metal accumulation and translocation. Such plants with increased accumulation and bioconcentration of metals in their shoot system are termed hyperaccumulators or metallophytes. After harvesting the biomass, they are exposed to combustion or incinerator burning, which is used for bioenergy production. After the complete burning, the bottom ash becomes a rich source of metals termed the bio-ore, used for extracting and recycling the metal (Li et al. 2020).

### 17.3.2 Bioenergy Plants Used for Phytoremediation

Different plants species can be utilized for the restoration of soil fertility by implementing effective remediation. For the feasibility and efficiency of phytoremediation, the plant species should be non-edible and have the ability to grow luxuriously in contaminated lands. Even though several reports regarding the potential of edible plants for phytoremediation purposes are coming into focus, it is better to use non-edible plants in order to avoid the risks of heavy metal entry into the food chain (Abdelsalam et al. 2019). In China, about 64 plant species are being introduced as potential bioenergy plants, but their growth and development largely depend on the biological and environmental suitability of the growing region and climate conditions (Li et al. 2010). Figure 17.1 represents the most commonly used promising energy crops with heavy metal hyperaccumulation potential.

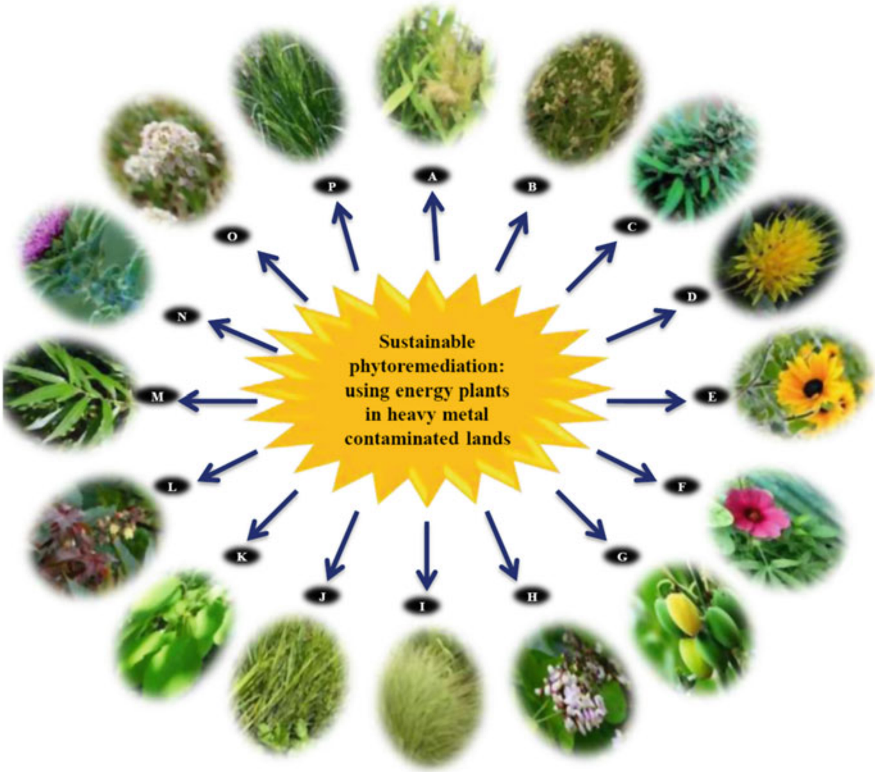

**Fig. 17.1** Dedicated energy plants used for coupled utilization of phytoremediation and bioenergy production: (**a**) *Arundo donax*, (**b**) *Azadirachta indica*, (**c**) *Cannabis sativa*, (**d**) *Carthamus tinctorius*, (**e**) *Helianthus annuus*, (**f**) *Hibiscus cannabinus*, (**g**) *Jatropha curcas*, (**h**) *Millettia pinnata*, (**i**) *Miscanthus species*, (**j**) *Panicum virgatum*, (**k**) *Populus deltoids*, (**l**) *Ricinus communis*, (**m**) *Salix alba*, (**n**) *Silybum marianum*, (**o**) *Thlaspi caerulescens*, and (**p**) *Vetiveria zizanioides*

The most popular biodiesel plant, *Jatropha curcas*, is a perennial oil yielding plant growing abundantly in tropical and subtropical regions. This plant is widely used for the decontamination of wetland ecosystems as well as for the revegetation of the mining dump sites and coal fly ash landfills (Abioye et al. 2017). In India and China, approximately 2.6 million hectares of land are used for the cultivation of *Jatropha* for the purpose of bioenergy production (Fairless 2007). *Ricinus communis* is an oilseed plant growing luxuriously in marginal lands with approximately 50% of seed oil content and oil productivity of 1414 kg/ha, having a wide range of industrial and pharmaceutical uses (Kaur and Bhaskar 2020). Castor oil has high lubricity characteristics such as viscosity, density, and thermal conductivity as compared to the standard lubricants (Patel et al. 2016). *Helianthus annuus*, an ornamental and biofuel crop, has the ability to stabilize, absorb, and accumulate heavy metals at higher concentrations from industrial effluents (Chauhan and Mathur 2020). The study conducted in relation to the production of biogas from plant biomass, which is collected from metal-contaminated and non-contaminated lands, indicates that *H. annuus* produces 134–154 ml $g^{-1}$ biogas with insignificant differences in the contaminated and non-contaminated lands (Hunce et al. 2019). A detailed analysis of the previous studies related to the phytoremediation and bioenergy production potentials in the non-edible energy plants has been carried out, which is summarized by citing the most recent articles in Table 17.1.

### 17.3.3 Bioenergy Production from Phytoremediation Biomass

The energy-enriched compounds generated from the bioresources directly through the biological processes or by chemical/biochemical processes are termed biofuels. Right from ancient to the present age, the advancement in the production of biofuels can be categorized as the first-generation to fourth-generation biofuels (Kour et al. 2019). The first-generation biofuels are mainly produced from food crops such as corn, barley, wheat, sugarcane, potato, sunflower, soybean, or other oil seeds. The fermentation of corn or sugarcane by using starch digesting fungi such as *Saccharomyces cerevisiae* and some species of *Rhizopus* results in the production of bioethanol (Vohra et al. 2014). Since the production of first-generation biofuels mainly includes food crops, they negatively affect food security. Industrial processing systems like enzymatic hydrolysis of sucrose or starch were used for the large-scale production of bioethanol (Sheldon 2018). The second-generation biofuels are generally produced from non-food plants or crop by-products, which include the production of bioethanol from organic waste materials and forest and waste wood residues. Any kind of lignocellulosic waste such as sawdust or straw is also used for generating second-generation biofuels (Pidlisnyuk et al. 2014). The production of biodiesel from cellulolytic bacteria, microalgae, and other microorganisms, which are characterized by fast growth rate and increased $CO_2$ fixation ability, comprises third-generation biofuels (Kour et al. 2019). The fourth-generation biofuels involve the metabolic engineering of microalgae with the help of post-genome technology (Abdullah et al. 2019). Even though genetically modified

**Table 17.1** Non-edible bioenergy plants with promising phytoremediation and bioenergy production potentials

| Energy plants | Phytoremediation potential | Bioenergy production potential |
|---|---|---|
| *Arundo donax* (giant reed) | Phytoremediation of Cr-, Pb-, and Zn-contaminated soil (Barbosa et al. 2015) | Higher biomass production in marginal lands and an energy yield of 4654.4 GJ/ha (Jámbor and Török 2019) |
| *Azadirachta indica* (neem) | Phytoremediation of As-, Cd-, Co-, Cr-, Cu-, Fe-, Mn-, Mo-, Ni-, Pb-, and Zn-contaminated soil (Abu-Abdoun and Al-Balawna 2019) | Biomass yield of 53.7 tons biomass/day, 756.538 $m^3$ of biogas with 5.674 MWh/day of electrical energy (Tizé et al. 2016) |
| *Cannabis sativa* (hemp) | Phytoremediation of Cd-, Co-, Cr-, Cu-, Fe-, Mn-, Ni-, Pb-, and Zn-contaminated soil and municipal sewage sludge (Zielonka et al. 2020) | Production of 53.33% of oil, 34.66% of biochar, and 12% gas from biomass harvested from Co- and Ni-contaminated lands (Tahir et al. 2020) |
| *Carthamus tinctorius* (safflower) | Phytoremediation of Ni-, Pb-, and Zn-contaminated soil (Al Chami et al. 2015) | Oil content of 26–45%, with enhanced performance, emission, and combustion characteristics of safflower biodiesel, suitable for ignition engine (Yesilyurt et al. 2019) |
| *Cynara cardunculus* (cardoon) | Phytoremediation of Cd, Cr, Cu, Ni, Pb, and Zn (Delgado et al. 2017) | Yield of 14 t dry matter $ha^{-1}$ $y^{-1}$ containing 8–10% oil fruits (Mehmood et al. 2016) |
| *Helianthus annuus* (sunflower) | Reclamation of As-, Cd-, Cu-, Fe-, Pb-, and Zn-contaminated industrial soil (Chauhan and Mathur 2020) | An average of 356–473 ml $g^{-1}$ biogas yields and 70.7% of $CH_4$ concentration from the seeds (Hunce et al. 2019) |
| *Hibiscus cannabinus* (kenaf) | Phytoremediation of As-, Cd-, Cr-, Co-, and Mn-contaminated soil (Nizam et al. 2016; Shehata et al. 2019) | The liquefaction of dried hydrolysate yield 77.2% oil, higher level of crude protein, and less fiber content (Meryemoğlu et al. 2014; Ammar et al. 2020) |
| *Jatropha curcas* (physic nut) | Phytoextraction of As-, Cd-, Cu-, Fe-, Pb-, and Zn-contaminated soil and mine tailings (Martín et al. 2020) | Oil content of 63–66% in seeds, with oil viscosity of 4.8 $mm^2/s$, density of 862 $kg/m^3$ (Hamzah et al. 2020) |
| *Millettia pinnata* (Indian beech) | Phytoextraction of Cr, Cu, Mn, and Pb from industrial wastewater (Kanwal et al. 2019) | Annual yield of seed oil is 1000–5000 L/ha, which is a rich source of oleic acid (Gresshoff et al. 2017). |
| *Miscanthus* species (Chinese silver grass) | Phytoremediation of Cr-, Pb-, and Zn- contaminated soil (Barbosa et al. 2015) | The biomass yield is 2.1–32.4 t $ha^{-1}$ $y^{-1}$, and the biomass may yield 183.9 TW h $y^{-1}$ electricity (Mehmood et al. 2016) |
| *Panicum virgatum* (switchgrass) | Phytoremediation of Cd-, Pb-, and bisphenol-A-contaminated soil (Guo et al. 2019; Phouthavong-Murphy et al. 2020) | A cellulosic bioenergy crop, yield 5.2–11.1 Mg $ha^{-1}$ biomass and 60 GJ $ha^{-1}$ $y^{-1}$ net energy (Mehmood et al. 2016) |

(continued)

**Table 17.1** (continued)

| Energy plants | Phytoremediation potential | Bioenergy production potential |
|---|---|---|
| *Populus deltoides* (poplar) | Phytostabilization of As-, Cd-, Cr-, Co-, Cu-, Ni-, Pb-, Sb-, Se-, Sn-, Ti-, and Zn-contaminated soil (Ancona et al. 2019) | Biomass has the cellulose content 42–49%, hemicellulose content 16–23%, and lignin content 21–29%, yields 39.8% bio-oil at 450 °C (Daniel et al. 2018; Bryant et al. 2020) |
| *Ricinus communis* (castor) | Phytoremediation of Cd-, Cu-, Ni-, Pb-, and Zn-contaminated soil and Cu mine slags (Palanivel et al. 2020; Yeboah et al. 2020) | Seed extracts 46–55% oil content containing 80–90% of total fatty acid (Osorio-González et al. 2020) |
| *Salix alba* (willow) | Phytostabilization and phytoextraction of Cd-, Cr-, Cu-, Ni-, Pb-, and Zn-contaminated soil (Korzeniowska and Stanislawska-Glubiak 2018; Malik et al. 2020) | Biomass yields 2.5–27.5 Mg/ha, with 81.23% volatile matter and the heating value 18.03 MJ $kg^{-1}$ (Mehmood et al. 2016; Rasool et al. 2018) |
| *Vetiveria zizanioides* (vetiver grass) | Phytoremediation of Cu, Fe, Mn, Pb, and Zn from metal-contaminated water (Suelee et al. 2017) | Biogas yield of 3266 $m^3/m^3$/t/day and biomass utilization of 58.46% (Li et al. 2014) |

algal biofuels are a potential alternative to fossil fuels due to their higher energy content and eco-friendly nature, large-scale production is not economically feasible (Villarreal et al. 2020). Therefore, in the present scenario, the production of second-generation biofuels is the most reliable strategy for the production of bioenergy.

Direct combustion of plant harvest is one of the traditionally practiced thermochemical methods, which include the burning of the biomass in the presence of oxygen, in which the chemical energy is converted into a gas (McKendry 2002). The combustion of the feedstock to produce electricity in biomass power plants has some similarities with the fossil fuel plants. Advanced technologies such as pyrolysis, fermentation, and anaerobic digestion can be used to produce electricity and biofuels from the phytoremediation biomass (Pandey et al. 2016). The complete characterization of the pyrolytic nature of the harvested biomass is essential for sustainable and economically viable energy conversion (Mehmood et al. 2016). After the harvest, the biomass is subjected to physical or chemical, or biological pretreatment in order to hydrolyze the complex lignocellulosic biomass into simpler forms like lignin, hemicellulose, and cellulose molecules. The detailed mechanisms involved in the production of the four different forms of bioenergy generations are represented in Fig. 17.2.

## 17.4 Bioenergy and Environmental Safety

Plants are considered as the potential feedstock for the production of renewable energy. The integrated approach of phytoremediation and subsequent bioenergy production from the phytoremediation biomass would be a major advance in relation

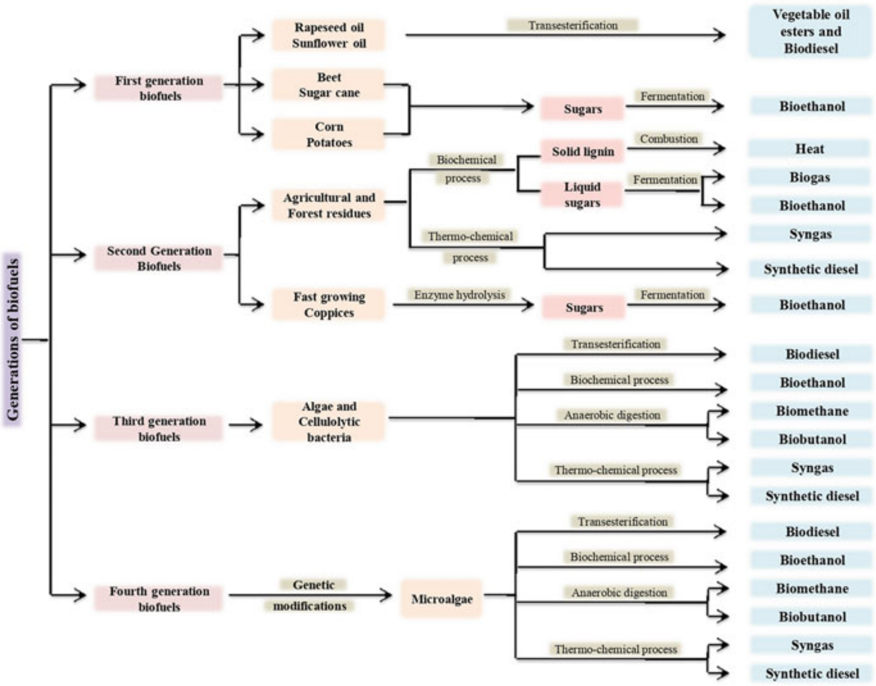

**Fig. 17.2** Mechanisms involved in the processing and production of the four different generations of biofuels

to sustainability in order to optimize the environmental and socioeconomic issues (Bauddh et al. 2017). Many countries are encouraging the use of biofuels in order to reduce the dependency on fossil fuels and also to attain energy security and mitigate the emission of $CO_2$. This coupled phytoremediation and bioenergy production will also help to restore the degraded lands and polluted water and reduce the greenhouse gas emissions and associated global climate change (Singh et al. 2019). Although many renewable energy sources are available abundantly and have considerable economic potential, they are exploited unsatisfactorily, and their contribution to the energy supply is very less. It was reported that the positive social impacts are not always coupled with the positive environmental impacts of biofuels production and vice versa (Rettenmaier and Hienz 2014).

### 17.4.1 Environmental Effects of Bioenergy

Even though bioenergy has a great significance in maintaining energy security, the production of biomass feedstocks and extraction of bioenergy potentially leads to some negative effects on the environment (Wu et al. 2018). Depending on the type of feedstock as well as the technology used, the nature and intensity of the environmental impacts of biomass energy are varied. Therefore, proper management of the feedstock grown in metal-polluted lands is essential in order to prevent the presence of metal in the final energy product. The amount of carbon released during biomass combustion is always equal to the amount of carbon absorbed during the growing period (Cherubini et al. 2011). Therefore, the net carbon release and global warming emissions are zero, indicating the sustainability of utilizing biomass energy.

In addition, growing-dedicated energy plants in marginal arable lands like short-rotation trees such as *Populus* and perennial grasses like *Miscanthus* and *Panicum* (a) do not directly compete with food crops, (b) require low fertilizer input as well as annual cultivation costs, (c) avoid the destruction of native forests, and (d) reduce the damages to the biodiversity, as compared to other energy plants (Ale et al. 2019). According to the observations of Padoan et al. (2019), the cultivation of short-rotation coppices (SRCs) such as *Populus*, *Salix*, and *Robinia* genotypes in urban Zn-contaminated lands was found to be the most productive plant species in terms of phytoremediation and bioenergy production. The best phyto-management practices will make the SRC phytoremediation an effective remedy to handle the urban contaminated lands along with the landscape benefits (Padoan et al. 2019).

### 17.4.2 Socioeconomic Effects of Bioenergy Production from Contaminated Lands

The socioeconomic impacts of bioenergy production from contaminated lands are many.

a. The utilization of the contaminated lands for bioenergy production can reduce the costs for ecological restoration (Singh et al. 2019).
b. The production of second-generation biofuels by using crop residues is regarded as economically feasible as it causes comparatively lesser impacts on food production (Ale et al. 2019).
c. Though bioenergy is generally more expensive than conventional fossil fuels, the reduction in the emission of greenhouse gases can reduce the cost gap (Duer and Christensen 2010).
d. The sustainable production of biofuels promotes efficiency in land management practices, which optimize the utilization of land, water, and solar energy and thereby guarantee food and fuel security, maximize carbon storage, and reduce greenhouse gas emissions (Acharya and Perez-Pena 2020).

## 17.5 Bioenergy for Sustainable Development

Since bioenergy comprises the principal part of the renewable energy sources, it is significant to support the UN Sustainable Development Goals (SDGs) in relation to climate change and energy security (IRENA 2016). The Brazilian government developed new policies and implemented a biodiesel program in 2004 itself to promote bioenergy as a clean source and thereby facilitate the development of rural areas (Finco and Doppler 2010). The majority of the developing countries in Southeast Asia have the tendency to export bioenergy rather than using it for domestic purposes, which negatively affects the sustainability of the bioenergy systems (Goh et al. 2020). Therefore, it is the need of the hour to explore the local bioenergy resources and utilization in meeting the domestic energy demands. Growing perennial energy crops in the edges of the arable lands or growing biofuel-producing cover crops in the field provide an additional source of income for farmers (Ale et al. 2019). Moreover, growing bioenergy plants using best landscape management practices can also contribute to the various aspects of sustainability, such as maintenance of healthy forests, reduction in carbon, and avoiding natural calamities.

## 17.6 Strategies for the Enhancement of Phytoremediation Potential of Bioenergy Plants

Various soil amendment practices are used for solubilizing the complex metal ions present in the soil, which will contribute to the enhanced phytoremediation potential of plants. The application of biodegradable metal chelating agents and micronutrients to the contaminated lands can increase the heavy metal uptake by plants. According to Chen et al. (2020), the exogenous application of citric acid, ethylenediamine disuccinate, and oxalic acid in cadmium- and uranium-polluted soils enhances the mobilization of these metals to the plant system. Similarly, abscisic acid (ABA)- and ABA-catabolizing bacteria-mediated heavy metal transport is a significant strategy for enhanced metal bioavailability and metal uptake in the hyperaccumulator plants (Lu et al. 2019). The list of bioenergy plants with enhanced heavy metal phytoremediation due to soil amendment practices is provided in Table 17.2.

Transgenic approaches are the accurate and advanced technology to improve the phytoremediation potential by introducing or over-expressing the genes involved in the heavy metal uptake, translocation, and sequestration mechanisms. The genetic manipulation in the hyperaccumulator plants with a slow growth rate and less biomass can also be used for improving the biomass accumulation and resulting in the enhanced phytoextraction (Saxena et al. 2019). The knockdown or over-expression of genes involved in the synthesis of phytochelatins and metallothioneins can specifically contribute toward the metal sequestration in plants. The studies related to transgenic approaches in heavy metal accumulating bioenergy plants for enhanced metal accumulation are included in Table 17.3.

**Table 17.2** Detailed list of bioenergy plants showing enhanced heavy metal phytoremediation in bioenergy plants due to soil amendment practices

| Energy plants | Amendment used | Heavy metals | Remarks | References |
|---|---|---|---|---|
| *Brassica napus* | Ethylenediaminetetraacetic acid (EDTA) | Pb | Enhanced plant growth, biomass, gas exchange characteristics, chlorophyll content and antioxidant enzymes activities, and improved metal accumulation | Kanwal et al. (2014) |
| *Carthamus tinctorius* | Melatonin | Pb | Modulation in activities of different antioxidant enzymes, enhanced phytochelatin and GSH contents, higher biomass production, reduced metal uptake and translocation, and enhanced phytostabilization | Namdjoyan et al. (2019) |
| *Helianthus annuus* | Citric acid, oxalic acid, and ethylenediamine disuccinate | Cd and U | Improved metal mobilization, enhanced metal removing efficiency, and increased phytostabilization | Chen et al. (2020) |
| *Jatropha curcas* | Corn biochar | Cd, Pb, and Zn | Enhanced biomass production, nutritional quality, improved physiological performance, and enhanced phytostabilization | González-Chávez et al. (2016) |
| *Miscanthus sinensis* | Bone mill, bottom ash, furnace slag, and red mud | Cd, Cu, Pb, and Zn | Reduced metal availability and mobility, restricted metal uptake, and increased plant biomass | Lee et al. (2014) |
| *Ricinus communis* | *Prosopis* biochar and rice husk | Pb | Improved soil pH, enhanced nutrient uptake and plant growth parameters, increased antioxidant enzyme activities, and increased metal tolerance | Kiran and Prasad (2019) |

(continued)

**Table 17.2** (continued)

| Energy plants | Amendment used | Heavy metals | Remarks | References |
|---|---|---|---|---|
| *Salix alba* | Biochar and garden soil | As, Cu, and Pb | Enhanced soil fertility, plant growth, and enhanced phytostabilization | Lebrun et al. (2018) |

**Table 17.3** Transgenic approaches in heavy metal accumulating bioenergy plants for enhanced metal accumulation

| Energy plants | Genes incorporated | Source | Remarks | References |
|---|---|---|---|---|
| *Brassica juncea* | Glutathione synthase (GS) and γ-glutamyl cysteine synthase (ECS) | Bacteria | Increased tolerance and accumulation of As, Cd, Cr, Pb, and Zn | Reisinger et al. (2008) |
| *Brassica juncea* | Adenosine triphosphate sulfurylase (APS), γ-glutamyl-cysteine synthetase (ECS), and glutathione synthetase (GS) | *Escherichia coli* | Increased tolerance and accumulation of Se in leaves | Bañuelos et al. (2005) |
| *Brassica juncea* | ATP sulfurylase (APS1) | *Arabidopsis* | Increased tolerance and accumulation of Cd, Cr, Cu, Mo, V, and W | Wangeline et al. (2004) |
| *Populus alba* | Metallothionein A1 gene (PsMT$_{A1}$) | *Pisum sativum* | Increased tolerance and accumulation of Cu and Zn, by regulating reactive oxygen species | Turchi et al. (2012) |
| *Populus alba* × *P. tremula* | Vacuolar transporter (YCF1) | Yeast | Increased tolerance and accumulation of Cu, Pb, and Zn, enhanced growth, and reduced toxicity symptoms | Shim et al. (2013) |
| *Populus tremula* × *P. alba* | Bacterial c-glutamylcysteine synthetase | Bacteria | Increased tolerance and uptake of Cd produce higher glutathione (GSH) in leaves, elevated transcript levels of genes involved in $Cd^{2+}$ transport, and detoxification | He et al. (2014) |
| *Populus tremula x P. alba* | gsh1 | Bacteria | Enhanced tolerance to drought and Pb levels and increased glutathione content | Samuilov et al. (2016) |

## 17.7 Challenges and Future Perspectives

The use of biomass and bioenergy enhances access to regional energy sources and thereby reduces the reliance on fossil fuels, providing affordable and high-quality energy services. The significant challenge regarding the production of bioenergy from phytoremediation biomass is the transfer of heavy metals to biofuels (Gomes 2012). In order to avoid metal entry into the aerial biomass, the phytostabilization process can be encouraged in the energy plants, and effective processing of the root biomass for extracting the pure metal can be employed (Zgorelec et al. 2020). In contrast, according to the observations of Palanivel et al. (2020), the translocation of the heavy metals in hyperaccumulator plants is potentially restricted into the seeds, and the presence of these toxic metal ions in the seeds and seed oil will be below standard toxicity level even in the case of plants grown in heavily contaminated lands.

For the large-scale production of bioenergy by utilizing heavy metal-contaminated lands, it is necessary to perform SWOT (strength, weakness, opportunities, and threat) analysis along with detailed cost–benefit analysis (CBA) and social impact assessment (SIA) to scrutinize the environmental sustainability and ecosystem services (Tripathi et al. 2016; Abdelsalam et al. 2019). Since many of the developed countries are following biomass energy utilization, the developing countries can emulate the experiences and achievements of these countries for sustainable energy utilization and thereby enhance energy self-sufficiency. The sustainable industrial utilization of biomass has to consider the ecological, financial, social, and technological aspects since they may show discrepancy among different countries, and therefore it is difficult to find a unique solution for global biomass utilization (Malico et al. 2019).

## 17.8 Conclusion

Bioenergy can be considered an effective and sustainable remedy for the ever-increasing demands for energy supply. Although fossil fuels provide the major source of global energy demands, they are non-renewable and also contribute significantly to the major portion of atmospheric carbon dioxide. The energy crisis and accelerated environmental pollution due to industrialization and the associated complex environmental problems can be attenuated by the sustainable interdisciplinary research in phyto-technology for large-scale bioenergy production. To overcome the food versus fuel debate that emerged due to the increasing population and food demand, it is significant to go for large-scale production of third- and fourth-generation biofuels in the near future. The inadequacies in the existing phyto-technologies must be analyzed in such a way as to improve the efficiency in multifunctional bioenergy production systems, and it is also imperative to design market-based mechanisms in order to promote investments in bioenergy for economic sustainability.

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# Role of Process Intensification in Enzymatic Transformation of Biomass into High-Value Chemicals

# 18

Parimala Shivaprasad

## Abstract

With an increased emphasis on green processing methods and a move beyond the use of petrochemical feedstock, it has become important to develop processes to maximize the utilization of existing resources. Biomass is a renewable and abundant source of fuels and chemicals. Enzyme-assisted valorization of biomass is a sustainable alternative to chemical routes due to the superior properties of enzymes such as high enantioselectivity and ability to operate under mild conditions. However, the challenges associated with enzyme de-activation and reusability often limit the application of enzyme catalysis on a commercial scale. This can be circumvented by immobilizing enzymes, which allow for easy recovery and reuse and higher storage and operational stability. Supported enzyme catalysis requires increased mixing, increasing energy costs, and catalyst disintegration, which until now limit the application of conventional batch reactor design. Process intensification (PI) is becoming increasingly popular in the chemical process industry as it allows for better control of chemical reactions on a molecular scale, resulting in enhanced reaction rate and minimizing waste generation. Recent advances in PI reactors such as micro-, membrane, and spinning disk reactors have increased the applicability of enzyme catalysis for production of high-value chemicals. This chapter will focus on enzyme-catalyzed valorization of biomass into high-value products using PI technologies for applications in the fine chemical industries. Opportunities and challenges for the commercialization of these technologies for the application on an industrial scale will also be discussed.

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**Keywords**

Reaction intensification · Enzyme catalysis · Catalytic reactors · Biomass valorization

## 18.1 Introduction

Biomass waste is generated at the rate of 100 billion annually and is usually disposed through incineration or landfills, leading to severe environmental pollution. These have often been regarded as a low-value feedstock to be used as raw material in chemical industries (Koutinas et al. 2014). However, recent advances in biomass valorization chemistry have shown the potential for transforming the diverse range of chemical compounds present in biomass as chemical building blocks to replace raw materials derived from petroleum feedstock (Cho et al. 2020). Enzymatic pathways offer an environmentally benign solution for decomposition and processing of biomass compared to traditional chemical routes. Additionally, enzymes are highly selective to the substrates, reducing the formation of side products. However, enzymes are expensive, and their application on an industrial scale is currently limited due to challenges associated with their recovery and reuse. Enzyme immobilization has been shown to overcome this challenge; however, the use of conventional batch technology for enzymatic biomass valorization is associated with additional issues such as slow reaction rates, product inconsistencies, and scale-up (Leak et al. 2014).

Process intensification (PI) in chemical processing is increasingly receiving commercial attention due to its potential to reduce process inefficiencies by achieving a homogeneous reaction environment for chemical processing (Gorak and Stankiewicz 2011). PI can be achieved either through novel/hybrid reactor design or a processing route (alternate energy forms and synergistic reactions). PI reactor configurations have been studied for over a decade and are transitioning from laboratory-scale studies to examples of commercial applications in the fine chemical industries.

The aim of this chapter is to highlight the application of PI reactor configurations for biocatalytic valorization of biomass into useful chemical intermediates. This chapter will provide an overview of enzymes as catalysts for biomass valorization and discuss the need for PI reactors for this application. Thereafter, different examples of PI reactors for biomass valorization will be discussed with an emphasis on using immobilized enzymes as catalysts. The chapter will conclude with an outlook for commercial opportunities for PI reactors in the area of biomass valorization.

## 18.2 Enzymes as Catalysts for Biomass Valorization

Enzymes are naturally occurring catalysts which have shown potential for enhanced chemical processing over chemical catalysts, are more cost-effective, are environment friendly, and hence are more sustainable. They are characterized by high regioselectivity and specificity, allowing for high reaction yields with minimum by-product formation. Free enzyme-catalyzed reactions are simple to operate but have key challenges such as loss of enzyme activity and limited recovery and reuse. Enzyme immobilization on suitable supports has shown to overcome this challenge as they are able to retain long-term operational/storage stability and can be recovered easily from the reaction mixture for reuse. The application of enzymes for biomass valorization is a rapidly developing area due to inherent process advantages. The use of cellulase to break down cellulose into small chain polysaccharides is well established. Further research has enabled the application of a cocktail of carbohydrate-active enzymes for further valorization of lignocellulosic biomass into high-value chemical intermediates (Arnoldi Pellegrini et al. 2020). New sources of enzymes have been identified and reported for valorization of lignocellulosic biomass to promote cost-effective solution for biomass valorization (Maitan-Alfenas et al. 2015).

## 18.3 Process Intensification of Biomass Valorization

Industrial application of enzymes for biomass valorization is limited by batch reactor design due to high mass transfer resistances (immobilized enzymes) and poor enzyme recovery and reuse (free enzymes). Hence, there is a need for novel reactor designs to facilitate easy and cost-effective scale-up of enzyme-catalyzed biomass valorization.

In recent years, process intensification (PI) has been adopted as a potential route for improved process efficiency by achieving better control over the chemical process and a reduction in waste production and energy consumption (Dudukovic 2010; Shivaprasad et al. 2017; Stankiewicz and Moulijn 2000). Process intensification can be achieved through the use of novel/hybrid reactor configuration and novel processing routes or through a synergy of both. PI reactor configurations such as microchannel and membrane reactors have been investigated extensively on laboratory scale and are now being employed for commercial-scale operations (Stankiewicz and Moulijn 2000; Shivaprasad and Emanuelsson 2018). The common characteristics such as improved mass and heat transfer properties, improved reaction kinetics, easy scale-up, inherent safety, and minimum downstream processing are attractive to the fine chemical industry and are competitive with the traditional stirred tank reactor configurations (Stankiewicz and Moulijn 2000; Shivaprasad and Emanuelsson 2018). The aim of this chapter is to showcase recent developments in reactor designs which allow for intensification of enzymatic biomass valorization.

### 18.3.1 Microreactors

Microreactors consist of channel or capillary-like configuration with internal channel diameter between 10 and 500 μm (Fig. 18.1). Wide range of materials like Teflon, PTFE, stainless steel, fused silica, and glass is reported in the construction of microreactors, based on the reaction environment. The microchannels have a large surface-to-volume ratio compared to traditional reactor configurations, allowing for higher mass and heat transfer properties. The reactors support both heterogeneous catalysis and homogeneous catalysis, and reactions can be scaled- up by stacking-up multiple microreactors into a reactor bundle for high-throughput production.

Microreactors are now employed in commercial-scale production of pharmaceuticals and are fast gaining attention for biomass valorization due to the following advantages (Borukhova and Hessel 2013):

- Superior process control improves reaction selectivity and accurate kinetic data crucial for further reaction optimization.
- Reactor miniaturization reduces the volume of reagents required while increasing the safety for hazardous reaction schemes.

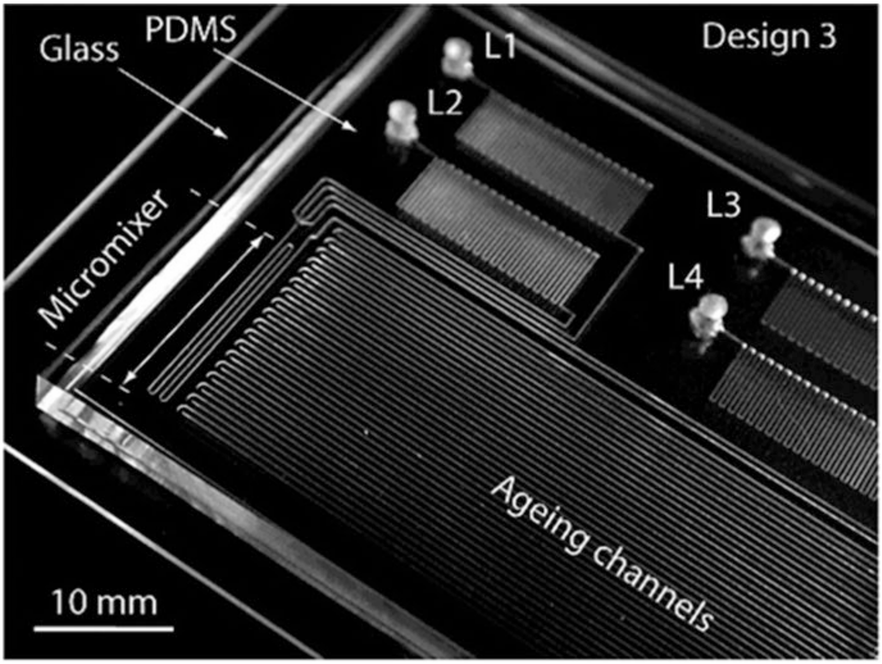

**Fig. 18.1** A PDMS and glass microreactor design for supported enzyme catalytic reaction. Reprinted (adapted) with permission from Khan et al. (2004). © 2007 American Chemical Society

- Continuous mode of microreactors and their simple configuration allows for fast reaction screening, in difference to the conventional batch reactors which are limited by long reaction times.
- Mass transfer rates of multi-phase reactions can be predicted accurately due to uniform flow patterns enabling greater insight into reaction kinetics.

Enzymatic transformation of biomass derivatives to platform chemicals in microreactors has been widely reported. Enzymatic conversion of carbohydrates into furan-based chemicals is a popular application of microreactors. Furans are a versatile platform chemical for the production of oligosaccharide and their derivatives that have applications in biomedical, cosmetic, and the pharmaceutical industries. Microreactor channels (glass, PMMA, and silicon) immobilized with β-galactosidase have been used for the synthesis of oligosaccharide derivatives. In all cases, the product yield was higher compared to the reactions carried out in batch reactors with catalyst reusability over multiple runs. The increase in reaction rate can be attributed to the efficient diffusive mixing obtained in the microreactor channel.

In another study, aerobic liquid phase oxidation of glucose catalyzed by glucose oxidase in aqueous phase was carried out using a falling film microreactor (Illner et al. 2014). Glucose conversion of 50% was achieved, which was ~25% higher than the bubble column reactor, due to improved gas–liquid mass transfer enabled by the large interfacial area of the microreactor. Hydrolysis of complex polysaccharides into simple monosaccharides improves their processability to high-value chemicals. Invertase immobilized on silica (in a packed bed microreactor) (Carvalho and Fernandes 2015) and capillary walls of a glass microreactor (Carvalho et al. 2017) has shown improved space-time yield for hydrolysis of sucrose into glucose and fructose. The reactor was operated in a continuous mode for 30 days with a 100% retention of the catalytic activity. Biomass transformations such as esterification for transformation of caffeic and levulinic acid have been shown to be intensified in a packed bed microreactor (microchannel packed with immobilized enzyme catalyst). The reactions benefited from a larger catalytic surface area and improved heat transfer and temperature control in comparison to the batch reactors. Recent advances include ring-opening polymerization of caprolactone into polycaprolactone, a key biodegradable polymer for biomedical applications. Enzymatic transformation offers a more sustainable route to the traditional metal-organic catalytic pathway, but is limited by enzyme deactivation and inconsistencies in process parameters. This has been alleviated by using a packed bed aluminum microreactor filled with Novozym 435 catalyst (Kundu et al. 2011). A conversion of 90% was achieved for a residence time of 5 minutes, a sixfold improvement in reaction time compared to the batch reactor system. Enzymatic synthesis of isoamyl acetate (Pohar et al. 2009; Cvjetko et al. 2012), a common flavoring agent in the food industry, has been successfully synthesized using Lipase B derived from *Candida Antarctica.* A two-fold increase in productivity was achieved compared to a well-mixed batch reactor system due to intensified emulsification resulting in well-defined flow patterns and in situ product extraction in the microreactor.

Microreactors have potential to intensify enzymatic transformation of biomass and its derivatives into high-value chemicals. However, there are key challenges that must be overcome to enable their implementation on a commercial scale such as (1) effective solid handling to prevent clogging of microchannels, (2) reactor configuration for supported catalytic process to minimize pressure drop issues and maximize catalytic activity, and (3) holistic process analysis to determine the reaction kinetics to enable the shift to pilot-scale operations (Denčić et al. 2013). Micro-biorefinery (Hommes et al. 2019) is a future prospect for the microreactor technology where the crude biomass can be converted into value-added compounds continuously. This can be achieved by operating microreactors in tandem for multiple synthesis and separation steps or by using one-pot multi-catalytic processes. Catalyst selection, accurate reaction kinetic studies, and economic analysis are necessary to achieve this on an industrial scale.

### 18.3.2 Monolithic Reactors

Monoliths are porous, structural materials usually produced using a mound tailored for required shape and size (Fig. 18.2). The porous space is characterized by a network of inter-connected channels, enabling fluid flow characterized by very low backpressures in difference to a conventional packed bed configurations. Typically,

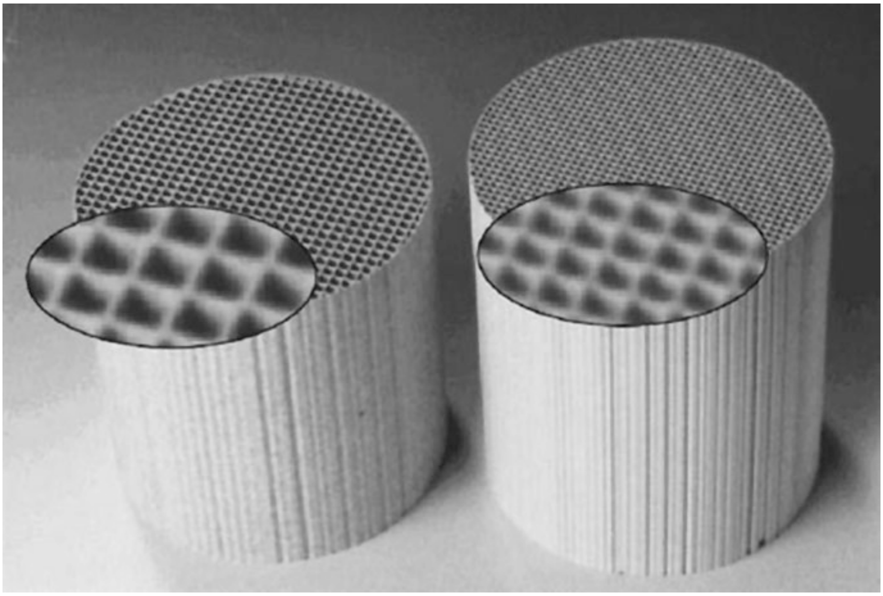

**Fig. 18.2** Monolithic supports for enzyme immobilization. Reprinted from De Lathouder et al. (2006). Copyright 2006, with permission from Elsevier

the inner walls of a monolith support are washed coated to support catalyst immobilization and maximize catalyst activity.

Monolithic reactors have traditionally found application in catalytic treatment of exhausts from chemical industries and automobiles. They are increasingly being investigated for biomass transformation due to the following advantages (Wood 2013):

- Increased surface area per unit volume of the reactor, typically 2–4 times higher than particulate catalysts.
- Reduced pressure drop for single- and multi-phase flows in difference to the traditional packed bed reactor.
- Improved catalyst efficiency and reaction selectivity as high mass transfer can be achieved due to short diffusion paths within the monolith.
- They allow for safer reaction environments and can be integrated with microreactors to enable plant miniaturization.

Monolithic supports can be either organic or inorganic, although the latter are gaining importance as they are biodegradable and easy to fabricate. L-aminoacylase was immobilized on cellulose acetate and investigated for biotransformation of over 15 substrates like benzoyl, acetyl, and chloroacetyl groups (Ngamsom et al. 2010). The enzyme activity was retained for up to 50 h at 50 °C, and longer operational and storage stability of the enzyme was observed when the reactor was operated at room temperature. The monolithic reactor was further integrated into a microchannel reactor enabling high-throughput screening of the substrates.

In another study (Sandig and Buchmeiser 2016), cellulose acetate beads were used as a support to immobilize lipase containing ionic liquids and embedded in a polyurethane matrix. The resulting hybrid monolith was operated in a continuous flow environment for enantioselective transesterification and amidations. In difference to the batch reactors, the productivity improved by a factor of 40 for the similar enantiomeric excess values. The monoliths were also successfully regenerated for multiple runs. Immobilized lipases on inorganic monolithic supports have been widely reported for transesterification reactions using biomass intermediates as starting materials to produce fragrance and flavoring agents (De Diego et al. 2005; Kawakami et al. 2007, 2009, 2012). The monolith catalyst was used in a continuous flow environment, and the conversion rate was found to be 1.5–2 times higher than the reaction in a batch slurry reactor, and catalyst activity was maintained under these conditions for 15 days. Another direct application of biomass valorization is the utilization of urban vegetable refused to synthesize organic monolithic supports. Magnacca et al. (2012) successfully produced a silica-based organic monolithic support for the immobilization of soybean peroxidase. The resulting monolithic support was used to carry out the oxidation reactions of commercially important organic molecules like DMAB and MBTH. Improved reaction kinetics were observed even on reusing the monolith indicating robust immobilization and retention of enzyme active sites due to the presence of mesoporous network within the monolith. Support-free monoliths using self-assembling decarboxylase hydrogels have been successfully implemented for the synthesis of biopolymers under flow conditions for more than 10 h with >98% reaction conversion (Mittmann et al. 2019).

Despite the numerous advantages of the IMERs over pellet-based catalysts, their application for enzymatic transformations on a commercial scale is very limited. One reason for this could be the high manufacturing cost of monolithic catalysts in difference to catalyst pellets. However, laboratory-scale studies have reported regeneration of monolithic support can reduce the overall cost of catalyst preparation. Research into alternate, cost-effective support materials and regeneration of monoliths can improve the commercial prospects for these reactors in the future.

### 18.3.3 Membrane Reactors

Membrane reactors are one of the earliest examples of achieving process intensification by combining reaction and separation steps in a single unit. The membrane reactor can be operated in three configurations: (1) selective removal of products from the reactor, (2) controlled addition of reactants to the reaction mixture, and (3) improve contact between the reactant and catalyst (catalytic membranes) (Westermann and Melin 2009; Shivaprasad and Emanuelsson 2018). Flow through catalytic membrane reactor is a popular configuration for heterogonous catalytic transformations due to high catalyst activity. The catalyst is present inside the membrane pores resulting in convective flow of reactants intensifying the contact between the catalyst and the reactants (Westermann and Melin 2009; Plakas et al. 2019; Bahadorikhalili and Mahdavi 2018). In traditional fixed bed reactor, the reaction rate is often limited by pore diffusion resistance. In addition to providing high-product selectivity and yield, they also minimize catalyst losses compared to traditional homogeneous catalyst systems (Plakas et al. 2019).

Enzyme membrane reactors (EMRs) have been widely reported for the production of oligosaccharides from biomass (Fig. 18.3). The enzymes are used either in their free or immobilized forms. Free enzyme EMR is characterized by utilizing small amounts of enzymes to reduce the formation undesired by-products. The membrane allows the reaction products to pass through, while retaining the free enzyme. In difference to the batch reactor, a higher degree of control is obtained over the product quality in an EMR. However, it is key to optimize the reaction time and the enzyme/substrate ratio to achieve maximum productivity. Enzymes can also be used in immobilized form, where they are either immobilized directly on the enzyme or through a support matrix. EMRs with immobilized enzymes enable activity retention for enzymes and reusability, although they are characterized by small losses in their activity. Reports of production of dextran oligosaccharide using dextranase immobilized on activated carbon (Torras et al. 2008) and modified magnetic membrane systems (Wang et al. 2018) showed better molecular weight distribution of the products and longer enzyme activity retention.

Lactic acid production from biomass is increasingly attracting interest due to its wide range of applications in the fine chemical industry. Conventional fermenters

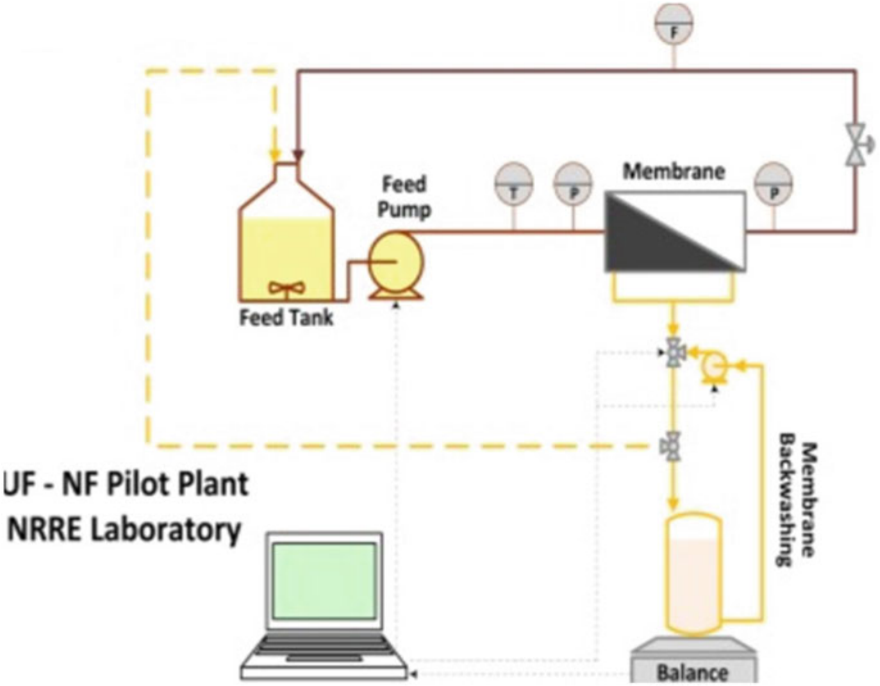

**Fig. 18.3** Schematic representation of enzyme membrane reactor set-up. Reprinted from Plakas et al. (2019). Copyright 2019, with permission from Elsevier

used for production of lactic acid are limited by downstream processing and purification. However, in recent years, reports have shown successful integration of micro-, nano-, and ultrafiltration membranes to achieve efficient separation of the products from the reaction mixture (Gonzalez et al. 2008; Li and Shahbazi 2006). Further, a combination of these membrane process in different configurations has shown higher productivity of lactic acid while allowing for environmentally benign processing (Pal et al. 2009). Valorization of lignin as a source of highly valuable feedstock has been successfully reported in EMRs (Busse et al. 2017). Versatile peroxidase was used to catalyze the lignin transformation in a continuous stirred tank reactor integrated with an ultrafiltration unit. A mathematical model was used to minimize the challenges associated with co-substrate inhibition of the enzyme and membrane fouling, and it was found that continued addition of enzymes resulted in a higher reaction rate. Lim and Ghazali (2020) reported the use of EMR for cellulose hydrolysis with intermittent product removal strategy to minimize enzyme fouling and improve product yield. It was found that intermittent product removal after 24 h resulted in a conversion of over 80% compared to product removal after 4 h. However, increase in substrate concentration has a negative impact of membrane flux and resulted in membrane fouling.

Enzymatic membrane reactors have been used successfully in various transformations but are still limited by challenges such as enzyme reusability and membrane fouling for industrial applications. These challenges can be overcome by choosing a suitable membrane, using membrane modifiers to reduce fouling, and optimizing temperature and pH to maintain enzyme activity and an optimal reactor design to support membrane configuration targeted for specific applications.

### 18.3.4 Ultrasound-Assisted Biomass Valorization

Another route to achieving reaction intensification is through the use of an alternate processing technique, such as ultrasound. Ultrasound is an acoustic wave that imparts large amounts of energy to the reaction medium through cavitation and other secondary effects. The chemical effects of ultrasound result in the production of local hotspots due to the collapse of cavitation bubbles resulting in a localized increase in temperature and pressure (Luo et al. 2014). This can result in the formation of reactive radicals and also can cause destruction of crystalline materials causing solid materials to fuse. The unique environment produced by ultrasound can be utilized for pretreatment, fractionation, and chemical reactions of biomass (Fig. 18.4) under conditions milder than thermochemical routes resulting in an increased reaction efficiency and catalytic activity (Luo et al. 2014).

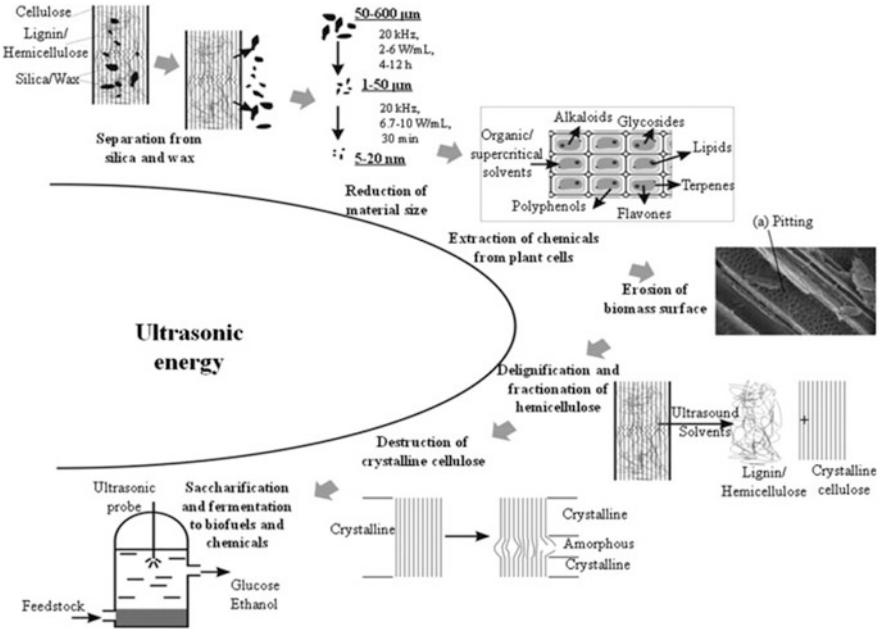

**Fig. 18.4** Potential pathways for pretreatment and transformation of lignocellulosic biomass. Reprinted from Luo et al. (2014). Copyright 2014, with permission from Elsevier

The combination of ultrasound and enzymes has been shown to intensify saccharification of lignocellulose due to increased accessibility of the substrates by the enzymes. Intensification was mainly observed through an increase in the reaction rate constant (Indra Neel et al. 2012), activation energy of the enzyme (Souza et al. 2013), and increase in the product yield catalyst (Velmurugan and Muthukumar 2012) over unsonicated routes. Additionally, sono-enzymatic saccharification of cellulose in ionic liquids has demonstrated improved reaction yield due to increased mass transfer facilitated by cavitation, in difference to using chemical solvents and conventional heating (Wang et al. 2012; Li et al. 2012). Another application of the synergistic combination of ultrasound and enzyme catalysis is production of biodiesel. Lipase-catalyzed hydrolysis of non-edible oil has shown improved reaction rate and a massive reduction in reaction times (50 h to 3 h) with minimum need for product purification compared to chemical catalyst routes (Fiametti et al. 2011). Reaction rates for transesterification of triglycerides were found to be 1.5–2 times quicker than conventional methods and were accompanied by minimum waste of unreacted starting materials due to low stoichiometric ratio of the substrates (Tupufia et al. 2013; Batistella et al. 2012). Pretreatment and extraction from algal cells for production of bioethanol have been reported. Depending on the algal species, the effect of sequential vs combined sonication and enzyme processing, sonication frequency, time period, and type of enzymes used had a significant effect on the extraction and reaction efficiency. (King Pm et al. 2012; Wang and Wang 2012; Kumari et al. 2011; Cheng et al. 2011). However, this technique is applicable only to a limited range of algal species and further research is needed to understand the impact of sonication on cell morphology and subsequent reactivity.

The synergistic combination of ultrasound at low-frequency range and enzyme catalysis can intensify biomass valorization, without adversely affecting the structural conformation and activity of the enzymes. However, enzyme deactivation was still observed due to localized heating effects in some enzyme species resulting in reduced reaction yield and product inhibition (Lopez et al. 1994; Kadkhodaee and Povey 2008). This can be overcome by further identifying optimum ultrasound parameters for a wide range of enzymes and understanding the effect of ultrasound irradiation on the morphology of the enzymes to maintain catalyst activity and promote reusability for consecutive reaction cycles.

## 18.4 Opportunities and Considerations for Commercialization

With an increasing awareness about resource use and government regulation, chemical industries are fast realizing the need to change their manufacturing processes to align with sustainable strategies. The products of biomass valorization are diverse and find application in a range of sectors within the chemical industry. The drivers for change from conventional batch reactors to PI reactors depend on the target sector for the valorization products. Economic drivers such as capital and utility costs and return on investment play a significant role in the petrochemical sector for biomass valorization using PI reactors. However, environmental drivers such as low greenhouse gas emissions and small chemical and equipment footprint for PI reactors have the potential to accelerate technology transfer from laboratory-scale to commercial-scale operations. In difference, the drivers for the pharmaceutical industries include fast and easy scale-up, consistent product quality, and inherent safety (for exothermic and high-pressure reactions). The production capacity in this sector is in the range of 200–500 tons/year for high-value, niche chemicals (Harmsen 2013a, b). Hence, savings on the capital cost of the equipment itself are not a significant driver, but key benefits of PI reactors to improve processing efficiency and product quality can improve the prospects for producing high-value chemical intermediates through biomass valorization in PI reactors on an industrial scale. Additionally, biomass valorization has inherent challenges associated with the scale of land area needed for cultivation, transportation of biomass to processing plants, and water separation cost from the end product. The modular and flexible nature of PI reactors can be harnessed to process biomass at the point of origin and integrating it with suitable in situ separation systems to reduce transportation and separation costs.

Despite the obvious advantages of PI reactors for biomass valorization, there are very limited examples of industrial applications. One of the main reasons for this is the novelty of biomass valorization as a process and the complexity of the feedstock, resulting in a slower commercial uptake in comparison to well-established petroleum–feedstock-based industries. Similarly, replacing batch reactors with novel reactor configurations are faced with barriers such as cost of change (capital expenditure and regulatory approvals), uncertainty, lack of technical awareness, and motivation for change. These barriers can be overcome by demonstrating PI reactors for industrially relevant biomass transformation processes, innovative business models to reduce the risk for chemical industries, financial support for high-risk innovations, and increased engagement of chemical industries with upcoming process innovations in biomass valorization using PI technologies.

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# Wetland Flora of West Bengal for Phytoremediation: Physiological and Biotechnological Studies—A Review

# 19

Gouri Das and Ashwani Kumar

## Abstract

Phytoremediation is a promising green technology for the remediation of various industrial effluents. Notably, aquatic plants are widely applied to remove dyes and toxic metals from polluted environments. Aquatic plants have attracted wide attention because of their low cost and high level of resource utilization and are promising green technology for the remediation of various industrial effluents. Water treatment, reuse, and reducing the nutrients loading to the aquatic environments are key ways to achieve sustainable aquaculture. A large number of plants are being used for phytoremediation. Water plants or hydrophytes are the most conspicuous and colorful element of any wetland system. In present investigation, the hydrophytes, phytoplanktons, and herbaceous plant life of the adjacent areas of Tapan Dighi in Dakshin Dinajpur District of West Bengal were explored, collected, and preserved. Altogether, 78 species under 37 families were observed during the study. Phytoplanktons, especially cyanobacteria and chlorophycean members (blue-green and green algae) showed rampant growth in late spring producing algal bloom. Macrophytes observed were of free-floating, rooted with floating leaves, submerged, and immersed with floating and submerged leaf types. Phytoremediation by aquatic macrophytes is a promising technology with higher efficiency. The data generated from the study will provide

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a meaningful insight into the vegetation, phytoremediation, and ecosystem of the study area. Key issues of phytoremediation are reviewed.

**Keywords**

Hydrophytes · Phytoplankton · Phytoremediation · Tapandighi · Wetland

## 19.1 Introduction

With the development of industrialization and urbanization, the abundance of heavy metals in the environment has increased enormously during the past decades, which raised significant concerns throughout the world. Merian (1984) reported that emissions of the heavier elements chromium, nickel, and cobalt with their short atmospheric life cycle play a more overpowering role in waters and soils. Merian (1984) further suggested that natural weathering of rocks and soils is in the order of one-sixth of the total emissions for cobalt, chromium, nickel, and arsenic, but is relatively smaller for beryllium, selenium, and cadmium. Sebastian and Prasad (2014) studied cadmium minimization in rice. However, volcanic emissions are globally of very low significance. Increasing use of metal contaminated underground water for agricultural purposes (irrigation in rice and other crop fields) in the arsenic-affected areas, especially in Bangladesh and West Bengal (India), resulted in the increased concentration of pollutants, especially arsenic in freshwater systems, which could potentially lead to arsenic entering the human food chain (Rmalli et al. 2005; Beebout 2013). Many countries like India, Bangladesh, Taiwan, Argentina, Hungry, Mexico, and Chile have reported extensive arsenic contamination in their groundwater. Indiscriminate use of arsenical pesticides, geogenic sources, and anthropogenic activities like fossil fuel burning, mining, and natural minerals are the main source of arsenic contamination in the environment. Contamination in freshwater systems poses a health threat not only to the aquatic organisms but also to the humans. High levels of arsenic have been reported in a number of aquatic plants grown in contaminated water bodies. Contamination of rice with toxic heavy metals is especially a health concern in developing countries. Tripathi et al. (2007) studied Arsenic tolerance and remediation by plants. Alarcón-Herrera et al. (2013) reported that several million people around the world are currently exposed to excessive amounts of arsenic (As) and fluoride (F) in their drinking water. Das et al. (2022) reviewed efficacious bioremediation of heavy metals and radionuclides from wastewater employing aquatic macro- and microphytes.

A large number of azo dyes including Reactive Red 120 (RR120) are extensively used in fabric manufacturing due to low cost, ease of preparation, fastness, versatility, and intensity of the colors (Mathur and Kumar 2013, 2016; Rawat et al. 2016). Certain azo dyes contain chemical groups, which have a high affinity for metal ions (Balapure et al. 2015; Hussain et al. 2016). These enhanced properties provide a high degree of biological, chemical, and photocatalytic stability. Nevertheless, their resistance to break down due to time, exposure to sunlight, detergents, water, and microorganisms results in poor degradation in the environment (Solís et al. 2012). The dye removal using phytoremediation has demonstrated its potential to degrade many recalcitrant dyes. Phytoremediation, a plant-based green technology, includes several processes namely phytoextraction, phytodegradation, rhizofiltration, phytostabilization, and phytovolatilization. It has received increasing attention after the discovery of hyperaccumulating plants, which are able to accumulate, concentrate, and translocate high amounts of certain toxic elements in their above-ground/harvestable parts (Mathur and Kumar 2013). The water treatment, reuse, and reducing the nutrients loading to the aquatic environments are key ways to achieve sustainable aquaculture (Sarkheil and Safari 2020).

The usage of phytoremediation is a widely studied and applied technology using aquatic plants, and their associated microorganisms is an effective and environment-friendly method for water treatment (Mathur and Kumar 2013; Bokhari et al. 2019). Both terrestrial and aquatic plants can remediate contaminated soils and waters due to their high adaptive and hyperaccumulation capability (Patel and Sahoo 2020).

Wetlands, the earth's most important freshwater resource, perform some of the important functions including water storage, flood attenuation, recharge of groundwater, water purification, biogeochemical filtration, agriculture, fisheries, wildlife resource and habitat, transport, and recreation. Wetland ecosystem, which has phytoplankton, zooplankton, plant community in succession, microorganisms, migratory flocks, and many others, is the common biotic elements, and is the most fragile and biologically diverse ecosystem. The vegetation type of such water bodies thus plays a very crucial role in these functions in the long run. Aquatic plants are widely applied to remove dyes and toxic metals from polluted environments.

Aquatic macrophytes are defined as emergent, floating-leaved, or submerged macroscopic plant species with distinct roots and shoots. Rooted plants include submerged (e.g., family Hydrocharitaceae, Ceratophyllaceae) and emergent (e.g., family Potamogetonaceae, Ranunculaceae, and Cruciferae) members. A number of aquatic plant species have been investigated for the remediation of toxic contaminants such as As, Cu, Zn, Cr, Cd, Pb, and Hg. This review provides insight into the process of phytoremediation and possible sources native and invasive plants from Dakshin (South) Dinajpur for current and future use.

Dakshin (South) Dinajpur District is traditionally known as a district of wetlands. The district is dotted with numerous natural water bodies each having its influence on life and professional activities on the areas around the wetlands. The vegetation of the wetlands and adjacent areas, therefore, need to be peered into in depth. There is a complete lack of data concerning the plant community present in and around the wetland ecosystem for South Dinajpur. This study was carried out with an intention to generate awareness and information on phytoplankton and plants available in and around one of the water bodies with historical importance. The data generated from the study will be collated for rendering a meaningful insight into the vegetation and ecosystem of the study area. It will certainly create awareness among the incumbents about the vegetation pattern and diverse plant groups of the water body and the need to conserve the biodiversity of the water body and adjacent area. Map showing physiographic divisions of West Bengal is presented here. Source: https://www.researchgate.net/publication/275833658_River_systems_and_water_resources_of_West_Bengal_A_review/figures?lo=1. This figure was uploaded by Sunando Bandyopadhyay (Fig 19.1).

Plants growing in wetlands and water are technically called hydrophytes (Tiner 1999). Hydrophytes (plants with perennating rhizomes or winter buds) and helophytes (plants with buds at the bottom of the water or in the underlying soil) were the two types of cryptophytes (plants with dormant parts below ground), while other wetland plants were included in other life forms, such as phanerophytes (trees and shrubs) as envisaged by Vymazal (2007).

***Life Forms*** Plants growing in wetlands and other moist soils may either be annuals or perennials. Many of the smaller and slender herbs are annuals. They flower, fruit, and disperse seeds and die in a single year. However, perennials have been growing for many years developing food storing woody structures such as rhizomes, corms, and stolons (Rao et al. 2008). Such plants provide a rich source of phytoremediation activities and have been used widely (Brunhoferova et al. 2021).

***Growth Forms*** There have been many attempts to classify aquatic plants according to their growth forms. A classification based on the response of the plant to milieu for growth and development was suggested rather than directly on its morphology or the way it survives adverse conditions (Cook 1996). According to this, the different types of growth forms are ephydates—bottom-rooted with floating leaves, haptophyte—attached to but not penetrating the substrate, hyperhydate—emergent aquatics (lower parts almost always in water), plankton—free swimming below the water surface, pleustophyte—free-floating (at the water surface), rosulate—submerged, bottom-rooted, leaves in a rosette, tenagophyte—juvenile submerged, adult usually terrestrial, and vittate—submerged, bottom-rooted, leaves cauline. However, many species change their dependence on water in regions with differing climates or with different hydrological regimes. Such plants can have more than one life form.

Brunhoferova et al. (2021) studied a mixture of 27 micropollutants (pharmaceuticals, pesticides, herbicides, fungicides, and others) and their removal from aqueous solution by phytoremediation. Phytoremediation contributes to removal of micropollutants from wastewater in constructed wetlands. *Phragmites australis*, *Iris pseudacorus*, and *Lythrum salicaria* in hydroponic conditions indicated leading capability for micropollutant uptake in *Lythrum salicaria*.

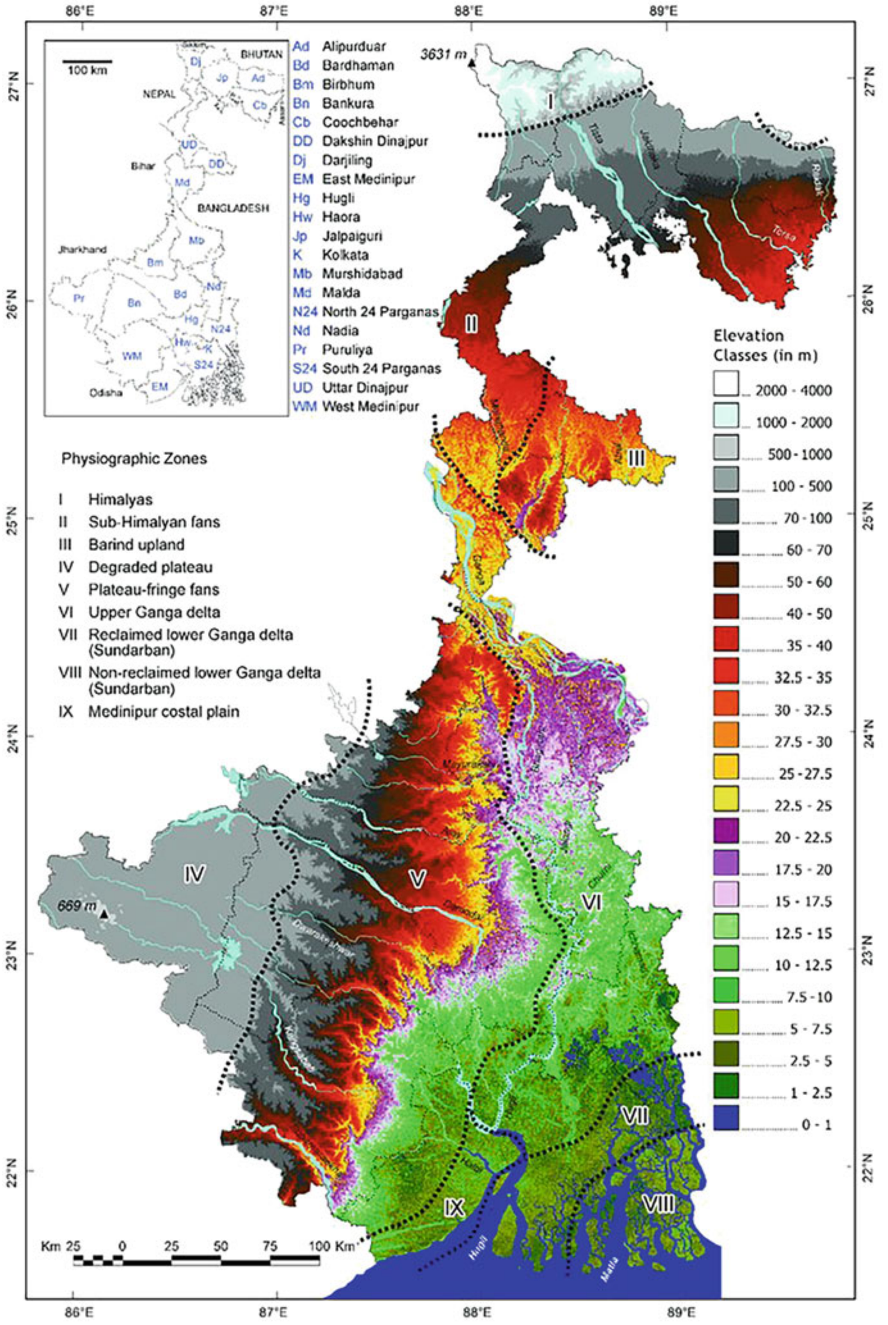

**Fig. 19.1** West Bengal: physiographic divisions. Districts shown in this and most subsequent maps are identified in the inset. (Source: Spot-heights from Survey of India. Other elevations from Shuttle Radar Topography Mission data of 2000; https://www.researchgate.net/publication/275833658_River_systems_and_water_resources_of_West_Bengal_A_review/figures?lo=1. This figure was uploaded by Sunando Bandyopadhyay)

Huang et al. (2021) reported vermifilter combined with wetland plant (VFP) is an eco-friendly and sustainable approach for recycling excess sludge by joint action of earthworms, wetland plants, and microorganisms. However, the effects of wetland plants on sludge decomposition and involved microorganisms remain unclear. The wetland plants' species of *Acorus calamus* and *Epipremnum aureum* were separately planted on the surface layer of vermi-reactors by earthworms *Eisenia fetida*. Wetland plants could significantly ($P < 0.001$) enrich the eukaryotic population, rather than bacterial population (Huang et al. 2021).

## 19.2 Materials and Methods

The study was a random opportunistic survey visiting the wetland locality of Tapan Dighi. The area adjacent to Tapan Dighi was also studied floristically. In wetland, plants were collected from different wetland zones such as deep waters, shallow water, fringes, and other nearby moist soils around the water body. All the collected specimens, identified and unidentified, were pressed for herbaria using a dry method and are kept in the department. Fresh specimens were identified with the comparison from relevant documents, books, and regional and other floras.

### 19.2.1 Study Area

Down the ages, Tapan Dighi has been the cynosure of many events of political and social importance. Many myths still can be heard among the locals. The wetland, Tapandighi, is situated 14 km to the south of Bangadh, a place of historic interest, and is 30 km away from Balurghat, the district headquarter of Dakshin Dinajpur. As per measurements taken by Mr. Hamilton, during the early nineteenth century, it measures 1420 yards in length and 400 yards in width (Fig. 19.1a–d).

## 19.3 Results

### 19.3.1 Habit and Habitat

Tapan Dighi offers a very rich habitat for phytoplankton (Fig. 19.1c). It is seen in the form of smelly filamentous mats on the surface of water, and some unicellular algae and members of Cyanophyceae occur in late spring season, which reduce clarity of the water body and often appear green or golden blue in color.

## 19.3.2 Survey and Collection

The pond typically contains three broad categories of phytoplankton—filamentous phytoplankton, macroscopic multi-branched phytoplankton, and unicellular phytoplankton. Macroscopic filamentous phytoplankton is composed of long chains of cells attached to a substrate, like submerged or emergent vegetation, rocks, or bottom of the Dighi. The rampant growth of these benthic filamentous algae becomes visible at the surface. Benthic filamentous algae often break and float on the surface as dense mats. Members of filamentous blue-green algae and green algae develop into nuisance filamentous mats on the surface of Tapan Dighi.

A thorough survey of the vegetation of Tapan Dighi and adjacent areas was carried out, and specimens were collected, studied, and recorded (Tables 19.1 and 19.2). The plants collected were pressed, dried, and preserved following proper technique for herbarium sheet preparation. The identification was made following relevant floras and manuals (Das 2004; Ghosh et al. 2008). Some of the plants recorded here are already in use for phytoremediation, and possibility of other plants for phytoremediation is very bright as they purify the water of wetlands (see asteristics).

## 19.3.3 Identification

**Table 19.1** List of plants collected from and around Tapandighi

| Cryptogams | | |
|---|---|---|
| Sl. No. | Name of the species | Family |
| 1. | *Anabaena* sp. | Nostocaceae |
| 2. | *Azolla pinnata* R.Br. (Robert Brown) | Salviniaceae |
| 3. | *Cladophora* sp. | Chadophoraceae |
| 4. | *Chara* sp. | Charophyceae |
| 5. | *Hydrodictyon* sp. | Hydrodictyaceae |
| 6. | *Microcystis* sp. | Microcystaceae |
| 7. | *Nostoc* sp. | Nostocaceae |
| 8. | *Oedogonium* sp. | Charophyceae |
| 9. | *Oscillatoria* sp. | Oscillatoriaceae |
| 10. | *Spirogyra* sp. | Zygnemataceae |
| 11. | *Ulothrix* sp. | Ulotrichaceae |

**Table 19.2** List of plants collected from and around Tapandighi

| Angiosperms | | |
|---|---|---|
| Sl. No. | Name of the species | Family |
| Magnoliopsida | | |
| 1. | *Acalypha indica* L. | Euphorbiaceae |
| 2. | *Achyranthes aspera* L. | Amaranthaceae |
| 3. | *Ageratum conyzoides* L. | Asteraceae |
| 4. | *Alternanthera sessilis* (L.) R.Br. ex DC. | Amaranthaceae |
| 5. | *Amaranthus spinosus* L. | Amaranthaceae |
| 6. | *Amaranthus viridis* L. | Amaranthaceae |
| 7. | *Argemone mexicana* L. | Papaveraceae |
| 8. | *Bidens pilosa* L. | Asteraceae |
| 9. | *Cassia tora* L. | Fabaceae |
| 10. | *Centella asiatica* (L.) Urb. | Apiaceae |
| 11. | *Chromolaena odorata* (L.) R.M. King and H. Rob. | Asteraceae |
| 12. | *Clerodendrum infortunatum* L. | Lamiaceae |
| 13. | *Croton bonplandianum*, Baill. | Euphorbiaceae |
| 14. | *Cyanthillium cinereum* (L.) H. Rob. | Asteraceae |
| 15. | *Dentella repens* (L.) J.R. Forst. and G. Forst. | Rubiaceae |
| 16. | *Dentella repens* var. *serpyllifolia* (Wall. ex Craib) Verdc. | Rubiaceae |
| 17. | *Digitalis ciliaris* Ehrh. | Scrophulariaceae |
| 18. | *Eclipta alba* (L.) Hassk. | Asteraceae |
| 19. | *Emilia sonchifolia* (L.) DC. ex DC | Asteraceae |
| 20. | *Enhydra fluctuans* Lour. | Asteraceae |
| 21. | *Evolvulus nummularius* (L.) L. | Convolvulaceae |
| 22. | *Glinus oppositifolius* (L.) Aug. DC. | Molluginaceae |
| 23. | *Ipomoea carnea* Jacq. | Convolvulaceae |
| 24. | *Leucas aspera* (Willd.) Link. | Lamiaceae |
| 25. | *Ludwigia repens* J.R. Forst. | Onagraceae |
| 26. | *Mikania micrantha* Kunth. | Asteraceae |
| 27. | *Mimosa pudica* L. | Fabaceae |
| 28. | *Nymphaea nouchali* Burm. f. | Nymphaeaceae |
| 29. | *Parthenium hysterophorus* L. | Asteraceae |
| 30. | *Persicaria hydropiper* (L.) Delarbre | Polygonaceae |
| 31. | *Persicaria orientalis* (L.) Spach. | Polygonaceae |
| 32. | *Phyllanthus niruri* L. | Phyllanthaceae |
| 33. | *Ranunculus aquatilis* L. | Ranunculaceae |
| 34. | *Rumex dentatus* L. | Polygonaceae |
| 35. | *Scoparia dulcis* L. | Plantaginaceae |
| 36. | *Scrophularia scorodonia* L. | Scrophulariaceae |
| 37. | *Solanum torvum* Sw. | Solanaceae |
| 38. | *Spermacoce ocymoides* Burm.f. | Rubiaceae |
| 39. | *Suregada multiflora* (A.Juss.) Baill. | Euphorbiaceae |
| 40. | *Trapa natans* var. *bispinosa* (Roxb.) Makino | Trapaceae |
| 41. | *Tridax procumbens* (L.) L. | Asteraceae |
| 42. | *Xanthium strumarium* L. | Asteraceae |

Angiosperms

| Sl. No. | Name of the species | Family |
|---|---|---|
| Liliopsida | | |
| 1. | *Alisma subcordatum* Raf. | Alismataceae |
| 2. | *Colocasia esculenta* (L.) Schott. | Araceae |
| 3. | *Commelina benghalensis* L. | Commelinaceae |
| 4. | *Cymbopogon martinii* (Roxb.) Wats. | Poaceae |
| 5. | *Cynodon dactylon* (L.) Pers. | Poaceae |
| 6. | *Cynodon dactylon* (L.) Pers. | Poaceae |
| 7. | *Cyperus halpan* L. | Cyperaceae |
| 8. | *Cyperus rotundus* L. | Cyperaceae |
| 9. | *Dactyloctanium aegyptium* (L.) Willd. | Poaceae |
| 10. | *Eichhornia crassipes* (Mart.) Solms. | Pontederiaceae |
| 11. | *Eleocharis acicularis* (L.) Roem. and Schult. | Cyperaceae |
| 12. | *Eleocharis dulcis* (Burm.f.) Trin. ex Hensch. | Cyperaceae |
| 13. | *Eleocharis parvula* (Roem. and Schult.) Link ex Bluff, Nees and Schauer | Cyperaceae |
| 14. | *Eleusine indica* (L.) Gaertn. | Poaceae |
| 15. | *Fimbristylis miliacea* (L.) Vahl | Cyperaceae |
| 16. | *Hydrilla verticillata* (L. f.) Royle | Hydrocharitaceae |
| 17. | *Imperata cylindrica* (L.) Beauv. | Poaceae |
| 18. | *Lemna minor* L. | Lemnaceae |
| 19. | *Panicum repens* L. | Poaceae |
| 20. | *Pistia stratiotes* L. | Araceae |
| 21. | *Potamogeton crispus* L. | Potamogetonaceae |
| 22. | *Potamogeton epihydrus* Raf. | Potamogetonaceae |
| 23. | *Saccharum spontaneum* L. | Poaceae |
| 24. | *Sagittaria sagittifolia* L. | Alismataceae |
| 25. | *Vallisneria spiralis* L. | Hydrocharitaceae |

## 19.4 Phytoremediation

Irrigation of agricultural soils with wastewater results in elevated uptake of metals in crops affecting food quality and poses health risks to the consumers (Murtaza et al. 2015). Jallad (2015) analyzed different types of rice grains for heavy metal analysis and then compared the daily dietary intake of toxic metals for the general population in 29 different countries around the world. Efforts are being made to minimize uptake through roots and translocation to grains of toxic heavy metals, especially Cd in rice. Genetic engineering is used as an approach to achieve this goal, and some transgenic rice varieties have been developed to meet the challenge (Cai et al. 2015).

### 19.4.1 Textile Waste

Textile manufacturing releases potentially toxic compounds, such as synthetic dyes, into the environment. Uncontrolled use of such dyes can negatively affect human health and the ecological balance (de Oliveira et al. 2015). Aquatic pollution caused by dyes has increased together with the growth of activities using colorants such as the food, textile, leather, and agrochemical industries (Hernández-Zamora and Martínez-Jerónimo 2019).

#### 19.4.1.1 Phycoremediation of Heavy Metals using Living Green Microalgae

Due to anthropogenic activities, the quantity of heavy metals in the environment has led to an increase in our exposure to the metals and, by consequence, an increase in heavy-metal-related diseases. Microalgae are generally endangered in aquatic ecosystems by the presence of hazardous materials like toxic metals. According to Javanbakht et al. (2014), the metals that are currently considered to be the most problematic are cadmium (Cd), copper (Cu), magnesium (Mn), chromium (Cr), lead (Pb) iron (Fe), zinc (Zn), mercury (Hg), and as these metals are toxic even in low concentrations. Microalgae have the potential to offer a new eco-friendly, efficient, and cost-effective solution to remove heavy metals from wastewater (Javanbakht et al. 2014).

Microalgae are ecologically important species in aquatic ecosystems due to their role as primary producers (Moorthy et al. 2020; Spain et al. 2021; Fig. 19.2). The textile-used, azo dye DB15, caused toxic effects of different magnitude on microalgae (Hernández-Zamora and Martínez-Jerónimo 2019). Textile dyes—Optilan yellow, Drimarene blue, and Lanasyn brown—cause toxicity to algal growth. However, high decolorization percentages achieved by *Chlorella vulgaris*, *Anabaena oryzae*, and Wollea *saccata* make them potential candidates for bioremediation and preprocessing to remove dyes from textile effluents. However, recently the most frequently used microalgae strains in the phycoremediation belong to the

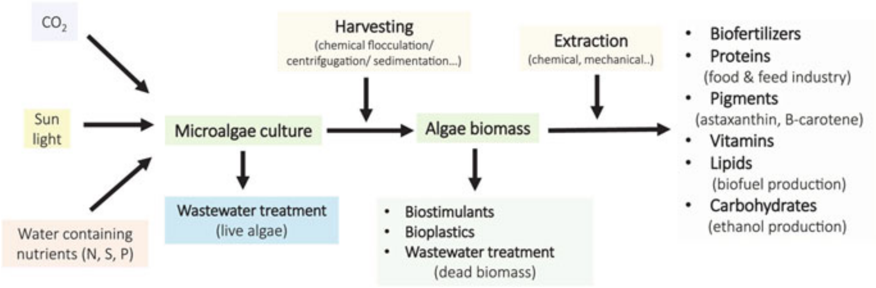

**Fig. 19.2** Schematic view of the product value chain of microalgae. Source: Spain et al. (2021). Reproduced under license number 5124011229845 dated 8 August

Chlorophyta phylum, particularly species of genera *Chlorella* and *Scenedesmus* (Spain et al. 2021).

The application of living microalgae for the phycoremediation of HMs can include both extracellular and intracellular bioremediation strategies, and Danouche et al. (2021) reviewed the different steps like

1. Michalak et al. (2013) defined biosorption as the rapid and reversible binding of ions from aqueous solutions onto functional groups present on the surface of biomass. The biosorption process includes the mechanisms: precipitation, complexation, ion exchange, transport across cell membranes, and physical adsorption (Javanbakht et al. 2014). HM biosorption indicates the physicochemical property of the microalgae cell surface, which allows the HM ions from the solution to bind to the cell surface without involving cell metabolism. This nonmetabolic process is highly dependent on a variety of different parameters (e.g., pH, temperature, concentration, biosorbant dosage, or contact time), with the microalgal strain, the contact time, and the pH being the most important aspects (Spain et al. 2021). Microbial extracellular polymeric substances (EPS) can enhance the aggregation of soil particles and benefit plants by maintaining the moisture of the environment and trapping nutrients (Costa et al. 2018).
   However, the extracellular polymeric substances (EPS) formed by microalgae in response to stress conditions depend on cell metabolism. EPS are biosynthetic polymers composed mainly of polysaccharides, structural proteins, lipids, nucleic acids, enzymes, and other compounds such as humic acids (Flemming et al. 2000). Costa et al. (2018) suggested that advances in modern techniques such as high-throughput sequencing, controlled low-strength material (CLSM), nuclear magnetic resonance, scanning electron microscopy, and association with classic microbiology techniques will characterize and develop new EPS with efficient function in water and soil ecosystem.

   The microalgal cells can regulate EPS synthesis and can also change the properties of these biopolymers as required (Naveed et al. 2019; Ubando et al. 2021). Naveed et al. (2019) reported applications of microalgal EPS to the remediation of metal(loid) polluted environments. Ubando et al. (2021) identified the materials produced by microalgae to facilitate biosorption. Industries may benefit from adoption of microalgal biosorption of heavy metals to treat their affluents.

   Spectroscopic studies have shown that functional groups including carboxyl, hydroxyl, sulfate, sulfhydryl (thiol), phosphate, amino, amide, imine, thioether, phenol, carbonyl (ketone), imidazole, phosphonate, and phosphodiester have the properties to be involved in metal binding (Fig. 19.3). Modification of surface reactive sites via surface grafting and/or exchange of functional groups could be helpful to improve biosorbent capacity (Javanbakht et al. 2014).
2. The complexation with extracellular polymeric substances was released by microalgae in the extracellular environment under stress conditions. The chelation and the complexation of HMs with active groups of the cell surface of microalgae incorporate ions such as $Na^+$, $K^+$, $Ca^{2+}$, and $Mg^{2+}$, which can be

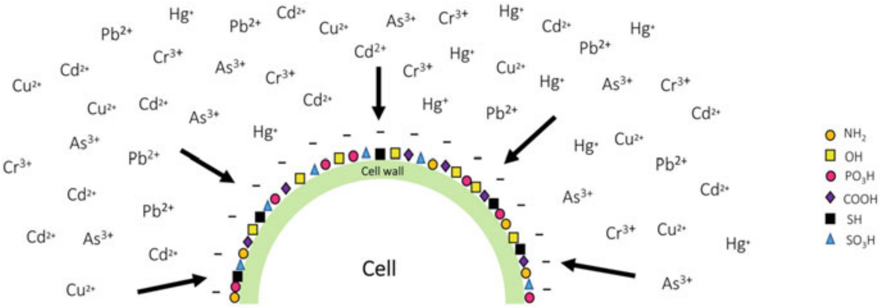

**Fig. 19.3** Process of metal ions to the functional groups on the microalgal cell wall (adapted from Javanbakht et al. 2014) Source: Spain et al. (2021). Reproduced under license number 5124011229845 from RightsLink dated August 8

reversibly substituted by other toxic HMs ions in solution, via a process called ion exchange (Danouche et al. 2021). According to Monteiro et al. (2012) when concentration of metal in the extracellular environment is significantly higher than the intracellular concentration, cations can be transported by the negative charged groups of the cell surface to reach the intracellular compartment via active transport across the plasma membrane after binding to thiol molecules primarily cysteine.

3. In contrast to the biosorption process, bioaccumulation is a dependent metabolic pathway. Metal transporters are involved in the intracellular bioaccumulation of HMs
4. Generally, most of the HMs are hydrophilic, and their transport across the plasma membrane (lipophilic) is mediated mainly through a specific protein called as metal transporters. Main intracellular mechanisms include compartmentalization in cell organelles, enzymatic biotransformation, or photoreduction of HMs (Fig. 19.4) (see review Danouche et al. 2021). The sequestration of the MT-HM complex in particular cell organelles, especially vacuoles, chloroplasts, and mitochondria, has prompted researchers to develop hypotheses about metal bioaccumulation pathways for the associated tolerance mechanisms. Thereafter, several detoxification pathways can take place in intracellular compartments (Leong and Chang 2020).

Figure 19.4 summarizes the main pathways involved in the bioremediation and mitigation of HMs (Danouche et al. 2021).

#### 19.4.1.2 Role of Microorganisms

García-García et al. (2016) suggested that bacteria, protists, and microalgae are able to compartmentalize heavy metal complexes into vacuoles, phytochelatin, and metallothionein biosynthesis, phosphate/polyphosphate metabolism; chloroplasts, and mitochondria. Recently, genetically engineered microorganisms with greater capacities and efficiencies for heavy metal recovery, recycling of heavy metals,

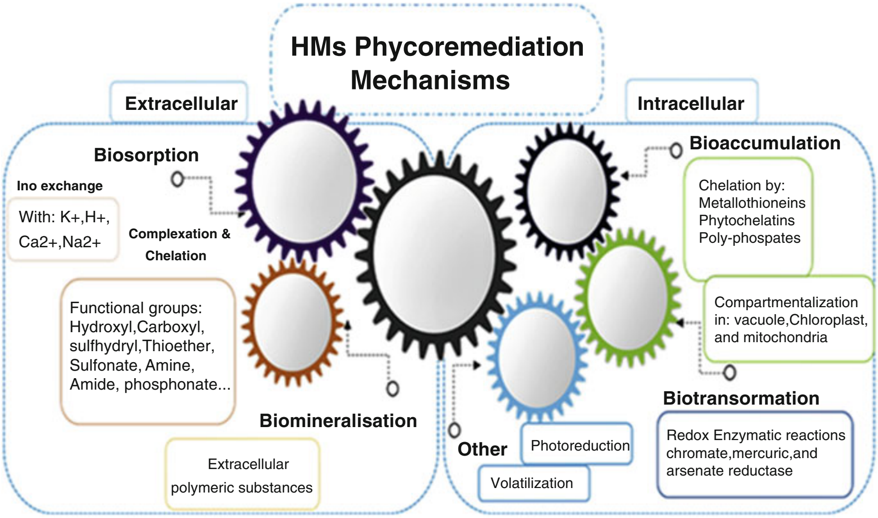

**Fig. 19.4** HM-phytoremediation mechanisms (modified from (García-García et al. 2016; Kumar et al. 2015)). Source: Danouche et al. (2021). Reproduced with licence number 5234950808694 dated 23rd January 2022

biosensing of metal ions, and engineering of metalloenzymes have been developed. Microorganisms are the most favorable convertor of azo dyes in comparison with other applications due to their practicality, productivity, simplicity, and inexpensiveness (Islahuddin et al. 2017; Manogaran et al. 2021). The application of microorganisms in azo dye remediation is one of the most favorable processes in comparison with other applications due to its practicality, productivity, simplicity, and inexpensiveness.

### 19.4.2 Hyperaccumulating "Monilophytes" or Ferns

Water fern, *Azolla pinnata*, *Azolla caroliniana*, *A. filiculoides*, etc., are found to possess capacity of toxic element uptake (Zhang et al. 2008). *Azolla filiculoides* bioaccumulates As, Hg, and Cd as depicted by Rahman et al. (2008), Rai (2008), Rai and Tripathi et al. (2008).

Current application of the synthetic chemical controls and its constant repetitive applications have resulted in resistant mosquitoes and environmental pollution (Ravi et al. 2018). *Azolla pinnata* extracts have shown the potential for developing natural products against *Aedes aegypti* and *Aedes albopictus* mosquitoe dengue vectors (Ravi et al. 2021). Their experimental test showed that *A. pinnata* plant extracts can be used for *Aedes* adulticidal activities by impregnated paper method (method similarly used to test chemical insecticides). Three important chemical compounds from *A. pinnata* extracts have been isolated such as 1-(O-alpha-D-glucopyranosyl)

(1,3R,25R)-hexacosanetriol, pyridate, and nicotinamide N-oxide. These are bioactive compounds that are responsible for adulticidal and ovicidal activity in *Aedes* mosquitoes and at the same time inducing repellence toward the mosquitoes. All these chemicals have also been used against mosquito vectors such as *Culex pipiens* and *Anopheles* spp. as reported by Ravi et al. (2021).

### 19.4.3 The Hyperaccumulating Angiospermic Plants

The hyperaccumulating plants, the major tools for green technology, are known to accumulate, concentrate, and translocate high amounts of toxic elements present in the environment. In aquatic phytoremediation systems, aquatic plants can be either floating on the water surface or submerged into the water. The floating aquatic hyperaccumulating plants absorb or accumulate contaminants by its roots, while the submerged plants accumulate metals by their whole body. Some aquatic macrophytes, like *Eichhornia crassipes*, *Lemna gibba*, *L. minor*, *Pistia stratiotes*. *Hydrilla* verticillata, *Spirodela polyrhiza*, *Lepidium sativum*, *and Ipomoea aquatica*, are employed for phytoremediation. Nahar and Hoque (2021) reported use of floating aquatic macrophyte, water lettuce (*Pistia stratiotes* L), to improve eutrophic ecosystem.

### 19.4.4 Aquatic Macrophytes for Phytoremediation

*Lemna minor* L. has been found to be a potent toxic accumulator in aquatic ecosystems. The prominent contaminants in this case are As, Cu, Zn, and Hg (Alvarado et al. 2008; Kara 2004, 2005). According to Bokhari et al. (2019), macrophytes like swollen duckweed (*Lemna gibba* Linn.) lesser duckweed (*Lemna aequinoctialis* Welw.) can accumulate heavy metals (HMs) in its root and shoot systems up to 100 times higher concentration than a surrounding environment without any production of toxic symptoms.

## 19.5 Removal of Various Pollutants

### 19.5.1 Herbicides

Glyphosate (Gly) is the most widely used herbicide in the world as it has broad spectrum and nonselective activity. Its indiscriminate use results in risks of contamination of water bodies and can affect living organisms. Their indiscriminate use results in their accumulation of water bodies and can affect living organisms, especially sensitive or resistant nontarget plants (Dosnon-Olette et al. 2011; da Silva et al. 2020). da Silva et al. (2020) studied mechanisms and phytoremediation potential of the macrophyte *Salvinia biloba* (Fig. 19.5). da Silva et al. (2020) exposed *Salvinia biloba* to different concentrations of a Gly commercial formulation (Gly-CF) and a Gly analytical standard (Gly-AS) and evaluated the physiological

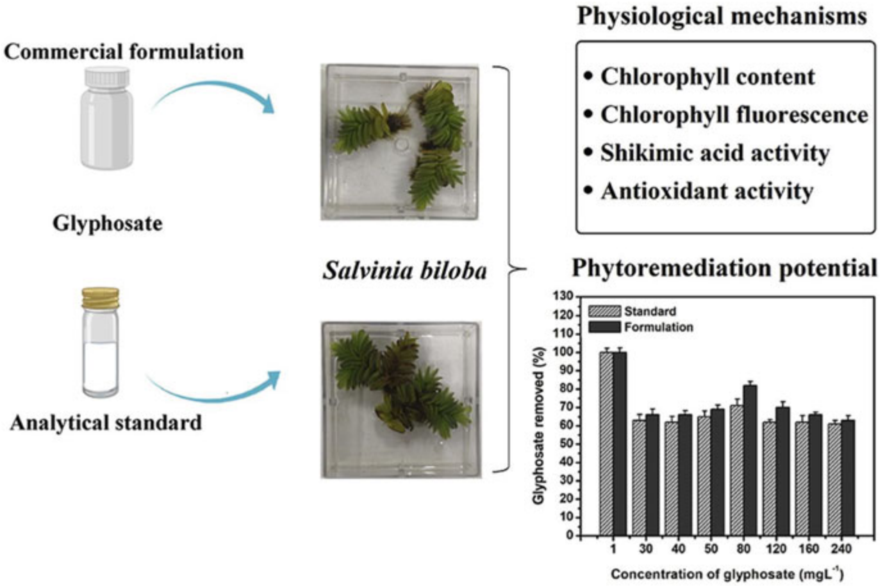

**Fig. 19.5** Bioremediation using *Salvinia biloba.* Source: da Silva et al. (2020). Reproduced by License Number 5123010494525 License date Aug 6, 2021

mechanisms of the aquatic macrophyte. *S. biloba* may be a potential phytoremediation agent for low Gly concentrations, since 1 mg $L^{-1}$ Gly was completely removed and exhibited low phytotoxicity (da Silva et al. 2020). Santiago (2020) also studied physiological mechanisms and phytoremediation potential of the macrophyte *Salvinia biloba* towards a commercial formulation.

Dosnon-Olette et al. (2011) focused on toxicity and phytoremediation potential of aquatic plants to remove phytosanitary products from contaminated water. *Lemna minor* (*L. minor*) could eliminate two herbicides isoproturon and glyphosate from their medium. Effect of *Salvinia molesta* on isolated and combined effects of glyphosate and its by-product aminomethylphosphonic acid was studied (Mendes et al. 2021). Kaushal and Mahajan (2021) studied phytoremediation ability of *Salvinia molesta* Mitchell and reported that it can be utilized for remediation of water bodies and wetlands contaminated with Direct Red 28 (DR28) dye wastewater in natural conditions.

Mendes et al. (2021) evaluated *Salvinia molesta* to remove glyphosate and its by-product aminomethylphosphonic acid (AMPA) from contaminated water.

Chen et al. (2019) reported herbicides (mesotrione and fomesafen) with long degradation cycles in water by water hyacinth (*Eichhornia crassipes*). They suggested that uptake by plants combined with degradation by plant-associated bacteria may be the dominant process in the removal of mesotrione and fomesafen by water hyacinth.

### 19.5.2 Pesticides

Only a small section of the microbiota has the ability to decompose and biotransform certain residual effluents, pesticides, and hydrocarbons (Bhattacharjee et al. 2020). Góngora-Echeverría et al. (2020) using a microbial consortium having *Pseudomonas nitroreducens* and *Ochrobactrum sp.* obtained highest degradations (>90%) of the five pesticides commonly used in Yucatan Mexico: atrazine, carbofuran, and glyphosate.

### 19.5.3 Heavy Metals

#### 19.5.3.1 What are Heavy Metals?

According to Csuros and Csuros (2002), a heavy metal is defined as "a metal with a density greater than 5 g/cm$^3$ (i.e., specific gravity greater than 5)."Very recently, we have proposed a broader definition for the term, and heavy metals have been defined as "naturally occurring metals having atomic number greater than 20 and an elemental density greater than 5 g·cm$^{-3}$" (Ali and Khan 2018). According to Duffus (2002) "the term "heavy metals" is often used as a group name for metals and semimetals (metalloids) that have been associated with contamination and potential toxicity or ecotoxicity."

HMs can also be classified into three categories: (1) toxic HMs, such as Hg, Cr, Pb, Zn, Cu, Ni, Cd, As, Co, and Sn; (2) precious metals mainly including Ag, Au, Ru, Pt, and Pd; and (3) radionuclides HMs such as Ra, Th, and U. On the other hand, it is commonly recognized that nonessential HMs have varying degrees of toxicity toward microorganisms, animals, plants, and humans even at very low concentrations (Ali and Khan 2018; Ali et al. 2019). Five heavy metals namely arsenic (As), cadmium (Cd), chromium (Cr), lead (Pb), and mercury (Hg) are carcinogenic and show toxicity even at trace amounts, posing threats to environmental ecology and human health (Leong and Chang 2020).

Examples of essential heavy metals are Mn, Fe, Cu, and Zn, while the heavy metals Cd, Pb, and Hg are toxic and are regarded as biologically nonessential (Ramírez 2013). The heavy metals Mn, Fe, Co, Ni, Cu, Zn, and Mo are micronutrients or trace elements for plants.

Hartl (2012) remarks that "metals, of natural or anthropogenic origin, are ubiquitous in the aquatic environment, and therefore understanding their behavior and interaction with aquatic organisms, particularly fishes, a major source of protein for human consumption, is of a great socio economic importance." Chronic exposure to heavy metals in the environment is a real threat to living organisms (Wieczorek-Dąbrowska et al. 2013).

#### 19.5.3.2 Environmentally Relevant Most Hazardous HMs and Metalloids

Environmentally relevant most hazardous heavy metals and metalloids include Cr, Ni, Cu, Zn, Cd, Pb, Hg, and As. Vehicle traffic is among the major anthropogenic sources of heavy metals such as Cr, Zn, Cd, and Pb (Ferretti et al. 1995). During coal combustion, Cd, Pb, and As are partially volatile, while Hg is fully volatile. Phosphate fertilizers are particularly rich in toxic heavy metals. Fertilizers also usually contain significant contents of Cr (Krüger et al. 2017). The natural sources of Cd in the environment are volcanic action and weathering of rocks, whereas an anthropogenic source is nonferrous metal mining, especially processing of Pb-Zn ores (Wang et al. 2015a, b). Anthropogenic increases in Cd concentrations are also caused by excessive application of chemical fertilizers (Grant and Sheppard 2008). The two main pathways for transfer of toxic heavy metals from phosphate fertilizers to the human body are shown below (Dissanayake and Chandrajith 2009). Combustion of leaded gasoline is also a source of Pb in the environment. Although use of the tetraethyl lead as an antiknock agent in gasoline has been banned, it is still used in some developing regions of the world (Palaniappan and Karthikeyan 2009).

The soil-to-plant transfer of heavy metals is a very important step in the trophic transfer of such metals in food chains. Consumption of cereals contaminated with toxic heavy metals may cause risk to human health (Orisakwe et al. 2012). The translocation of heavy metals from roots of the rice plant to stem, leaves, and rice grains is of human health concern. Rice crop is especially susceptible to heavy metal contamination because it needs water during most of its growth period. The trace elements, Cd, Pb, Hg, and As, are ubiquitous in the environment with harmful effects on human health. Regarding their presence in rice as a public health concern, As is on top followed by Cd (Beebout 2013) Human intake of Cd has been reported to be highest through consumption of rice (Cai et al. 2015).

Aquatic macrophytes have been widely employed for in situ phytoremediation of cadmium (Cd)-polluted sediments (Yuan et al. 2021). Eelweed, *Vallisneria spiralis* L., can also degrade Cu, Cd, along with Hg as opined by Rai and Tripathi (2009). Bioaccumulation and toxicity of mercury in rooted submerged macrophyte, *Vallisneria spiralis*, *were* studied by Gupta and Chandra (1998). Dixit and Dhote (2010) suggested that *Hydrilla verticillata* commonly known as Esthwaite waterweed would be a good option for phytoremediation of contaminated water. Pb, Zn, and Cr are the contaminants, which are corrected by *Hydrilla verticillata.* Accumulation of heavy metals in water spinach (*Ipomoea aquatica*) cultivated in the Bangkok region showed As, Cd, Pb, Hg, Cu, and Zn as the major heavy metals.

Gothberg et al. (2002, 2004), Hu et al. (2008). The tolerance of plants to Cd is a scientific and interesting issue for phytoremediation. The absorption of Cd is mainly retained in the root of *E. crassipes*. It will be effective in remediating sites with moderate pollution ($\leq 2$ mg/L). Maine et al. (2004), enumerated the role of Water lettuce *Pistia stratiotes* in As, Cr, Pb, Ag, Cd, Cu, Hg, Ni, and Zn accumulation. Significant enrichments in agricultural soil for As, Pb, and Zn (in urban area), Cd, Cu, and Ni (in a copper mine area), higher availability detected in developing world, was ascribed to both lithogenic and anthropogenic elements (Balabanova et al. 2015). Availability of metals in a potentially polluted soil and their possible transfer and bioaccumulation in sorrel (*Rumex acetosa*), spinach (*Spinacia oleracea*), and common nettle (*Urtica dioica*) was examined (Balabanova et al. 2015). Measurement of eight potentially toxic elements (As, Cd, Cr, Cu, Mn, Ni, Pb, and Zn) in sediment and plant tissues of *Typha latifolia* L. showed good phytostabilization capability of *Typha latifolia* L. for Cd, Cu, and Pb, and phytoextraction capacity for Zn (Haghnazar et al. 2021). Thus, phytoremediation using *Typha latifolia* L. could be a practical method for uptake and removal of potentially toxic elements from aquatic environments (Haghnazar et al. 2021).

Ameh et al. (2021) reported that *Ageratum conyzoides* Linn (ACL), *Desmodium velutinum* (DV), *Triumfetta rhomboidea* Jacq. (TRJ), *Gleichenia linearis* (Burns) (GL), *Selaginella myosurus* (SM), and *Sida linifolia juss.* excav. (SL) had potential as hyperaccumulators of nickel. *Eclipta alba* (L) Hassk (EAH) and *Triumfetta rhomboidea* Jacq were phytostabilizers for Pb. Most of the plants were found to be useful for phytoremediation of the soil.

## 19.6 Combination Treatment

Microbial-assisted phytoremediation and reclamation are both potential contaminated soil remediation technologies, but little is known about the combined application of the two technologies on real contaminated soils.

### 19.6.1 Macrophytes and Algae

Tabinda et al. (2019) used two macrophytes *Pistia stratiotes* and *Eichhornia crassipes* and an alga (*Oedogonium* sp.) were used to treat textile effluents rich in COD, BOD, dyes, and heavy metals (Pb, Fe, Cd, Cu). They reported that *Oedogonium sp*. was the best for COD removal and decolorization. However, *Eichhornia crassipes* was the best for BOD and heavy metal removal, while *Pistia stratiotes* was found to accumulate more concentrations of Pb and Fe (Tabinda et al. 2019). Bokhari et al. (2019) reported the heavy metal phytoextraction potential of swollen duckweed (*Lemna gibba* Linn.) and lesser duckweed (*Lemna aequinoctialis* Welw.) for removal of nickel (Ni), lead (Pb), and cadmium (Cd). Gul et al. (2019) assessed EDTA-assisted Pb and Cd phytoextraction potential of locally grown *Pelargonium hortorum* and *Pelargonium zonale*. They reported that overall, the performance of *P. hortorum* was better than that of *P. zonale* for EDTA-assisted phytoextraction of Pb and Cd (see also Manzoor et al. 2019) .

### 19.6.2 Macrophytes and Bacteria

The plant-associated bacteria to enhance the phytoremediation efficiency of the heavy metals from polluted water are an emerging area of research. Bacteria-assisted phytoremediation is cost-effective strategy and metal sequestration mechanism that hold high metal biosorption capacities.

Application of plant-microorganism-based environmental remediation is gaining momentum. The roles of plant root exudates and rhizosphere microorganisms in the remediation of ecology and environment have also been discussed (Yang et al.

2021). Yang et al. (2021) described the composition, secretion mechanism, and functions of root exudates and summarized the functions of root exudate in heavy metal absorption, allelopathy, interaction between roots and rhizosphere microorganisms, and changes in soil physical and chemical properties.

Casella et al. (1988) proposed that rhizobia can circumvent exposure to the heavy metal by entering the plant roots. They reported that the nodulated plants, grown in the presence of 10 ppm of chromium, had an increased nitrogenase activity compared to the control plants. However, the effect of metal toxicity on the interaction between legumes and rhizobia is not clear (Hao et al. 2014). Yuan et al. (2021) studied responses of rhizosphere bacteria and their interspecific interactions to phytoremediation.

Legumes are important for nitrogen cycling in the environment and agriculture, and they have been reported to be dominant species in metal contaminated soils (Hao et al. 2014). They reviewed the potential role of legume–rhizobia symbiosis in aiding phytoremediation of such soils. The rhizobia used for phytoremediation could act on metals directly by chelation, precipitation, transformation, biosorption, and accumulation. The plant growth-promoting (PGP) traits of rhizobia including nitrogen fixation, phosphorus solubilization, phytohormone synthesis, siderophore release, and production of ACC deaminase and the volatile compounds of acetoin and 2,3-butanediol may facilitate legume growth while lessening metal toxicity. Naturally resistant rhizobia or recombinant rhizobia and co-inoculation with other plant growth-promoting bacteria (PGPB) may further increase metal detoxification process.

Yuan et al. (2021) suggested phytoremediation of cadmium-contaminated sediment using *Hydrilla verticillata* and *Elodeacanadensis* harboring two rhizobacteria *Pedosphaeraceae* and *Parasegetibacter*. The results showed that a group of specialized sediment bacteria were assembled in the rhizosphere zones closely associated with different host macrophytes. They benefited from the nutrients supplied from macrophyte roots, and thus, more bacterial species survived in the highly Cd-contaminated sediments (50 mg $kg^{-1}$). Fang et al. (2021) studied the effects of *Vetiveria zizanioides* L and assisted by *Herbaspirillum* sp. p5-19 for soil improvement and phytoremediation of copper stress tolerance and enhancing the accumulation of Mn, Cu, Zn, and Cd by *Vetiveria zizanioides* L. in copper tailings. Meanwhile, photosynthetic pigment contents were enhanced in co-inoculation treatment (p5-19 with alien soil improvement). In addition, the malondialdehyde (MDA) content was decreased, and the activities of antioxidant enzymes such as ascorbate peroxidase (APX), superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) were increased in p5-19 treatment, thereby alleviating the oxidative stress. These results provided the basis for the change in phytoremediation ability of *V. zizanioides* after inoculation. It was concluded that p5-19 assisted with alien soil improvement was a potential strategy for enhancing phytoremediation ability in tailings.

## 19.7 Eutrophication in Water Bodies and Nutrient Removal.

Shuai et al. (2019) reported that increased nitrogen and phosphorus pollution causes eutrophication in water bodies (see also Walter et al. 2016). Compared to native plants, the invasive plants show much higher nutrient removal efficiency with their high nutrient uptake capacity and thereby helping in the water purification process (Prabakaran et al. 2019). Aquatic macrophytes, such as water lettuce (*Pistia stratiotes*), water hyacinth (*Eichhornia crassipes*), and duckweed (*Lemna minor*), are ideally suited. Zhou et al. (2016) tested four plants, e.g., for *Acorus calamus*, *Typha orientalis*, *Lemna minor*, *and Ceratophyllum demersum* for phytoremediation. The results showed that the concentration of total nitrogen (TN), total phosphorus (TP), and chemical oxygen demand (COD) decreased sharply at the beginning of the test but decreased later. The wetland plant species were more effective in reducing TN when used in combination than used alone, and the combination of *T. orientalis*, *L. minor and C. demersum* had the highest efficiency in removing TN. A thorough understanding of mainly the agronomic, biochemical, physiological, and genetic aspects of phytoremediation is essential to develop the technology as envisaged by the phytoremediation researchers. Shuai et al. (2019) suggested six plants, e.g., *Polygonum orientale*, *Juncus effusus*,, *Iris pseudocorus*, *Phragmites australis*, *Iris sanguinea*, and *Typha orientalis*, are suitable to remove excess of N and P nutrients from water. Xu et al. (2021) suggested two plants *Iris ensata* Thunb. and *Potamogeton malaianus* Miq for the removal of different concentrations of wastewater and the effect of pollutants on plant growth.

## 19.8 Genetic Engineering for Phytoremediation

Jha (2020) reported that natural hyperaccumulator plant species mostly suffer with limitations of having low biomass and have less efficiency for uptake, accumulation, and degradation of xenobiotics. Genetic engineering of plants can improve their capacity of phytoremediation. *Festuca arundinacea* Schreb (Tall fescue) shows huge potential for lead (Pb) phytoremediation and overexpression of *FaHSP17.8-CII* gene improved Pb phytoremediation efficiency in tall fescue (Wang et al. 2021). The genetic engineering strategy to obtain transgenic tall fescue overexpressing a class II (CII) *sHSP* gene *FaHSP17.8-CII* exhibited 36.3% and 46.6% higher shoot Pb accumulation relative to the WT grasses. Furthermore, according to Wang et al. (2021), overexpression of *FaHSP17.8-CII* improved the synthesis of chlorophyll and transcript abundance of *FapsbC*, *FapsbD*, and *FapsbE*, and alleviated the photoinhibition of PSII in tall fescue under Pb stress.

Danouche et al. (2021) reviewed the future perspectives of physicochemical and genetic approaches, which can be used for the phytoremediation process in terms of selectivity for a targeted metal, removal efficiency, or reduction in treatment time and cost. Danouche et al. (2021) reported surface engineering for a target metal biosorption by algal surface (Fig. 19.6). Yen et al. (2017) reported that *Chlorella vulgaris* strains have the potential to convert Cr(VI) to Cr(III) through an enzymatic

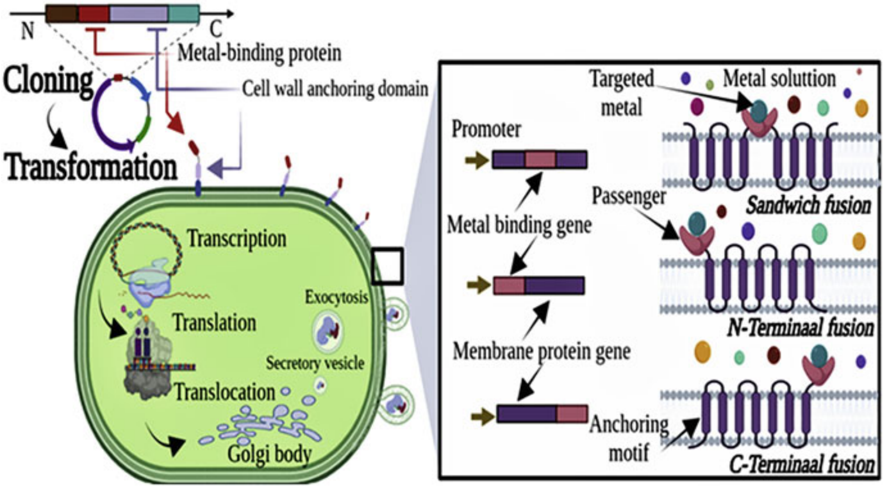

**Fig. 19.6** Procedure of cell surface engineering for a target metal biosorption. Source: Danouche et al. (2021). Reproduced under licence number 5234950808694 dated 23rd January 2022

reaction catalyzed by the chromate reductase. Of the two predominant forms of chromium, Cr(III) has only about one-thousandth the toxicity of Cr(VI).

The arsenate reductase has also been found in the green microalgae *Chlamydomonas reinhardtii* (Yin et al. 2011) (Fig. 19.7). Figure 19.7 shows the pathways of biotransformation of As (V and III) by microalgae. Generally, As species are present in different cellular fractions of cells of microalgae including the lipid, cytosolic, cell membranes, and debris fractions. Recent studies have shown that algal strains can grow in 500–2000 mg/l of As waters and can remediate a substantial quantity by rewiring their cellular physiology (Arora et al. 2018). The detoxification pathway begins with the reduction of As(V) into As(III) form and ultimately conversion to a range of organoarsenicals such as arsenolipids, arsenosugars, arsenobetaine, and arsenoribosides (Arora et al. 2018).

Proposed biotransformation pathways of Cr(VI) developed from the finding of Deng et al. (2006), Lee et al. (2017), Rahman and Thomas (2021), Yen et al. (2017).

Artificial proteins can be created through genetic and protein engineering (Agapakis and Silver 2009). Danouche et al. (2021) illustrated the cell surface engineering procedure for a target metal biosorption. First, the coding DNA of the target metal-binding peptide or protein was obtained via whole sequence synthesis or PCR amplification from genomic or plasmid DNA. Next, it can be transformed to the host in the form of fusion of a protein under specific induction. The metal-binding protein/peptide can be displayed in the form of fusion of an anchor protein after transcription, translation, and translocation. The secretory vesicles encompassing the passenger and carrier proteins pass through the cell membrane and anchor the passenger proteins to the surface of the cell wall (Kuroda and Ueda 2011; Li and Tao 2013; Wang et al. 2021).

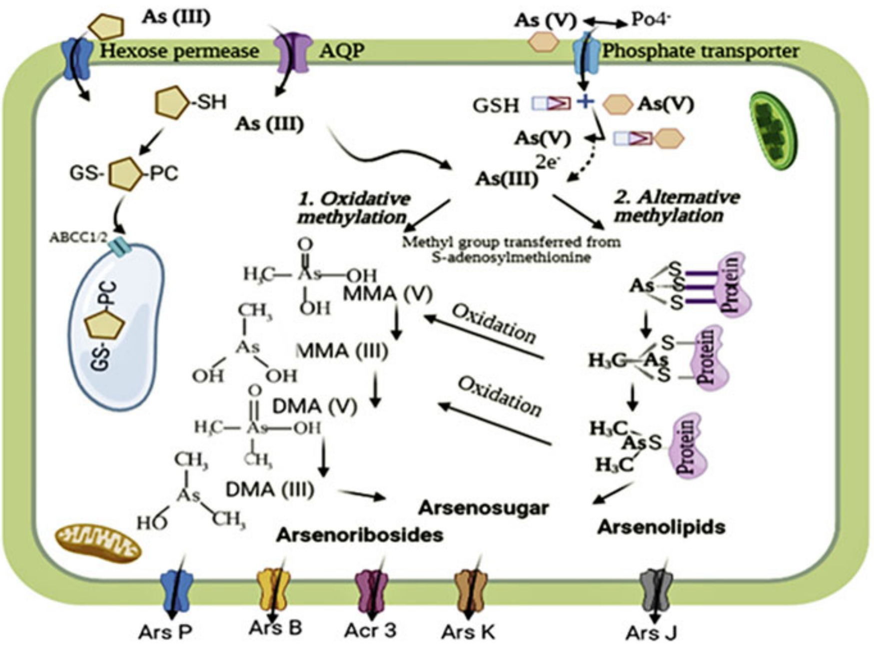

**Fig. 19.7** Proposed biotransformation pathways of As (V and III) developed from Arora et al. (2018), Garbinski et al. (2019), Wang et al. (2015b). Source: Danouche et al. (2021). Reproduced under licence number 5234950808694 dated 23rd January 2022

## 19.9 Discussion

Aquatic ecosystems, both freshwater and marine, are vulnerable to pollution. Contamination of water resources by heavy metals adversely affects plants, animals, and human health (Rezania et al. 2005). Heavy metals are extremely toxic to aquatic organisms even at very low concentrations (Akif et al. 2002). These elements can cause significant histopathological alterations in tissues of aquatic organisms such as fish (Ahmed et al. 2014). The release of industrial effluents without treatment into the aquatic bodies is a major source of pollution of surface and groundwater water (Afzal et al. 2018). Pollution of water bodies with heavy metals is a worldwide problem because of the environmental persistence, bioaccumulation, and biomagnification in food chains and toxicity of these elements (Rajaei et al. 2012). Ecological and environmental problems including heavy metal pollution have received increasing concerns (Yang et al. 2021).

The As and F contamination results from water–rock interactions and may be accelerated by geothermal and mining activities, as well as by aquifer overexploitation. Although the individual toxic effects of As and F have been analyzed, there are few studies addressing their co-occurrences and water treatment options. Enrichment of F is generally related to fluorite dissolution, and it is also associated with high Cl, Br, and V concentrations. It is still urgent to develop technologies and methods capable of monitoring and removing both of these contaminants simultaneously from water. Alarcón-Herrera et al. (2013) suggested that As and F co-occurrence in groundwater is linked to volcanism, geothermal, and mining activities. As and F co-occurrence are particularly pronounced in arid and semi-arid regions. As and F are generally associated with high concentrations of $Na^+$ and $HCO3^-$. Technology is required to simultaneously remove As and F from drinking water.

Remediation of the polluted ecosystem is important to lessen the detrimental effects of discharge of pesticides and heavy discharge of industrial effluents on a long-term basis (Bhattacharjee et al. 2020). Chemical treatment of this discharge using electrochemical removal, ion exchange columns, alkaline precipitation, filtration, and membrane technologies are the currently available technologies for heavy metal removal. These conventional technologies are not economical and may produce adverse impacts on aquatic ecosystems. Phytoremediation of metals is a cost-effective "green" technology based on the use of specially selected metal-accumulating plants to remove toxic metals from soils and water (Rai 2008). It is a promising cleanup technology for contaminated soils, groundwater, and wastewater that is both low tech and low cost (Farid et al. 2014). Sequential phytoremediation with a mixture of plants was more effective than that relying only on a single plant species (Farid et al. 2014). Burges et al. (2018) suggested that since the emergence of phytoremediation, much research has focused on its development for (1) the removal of metals from soil and/or (2) the reduction in metal bioavailability, mobility, and ecotoxicity in soil. They further suggested that the combination of these phytotechnologies or phytomanagement provides certain benefits for the restoration of important ecosystem services, e.g., nutrient cycling, carbon storage, water flow regulation, erosion control, water purification, and fertility maintenance.

## 19.10 Conclusion

Heavy metals are non-degradable by any biological or physical process and are persistent in the soil for a long period, which pose a long-term threat for the environment (Suman et al. 2018). According to their role in biological systems, heavy metals can be grouped as essential and nonessential. Essential heavy metals such as Cu, Fe, Mn, Ni, and Zn are required for physiological and biochemical processes.

Aquatic macrophytes have been widely employed for in situ phytoremediation of heavy metals. Wetlands characteristically offer a varied degree of wetness, thereby supporting a diverse group of plants. In wetlands a number of plants carryout the function of phytoremediation. The local availability of water is seen to encourage transition between annual and perennial growth forms. Some of the algae in wetlands can be helpful in bioremediation. Microbial remediation is reported, but microbe plus plants are recent reports. Combination of bacteria and plants causes bioremediation. Applying lower group plants, like *Azolla* while the wetland supports plant diversity and conservation in the local ecological domain, a number of human needs are also catered to. Shrinkage of the waterline as evident in recent times is a result of rapid urbanization. However, a silver line has been seen with the currently ongoing process of revamping the entire water body by the State Government. This is heralded as a leap forward toward suitable management, welfare, and conservation of flora of Tapan Dighi.

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# 20

# Vertical Cultivation: Moving Towards a Sustainable and Eco-friendly Farming

Subhrajyoti Mishra, K. M. Karetha, Yuan-Yeu Yau,
and Mona Easterling

## Abstract

Vertical farming, popularly known as sky farming, is a recently emerging technology for cultivating many herbacious crops. As the world's population increases exponentially, arable land is diminishing. The vertical farm concept was developed to remedy the spatial land utilization crisis. This method utilizes conventional farming methods such as hydroponics, aquaponics and aeroponics to gain greater productivity. Design, layout and configuration of these high-tech farms provide optimal light exposure, precisely measured nutrients for each plant and ability to grow in a controlled, closed-loop environment. These farms eliminate the need for harmful herbicides and pesticides, maximize nutrition and increase food value in produce. Vertical cultures under protected structures also enhance taste to cater to consumers preferences. In horticultural crops like berries, leafy vegetables, spinach and some crucifers, this technique has already been adopted. It provides a tremendous yield, improved quality assurance and sustained production capacity. Start-up costs are higher than traditional options, but these can be quickly overcome with increased productivity providing profit. Scientists are continuing to modify technologies to boost profits to users. Indoor horticulture production systems can be advanced and automated by vertical farming techniques to enhance organic farming concurrently with improved soil health and sustainable farming.

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**Keywords**

Vertical farming · Sky farming · Sustainability · Indoor gardening · Roof top farming · Quality foods · Urban farming · plant response · photochemistry · secondary metabolites

## 20.1 Introduction

Crop production is under threat as a result of extreme weather variables, biotic and abiotic factors and diminishing land resources. The global resources of land and clean water are diminishing daily. By 2050, predictions indicate a 60% increase in food production will be required (Tilman et al. 2002; Green et al. 2005; Alexandratos and Bruinsma 2012) to feed the projected human population of 9.5 billion. This will not be possible using conventional technologies; hence a fundamental change in food production is needed (Corvalan et al. 2005; Healy and Rosenberg 2013; Thomaier et al. 2015; Despommier 2010). Supply must meet the demand of an expanding population. Crop productivity must be enhanced significantly to achieve this goal. Undernourishment remains a hurdle to face globally (FAO 2020). As per a 2019 UN report, the number of people living in urban areas will rise to more than 6 billion with 90% of the demographic shift in developing countries. Recurrent, unprecedented population explosion and growth of mega-cities worldwide could prove unsustainable and ecologically disastrous (Banerjee and Adenaeuer 2014). In 2000, the world's mega-cities accounted for just 2% of land surface of Earth, but they consume roughly 75% of industrial wood and 60% of water to produce almost 80% of human-produced carbon emissions (UNPD 2008). Expanding human population and urban residency have a destructive influence on the environment. Urban centres concentrate service industry options for quick and affordable goods and services; this demand tends to increase vehicular emissions. Adoption of efficient tools and techniques can provide a means of maintaining the environmental and anthropogenic harmony.

Vertical gardening in urban and semi-urban areas could be an efficient method to merge food production and consumption locally (Al-Kodmany 2018b). Land becomes a vital resource due to limited availability and higher cost. Using novel food production specially might provide city dwellers with quality, poison-free, farm fresh produce that is less expensive. Recent advances in greenhouse technologies, soil-less farming, aeroponics, hydroponics and aquaponics have provided a promising future to the vertical farm concept. These high-tech systems represent a paradigm shift in farming and food production systems that offer sustainable and efficient methods for city farming by minimizing maintenance and maximizing yield. Reduction in application of poisonous agrochemicals in this type of farming also increases interest in health cautious communities. Rooftop farms using vertical concepts could feed a family size of 3–5 by meeting daily vegetable needs. This technology is in a juvenile stage and gaining interest from businesses, scientists and young people engaged in prototype development, with high resource use efficiency, low labour hinderance and greater productivity.

## 20.2 What Is a Vertical Farm?

The term vertical farm is self-explanatory with a literal meaning of farming vertically, up from the ground surface. Conventional farming can be broadly considered horizontal farming. Vertical is farming up rather than farming out (Fig. 20.1). There are three main types of vertical farming (Despommier 2014; Touliatos et al. 2016; Muller et al. 2017; Al-Kodmany 2018b) based on construction. The first type of vertical gardening is preferred by many industries and involves the construction of tall structures containing several levels of growing beds, often lined with artificial lights. Multi-coloured LED (Light Emitting Diode) lights are commonly used for crop-specific phenophases. The second type of vertical farm is built on the rooftops of old and new buildings. These may be constructed on commercial and residential structures, such as restaurants and grocery stores (Despommier 2014; Touliatos et al. 2016). The third type of vertical farm is a visionary, multi-story building with automated monitoring systems and fewer human interventions. The third type is the most advanced form of vertical gardening technology, but it is relatively rare. Vertical farming is the practice of growing or producing crops in vertically stacked layers. These farm comes in different shapes and sizes, from simple two-level structures or wall-mounted systems to large warehouses several stories tall. Vertical farming typically uses a mix of natural and artificial light. Artificial lighting is often LED-based and may be driven by a renewable power source such as solar power or

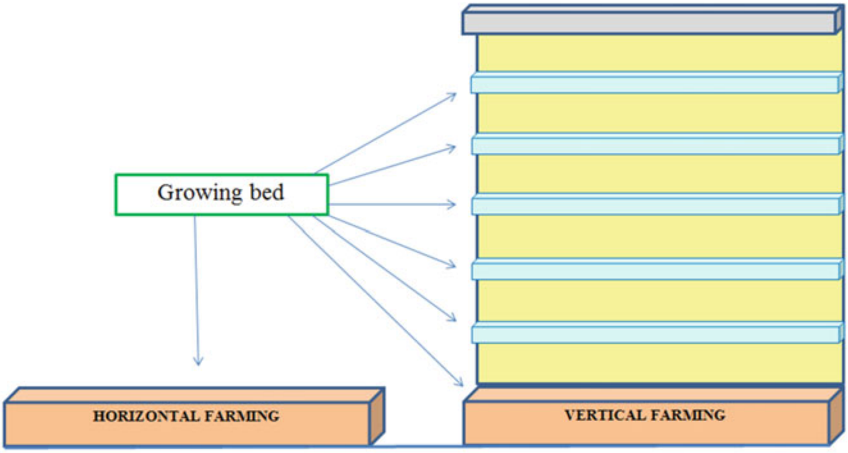

**Fig. 20.1** Generalized view of vertical garden versus horizontal garden. Horizontal gardening involves crops grown in soil as a single bed system, but vertical gardening increases the number of growth beds per land plot to accommodate more plants and potentially increase yield per plot

wind turbines. It may be very simple system and function only with available natural resources (outside greenhouse). The system may be highly sophisticated and driven by use of LED, aeroponics, hydroponics and aquaponics, including fully controlled growth conditions, automated irrigation, fertigation, plant health management, sensor-based harvesting operations and other technologies. Depending on human intervention, vertical gardens can be of two types. “Mixed used skyscrapers” developed by Ken Yeang have both plants and humans living together in natural environments. “Despommier skyscrapers” by Despommier contain plants grown in fully controlled environmental conditions with less human intervention. There are also two main types of growth media or root holding chamber used. One type comprised of multiple levels of traditional horizontal growing platforms, and the other type with crops grown on a vertical surface (Beacham et al. 2019). Using advanced greenhouse technologies such as soil-less culture, hydroponics, aquaponics and aeroponics, the vertical farm could theoretically produce the daily human requirements for fish, poultry, fruit and vegetables (Despommier 2010). The vertical farm is considered to promote sustainable agricultural practices beyond conventional farming, which relies on large scale, outdoor agriculture engaging heavy irrigation, intensive tillage and excessive use of fertilizers, pesticides and herbicides (Healy and Rosenberg 2013). Vertical farming provides adequate amounts of high quality foods and helps the environment by reducing agrochemicals and recycling water regularly.

## 20.3 Historical Background of Vertical Farming

Vertical farming is not an entirely new prospect. Examples of it can be traced back to the Hanging Gardens of Babylon in ancient era, one of Philon’s Seven Wonders of the Ancient World which was built around 600 BC. The term “vertical farming” was coined by Gilbert Ellis Bailey in 1915 who also wrote a book titled “Vertical Farming”. He also argued farming hydroponically in a controlled vertical environment would provide economic and environmental benefits. In the early 1930s, William Frederick Gericke of University of California at Berkeley pioneered hydroponics and became the father of that technology. In the 1980s, Ake Olsson, a Swedish ecological farmer, also proposed vertical farming as a means for producing fresh vegetables in cities. He is known as the inventor of a spiral-shaped rail system for growing plants (Corvalan et al. 2005; Healy and Rosenberg 2013; Thomaier et al. 2015; Despommier 2010, 2013, 2014). Around the turn of the century, Dickson Despommier, an American ecologist and professor of public health, passionately revived the concept of vertical farming. He described the vertical farm as “the mass cultivation of plant and animal life for commercial purposes in skyscrapers.” In India, vines are grown on rooftops, as shown in Fig. 20.2, as a very beautiful example of vertical-green wall.

**Fig. 20.2** Growing of cucurbits on the roof of thatch house in India. Figure adopted from https://www.outlookindia.comtravellermpvisual-escapephoto-gallerytraditional-architecture-madhya-pradesh

## 20.4 Concept and Technology Involved in Vertical Farming

Vertical gardening started in earnest during the 1990s and has expanded to metropolitan areas globally. Food production businesses searched for less intensive but highly profitable production systems. Vertical gardening became one viable option. Despite being an emerging technology, the diverse workability and expansion potential do not hamper it. Numerous organizations have proposed prototypes, such as Vertical Farm stands of Suwan in South Korea, the plant vertical farm of Chicago, Nuvege plant factory of Japan, Vertical harvest plans 2 of USA, eroFarms of New Jersey and the recent addition Plantagon, Urban Kissan of Hyderabad, India, City Greens of Ahmedabad, India. Designs differ, but vertical farm concepts remain the same with the general idea of growing crops in an upward direction rather than horizontally. Hydroponic, aeroponic, aquaponic and soil-less farming techniques are integral methods for holding and feeding vertical garden plants. The new technology is built on its theoretical concept. Vertical farming aims to increase crop yield per unit area of land, and this idea is currently gaining momentum (Agrilyst 2017). Here are a few common questions. (1) Can conventional farms growing crops only vertically be called vertical gardens? (2) Are all crops suitable for this technique? What about the other perennial crops like mango, jackfruit, persimmon and apple?

(3) What is the growth media used in this type of farming? Is it different than soil? Many additional questions related to crop environment, irrigation, nutrition and plant growth will depend on the questioner's interest and knowledge. To answer, vertical cultivation is growth of short duration crops in a climate controlled, fully automated environment using sensors and employing highly skilled workforce. In general, mango or any perennials are not cultivated in this method, but greens, vegetables, small berries, annuals and other herbaceous plants are cultivated. Because hydroponic, aeroponic, aquaponic and soil-less farming techniques are integral part of this system, these methods provide plant anchorage, nutrient uptake and water uptake. The vertical farm must provide optimal conditions for crops to transition from seedlings through germination, vegetative, reproduction and harvest phases. A major prerequisite is temperature and relative humidity-controlled growth chambers. Additionally, controlled and elevated $CO_2$-levels have been shown to maximize biomass yield. The closed system can filter out contaminants and trace gases, such as ethylene, which are released into the air by plants.

## 20.5 The Musts in Vertical Farm

This technology is new and outside general cultivation practices of traditional or conventional farming. Full expertise limits failures of technology. Certain listed critical factors determine cost effectiveness of vertical farming.

- Evaluate feasibility of vertical farming technology for climate/geographical areas
- Prioritize technologies by their type prior to establishment
- Become knowledgeable regarding vertical farm implementation
- Analyze availability of space, electricity, instruments, other resources
- Maintain hygienic and use of low weight, soil-less media
- Understand crop phenology and environmental requirements
- Find urban or semi-urban location with good transportation and water availability
- Obtain storage unit and start the cropping with few prior bookings
- Streamline the sell to catch the market
- Accommodate the maximum number of crops rather than one
- Find skilled and semi-skilled labours

### 20.5.1 Factors Affecting Design of Vertical Garden

The various factors which might affect the design and structure of vertical garden are listed here.

- Available materials for structural construction—locally available materials like bamboo, lumber, must be used for the early period with the growth of the farm they can be replaced by costly materials like stainless steel.

- Local preferences—crops suitable for the particular locality must be considered first. They should be tested in trial runs. If there is no profit, farm must shift to alternatives.
- Creativity and imagination—construction of the farm depends on maximum use of land and solar radiation.
- Crop management—care in selecting space for each crop.
- Resources—available energy or electricity. Determine need for solar system or any other source of energy.
- Space—total land availability.
- Climate—conditions of locality and more.

## 20.6 Environment and Plant Response to Vertical Garden

Environment plays a crucial role in all aspects of plant growth and development. Plant behaviour is the result of the climate in which it is grown. Light, humidity and temperature are the fundamental modifiers of plant responses. Plants grown in humid regions will have a larger leaf for maximum transpiration compared to those grown in dry climates. Likewise, plants under cold conditions preserve more food in the hard stem. Evergreens growing in tropical regions receive good amounts of solar temperature. Plants with poor irradiation are slightly pale green in colour compared to those grown under full solar light. Most vertical farming photosystems differ from the normal solar radiation used in greenhouses. The maximum impact of photon energy on variation of growth is prominent, including modification in plant physiology, biology, morphology and photosynthesis. Temperature variation is prominent moving in the vertical direction, making plant response to vertical farm raised temperatures a general concern. Therefore, the following subheadings highlight crop responses under vertical farming with various alternations in morpho-physicochemical activities inside and outside the plant.

### 20.6.1 Photo-biology

Plant biological processes are highly regulated through different light spectra. Photoreceptors function as light-absorbing molecules that determine the wide range of light or photo spectra and utilize them for specified plant responses. Photoreceptors are light-sensing proteins containing a chromophore molecule sensing and responding to specific wavelengths of light. Examples of these molecules include cryptochromes, zeitlupes, phototropins, phytochromes, UV RESISTANCE LOCUS 8 (UVR8) and too many others to list. Vertical farming allows for full control over plant light supply. Manipulation of light spectrum intensity and timing may help obtain or regulate specific plant physiological processes (Pattison et al. 2018). Plant use different photoreceptors to detect various spectra of light (Table 20.1). Research into the signalling role of individual photoreceptors is ongoing (Sanchez et al. 2020). The interplay and the crosstalk among the

**Table 20.1** Specified photoreceptors for different light spectrum (Smith et al. 2017)

| Sl. No. | Designated light spectra | Photoreceptors |
|---|---|---|
| 1 | Blue | Cryptochromes, phototropins and zeitlupes |
| 2 | Red: far red | Phytochromes |
| 3 | Green light | Cryptochromes and unknown receptor |
| 4 | UV-A, UV-B | UV RESISTANCE LOCUS 8 (UVR8) |

photoreceptors require more scientific attention. Light adjustments generate vertical farming possibilities, for example blue-red light opens of stomata, red light initiates photosynthesis, far red promotes stem elongation and blue light triggers floral induction (Sharath Kumar et al. 2020). In some short-day or long-day plants, floral induction is started by proper photon spectra. The dynamic role of light may boost yield and crop quality with proper implementation, using plant circadian clock mechanisms that regulate translational and transcriptional feedback loops (Sanchez and Kay 2016).

### 20.6.2 Photomorphogenesis

Photomorphogenesis refers to plant morphological responses induced by light. Plants intercept light, and efficient utilization depends on crop morphology specifically leaf morphology. Larger leaf surfaces intercept more light for photosynthetic processes. That results in more photosynthate and dry matter production to improve yield and quality. Not only leaf size but also its orientation, or phyllotaxy, impacts the efficient use of light energy by the plant system. Spectral effects of light have a major impact on dry matter partitioning which can improve the harvest index (HI) of crop plants. Morphogenesis and metabolic processes provide a holistic approach for improving yield (Fernie et al. 2020) as do important hormones responsible for flowering like florigen and gibberellin (Eshed and Lippman 2019). Hormones can modify plant architecture, synchronous flowering, fruiting and yield of crops for vertical farming.

### 20.6.3 Photosynthesis

Light is the major factor in plant photosynthetic processes both in spatial and temporal distribution. The importance of the light energy is well known in both horizontal and vertical farming plants. In vertical farming where solar radiation is the sole source of light, plant growth is reduced in lower layers due to reduced light. Photosynthesis enhancement is essential, but practical application is limited to balanced source and sink strength at the whole-plant level. Although leaf photosynthetic efficiency of green light is usually lower than that of red and blue due to higher reflection, green light penetrates deeper leaves and canopies benefiting crops. Additionally, far-red may play a role in photosynthesis and has been proposed to be photosynthetically active radiation (Zhen and Bugbee 2020). Vertical farms will enable us to grow plants at continuously saturating $CO_2$ to obtain greater production in less time, but most research has been conducted at ambient or moderately elevated $CO_2$.

### 20.6.4 Secondary Metabolites Production

Secondary metabolite production is not part of the general plant response. Certain conditions like extreme weather, temperature and light spectrum produce those plant metabolites. These are reported to be highly beneficial, rich in nutraceutical, dietary value and enhanced quality. LED lighting in vertical production system could enable exposure to diverse light spectrum, helping produce of these compounds, even with short illumination before harvest. Biosynthesis and breakdown of secondary metabolites depend on light-regulated transcriptional factors (Sharath Kumar et al. 2020). For example, light-regulated transcriptional factor R2R3- MYB for phenylpropanoids (Liu et al. 2015) and phytoene synthase activity (PSY) for carotenoids (Llorente et al. 2017) are already well described. Ntagkas et al. (2018) reported that the production of this metabolite is directly linked with photosynthesis and respiration or indirectly to subsequent effects on sugar availability. Photosynthates or sugars can serve as substrates and signalling molecules for secondary metabolite biosynthesis (Sharath Kumar et al. 2020).

### 20.6.5 Thermomorphogenesis

Vertical farming grows compact plants. In this system, diel temperature patterns, i.e. more temperature in night than day temperature or specific light spectra needed for particular growth could provide dense plant growth (Sharath Kumar et al. 2020). It is already known that PhyB acts as a thermosensor and PIF4 is a transcription factor serving as a central regulator of thermomorphogenesis (Casal and Balasubramanian 2019). The morphogenesis of any plant is a result of interaction of light spectra, photoperiod and temperature (Sharath Kumar et al. 2020). Temperature in vertical farming must be adopted based on the crop grown and its growth stage.

## 20.7 Proposed Design of Vertical Farm

The design and working principles of vertical farm are numerous. There are also many types of vertical farms and prototypes. Here is a generalized design for a vertical garden proposed by the authors. This vertical farm consists of the desired numbers of growing beds constructed under a highly controlled and automated greenhouse. For raising plants, the vertical trays (in hydroponics) or the bed (in drip system; as shown in Fig. 20.3) is used. The laterals from the submain line

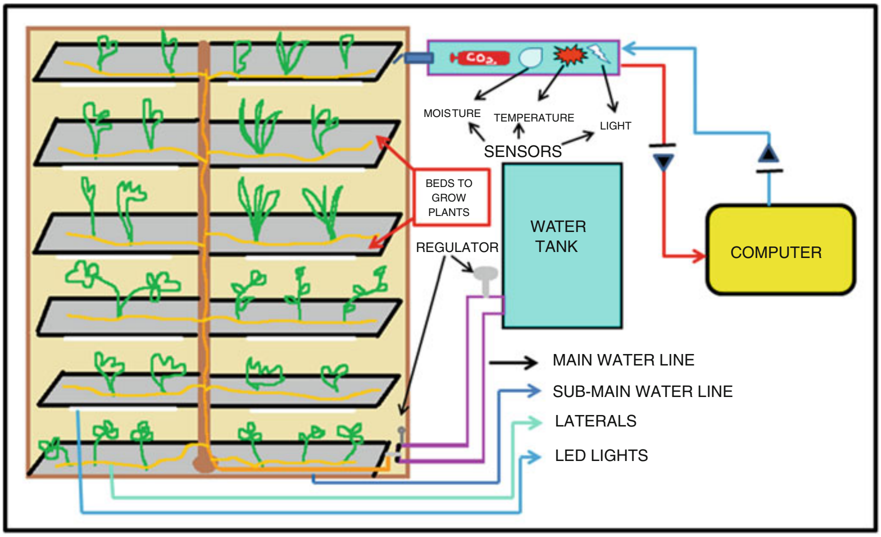

**Fig. 20.3** Proposed model of an automated vertical garden design (drawn by author)

are spread on the bed with a sufficient number of emitters placed at equal distances. Plants are fed with the nutrient solution as fertigation at frequent intervals. The greenhouse or farm has sensors to detect humidity, plant root zone moisture, temperature, $CO_2$ concentration and light intensity and can be modified to any desired level using computer programs. LED lights provide even and specific distribution of light for each crop growth stage. Energy must be harvested from renewable energy sources for maximum economy. Crop residue might be composted or used to generate hydro energy by burning.

## 20.8 Sources of Photosystem and Importance of Green Energy

Vertical farming needs artificial lightning as irradiance is plentiful in the upper growing bed but decreases sharply moving down in the beds. Various sources of energy like electricity, solar energy, wind energy or hydro energy will fulfil the desired light requirement in the farm. LED technology is the best for artificial lighting as it emits a low level of thermal radiation, contains no hot electrodes and no high-voltage ballasts. LED also has a long operating life that makes it a practical alternative for long-term use like vertical farming. Most importantly, it is possible to modify the irradiation output to approximate the peak absorption zone of chlorophyll. Furthermore, LED can be operated in a shutter sequence to save power. A shutter sequence means LED is frequently turned on and off with a defined frequency. Investigation in plant response has shown shuttering of LED do not affect plant growth and development but can drastically reduce required electrical energy (NASA 2004). Green energy referrers to energy with zero pollution. That includes the solar energy, wind energy or hydro energy. A highly automated greenhouse requires more energy. Non-renewable sources are diminishing at a rapid rate. Adoption of green energy could minimize electricity cost and simultaneously reduce environmental pollution. The efficiency of green energy to operate and illuminate the vertical farm needs research and development.

## 20.9 Is Vertical Farm Viable?

The viability of any business depends on work environment, functioning and efficiency. To consider farm viability in present context, a discussion of vertical farm establishment and advantages follows with a conclusion concerning long-term viability.

### 20.9.1 Why Vertical Farming Must Be Adopted?

Adaptation of vertical farming is a means to cope with land degradation, climate change, the need for sustainable farming, demand for quality food, providing local food security and increasing cultivable land. Selected consideration of future food production issues follows with a detailed description.

#### 20.9.1.1 Climate Change

Climate change is a major factor contributing to the reduction of arable land. Both natural disasters like flooding, hurricane, storms, drought and man-made hindrances such as over use of fertilizers and agrochemicals decrease valuable agricultural land drastically, thereby damaging the global economy (Despommier 2010; Muller et al. 2017; UN 2017; Kalantari et al. 2017). Climate change and adverse weather conditions are present today and might occur at an increased rate in the future. Over time, these events might render arable land useless for farming. Prioritization of productivity over sustainability has left the soil simultaneously hungry and thirsty. Popularity and provision of crop insurance subsidies by government following natural catastrophes might help traditional farms to plan outside the current scenario (Sanye-Mengual et al. 2013; Thomaier et al. 2015; Padmavathy and Poyyamoli 2016). Traditional farming requires more fossil fuel, more manpower and more human interventions to conduct agricultural operations. Traditional farming faces problems of pest management and low productivity. Distance travelled by crops or "food miles" contributes to environmental pollution and reduces food freshness. On average, food travels 1500 miles from farm to dining table (Despommier 2013; Astee and Kishnani 2010). A study conducted during 2008 at Carnegie Mellon concluded food delivery is responsible for 0.4 tons of carbon dioxide emissions per household per year (FAO 2013). Sadly, resulting greenhouse gas emissions from food transport and agricultural activities have contributed enormously to climate change. Adaptation of indoor gardening and roof top gardening with vertical technology or vertical farms in urban and semi-urban areas carries the possibility of minimizing these effects.

#### 20.9.1.2 Ecosystem Sustainability

Over millions of years, traditional agriculture is believed to infringe upon natural ecosystems. Over-cultivation is believed to be a primary cause of soil erosion. Dickson Despommier states, “Farming has upset more ecological processes than anything else which is the most destructive process on earth”. Brazilian rainforests recorded noticeable impact with around 1,812,992 $km^2$ of hardwood forest being destroyed for farmland (Corvalan et al. 2005) over the past half century or so. According to Despommier, encroachment on ancient ecosystems leads to climate change. As a consequence, indoor vertical farming could substantially reduce the harmful agricultural impact on ecosystems by conservation and restoration of biodiversity and reduction of harmful influences on climate change. According to Al-Kodmany (2018b), urban vertical farms producing merely 10% of the ground area they consume might reduce $CO_2$ emissions enough to sustain long-term biosphere improvement. Fertilizer runoff and pesticide residue would not hamper the ecosystem and its organism. Wood et al. (2001) summarizes this point stating, “The best reason to consider converting most food production to vertical farming is the promise of restoring the services and functions of ecosystems”.

#### 20.9.1.3 Food Security

Food security has become an increasingly important issue in recent days. Demographers anticipate a dramatic increase in the urban population as a consequence of job migration and quality lifestyle. Land specialists viz., agronomists, ecologists and geologists warn of rising farmland shortages (Corvalan et al. 2005; Healy and Rosenberg 2013; Thomaier et al. 2015) with the potential for demand surpassing supply resulting in global hunger. The United Nation (UN) estimates a 40% increase in world’s population, exceeding 9 billion people by 2050 (UN 2017). The UN report also projects 6 billion people will reside in cities by that time. Again to feed an increased population of 60% (Tilman et al. 2002; Green et al. 2005; Alexandratos and Bruinsma 2012) requires 70% (UN 2017) increase in food production. Food prices have skyrocketed in past decades and have the potential increase as a result of the COVID-19 pandemic. Farmers predict increased prices due to in climbing oil costs and decreased availability of water, energy, farmland and agricultural resources (Despommier 2010, 2013, 2014; Touliatos et al. 2016). The logic of vertical farming is very simple, i.e. to produce more food on less land (Touliatos et al. 2016; Muller et al. 2017), with the same concept in which we prepare our buildings. Adaptation of vertical farming could provide easy access to quality foods free from residual toxicity of agrochemicals. Efficient and sustainable production system management could help address multiple concerns arising from pollution, climate change, pest load, as it simultaneously minimizes energy use, saves water, reduces fertilizer and agrochemical use, restores ecosystems, upgrades financial status, provides employment opportunities and finally helps supply good quality foods locally (UN 2017). In general, indoor farming offers a healthier environment for food growth (Healy and Rosenberg 2013; Mukherji and Morales 2010). Since indoor farming is independent of weather conditions, year-round operations could provide greater yields and income (Katz and Bradley 2013). Indoor vertical farm could help reduce travel time, cost and subsequent greenhouse gases and other airborne pollutants (Mukherji and Morales 2010; Astee and Kishnani 2010). Food costs could decrease as a result of reductions in travel and packaging cost. Vertical farming can boost the local economies by granting job opportunities (Mukherji and Morales 2010; Healy and Rosenberg 2013), and directly help address local food security. Vertical farming also helps address the problem of farmland shortages (Corvalan et al. 2005; Healy and Rosenberg 2013; Despommier 2010). According to the United Nations' Food and Agriculture Organization (FAO), there was 0.42 ha (1 ac) of arable land per person on earth in 1961. Because of population growth and urbanization, the number had dropped by nearly half (0.23 ha or 0.5 ac) in 2002 (Healy and Rosenberg 2013). An estimated 28 $m^2$ of intensively farmed indoor space is enough to produce food supporting a single individual in an extra-terrestrial environment. In a space station or space colony, this supplies around 3000 kcal of energy per day (Mitchell 1994). In 2011 from the global assessment of planetary land resources, the United Nations found a quarter of arable land to be highly degraded. Dickson Despommier highlighted current agricultural supply will soon become largely inadequate for the growing population. On average, every human being needs 1500 calories daily. In order to meet such demand by 2050, an area of land equal to the size of Brazil will need to be added to existing agricultural land area (Despommier 2010). Vertical farming could be one means to consider for achieving this goal.

#### 20.9.1.4 Health

Conventional farming stresses the economics of agriculture causing unnecessary harm to both environment and animals (Despommier 2010, 2013; Touliatos et al. 2016). Current processes depend on use of highly toxic agrochemicals *viz*., fertilizers, fungicides, synthetic growth regulators, insecticide and other chemicals having residual impact. These practices repeatedly cause erosion, contaminate soil and generate excessive waste water. Regarding human well-being, the World Health Organization has determined that over half of the world's farms still use raw animal waste as fertilizer which may attract flies and may contain weed seeds or disease transmissible to plants (Al-Kodmany 2018a). Consequently, these practices compound negative health outcomes for people consuming such produce. Crops grown in controlled indoor environments could reduce excessive use of pesticide and herbicide to reduce risk of agricultural runoff pollutants. In a contained environment, pests, pathogens and weeds have a much harder time infiltrating and destroying crops. When excessive fertilizer nitrogen and phosphorous compounds wash into water bodies (e.g. rivers, streams and oceans), a high concentration of nutrients is created (called eutrophication). This disturbs ecological equilibrium causing dead aquatic zones (Despommier 2013). As of 2008, there were 405 dead zones counted around the world. Further, indoor vertical farming employs high-tech growing methods using little water (about 1/10th of that used in traditional farming) by offering precision irrigation and efficient scheduling (Wood et al. 2001). This significantly minimized fertilizer erosion to water bodies, diminished environmental pollution. An ameliorative effect is predicted, since demands on water increase as urban populations grow. As use of agrochemicals, pesticides and herbicide is reduced, pesticide residual toxicity concerns will diminish and provide consumers with healthy, nutritious food. Urban or semi-urban vertical farming preserves produce integrity as vegetables and fruits require less time to transportation and more time to provide fresh foods.

#### 20.9.1.5 Urban Density and Food Production System

Vertical farming offers numerous advantages over horizontal or conventional farming. It offers more land for non-agricultural activities viz., housing, services and amenities (Despommier 2010). Urban areas are overloaded with population numbers, and there is less available arable land. The vertical farming concept could reduce pressure for land requirements for agricultural activities. Low density populations incur higher energy use and generate more air and water pollution. Serious steps are needed to feed a rising global population by 2050 requiring around 2.1 billion acres of land (Despommier 2009). Despommier suggested the space efficiency of vertical farms that a 30-story building of about 100 m high with a basal area of 2.02 ha (5 ac) would be able to produce a crop yield equivalent to 971.2 ha (2400 ac) of conventional horizontal farming around 480 times greater than the conventional farming method. The above example shows production of one high-rise farm is manyfold than conventional horizontal farms, which could produce several times more than horizontal farms.

#### 20.9.1.6 Efficiency and Economics

Vertical farm could yield competitive food prices (Al-Kodmany 2016). The urban vertical garden may provide both jobs to support the local economy and quality foods to the neighbourhood. In certain instances, the food from these gardens would be sold locally without middlemen and rigorous transportation from production to distribution site. This transport cost constitutes a greater amount, up to 60%, of the final product price (Al-Kodmany 2016). The increasing expense of traditional farming is quickly narrowing the cost gap. For example, when vertical farms are located strategically in urban areas, it would be possible to sell produce directly to the consumer, reducing transportation costs by removing the intermediary. Start-up costs and site selection of vertical farming systems have been major constraints (Benke and Tomkins 2017), causing recent demand for streamlined government policies and intervention. Use of advance technology and intensive farming methods in these types of farms can exponentially increase production. As this technology is new, researchers have been engaged in optimizing indoor vertical farming by consideration of a wide range of variables including light intensity, light colour or spectrum, space, temperature, crop shoot and root, $CO_2$ contents, soil, water and air humidity (Harris 1992; Al-Kodmany 2016), etc. Abandoned, old or unused urban buildings could be converted into vertical farms to provide healthy food in neighbourhoods where fresh produce is limited. Finally, this farming technology could reconnect urban people with nature through the activity of farming (Al-Kodmany 2016). Use of advanced technology like full automation can make this farming fun and increase yield. Banerjee and Adenaeuer (2014) declared land productivity of vertical farming to be double that of traditional agriculture. With only 0.25 ha, a farm was built and recorded an increase of 516-fold in total yield compared to traditional agriculture through stacking production. In total, this leads to an estimated production of 3573 tons of edible fruit and vegetables.

Depending on the present scenario of fluctuating calamities, health consciousness, need for sustainable farming, personal economy and present and futuristic demand for food security could emphasize the need for vertical farming. Though this is an emerging technology, it still has a prominent impact on young minds. The present cost of construction for vertical farms is a concern. Scientific intervention is needed to standardize this to low-cost technology which should be region specific. Recent agriculture technology, streamlined research, technology transfer and field trials could make vertical farming a huge success. A fully enclosed and controlled environment and complete or partially automated system to monitor the crop growth and environmental parameters like humidity, $CO_2$ concentration, temperature and light make this technology a long-run prospect for sustainability.

### 20.9.2 Benefits of Adopting Vertical Farming

The following are few listed benefits of adopting vertical farming technology.

- More crop per space, as the vertical space is judiciously being used for raising more crops.
- More crop productivity or increased production per resources used.
- Year round yield for indoor or greenhouse growth condition.
- Full control over the plant growth using desired light, nutrient, moisture and $CO_2$.
- Minimized use of dangerous agrochemicals to stabilized climates and ecosystems.
- Reduced global carbon foot print when located at urban or semi-urban localities.
- Improved quality of food to boost healthy life.
- Less labour force, as this technology performs better in automated controlled conditions.
- Less crop loss as a result of natural calamities, invasion by insect, pests, diseases and weeds are minimized.
- Reduced transportation cost for local produce to benefit consumers, grow local economies and reduce environmental emissions.
- Protect crops from weather-related problems as well as pests and diseases.
- Minimize agricultural water requirements.

### 20.9.3 Demerits in Vertical Farming

Though this farming has numerous prospects, some shortfalls are listed here.

- Requires significant financial investment.
- Profit of this system depends directly on the patience of the grower.
- Supply of electricity 365 × 7 × 24 is a must that could be filled by installing various renewable sources.
- Disease may spread quickly through any closed system and result in 100% crop loss.
- Requires well-trained technical staff, as most of the operations are highly advanced.
- Direct marketing is possible in urban, but rural or peri-urban areas require an investment for marketing.
- Selection of crop on the basis of local demand, feasibility of growing structure must be worked out prior to installation.
- Structural integrity of the vertical frame work needs research and standardization, because frames are prone to humidity damage or rust in long run due to the controlled climate.

## 20.10 Insect and Pest Concern Under Vertical Farm

The vertical farm is an enclosed space, which restricts the entry or growth of harmful pests. Farming conditions are clean and maintain sanitary conditions. Crop residues are removed from the greenhouse. In total, the plant growth process begins in laboratory conditions and ends the same. For example, selected seeds are inspected and treated in laboratory conditions, grown in humid chambers and taken directly to the environmentally controlled greenhouse for planting in the vertical farm. So, there is very little chance of contamination. The entire process of farming is carried out in an organic manner (Kalantari et al. 2018). One advantage of vertical farms is significant reduction or omission of agrochemicals. Attempts can be made to find natural solutions for problems, by utilizing useful insects, i.e. predators. As previously mentioned, all vertical farms benefit from controlled conditions void of hazardous pests (Despommier 2010, 2011; Ellingsen and Despommier 2008; Germer et al. 2011) eliminating the need for pesticides. In the vertical farm, there is no need for burning fields, grass or waste for pest control, other methods of pest control can be employed. All materials are sanitized or washed prior to use. Frequent checks control the spreading of harmful organisms before they spread in the closed environment. Water may act as a contamination source and must be treated before use.

## 20.11 Recent Advancement

New horizons in agriculture are generally slow to emerge but to keep pace with exponential growth potential of any green revolution the grower can efficiently modify systems as he becomes acquainted with them. In the recent past industry, advances are rising to support this emerging technology. However, vertical farming is still not a common grow practice. Many industries have tried various crops (Table 20.2) and gained good results. Again this technology needs specificity in terms of locality, crops, climate, economy and more.

## 20.12 Conclusion

Vertical Farming is an emerging technology with huge potential for increasing crop production per unit land area in response to the pressure of population growth on agricultural production. The design, layout and configuration of these high-tech farms would provide optimal light exposure, along with precisely measured nutrients for each plant, designed to grow in a controlled, closed-loop environment. These farms would eliminate the need for harmful herbicides and pesticides, maximizing nutrition and food value in the product produced. Vertical culture in a protected structure could also enhance the taste of produce to cater to consumers preferences. In horticultural crops like berries, green leafy vegetables, spinach and some crucifers, this technique is already adopted, providing a tremendous result in terms of yield, quality and sustained production. Vertical farming is an expensive method for growing crops on designated bed, under the greenhouse or controlled environment. A combined technical approach is required to accommodate factors including lighting, growing system, crop nutrition, energy efficiency, construction and site

**Table 20.2** Crops selected for growing under vertical farming system

| Sl. No. | Crop | References |
|---|---|---|
| 1 | Micro greens | Growing Underground (2020), Verti Crop (2020) |
| 2 | Salad leaves | Growing Underground (2020), AeroFarms (2020), Verti Crop (2020) |
| 3 | Strawberry (*Fragaria* spp.) | Murthy et al. (2016), Saturn Bioponics (2020), Verti Crop (2020) |
| 4 | Lettuce (*Lactuca sativa*) | Sky Greens (2020), Touliatos et al. (2016), Saturn Bioponics (2020) |
| 5 | Spinach (*Spinacia oleracea*) | Sky Greens (2020) |
| 6 | Tropical leafy vegetables | Sky Greens (2020) |
| 7 | Assorted leafy vegetables | Song et al. (2018) |
| 8 | Culinary herbs | Saturn Bioponics (2020), Verti Crop (2020) |

selection. This system has been shown to have a huge potential for the production of a wide range of crops but technical and economic optimizations are required. Research regarding maximized productivity and system cost reduction is in the forefront. Though start-up costs are high, increased input cost can be overcome by increased productivity. Scientists are looking to modify economic aspects of this technology. Indoor horticulture production systems can be boosted by advanced and automated vertical farming techniques to fulfil the current prospect of adaptive organic farming while concurrently improving soil health and maintaining sustainable farms.

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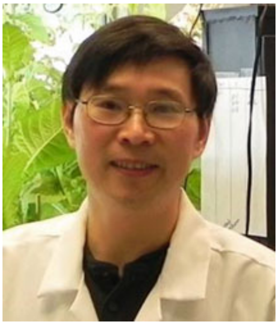

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# Climate Change and its Effects on Global Food Production

# 21

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## Abstract

Earth's population is increasing each year and it has drastic effects on the environment as well as on the global food production. Climate change unfortunately, affects not only humans' lives but also affects the ways through which we produce our food and system of agriculture. Many studies revealed that yield of green vegetables is expected to decrease by 35% due to increased emission of

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greenhouse gases. Many factors like warmer temperature, increased $CO_2$ concentration, pathogens, and decreasing pollinators affect the global food production. Fish catch has been decreased in recent decades due to acidification of the water and warmer temperature while the production of livestock has also been decreased significantly because of climatic changes. Our ability to ensure food security and adequate nutrition in a rapidly changing biophysical environment will be the determining factor in the global disease burden in the next century. There is a direct relation between the climate and the global food production. This chapter is intended to provide information regarding the drastic effects of climate change that could be faced in future.

**Keywords**

Climate change · Food security · Global environmental effects · Global health · Malnutrition

## 21.1 Introduction

Climate change is a serious environmental concern of current era possessing a serious threat to sustainable development in world and specifically in developing countries. Climate change affects the water resources, health, food, and ecosystems (Pedersen et al. 2021). Despite of increase in global food production in the recent past, the problem of malnutrition has been increased. It is estimated that 462 million people are underweight whereas 1.9 million have the problem of obesity (WHO 2020). Increased food production has been achieved by the technological advancement, including but not limited to development of high yield varieties of grains, synthetic pesticides and fertilizers, and mechanization of the agricultural sector. All this was possible to achieve by a large portion of natural reserves of the Earth. Around 40% of the ice-free land is being used for the production of crops (Foley et al. 2011). Irrigation system utilizes about 66% (approximately 2000 $km^3$) of annual withdrawn water and it is the single principal use of water by the humans (Brauman et al. 2016). Looking forward into the future, global food requirements are expected to rise continuously at historically sheer speed starting in early 1950s (Fróna et al. 2019). However, contrary to 1950s, constraints are being faced in this era because of limited availability of new land and water as well as fisheries to cope with increase demand of food. Moreover, a new challenge that could be added to these existing one is the change in environmental condition that is rapidly developing, in which we had to grow the food for our consumption. It is a great humanitarian challenge that is being faced in the twenty-first century to cope with the increasing demand of increased nutritional demands in terms of limited available natural resources to produce the food and the climate change.

Climate change is linked to increase in temperature and heavy rainfall that lead to alteration in the relationship of crops, pathogens, pests, and weeds. It also aggravates many patterns including the reduction in pollinating insects, increased ozone concentration at ground level, rise in water scarcity, and decline in fishery. Contrary to this, there are some benefits in terms of high yield due to increased $CO_2$ concentration (Ebi et al. 2021) as per the Blackman's law of limiting factor (Farazdaghi 2011). There are researches that cover the prospective impact of climate change in the nutrition and mortality but inevitably involve significant uncertainty, mainly due to constraints in our knowledge regarding complex and engaging pathways whereby climate change may affect food security and health.

## 21.2 Agriculture

Agricultural history involves the continuous vanquished limitations and attainment of high production of food by increase in area of the cultivated land and strengthening cultivation by employing advanced agricultural technologies (Rotz et al. 2019). Nevertheless, the nutritional quality and quantity of the agricultural yield relies on the sprightly equilibrium of proper biophysical resources, e.g., sunlight, $CO_2$, temperature suitability, water availability, and to some extent, on pollinator profusion (Yano and Cossu 2019). Production dwindles under specific extreme weather conditions and from pathogens, pest, and air pollution (viz. tropospheric ozone) (Storkey et al. 2019). At certain areas of world, agricultural production is greatly dependent on labor (Giannakis and Bruggeman 2018; Van der Ploeg and Long 2019). Climate change is anticipated to have influence on each of these dimensions of agricultural yield, yet, poorly characterized.

### 21.2.1 Temperature, Water, and $CO_2$

Average global land temperature has been increased to 1.0 °C in 2006–2015, which is higher than the previous century average (NOAA 2020). The atmospheric level of $CO_2$ is expected to rise beyond 400 ppm than 280 ppm baseline if the same scenario of greenhouse gas emission continues at the value of representative concentration pathway (RCP), i.e., 4.5, it can go to 540 ppm by 2100 (Zeleke 2021). Climate's computer modeling suggests in further increase in global land temperature of 1.9–4.0 °C (NOAA 2020). Under higher RCP 8.5, $CO_2$ level could be reached to 940 ppm in 2100 with increased land temperature of 4.0–6.8 °C (NOAA 2020). If we expect a moderate emission situation, it is speculated that average temperature in summer will increase than currently being experienced high temperatures around the globe.

Climate change will also affect the availability of water for the agricultural use in many ways such as switching precipitation patterns, early seasonal snow melting with or without glaciers loss, and saltwater intrusion in coastal aquifers. Climate modeling projection indicates the higher precipitations in polar latitudes while low precipitation in arid and semiarid regions of the world (Collins et al. 2013). Rainfalls are being foreseen to become more severe, resulting in increased overspill and flooding (Collins et al. 2013).

Crop production is greatly sensitive to water availability and change in environmental temperature. Cultivars and other environmental variables can vary the optimum growing temperatures; however, the air temperature above 30 °C is usually linked to reduced crop production for rainfed crops (Black et al. 2013). Although, high temperatures can accelerate the crop development but it can directly damage the plant cells (Sánchez et al. 2014) ultimately resulting in low crop yields. Crops exposure to this harmful temperature rise will be due to global temperature increase, nevertheless these trends will differ in different regions of the world and can be locally mitigated by irrigation or modifications in agricultural practices (Davin et al. 2014).

Water stress to crop also plays a major role in reduced crop production and is usually coupled with increased temperatures both with increased crop water depletion (Lobell and Gourdji 2012) and decreased evaporative cooling from the landscape due to low moisture level (Mueller and Seneviratne 2012).

Even though the increased level of atmospheric $CO_2$ is the primary factor for the harmful anthropogenic climate change, it can result is enhanced crop performance by increased photosynthesis rate and water use. Crops such as rice, wheat, and soybean that utilize the C3 photosynthesis, undergo better growth stimulation by increased $CO_2$ rather than the crops with C4 photosynthetic pathway like sorghum, maize, and sugarcane (Leakey et al. 2009).

There is limited data available of the consequences of $CO_2$ concentration and changing temperature on management driven crops. Climatic changes may either decrease or boost the ongoing crop production. Existing data indicate that the climatic shifts have been resulted in decrease of 5% of global production of wheat and maize since 1980, while net global production of rice and soybeans has not been affected by the climatic shifts, although there are regional losses and gains (Lobell et al. 2011).

Further climatic change scenarios estimates indicate that the yields of wheat and maize will be depressed, with greater production losses in tropical regions, while rice production seems to be less sensitive to expected changes (Rosenzweig et al. 2014).

Models of crop growth that includes the effects of $CO_2$ concentration in conjunction with effects of nitrogen limitation, water availability, and temperature indicate 15% average production losses of wheat for low latitude and 25% average production losses of maize for low latitude whereby increase in global temperature by 4 °C by 2100 (Rosenzweig et al. 2014).

Different individual models suggest different outcomes of the climate change, nevertheless, some models forecast the double losses and other predicts a slight increase in production in low altitudes.

Moreover, these models did not definitely adapt the phenomenon like pollinators, pests, agricultural labor, and ground level ozone level. Adaptation of farmers to the new climatic conditions possesses assurances for mitigating agricultural production losses, even though the extent of adaptation is a topic of continuous debate (Moore and Lobell 2014). Under a specific crop management system, farmers can change the plantation and harvesting dates, adjust the irrigation system and changing the crop varieties. A current meta-analysis identifies the benefits from such changes in terms of production increase of 7–15% in contrast of absence of adaptation. Adaptation tends to work in temperate areas with increase in production; however, the gains were minimal for tropical wheat and maize production (Challinor et al. 2014). Farmers can also adapt to these climatic conditions by transferring to completely different crops or reallocating land to grazing from crop production.

### 21.2.2 Ground Level Ozone

Chemical reactions in anthropogenic emissions result in the ground level ozone (Ainsworth et al. 2012). Ozone development enhances with increasing temperature, especially beyond 32 °C. Besides being harmful to human cardiorespiratory system, ground level ozone is also toxic to plants in terms of creating hindrance in photosynthesis of crop and growth, along with reduced grain weight and production. Current ozone pollution level is found to have a 57% decrease in the rice crops reduction in India (Saxena et al. 2020) while 15–35% reduction in wheat production in Southeast Asia (Sarkar et al. 2021). Even though current governmental regulation in developed countries is expected to decrease the ozone population in coming decades, many developing countries, especially in Asia and Africa can forebode an increase in ozone levels because of increased emission and global warming issues.

### 21.2.3 Pests

Pathogens, fungi, insects, and weeds are identified to be a reason for the reduced crop production of major crops ranging from 17 and 23% for major crops, i.e., wheat, potato, maize, and soybean, except rice, for which it is estimated to be 30% (Savary et al. 2019). Annual global dietary energy availability is estimated to be decreased by 8.5% by fungi infestation (Fisher et al. 2012). Herbivory rate and winter survivability chances of pests have been increased because of increasing warmer temperature. Change in temperature is also shifting the latitudinal range of crop pathogens and pests. About 612 pathogens and pest species are found to be poleward shifted to 2.7 kilometer (1.67 miles) per annum since 1960 (Bebber et al. 2013). Crops usually lack defense mechanism against exotic pathogens and pests, hence requiring a continuous effort from experts on management and breeding to overcome such threats. Biological control system is undermined in case of spatial mismatch among pests and predators.

Intense weather conditions can subvert agricultural systems, palliating crop defense mechanism, and establishing a niche allowing pests and weeds to grow on their own. But on contrary, extreme weather conditions may also result in a serious threat to pests and weeds in terms of growth and survivability and boosting the competitive capability of crops to survive (Young 2015). Besides the changing climate’s effect, scientists foresee that increased $CO_2$ level will result in intricated changes in weeds composition and plant’s defense mechanism against pathogens and pests. Furthermore, herbicides are found to be less effective against increased weed biomass control caused by increased levels of $CO_2$.

### 21.2.4 Pollinators

Climate change is also affecting the production of flowering species thus resulting in reduction of the pollinating insects and disturbing their regional distribution (Abrol 2012). Increasing global temperature results in delay of flowering and causing plant communities to relocate poleward. These migrations may cause mismatch in mutualism relationship between plant and pollinator pairs, therefore disturbing mutual interaction and ecosystem functionality. Moreover, decreased interaction among flowering timing and pollinator insect's emergence can decrease the extent of pollinators diet, leading to a decreased pollinators profusion and increase in the probability of extinction of both pollinator and plants.

Increased atmospheric $CO_2$ level is also resulting in change of the nutritional values of the forages that are necessary for pollinators, with indeterminate impact on health of pollinators. It is found in a study that the protein content of the goldenrod pollen has been decreased to one-third since late 1950. Study further indicates a decline in concentration in protein content over time with increasing $CO_2$ concentration by chamber experiment (Ziska and Goins 2006).

Even though the overall climatic change effect on pollinators is uncertain, scientists believe that a decline in insect pollination will reduce production of various pollinator-dependent food crops that will drastically affect the provision of food to humans. Modeling suggests that a global decrease in pollinator will result in birth defects and child mortality from increased folate and vitamin A deficiency, respectively. It will also result in increased risk of stroke, heart disease, certain cancers, and diabetes in adults due to decreased dietary consumption of vegetables, fruits, seeds, and nuts (Smith et al. 2015).

### 21.2.5 Nutrient Losses

Increased $CO_2$ concentration is also resulting in change in crops' nutritional composition. A reduction in protein content of edible portion of food crops was recorded in the experiments in which these plants were grown in enhanced $CO_2$ concentration both in field conditions and closed chamber.

Contrary to C4 crops and C3 legumes that show insignificant reduction, C3 tubers and grains including potatoes, wheat, rice, and barley are found to have 7–15% decrease in protein content (Myers et al. 2014). If these nutritional changes are modeled among current diets, it is estimated that about 200 million people will fall below the threshold level of recommended protein intake, while this level will be worsen for those who are already below this threshold level (Medek et al. 2017).

Crops cultivated at increased $CO_2$ concentration also revealed a significant lower concentration of some important minerals. $CO_2$ level of 550 ppm can entail a 3–11%

reduction of iron and zinc concentration in legumes and cereal grains (Myers et al. 2014). Moreover, at $CO_2$ level of 690 ppm, the concentration of potassium, phosphorus, sulfur, magnesium, calcium, copper, manganese, zinc, and iron decreased to 5–10% across many crops (Loladze 2014). This decline in zinc concentration will put 150–200 million people at risk of zinc deficiency and aggravate present zinc deficiency in more than 1 billion people (Myers et al. 2014). Furthermore, approximately 1.4 billion children (of age 1–5 years) and women (of childbearing age i.e., 15–49 years) in countries with more than 20% anemia prevalence, dietary iron intake is anticipated to decrease to more than 3.8% due to these $CO_2$-associated nutrient changes (Smith et al. 2017). In general, rising $CO_2$ level will put hundreds of millions of people at risk of protein, iron, and/or zinc deficiency, and estimated more than 2 billion people with iron or zinc will face worse condition aggravated by such environmental change.

### 21.2.6 Agricultural Labor

Less developed countries heavily rely on physical human labor rather than mechanization for their food production. However, this labor is limited to regulate their body temperature under such conditions like high ambient temperature, high humidity, low wind, and increased radiation. Increased temperature is already responsible for limited agricultural labor work in subtropical and tropical regions at particular time of the year, and climate change will worsen this condition by putting further constrains on humans to work in such high temperature (Kjellstrom et al. 2016).

Under moderate RCP4.5 emission, it is expected that outdoor labor activity would be restricted to 50% during hottest season in most of Australia, sub-Saharan Africa, and India by late century. These restrictions will be further worsened at RCP8.5 at end of century in subtropical and tropical regions (Dunne et al. 2013).

Temperate regions labor is less likely to be affected by such changes, but one economic evaluation estimated that US labor productivity will be decreased by 0.6–3.2% in agricultural or other related sectors at the end of century under high emission scenario (Houser et al. 2015). Increased mechanization could help to reduce the work loss by human produced in heat stress, although in some developing countries this will have less effect on the economic potential for such potential (Kjellstrom et al. 2016). It is still unclear that how climate change will affect the working capacity of humans in terms of agricultural practices and overall production; however, there is increasing concern about heat stress and labor working in subtropical and tropical areas of the world, because they play major role in overall productivity.

## 21.3 Animal Husbandry

Livestock is a major agricultural commodity of global food security because it provides 33% protein and 17% kilocalorie of global consumption (Samuel et al. 2019). Livestock provides employment to 1.1 billion people while feeding one billion people simultaneously in poorest countries (Hurst et al. 2005). There is increasing demand of livestock products in developing countries. Worldwide meat production is expected to rise to 455 million tons in 2050 from 258 million tons in 2006, while milk production is projected to increase to 1077 million tons in 2050 from 664 million tons in 2006 (Alexandratos and Bruinsma 2012). However, production of livestock products is expected to be severely affected from climatic changes.

### 21.3.1 Water

Availability of water will affect the livestock sector, which consumes water for animals' drinking, product processing, and feed crops and it accounts for 8% of the total human water consumption and warmer temperature can result in increased water consumption of animals by a factor of 2 or 3. More salt water could be mixed with fresh water, if the sea level rises in future resulting in more salination of biological and chemical contaminants and increased concentrations of heavy metals that will affect the livestock production (Lacetera 2018). This will affect metabolism, digestion, and fertility of the animals. Heavy metals and chemical contaminants may alter respiratory, cardiovascular, nervous, excretory, and skeletal systems, and undermine hygienic production of livestock products (Lacetera 2018).

### 21.3.2 Livestock Diseases

Geographical location, animal susceptibility, and disease characteristics are the driving factors of livestock diseases caused by climate change. Health of the animal affected directly or indirectly by the climate change, especially due to rising temperature (Lacetera 2018). Direct effects linked to increased temperature include the potential of morbidity and mortality, while indirect effects include the impacts of microbial communities (parasites and pathogens), vector-borne diseases spread, host resistance, food-borne diseases, and water and feed shortage (Lacetera 2018). Increased temperature can hasten the pathogens or parasites growth (part of live cycle that is outside of animal's body) which can negatively impact on health of livestock population (Rojas-Downing et al. 2017). Climate change can cause an outbreak, shift in disease spread or introduce a new disease in animals that are already not present in that species. Climate change can also change the precipitation and spread of vector-borne pests such as mosquitoes, flies, ticks, and mites. There is also an increased chance of new emerging infectious disease because of new genetic makeup and transmissibility (Lacetera 2018; Rojas-Downing et al. 2017).

### 21.3.3 Heat Stress

All animals have a range of ambient environmental temperature that is helpful to perform their physiological functions. Animals can keep their body temperature within a range of ±0.5 °C (Henry et al. 2012). When temperature rises above their upper critical limit, animals start to suffer from heat and show signs of increased water intake, reduced feed intake, and altered physiological parameters such as increased respiration, and productive and reproductive efficiency. For poultry, heat stress reduces growth, meat quality, egg quality, and yield (Lara and Rostagno 2013).

### 21.3.4 Quantity and Quality of Feeds

Quantity and quality of feed will be affected by the local interaction of temperature, $CO_2$ levels, and precipitation. Increasing $CO_2$ concentration is predicted to have a positive impact on the pasturelands, while increased temperature can have a negative or positive effect, according to dubious changes in precipitation and soil water availability (Izaurralde et al. 2011). Increased $CO_2$ levels may have positive affect on C3 plants by reducing transpiration by partial closure of stomata and improving water usage efficiency. However, it can also result in decreased protein content of certain plants by changing carbon to nitrogen ration (Gamage et al. 2018). Methane emission per unit of gross energy consumed can be increased due to decreased forage quality (Eugène et al. 2021). Thus, replacement should be provided in form of grains, in case of increased methane production due to decreased forage quality.

## 21.4 Fisheries

Even though, agriculture is a major part in global food production in terms of total dietary energy, seafood is also an important in supply of vitamins, proteins, fatty acids, and minerals for many people around the world (Golden et al. 2016). Current analysis indicates that decreasing fish harvesting will result in deficiency of vitamin A, zinc, and iron to around 845 million people while omega-3 long-chain polyunsaturated fatty acids and vitamin B12 deficiency to 1.4 billion people (Golden et al. 2016). Poor population of the world is at higher risk of nutrient deficiencies due to their limited resources for acquiring alternative dietary products such as fish and livestock products, nutritionally fortified foods, and vitamin supplements.

Regardless of the fact of change in climate, recent fish catch trajectory is alarming. Current estimate of the Sea Around Us project shows that fish catch has been decreased by 1.22 million metric tons per year since its peak in 1996, which is three times faster decline as reported by Food and Agriculture Organization (FAO) (Pauly and Zeller 2016). Another analysis showed that about 68% of the global fish stock has been decreased below the biomass target for sustainable yield and expected to decrease to 88% in 2050 (Costello et al. 2016).

### 21.4.1 Rise in Sea Temperature

Climate change is anticipated to acidify, deoxygenate, and warm the ocean. This will alter the production and poleward habitat displacement. Increased stratification of the marine layer is resulting because of the warming, hence resulting in reduced ascending flux of nutrients to euphotic zone that leads to variation in phytoplankton production. A recent analysis indicates that response of phytoplankton to changing environment will be variable depending upon their location and nutrients availability (Lewandowska et al. 2014). Such changes in the plentitude and dispersion of phytoplankton are crucial since they make the foundation of marine food web.

Global fish catch is expected to decrease by 3–13% by 2050 under high emission RCP8.5, as compared to recent decade because of change in size and dispersion of phytoplankton (Cheung et al. 2016). Moreover, another study suggests that the tropical fish communities' biomass will be decreased about 20% until 2050, in case of high emission situation, because of warming and decreased oxygen level of ocean (Cheung et al. 2013). Decrease of 30–60% has been indicated for some upwelling and tropical areas.

The overall impact of climate change on aquaculture is not completely characterized and seems to be diverse. Some benefits are also expected for the aquaculture due to this climate change in terms of better growth rate and food conversion efficiency in increased water temperature, prolonged growing season, and increased aquaculture operation potential at high altitudes due to decrease in sea and lake ice cover. But high temperature may also result in increased infection rate in fishes, enhanced hazard of harmful algal bloom, and hasten the heavy metals and toxins uptake in freshwater shellfish.

### 21.4.2 Ocean Acidification

Current knowledge of acidification impact on ocean production is very limited. But it is obvious that coral reef will be heavily affected by ocean's warming and acidification. It is estimated that 92% coral reef habitat will be decreased by 2100 (Speers et al. 2016).

### 21.4.3 Nutrient Quality

Climate change can also result in change in nutrient contents of seafood by changing nutrition composition of phytoplankton, ultimately affecting the food chain. Increase in water temperature results in decrease in uptake of long-chain polyunsaturated fatty acids of phytoplankton (Hixson and Arts 2016) and in cold water pelagic fish, e.g., anchovy and sprat. It is also suggested that mineral uptake like iron becomes limited in acidic and warmer water (Chavez et al. 2010); however, further analysis of impact of micronutrients is required to know their impact on composition.

Like agriculture, direct impact of $CO_2$ emissions coupled with accompanying climatic changes leads to significant uncertainties in terms of food and nutrition availability. For fisheries, although, the combining intricacy of how the whole marine food web will be modified results in higher uncertainty.

## 21.5 Effects on Food Security and Nutrition

Overall, global food production will be affected by many climatic changes affecting quality and quantity of the food produced in agriculture, livestock, and fisheries sectors. Even though accurate analysis of their impact is far beyond of our recent knowledge, there is a disturbing aspect of disrupting supply chain of the nutritious food supply. If we do not do that, then wealthier states of the world will ensure the supply of food to their own wealthy people because of their high purchasing power, leaving behind the poor with lack of proper food supply. Indubitably, food security is determined not only by the whole supply, but also by peoples' ability to access, buy, and use of food.

### 21.5.1 Conflicts

Politics and economics also play a driving force for food access. Discrimination, particularly in the wake of gender, caste, ethnicity, and wealth hinders participation in markets, legal acquisition of asset and land ownership, and other rights necessary for food security. Climate change can hinder social exclusion through increased competition for use of natural resources and compelling migration. These factors played a major role in past decades during civil conflicts of Middle East and sub-Sahara African countries for restricted food access (Hendrix and Salehyan 2012). Although the link between violence and climate change is controversial, but the evidence is increasing. A recent review suggests that there is strong relationship between these two phenomena (Hsiang et al. 2013), indicating a rise in violence among groups due to increased temperature especially in severely affected region of food shortage, i.e., South Asia and sub-Saharan Africa. Such type of intense conflicts along with population displacement will result in different health issues related to malnutrition.

### 21.5.2 Price Hike of Staple Foods

Climate change will exert a serious pressure on food access. It is estimated that by 2050, the prices of rice, maize, and wheat will be increased to 31–60%, while population growth, income growth, and climate change mitigation efforts would be factors affecting on these estimated values (Nelson et al. 2010). It could be true that the increased income would overcome the cost of expensive food and increased wages to the workers working at the farm. However, increased food prices will result in increase in poverty and food security both for the people of rural and urban areas.

### 21.5.3 GDP Growth

Food prices are also dependent on the gross domestic product (GDP). One recent study identifies that there will be 75% decline in income if this situation keeps growing without any climate change mitigation by 2100. While, at RCP8.5 43% of all countries around the globe would become poorer than they are now by the end of century (Burke et al. 2015). There could be an immense loss in economics of developing countries because of unmitigated climate change, that may result in poor purchasing power of food. Despite the fact of improved crop production in developing countries, food system will continue to struggle because of poor food delivery system in poor countries. These threats coupled with demographic reality of population growth of 2.5–3.0 billion people in coming decade would worsen the conditions in developing countries.

### 21.5.4 Food Consumption and Disease

Food security is not just based upon demand and supply in markets rather it goes beyond extending to food consumption, including safe cooking and nutritious meals, and protecting the food from spoilage and pests during storage. If food is not cooked properly, pathogenic bacteria, viruses, parasite, and mycotoxins get entry into the body (Cissé 2019). This will result in diarrheal and infectious diseases that have esoteric impact on child growth and development (Ngure et al. 2014). An analysis of surveys that were conducted in 171 different countries between 1986 and 2007 indicated that water was linked with stunting growth in children under 5 years of age (Fink et al. 2011).

### 21.5.5 Volatility

Future projections of food availability and utilizations will depend upon the mean values of production, income, disease, etc. because these parameters change over time. Because change in climate will ultimately alter the spatial and temporal fluctuation in food production and prices may also change more frequently.

## 21.6 Conclusion

There are significant doubts about the effects of environmental changes, including the response of plants, animals, fisheries, and farm labor as well as their adaptation to these modifications. Even though these uncertainties picture the future food prediction tough, the evidence-based analysis strongly encourages to prepare for the possible consequences. Moreover, further research will reduce these uncertainties in the results. Analysis of FAO data has already been described to cause a serious impact in form of reduced fish catch, decreased pollinators, and health effects from increased $CO_2$ concentrations. But these analyses have many shortcomings. First, these estimates do not focus on actual intakes but rather rely on availability. Second, these estimates lack data about food distribution among sex, age, and income groups as well as food distribution among subnational populations. Third, these estimates have inadequate information for wild harvested foods including bushmeat and fish.

Furthermore, there is limited data available regarding nutritional values of the food available in scientific databases, and many of them have not been updated since years. This result in lack of our knowledge about what people are eating, and what is the impact of the changing environment on the nutritional composition of these foods and their affects on the body.

Many countries have increased their crop production after adopting modern crop varieties, and greater irrigation. Decreasing food loss and waste can help to fulfill future demand. About 1.4 billion tons of food produced globally is wasted annually worldwide. Food loss in developing countries is linked with fungus growth or from pests while in developed countries, this loss is associated with consumer households. Better mitigation of environmental change would alleviate stress from food system, and this can be achieved by the decreasing emission of greenhouse gases and other pollutants in the environment, improving efficiency of agricultural land and water use, as well as sustainable livestock and fish farming practices. Finding the right balance and scope of action among a variety of policy priorities requires a better understanding and more accurate explanation of how environmental change shapes food production and global health.

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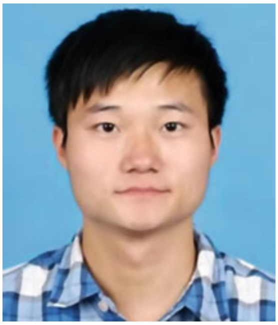
**Muhammad Fakhar-e-Alam Kulyar** has broad and acute interest about latest diagnostic tools, nutritional management, quarantine management, and disease control systems. He has abilities in gene expression studies especially related to clinical diseases in veterinary sector.



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# 22

# Genetically Modified Crops to Combat Climate Change and Environment Protection: Current Status and Future Perspectives

Riti Thapar Kapoor

## Abstract

Agriculture sector plays significant role in ensuring food security and sustainable development. The urbanization, industrialization, pollution, release of greenhouse gases, and adaptation of agrochemical-based crop production practices have increased heavy metals and other contaminants in the environment and food chain. The use of genetically modified stress-resistant crops reduces the impact of climate change. Genetically modified organisms are being made by inserting a gene from an external source such as microbes, animals or plants into generally unrelated species. The advantages of genetically modified crops for a society to solve the problem of food insecurity and malnutrition have been well established. Other benefits such as high nutritional value, herbicide tolerance, tolerance against abiotic and biotic stresses with high shelf life of crop products may be useful for farmers. Genetically modified crops are not a panacea but they have potential for achieving food security and can make a significant contribution to poverty alleviation in developing countries by increasing crop productivity under adverse climatic conditions.

## Keywords

Climate change · Environment · Food security · Genetically modified crops

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## 22.1 Introduction

Sustainable agriculture and food security are the main concerns for any nation in the world. Indian economy is directly or indirectly dependent on agriculture as 65% population living in rural areas are dependent on agriculture for their livelihood. India has experienced Green Revolution during 1970s which makes us self-sufficient in agriculture but the situation has changed due to climate change and increasing population pressure. The excessive utilization of chemical fertilizers has decreased soil fertility, enhanced air and water pollution, and release of greenhouse gases. Climate change associated factors like rise in temperature, change in rain fall pattern, loss of natural resources, pests, and diseases negatively influence agricultural yield. The global human population is continuously growing whereas arable area is reducing, so technological innovations are required in agriculture to increase crop production and reduce food losses. Genetically modified plants are divided into three groups such as first generation transgenic plants in which traits are related to increase insect and herbicide resistance in plants. Transgenic plants of second generation shows the traits associated with direct benefit to the consumer and value has been added to the end product by enhancement of nutrients. Genetically modified plants of third generation can be regarded as green factories and be used in the production of novel products such as pharmaceuticals and biofuel (Caserta and de Souza 2017; Turnbull et al. 2021). The adoption of transgenic crops can play significant function in increasing agricultural productivity. Transgenic technology can make significant contribution to achieve United Nations Sustainable Development goals such as reduction in hunger, improved nutrition, sustainable agriculture, and poverty alleviation (UNDP 2018). Klümper and Qaim (2014) reported that transgenic crops are grown by millions of farmers in all the parts of the world and have shown 37% reduction in chemical application, 22% increased yield, and 68% increase in farm profits.

Genetic engineering has played a pivotal role in crop improvement via introducing beneficial foreign genes or silencing the expression of endogenous genes in crop plants. The inserted gene is known as transgene which may come from an unrelated plant, bacteria, virus, fungus or animal species to improve the existing traits or for introduction of a new trait that does not occur naturally in the crop species. Genetically modified crops are the most expeditiously adopted technology in the history of agriculture and now reached 25 years of commercial production (Smyth 2020). In 1977, the natural ability of *Agrobacterium tumefaciens* to insert Ti plasmid DNA into host plant cell genome was discovered. The first transgenic plants such as antibiotic-resistant tobacco and petunia were developed. Transgenic tomato named as Flavr Savr was developed by Calgene (Monsanto) in 1994 with the property of delayed ripening and long shelf life and it was approved by Food and Drug Administration in USA. Transgenic crops such as canola, Bt potato, Bt maize, Bt cotton, and glyphosate-resistant soybean have received approval for their commercialization. According to ISAAA Database (2019), total 525 transgenic events in 32 crops have been commercialized in which maize accounts for maximum (238), cotton (61), potato (49), canola (42), soybean (41), and carnation (19). The

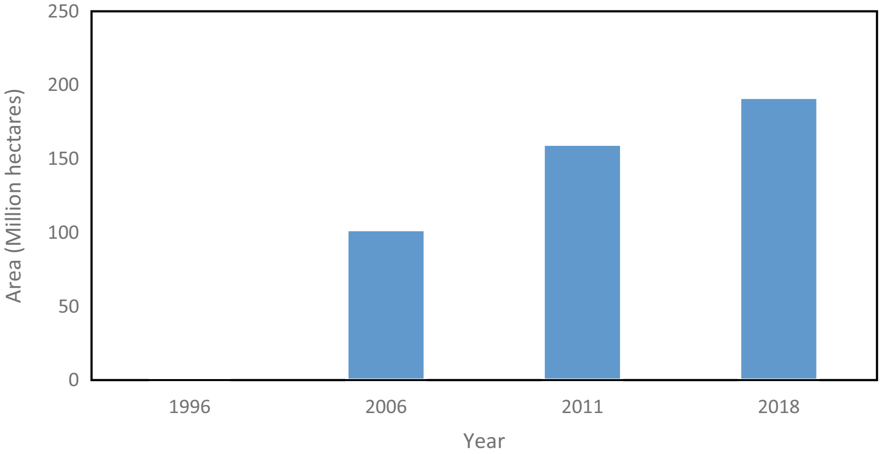

**Fig. 22.1** Global area covered by biotech crops (1996 to 2018)

herbicide and abiotic stress tolerance, insect and disease resistance, and improvement in nutritional contents were the major traits for which transgenic crops were approved for commercialization. Figure 22.1 indicates approximately 113-fold increase in the global area of transgenic crops, i.e., 1.7 million hectare in 1996 to 191.7 million hectares in 2018 (ISAAA 2018). Genetically modified crops were grown by 17 million farmers in 26 countries in the year 2017–2018 and their global market value was around US$18.2 billion (ISAAA 2018). Ghana and Uganda are planning to start field trials for its commercial cultivation and Burkina Faso wants to conduct trials with Bt cowpea (Komen et al. 2020).

There are some nongovernmental organizations and activist groups which are against the commercialization of genetically modified crops. They argue that farmers may lose their rights over the seeds and genetically modified crops will push the farmers into dept. Farmers will be forced to buy transgenic seeds from big multinational companies. They have also expressed their concerns regarding the biosafety issues and ethical acceptability of transgenic crops in society. The debate on the consumption of genetically modified crops will never end although there is no adequate scientific evidence against the safety of transgenic crops. Norman Borlaug also known as father of Green Revolution supported the production of genetically modified crops to remove hunger from the world and during his visit to India in 2005, he said “It is better to die eating genetically modified food instead of dying of hunger.” Our renowned agricultural scientist Dr. M. S. Swaminathan has given his recommendation in favor of approval process for the field trials of genetically modified crops in India. Recently, most of the Nobel laureates signed a letter and sent to Greenpeace, NGO to stop its opposition against genetically modified organisms (Sudha et al. 2018). Present review highlights the current status of transgenic crops and their benefits, public concerns, and biosafety-related regulations in India for the use of transgenic crops.

## 22.2 Genetically Modified Plants

Transgenic or genetically modified plants are produced by the transfer of gene between unrelated species by the tools and techniques of biotechnology.

### 22.2.1 Merits of Genetically Modified Plants

The significant amount of nutritionally balanced foods is needed for growth and to combat diseases (Datta 2012). The enhancement of the nutritional value of staple crops is a major consideration for global food production and nutritional security. Biotech crops have been cultivated globally for last 20 years with no adverse impact on the health of human beings, animals, and environment. The technology has a potential to produce crops with desired traits that could be beneficial for consumers such as:

#### 22.2.1.1 Agronomic and Economic Benefits

The farmers of our country lose approximately 50,000 crores every year due to attack of pests and diseases. Abiotic stresses such as drought, erratic rainfall pattern coupled with a lack of irrigation facilities are exacerbating the problem. Brookes and Barfoot (2014) reported that biotechnology was responsible for additional global production of 138 million tons of soybean, 274 million tons of corn, 21.7 million tons of cotton, and 8 million tons of canola for the period 1996–2013. If transgenic technology had not been available then to maintain production level would have required an increment of 11% of arable land in US and 32% of the cereal area in EU. The global increase in the farm income from transgenic crops had reached $116 billion, almost triple that of last 10 years from 2006–2012. Approximately 42% of economic benefit was from the increased yield due to resistance against pests and 58% was due to less production cost due to less application of herbicides and pesticides in agricultural fields (Zhang et al. 2016).

#### 22.2.1.2 Nutritionally Improved Transgenic Crops

Malnutrition is a problem in the developing and under-developed nations where most of the people rely on a single crop as a main staple food for their diet. Transgenic crops production can improve the quality of food by increasing macronutrients and micronutrients, promoting digestion by lowering glycemic index, reducing allergens content, and possibility of diseases such as cancer, diabetes, cardiovascular disease, and hypertension in human beings (Wendt and Izquierdo 2003). Iron rice was developed with increased iron content by inserting a gene from *Aspergillus niger* into the rice genome.

The deficiency of vitamin A may lead to blindness. The precursor molecule required for vitamin A biosynthesis is β-carotene but it does not occur naturally in staple food crop. Transgenic rice is enriched with provitamin A in its endosperm in which β-carotene biosynthesis was developed. Two foreign genes such as psy gene encoding for phytoene synthase from daffodil and crtI gene encoding for carotene desaturase from *Erwinia uredovora* bacterium reconstitute the carotenoid biosynthetic pathway within the rice endosperm and introduced in *Japonica* rice cultivar Taipei309. The engineered rice was named as “Golden rice” due to its yellow color and up to 1.6 μg/g total carotenoid accumulation was recorded. Syngenta introduced psy and crtI transgenes under the control of an endosperm-specific promoter in American rice variety Cocodrie and produced Golden Rice 1 which accumulates up to 6 μg/g carotenoid in endosperm. In 2005, Syngenta introduced maize psy gene along with bacterial crtI gene into American rice variety Kaybonnet and produced Golden Rice 2 which accumulated up to 37 μg/g total carotenoid which is about 23 times more than original golden rice (Paine et al. 2005). International Rice Research Institute, Philippines introduced maize psy1 and *Pantoea ananatis* crtI genes into rice and named as Golden Rice 2E. In 2017–2018, GR2E was approved for use as food in Australia, New Zealand, Canada, and United States under the Golden Rice trade name (ISAAA Database 2019) and this research work was funded by Rockefeller Foundation. Recently corn was developed with 169 times more beta-carotene, six times more ascorbic acid, and twice as much folate and cassava with 30 times more beta-carotene and four times more iron (Zilberman et al. 2018).

Protein deficiency is the most crucial problem that adversely affects physical growth and development and also increases morbidity. The amaranth seeds are rich in proteins (17–19% of seed dry weight) with balanced amino acid composition. The protein of this pseudocereal is rich in many essential amino acids such as lysine, tryptophan, and sulfur-containing amino acids. Starchy crop is generally poor source of many essential amino acids such as lysine, tyrosine, methionine, and cysteine which limits its nutritive value. The gene AmA1 was isolated from *Amaranth* that encodes a 35-kDa protein and rich in essential amino acids was expressed in potato which resulted in a significant increase in the concentration of essential amino acids (Chakraborty et al. 2010). The transgenic potato expressing AmA1 showed enhanced rate of photosynthesis and increase in tuber yield. The biosafety assessment of potato containing AmA1 gene reported that transgenic protein-rich potato is safe for human consumption and suitable for commercial cultivation. The 60% increase in total protein content of transgenic tuber was recorded and due to the nonallergenic nature of AmA1 it would have better acceptability.

#### 22.2.1.3 Modification in Fatty Acid Content

Metabolic engineering of oilseed crops through the transgenic approach has been utilized extensively for improving the nutritional qualities of seed oil such as altering the endogenous fatty acid composition to make it free of trans fats for health benefits and for increasing the shelf life of oils. The oils with low-saturated fatty acid content with higher proportion of polyunsaturated fatty acids (PUFAs) are considered better for human consumption.

*Camelina sativa* has been engineered with genes from marine microbes to produce high levels of omega-3 long-chain polyunsaturated fatty acids (PUFAs) like eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) same as present in fish oil (Usher et al. 2017). The omega-3 fatty acids play a significant role in brain development and reduce risk of cardiovascular diseases. Argentine canola was genetically transformed by incorporating seven genes taken from yeast and marine microalgae for the production of omega-3 fatty acid-DHA.

#### 22.2.1.4 Reduction in Antinutritional Factor and Resistance to Biotic Stress

Fruits and vegetables are important sources of vitamins and minerals but they also contain oxalic acid as an antinutritional factor. The high oxalic acid content is detrimental to human health as it may lead to the precipitation of calcium oxalate in kidney which may cause hyperoxaluria and destruction of renal tissues. The oxalic acid has been found linked with the pathogenesis of several phytopathogenic fungi which causes yield losses. The accumulation of high concentration of oxalic acid in leaves results in wilting and leaf death. Oxalic acid also serves as a substrate for synthesis of neurotoxin, b-N-oxalyl-La, b-diaminopropionic acid present in *Lathyrus sativus* and consumption of *L. sativus* leads to neurolathyrism which is characterized by spasticity of leg muscles, limb paralysis, and death.

The functional expression of oxalic acid degrading enzymes in crops is an important strategy for crop improvement by reducing the antinutritional factor and conferring resistance against fungal pathogens. The oxalate decarboxylase was isolated from *Flammulina velutipes* and OXDC gene was cloned from *C. velutipes*. The expression of OXDC gene in tomato and tobacco produced transgenic lines with improved resistance against infection of *S. sclerotiorum*.

#### 22.2.1.5 Enhancement in Shelf Life of Vegetables and Fruits

The postharvest losses of fruits and vegetables are approximately 35–40% in India. The main factor that determines the shelf life of fruits and vegetables crops is the rate of softening during ripening and softening of issues causes physical damage during transportation and increases their susceptibility to pathogens. Datta (2012) reported that α-mannosidase (α-Man) and β-D-*N*-acetylhexosaminidase (β-Hex) were used for the genetic manipulation to control fruit softening during ripening. The silencing of α-Man and β-Hex genes in transgenic tomato plants through RNAi technology resulted in enhanced fruit shelf life, due to reduced rate of fruit softening. Transgenic RNAi tomatoes exhibited prolonged shelf life of around one month with twofold firm fruits with α-Man and β-Hex suppressing RNAi lines (Meli et al. 2010). RNAi-mediated suppression of α-Man and β-Hex in capsicum also reflected enhancement in fruit shelf life (Ghosh et al. 2011). The introduction of cyclodextrin glycosyltransferases gene from bacteria in potato enhanced brightness and attractive appearance of potato. US Food and Drug Administration has approved first genetically engineered fast-growing AquAdvantagea salmon for human consumption in United States as it grows to full size only in 18 months rather than 3 years (Zhang et al. 2016).

#### 22.2.1.6 Disease-resistant Transgenic Crops

Diseases caused by pathogens such as fungi, bacteria, viruses, and nematodes cause extensive loss in the crop yield. Application of agrochemicals causes the environmental hazards so there is a need of exploration of alternative strategies to tackle plant diseases. Most of the virus-resistant transgenic crops have been developed via gene silencing methods such as co-suppression/RNAi and antisense RNA targeted against viral genes (Yan et al. 2007). The resistance was obtained against viruses such as cucumber mosaic virus, zucchini yellow mosaic virus, and watermelon mosaic potyvirus 2 by expressing the viral coat protein gene. Transgenic technology was used for resistance against papaya ring spot virus in papaya and cucumber mosaic virus in sweet pepper and tomato via the expression of viral coat protein. Guo et al. (2009) reported the use of replicase gene to produce virus-resistant papaya which was commercialized as Huanong No. 1 papaya.

#### 22.2.1.7 Abiotic Stress-tolerant Transgenic Crops

Abiotic stresses such as drought, flood, salinity, and temperature are limiting factors for the growth and development of crop plants and reduce crop yield (Suzuki et al. 2014). Under the changing climate conditions, the adverse impact of the abiotic stresses is believed to be increasing (Tuteja and Gill 2014). To combat abiotic stresses, plants alter their metabolism in different ways such as by activation of regulatory proteins, modification in antioxidant defense system to maintain cellular homeostasis and accumulation of compatible solutes, etc (Raza et al. 2019). The abiotic stresses cause changes in gene expression at the molecular level. Transgenic tomato that overexpresses the AtNHX1sodium/proton antiport pump gene obtained from *Arabidopsis* was able to grow under saline condition. Genetically modified tomato can be safe for human consumption because they contain high sodium concentration in their leaves but not in fruits.

Chandler and Tanaka (2018) reported that FRI gene can improve drought resistance capacity in different crop plants. *Agrobacterium*-mediated transformation was used to transfer gene from barley (HVA1) that codes for late embryogenesis in mulberry plants and improved water and salinity stress (Checker et al. 2012).

An antifreeze gene from cold water fish was introduced into plants such as tobacco and potato. Due to antifreeze gene, plants were able to tolerate low temperature conditions. The heterologous expression of sunflower Hahb-4 *Helianthus annuus homeobox-leucine zipper gene* in transgenic soybean was approved for cultivation in Argentina in 2015 and in USA and Brazil in 2019 under the name as Verdeca HB4 soybean. The transgenic HB4 soybean exhibited up to 14% increase in yield under field trials with less availability of water in Argentina and USA**.**

#### 22.2.1.8 Development of Colored Flowers

Efforts have been made to engineer a wide variety of esthetic traits in the floriculture industry. The cultivation of horticultural crops for esthetic values depends on the customer's choice on the flower color. Most of the roses are pink or red in color but after 13 years of collaborative genetic engineering research by an Australian company, Florigene and Japanese company, Suntory, rose containing blue pigment delphinidin was developed in 2004 (Chandler and Tanaka 2018).

#### 22.2.1.9 Herbicide-resistant Transgenic Crops

Weeds compete with crop plants for nutrients, water, space, sunlight, and cause yield losses. Most of the weeds are herbaceous plants and selective killing of the weeds in agricultural field is not practically possible. The herbicide-tolerant transgenic plants have been developed to tolerate glyphosate and glufosinate. Glyphosate inhibits 5-enolpyruvyl shikimate-3-phosphate synthase enzyme which is involved in the shikimate pathway of aromatic amino acid biosynthesis. Shikimate pathway is not present in the animals so glyphosate is not harmful for human beings. Glyphosate-tolerant Roundup ready soybean containing cp4epsps gene was commercialized as the first herbicide-tolerant transgenic crop in 1996.

Glufosinate inhibits glutamine synthetase enzyme which leads to accumulation of ammonia and inhibits photosystem I and II reactions. Two bacterial genes namely pat and bar isolated from *Streptomyces* species were utilized for the development of glufosinate-resistant crops. Both of the genes encode phosphinothricin acetyltransferase enzyme which detoxifies glufosinate herbicide by acetylation.

Total 351 herbicide-tolerant events have been approved from the year 1996 to 2018 (ISAAA Database 2019). The herbicide-tolerant transgenic crops occupy the largest area amongst commercialized transgenic crops and it has benefited farmers in many ways such as yield increase due to more efficacious weed management without any harm to environment (Brookes and Barfoot 2018). It was estimated that in glyphosate-resistant soybean, 38% of the economic benefit was due to increase in yield whereas rest 62% was due to reduction in cost for weed control (Brookes and Barfoot 2018).

#### 22.2.1.10 Insect-resistant Transgenic Crops

Insect pests cause severe crop loss and they also act as carrier of various plant pathogens (Rahman et al. 2012). Farmers rely on insecticides for the control of insect pests but this method of crop protection is not ecofriendly, cost-effective which imparts health hazards and economic burden on farmers. The cry genes isolated from soil bacteria *Bacillus thuringiensis* (Bt) are highly exploited genes for developing insect-resistant transgenic crops (Table 22.1). The cry genes produce Cry protein which gives insecticidal activities to *B. thuringiensis* but it is not toxic to mammals. Cry genes provide resistance against different insect pests such as lepidopterans, dipterans, and coleopterans. Cotton was the first commercially successful crop in which cry genes were incorporated to provide resistance against lepidopteron insect pest. The cry genes have been incorporated in various crops such as soybean (Dang and Wei 2007), maize (Gassmann et al. 2011), chickpea (Mehrotra et al. 2011), alfalfa (Tohidfar et al. 2013), and tomato (Koul et al. 2014). The VIP genes which encode vegetative insecticidal proteins have also been expressed in cotton and maize (ISAAA Database 2019).

Protease inhibitors are defense-related proteins in plants which are released in response to insect attack. Protease inhibitor works by inhibiting proteolytic enzymes present in insects and their larvae gut. The protease inhibitor-encoding genes were used to confer resistance against a wide range of insect pests such as cptI gene from *Vigna unguiculata* introduced in cotton, api gene from *Sagittaria sagittifolia*

**Table 22.1** Genetically modified plants approved for field trials in India (2014–till date)

| S. No. | Plant | Trait | Institutions involved in R & D |
|---|---|---|---|
| 1. | Wheat | Effect of mutant strain *Azotobacter* | National Research Center on Plant Biotechnology |
| 2. | Corn | Insect resistance, Herbicide tolerance | Monsanto, Pioneer, Dow Agrosciences |
| 3. | Rice | Insect resistance, disease resistance, nutritional enhancement | Indian Agricultural Research Institute, Tamil Nadu Agricultural University, Bayer Bioscience, MAHYCO |
| 4. | Potato | Fungal resistance, tuber sweetening | Central Potato Research Institute |
| 5. | Cotton | Insect resistance, Herbicide tolerance | Central Institute of Cotton Research, MAHYCO, Dow Agrosciences |
| 6. | Sugarcane | Insect resistance | Sugarcane Breeding Institute |
| 7. | Tomato | Insect resistance, Virus resistance, fruit ripening | Indian Institute of Vegetable Research, National Research Center on Plant Biotechnology |
| 8. | Mustard | Hybrid seed production | University of Delhi South Campus |
| 9. | Chickpea | Abiotic stress tolerance, insect resistance | Indian Institute of Pulses Research, ICRISAT, MAHYCO |
| 10. | Groundnut | Abiotic stress tolerance, Virus resistance | ICRISAT |

introduced in poplar, and pinII gene from *Solanum tuberosum* introduced in maize (ISAAA Database 2019).

#### 22.2.1.11 Reduction in Pesticide Poisoning

Crop losses from insect and pests can cause significant financial losses for farmers. Farmers are heavily dependent on pesticides (Schreinemachers et al. 2017) and approximately 2.5 million tons of pesticides are used in crop fields annually (Abrol and Shankar 2014). The crop plants treated with pesticides cause potential health hazards to animals and human beings and contaminate soil, ground water, and environment and may enter in food chain. Studies have revealed close relationship between pesticides exposure and development of malignant growth such as leukemia, neuroblastoma, soft tissue sarcoma, and tumor of lung, rectum, stomach, colon, and bladder in both children and adults (Bonner et al. 2016).

Genetically modified crops have shown significant reduction in pesticide poisoning cases due to reduced application of pesticides in agricultural fields. Bennet et al. (2003) reported that in South Africa, farmers reduced pesticide application from 11.2 per year to 3.8 which reduced pesticide poisoning cases from 50 per year to less than 10 in the first four years of Bt-cotton adoption. The report of medical test of 246 Chinese farmers associated with the production of non-Bt cotton had shown damage of liver function due to spray of fungicides whereas application of insecticides was related with nerve damage (Zhang et al. 2016). The farmers growing Bt cotton have experienced less cases of pesticide poisoning globally. Soil and ground water pollution continues to be a problem in all parts of the world. Recently poplar trees have been genetically modified and they can absorb heavy metals from contaminated soil.

#### 22.2.1.12 Development of Therapeutic Products

Medicines and vaccines are costly to produce as they require specific storage conditions which are not easily available in third world countries. The genetic engineering technique enables the expression of viral or bacterial antigen in the edible part of plant cells. Transgenic food can serve as an oral vaccine which will be more easier to store, transport, and capable of stimulating immune system to produce antibodies. Scientists are working to develop edible vaccines in tomato, rice, maize, soybean, potato, and banana against different infections caused by rabies virus, type B viral hepatitis, and *Helicobacter pylori* (Aggarwal 2012).

The edible vaccines are cheaper and safe as compared to the traditional vaccines and are being used in various countries (Ankita et al. 2016). Further researches are needed to determine human exposure level so that upper limits can be set by the application of pertinent safety regulations at which adverse effects are not present.

#### 22.2.1.13 Lowering of Cancer Cases

Development of insect-resistant crops has ability to improve human health through the reduction in cancer rate. The study of 21 years of maize production reported that Bt maize contained low concentration of mycotoxins (29%), fumonisins (31%), and thricotecens (37%) (Pellegrino et al. 2018). Mycotoxins are carcinogenic to animals and human beings whereas fumonisins may be responsible for neural tube defect (Missmer et al. 2006).

#### 22.2.1.14 Reduction in Mental Stress

Stress in agriculture is same as it is observed in other sectors and mostly associated to crop failure and financial debt which may cause stress burden on farmers. Due to the desired properties of genetically modified crops, farmers become assured that their crop will not fail due to insects, weeds and become more resilient against environmental stresses (Klümper and Qaim 2014). In this way, cultivation of genetically modified crops reduces mental stress in farmers.

#### 22.2.1.15 Less Cases of Farmers Suicide

The access to mental health support system in villages can be problematic within the agriculture sector due to rural areas, remote locations, and lack of proper facilities. A study was conducted to check the relationship between farmer suicide and adoption of genetically modified crops. Gruere and Sengupta (2011) reported decrease in the suicide rate after commercialization of Bt cotton as the number of farmer’s suicide was around 15,000 per year in 2004 but it was 25% reduced by the year 2007 in India. The reduced rate of farmer’s suicide was associated with the adoption of Bt cotton by the farmers and it prevented more than 70,000 farmer’s suicide.

### 22.2.2 Concerns Associated with the Genetically Modified Crops

The global adoption of transgenic crops in past two decades has shown economic and environmental benefits in terms of enhanced crop yield and farmer's income, reduced application of chemical insecticides and herbicides, reduced $CO_2$ emission, and improved nutritional quality (Brookes and Barfoot 2018; Kumar et al. 2020). However, some concerns that have been raised against transgenic crops are given below.

#### 22.2.2.1 Transfer of Gene in Nontransgenic Plants

Gene transfer is mainly of two types such as vertical gene transfer and horizontal gene transfer. The genetic material is transferred from parents to offspring by asexual or sexual reproduction in vertical gene transfer. In horizontal gene transfer, gene transfer from cultivated species to wild relatives takes place (Han et al. 2015). It may be a hidden hazard of transgenic crops and can lead to gene flow from species to species (Jangra et al. 2016). The transgene can be transferred from transgenic crops to other sexually compatible species and can impact environment by production of hybrids.

Quist and Chapela (2001) observed the presence of DNA segment of insect-resistant maize in the DNA of organic maize. Dyer et al. (2009) collected seeds from more than 1700 households in Mexico were contaminated with transgene. Recently, concern was raised about the horizontal transfer of antibiotic-resistant marker gene from transgenic food to animals and human gut microbes which may cause antibiotic resistance in the gut microflora (Keese 2008). However, possibility of this type of gene transfer is very less; now efforts have been made on the development of marker-free transgenic plants (Tuteja et al. 2012).

#### 22.2.2.2 Adverse Effect on Health

Concerns have been raised for biosafety of transgenic crops like toxicity and allergenicity to animals and human health. Lepidopteron insect-resistant corn known as Starlink containing Cry9c gene was approved for animal feed in USA in 1998 but it was not approved for human consumption due to its allergic reactions which may affect immune system (Bucchini and Goldman 2002). Later it was recalled in the year 2000 due to detection of Cry protein residues in food products (EPA US Environmental 2017). Direct link could not be found between Cry9c and allergic reactions in consumers. Another controversial study known as "Seralini affair" has reported health-related issues such as tumor, kidney disease, increased liver congestion, necrosis in male, and enhanced female mortality in rats fed with transgenic NK-603 Roundup Ready corn (Seralini et al. 2012). This study faced criticism from scientific community due to its imprecise experimental design and inappropriate statistical analysis. Seralini et al. (2014) published their work in expanded form and highlighted the need for conducting long-term feeding trials to assess the safety of transgenic crops. Most of the studies related to health hazards of transgenic crops on animals have not reported any adverse effect of transgenic food on animal health (De Vos and Swanenburg 2018).

#### 22.2.2.3 Impact on Soil Texture

Genetically modified plants may secrete toxin which can be absorbed by soil and bind to the surface particles and persist in soil for long duration. Vardha region of India was selected to study Bt-cotton effect on crop fields after growing the crop for three consecutive years. The decline in carbon and nitrogen biomass (8–9%) was reported in soil but no modification in microbial population and activities of esterase and alkaline phosphatase was observed in Bt-cotton fields (Tarafdar et al. 2012).

#### 22.2.2.4 Effect on Biodiversity

The extensive cultivation of transgenic crops may enhance chances of resistance development in insect population and weeds. High selection pressure may lead to the development of new insects and superweed with resistance against transgenic technology (Bawa and Anilakumar 2013). The continuous cultivation of transgenic crops may lead to the development of superweeds because the weeds may develop resistance against herbicides and may be problematic for the farmers. Gilbert (2013) reported that Roundup ready-resistant amaranth was first reported in Bt-cotton fields of Georgia, USA in 2004 and competed for water, sunlight, and nutrients with cotton plant.

#### 22.2.2.5 Adverse Impact on Nontarget Organisms

The adverse effect of transgenic crops on nontarget organisms has been reported in some studies. High mortality of monarch butterfly larvae fed on milkweed leaves dusted with genetically modified Bt maize in comparison to control under laboratory condition. This report was controversial due to error in the experimental design of laboratory bioassay tests. The subsequent study reported no harmful effect of Bt maize on monarch larvae (Dively et al. 2004). The decrease in population of monarch butterfly was also reported in Mexico and USA due to adoption of glyphosate-resistant transgenic crops. Brower et al. (2012) stated that the reason for decline in reproduction capacity of monarch butterfly was due to loss of milkweed plant by increased application of glyphosate.

#### 22.2.2.6 Cost for Commercialization

Major limiting factor for development of transgenic crop is an expensive procedure of safety assessment and time-consuming complex regulatory approval process (Davison 2010). It has been reported that the total time period required from initiation of a transgenic crop development project to its commercialization is at least thirteen years. Smart et al. (2017) reported the approval time for transgenic crops to pass through the regulatory procedures of European Union and United States was about 5 and 7 years, respectively. The average cost for approval of regulatory processes and global registration of genetically modified crops was approximately US$ 35.01 million. The development and commercialization of transgenic crops are not affordable by public institutions and small companies due to the high cost of development procedures.

#### 22.2.2.7 Role of Multinational Companies

Genes applied for development of crops with improved traits are patentable. It has been observed that most of the genetically modified crops are being developed and patented by few multinational companies. It may be due high cost related with research, development, and regulatory process of transgenic crops. Companies such as Monsanto, Syngenta, Bayer CropScience, Dupont, and Groupe Limagrain hold around 70% of transgenic seed market (Business Wire 2016). Therefore, farmers have no option to obtain seeds in the market and it raises the concern about the exploitation of farmers by few multinational companies as they charge high cost for genetically modified seeds.

### 22.2.3 Regulation of Genetically Modified Organisms in India

The regulation of genetically engineered organisms is described in rules notified by the Ministry of Environment, Forests and Climate Change, Government of India on December 5, 1989 under the Environment Protection Act 1986. It covers the import, research, and release of genetically modified organisms and derived products in India. The rules, 1989 have been implemented by MOEFCC jointly with Department of Biotechnology, Ministry of Science and Technology, and state government. Six regulatory bodies have been constituted under the rule 1989 which include rDNA Advisory Committee (RDAC), Institutional Biosafety Committee (IBSC), Review Committee on Genetic Manipulation (RCGM), Genetic Engineering Appraisal Committee (GEAC), State Biotechnology Coordination Committee (SBCC), and District Level Committee (DLC). RDAC is advisory in function; IBSC, RCGM, and GEAC are regulatory in function whereas SBCC and DLC are made for monitoring purposes (Table 22.2). FSSAI is food safety regulatory and monitoring body and its main function is formulation of food safety standards based on modern science and to regulate the food sector.

**Table 22.2** Regulatory authorities for approval of genetically modified crops in India

| S. No. | Regulatory authority | Functions |
|---|---|---|
| 1. | rDNA Advisory Committee (RDAC) | Advise on biosafety of emerging technologies |
| 2. | Institutional Biosafety Committee (IBSC) | R & D and contained research experiments (laboratory and greenhouse) |
| 3. | Review Committee on Genetic Manipulation (RCGM) | Risk assessment of plants, animals, microbes, and guidelines |
| 4. | Genetic Engineering Appraisal Committee (GEAC) | Approval for environmental release including confined field trails |
| 5. | State Biotechnology Coordination Committee (SBCC) | Monitoring and supervision at state level |
| 6. | District Level Committee (DLC) | Local supervision and compliance |
| 7. | FSSAI | Approval for commercial release of GM foods (processed) |

### 22.2.4 Conclusion

Genetically modified crops have tremendous potential to solve the problem of global hunger and malnutrition. Politics has also been involved for commercialization of genetically modified crops. There is an urgent need to keep politics separate from the science of transgenic technology. It has been observed that the negative perceptions about transgenic crops have been made in society, which have no scientific evidences but unfortunately general public can easily believe on them. The farmers of our country can rely more on transgenic crops as compared to the conventional crops to get consistent yield under adverse climate conditions such as unreliable weather conditions and rainfall pattern. National Academy of Agricultural Sciences (NAAS) of India already stated that “It is high time to approve environmental release of genetically modified varieties, which have been tested to be bio-safe for use of farmers and consumers without any delay”. The scientific community should initiate awareness programs to clear the doubts and concerns of public regarding the genetically modified crops. The government, nongovernmental organizations, and private research institutions should work together to educate farmers and youth generation of the society about the merits of transgenic crops. Hence, government of India should provide an enabling environment for adoption of genetically modified technology to ensure improved agricultural productivity, profitability, and food security. It will be a mistake to check the research in the area of genetically engineered technology, scientist should promote and society must accept new technologies and utilize them for the benefit of humanity and environment.

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# Efficacy of Algae in the Bioremediation of Pollutants during Wastewater Treatment: Future Prospects and Challenges

# 23

Saurabh Dhakad, Parul Chowdhury, and Sudipti Arora

## Abstract

Every nation wants to focus on reducing their carbon footprints and improve the ecological status of water quality, in particular by reducing the amount of nitrogen (N) and phosphorus (P) concentration in wastewater effluent. There are many technologies and materials which can treat the wastewater, but in recent years, algae have received huge attention in their use as a part of wastewater treatment. The mechanism is that algae use the inorganic nitrogen and phosphorus for their growth and generate biomass which can be used as a raw material for secondary process. This chapter aimed to focus on the importance of the algae for wastewater treatment by reducing N, P, chemical oxygen demand (COD), biochemical oxygen demand (BOD), heavy metals (HMs), and other parameters. Many types of wastewaters can be treated with the help of algae. Here, in this chapter, the principles of wastewater and bioremediation with various treatment strategies for the different types of wastewater parameters and the abiotic and biotic factors that affect the mechanism of the algae for treating the wastewater will be introduced. This chapter also describes the mechanisms involved in the treatment of different types of pollutants and the future prospects and challenges related to this particular approach.

## Keywords

Algae · Bioremediation · Organic load · Wastewater treatment

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## 23.1 Introduction

Water is one of the most important natural resources for sustaining life, and it is expected to become critically short in the next decades by the rising demand, rapid population growth, and the increasing economy of the country (Kumar et al. 2005). Although water covers almost 71% of the earth's surface, usable water for humans amounts to less than 2% of the total water on the planet. India accounts 2.45% of the world's land area and 4% of the world's water resources, yet it has 16.5% of the world's people. Its per capita water availability (PCWA) is roughly 1100 cubic metres ($m^3$), far below the globally acknowledged water stress level of 1700 $m^3$ per person and critically near to the 1000 $m^3$ per person threshold for water scarcity (Kaur et al. 2012; World Bank 2019). Water consumption will double in 2050 compared with 2010 due to significant population growth (CWC 2010) (see Fig. 23.1). The concerns about the quantity and quality of solid waste generated and directed towards natural waterbodies have recently prompted the development of new solutions to manage regional water quality issues. Bathing, toilet flushing, laundry, dishwashing, and other daily activities generate wastewater.

Mainly, wastewater is divided into three types: grey water, black water, and yellow water. Wastewater has 99.9% of water. The other 0.1% is what is cause for concern. That 0.1% of wastewater includes phosphorous, nitrogen, fats, oils, grease, cooking oils, body lotions, pathogens, disease-causing bacteria, viruses, and other solids. So, before the disposal of wastewater, its treatment is mandatory. There are some constituents that are point of concerns in wastewater: (1) total solids—composed of floating matter, colloidal matter, settable matter, and matter in solution;

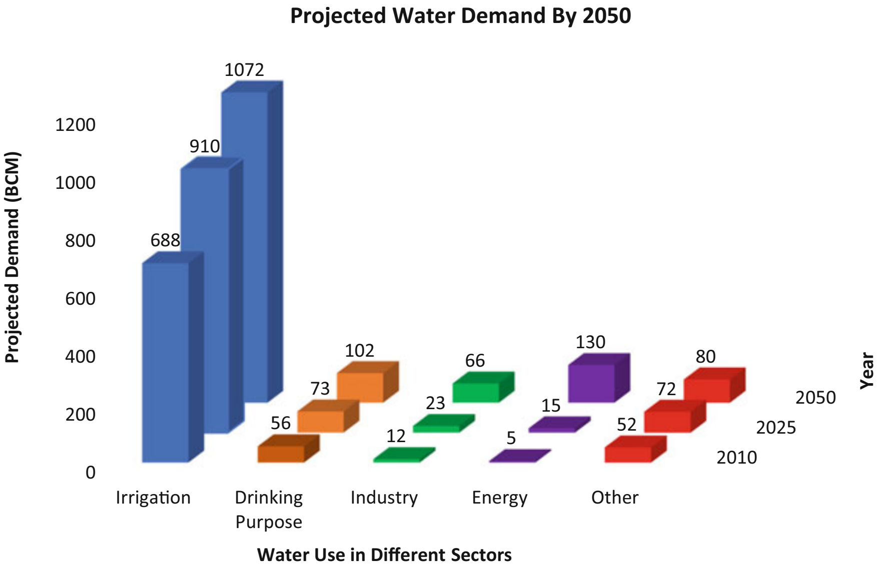

**Fig. 23.1** Projected water demand by different sectors (CWC 2010)

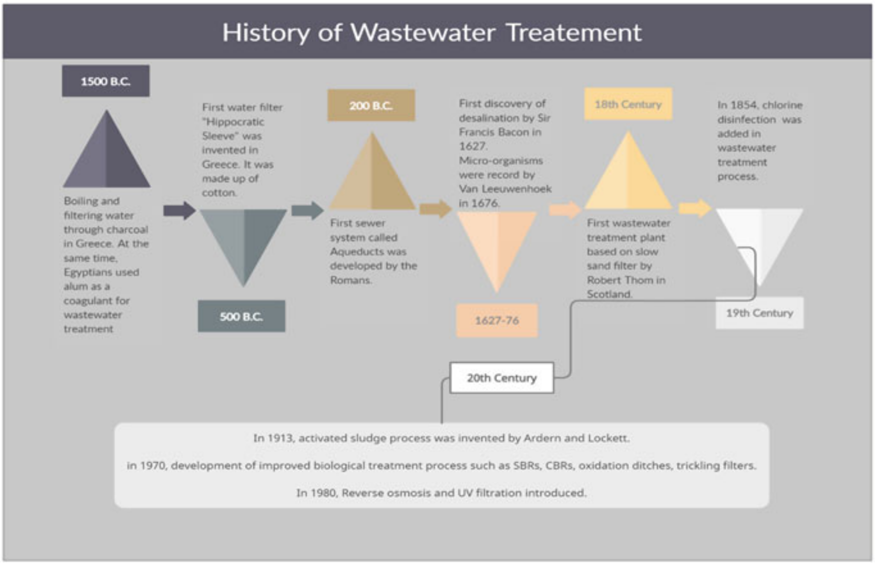

**Fig. 23.2** History of the wastewater treatment

(2) nutrients—nitrogen and phosphorus that lead to eutrophication; (3) pathogens—many of them can be the host of the disease; (4) priority pollutants—carcinogens and highly toxic; (5) refractory pollutants—pesticides, surfactants, and phenols; (6) organic pollutants such as oil; (7) heavy metals that are very harmful to animals, humans, and the environment; (8) dissolved organics such as calcium, sodium, and sulphate; and (9) pharmaceuticals that affect the living creatures and the environment.

Almost all the activities done by humans generate wastewater; therefore, treatment of this water is essential for people and the environment. If we look back in history, we find people treated wastewater by simple boiling in 1500 BC in Greece. With time, many advances came in wastewater treatment technologies (see Fig. 23.2).

The wastewater treatment involves many chemical and physical methods. Unfortunately, they are either unsustainable or inefficient in terms of energy use, but biological techniques such as bioremediation are available. Bioremediation is a method of removing nutrients from wastewater by using the microorganisms and other elements of the environment naturally. Bioremediation has the potential to be less expensive than other wastewater treatment methods for hazardous waste cleanup (Validi 2001). Bioremediation of wastewater using algae, in particular, has important benefits such as high pollutant removal capability and by-products such as biofuel, protein-rich food, and fertilizers. Bioremediation process uses microorganisms to reduce, eliminate, or transform to benign contaminants present in soils, sediments, water, and air (Adams et al. 2015). Because bioremediation is a very natural process, the society views it as an acceptable waste treatment method for contaminated materials such as soil. When the contaminant is present, the number of microbes capable of degrading it increases; when the contaminant is degraded, the biodegradative population decreases. The treatment residues are typically harmless by-products such as $CO_2$, water, and cell biomass (Validi 2001).

## 23.2 Algae

Linnaeus coined the term “algae” in 1753, and A.L. de Jussieu separated algae from the rest of the plant kingdom. The majority of algae are aquatic; however, some are also found on land. They dont have real tissue system like root, shoot and leaves, their plant body have uniceluar to multicelluar structures with no vascular system and therefore referred to as thallophytes. Seaweeds can range in size from a single cell to huge seaweeds that can grow to be more than 60 m in length. Algae are classified based on their morphology, pigments, cell walls, stored food materials, reproductive structure, and life-history patterns into seven major groups (Hallmann 2015):

- Rhodophyta
- Charophyta
- Chlorophyta
- Euglenophyta
- Chrysophyta
- Phaeophyta
- Pyrrophyta

Human population is increasing rapidly, so wastewater treatment can be a sustainable solution to handle the influx of the human waste without harming the environment. Firstly, in the starting of the twentieth century, microalgae were noted to treat the wastewater in a highly efficient manner by aerating the water and by eating the waste. By mid-century, microalgae grown in wastewater were introduced as a biomass source for fuel, food, and biomaterials. As we know, fossil fuel resources are limited on the earth, during the oil crisis, algae were introduced as a biofuel source as an alternative fuel source. Microalgae wastewater treatment can cut the greenhouse gas emissions and can produce sustainable biofuel source to curb global warming (Paddock 2019). Microalgae are able to consume large amounts of nitrogen, phosphate, and carbon dioxide that are converted into biomass, thereby making these species attractive for carbon dioxide mitigation and pollution reduction in waterbodies, even for reduction in toxic materials (Park et al. 2011).

### 23.2.1 Algal Bioremediation

Bioremediation is a biological method of removing unwanted compounds from the environment (Gianfreda and Rao 2004). In terms of cost-effectiveness, convenience, total removal of organic contaminants, and lack of collateral destruction of site materials or impact on indigenous flora and fauna, it offers several clear advantages over physicochemical remediation methods. Bioremediation has become one of the most important developing disciplines for environmental restoration. The use of microorganisms in bioremediation reduces the concentration and toxicity of numerous chemical pollutants such as heavy metals, dyes, and pesticides. A lot of work has gone into establishing a low-cost, environmentally friendly bioremediation method that can successfully immobilize dissolved hazardous metals; a range of living biomass has been investigated for metal removal and/or recovery for their potential reuse. Algal biomass has caught the focus of the researchers all around the world due to its high metal adsorption capability, low cost, and widespread abundance. In algal bioremediation, various types of either living or dead biomass have been used to absorb dissolved toxic metals.

### 23.2.2 Advantages of Using Algae

- For growth and survival, blue-green algae (cyanobacteria) rely on light energy and $CO_2$. As a result, it helps in the carbon sequestration process ultimately global warming reduction.
- Cost-effective and environmentally friendly.
- They also fix atmospheric nitrogen, allowing them to thrive in environment which is nutrient-limited.
- Microalgae cultures can be grown in open ponds or large-scale reservoirs. At the same time, algal growth in the laboratory provides a consistent and reliable supply of biomass.
- They produce less chemical and/or biological sludge that must be disposed of.
- They have a high-surface-area-to-volume ratio and are efficient in dilute effluents.
- They have the capacity to treat polluted sites that have been polluted by more than one type of pollutants.

Microalgae-based technologies are very sustainable because they can remediate wastewater and use less energy due to $O_2$ aeration. It helps in the removal of $CO_2$ from the atmosphere and provides biomass for the production of a variety of bioproducts.

### 23.2.3 Factors Affecting Algal Growth and Nutrient Removal

Algae treat the wastewater naturally so both biotic and abiotic factors affect the efficacy of algae in wastewater treatment. There are many parameters such as dissolved oxygen (Dissolved oxygen (DO) is the amount of oxygen that is present in water for the growth of the algae.) and organic matter contents, light, pH, growth of algae, temperature, and salinity of the any wastewater treatment unit. Table 23.1 shows all these parameters and their effects on the algal framework.

**Table 23.1** Factors affecting algal growth and its framework (Goffin et al. 2018; Gonçalves et al. 2017; Ye et al. 2018; Judd et al. 2015; García et al. 2018)

| S.N. | Factors | Effects on the algal framework |
|---|---|---|
| 1 | Dissolved oxygen and organic matter | Affects the algae-bacteria consortium decomposition of algae |
| 2 | pH | Alters the physiological characteristics of algae |
| 3 | Light supply | Affects the growth and reproduction of algae |
| 4 | Algal growth | Decreases the light supply to the inner cells of the algae culture |
| 5 | Temperature | Affects algal growth, intracellular enzyme activity, and biomass productivity |
| 6 | Salinity | Affects the growth and composition of algae |

### 23.2.4 Algae for Wastewater Treatment

In the last 75 years, mass production of many different algal strains has introduced with the commercial implementation of the algal cultures for the wastewater treatments such as *Chlorella* and *Dunaliella*. There are many algae strains that can be used to treat different types of pollutants in wastewater (Table 23.2).

### 23.2.5 Phycoremediation

The term "phycoremediation" is relatively new, coined in 1991. Phycoremediation is a subdivision of bioremediation where algae are being used for the removal or biotransformation of pollutants, including nutrients, xenobiotics, and carbon dioxide. It simply offers clean-up methods, which are low in cost, non-intrusive, and very safe. In many terms, phycoremediation is better than conventional wastewater treatment. Phycoremediation is more sustainable than conventional wastewater treatment in both economic and environmental prospects (Table 23.3).

### 23.2.6 Algae and Wastewater Treatment

Wastewater treatment involves the physical, chemical, and biological removal of organic carbon, nitrogen, phosphorus, heavy metals, and other contaminants. Wastewater treatment with algae is a hopeful method for the removal of contaminations and converting them into products which can be further used (Liu et al. 2020). According to Hoffmann, wastewater treatment with algae has been proposed as a more environmentally friendly approach, particularly for the removal and recovery of nitrogen and phosphorus (Hoffmann 1998). Algae generate oxygen through photosynthesis and assimilate carbon dioxide ($CO_2$). This process supports a cycle between bacteria and algae, and both are beneficiated by each other. Oxygen

**Table 23.2** Algal strains and their functions in wastewater treatment (Saravanan et al. 2021)

| S.N. | Algae strains | Functions |
|---|---|---|
| 1 | *Chlorella* | • Removes lead (II) ions from wastewater |
| | | • Removes nutrients (N and P) from domestic wastewater<br>Detoxifies cyanide from wastewater |
| 2 | *Pithophora* sp. | • Removes malachite green dye from wastewater |
| 3 | *Scenedesmus abundans* | • Removes cadmium (Cd) and copper (Cu) present in contaminated water |
| | | • Detoxifies cyanide present in wastewater |
| 4 | *Sargassum muticum* | • Removes methylene blue dye from wastewater |
| 5 | *Spirulina* sp. | • Biosorption of heavy metals like antimony and chromium present in wastewater |
| 6 | *Botryococcus braunii* | • Removes N, P, and other simple inorganic nutrients from industrial wastewater, especially in piggery wastewater |
| 7 | *Dunaliella salina* | • Removes Cu, Cd, Co, and Zn from wastewater |
| | | • Treatment of hypersaline wastewater |
| 8 | *Ankistrodesmus* sp. | • Removes mercury, arsenic, and selenium through methylation |
| 9 | *Actinastrum* sp. | • Removes copper (Cu) from wastewater |
| 10 | *Micractinium* sp. | • Removes zinc (Zn) and cadmium (Cd) from wastewater |
| 12 | *Pediastrum* sp. | • Indicators of organic compounds in wastewater |

generated by algae is used by bacteria to oxidize organic carbon, and the carbon dioxide generated by bacteria is assimilated by the algae (see Fig. 23.3).

The concept of using algae for wastewater bioremediation dates back to 1957 when Oswald and his co-workers reported the potential of photosynthetic nutrient removal in outdoor waste stabilization ponds (Oswald et al. 1957). Currently, algae-based wastewater treatment processes have been successfully demonstrated at the laboratory (Mennaa et al. 2019), pilot (Arbib et al. 2017), and demonstration scales (Delrue et al. 2016). High-rate algal ponds (HRAPs) have received considerable attention in recent years due to various advantages such as low input energy, high-quality effluent (treated wastewater), production, and recovery of algal biomass for diverse applications such as biofuels, nutraceuticals, protein-rich feed, and as fertilizer supplement (Craggs et al. 2012; Chinnasamy et al. 2014; Ansari et al. 2019; Ruedaa et al. 2020). Other significant advantages of using algae for wastewater treatment include their capability of $CO_2$ sequestration and their ability to survive in extreme environmental conditions by switching the metabolism between autotropism, heterotropism, and mixotropism (Terrado et al. 2017).

#### 23.2.6.1 Removal of Coliform Bacteria

Human activities have an ongoing impact on the quality and quantity of water (Ruiz-Marin et al. 2010). The majority of pathogenic agents found in municipal wastewater

**Table 23.3** Differences between phycoremediation and conventional wastewater treatment

| S.N. | Phycoremediation | Conventional wastewater treatment |
|---|---|---|
| 1 | Relies on microalgae and macroalgae | Relies on physical and chemical treatments |
| 2 | Utilizes natural oxygen generated from photosynthesis for oxidation of pollutants | Utilizes artificial oxidation process such as stirred tank reactors and activated sludge process |
| 3 | Generally in situ process | Either in situ or ex situ process |
| 4 | Compatible with conventional processes | Highly specific and noncompatible |
| 5 | Single-step processes for pH correction, TDS reduction, COD/BOD removal, color and odor removal | Multistage process for each parameters |
| 6 | Negligible generation of sludge | High sludge generation |
| 7 | Waste remediation can be coupled with biomass valorization | Sludge used as fertilizers or as landfills |
| 8 | Process can be linked with algae-based bioenergy production | High energy consuming |
| 9 | Lower capital and operational costs | High capital and maintenance costs |

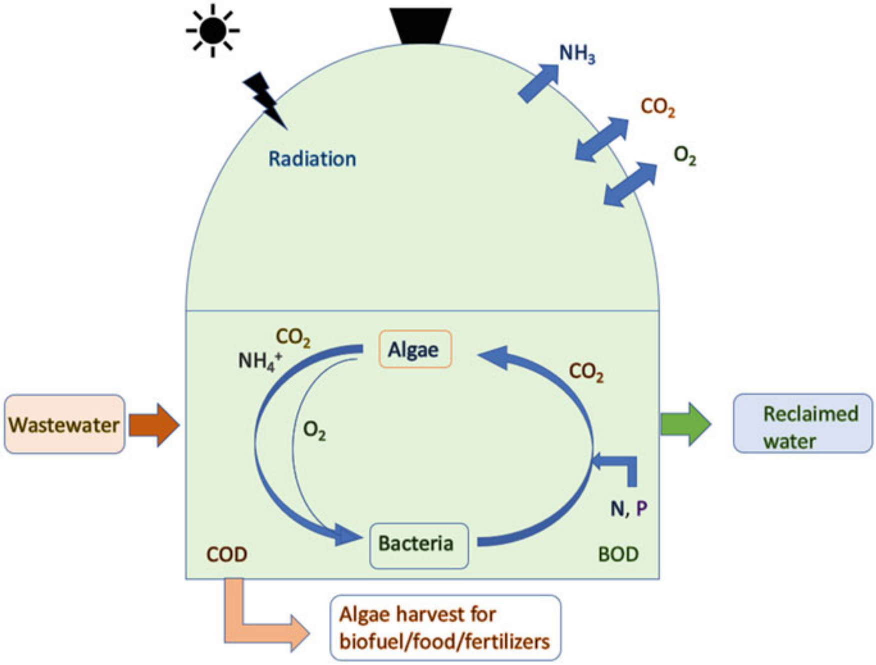

**Fig. 23.3** Treatment of wastewater through the combination of algae and bacteria

are bacteria, helminths, viruses, and a variety of protozoa and fungi. These pathogens cause a variety of health issues, including water-borne diseases, particularly in developing countries. There are a variety of harmful diseases that can be caused by these pathogens, which are commonly found in municipal wastewater. Bacterial pathogens are extremely harmful to health and are a leading cause of death, particularly in areas where sanitation is inadequate or non-existent (Awuah 2006). Coliform bacteria are a type of bacteria that are used as indicators for various disease-causing pathogens. This group of indicator bacteria includes *Escherichia coli* as well as other types of bacteria found in human faeces or originating from other sources. The presence of faecal coliform bacteria, which are commonly used as an indicator to assess water contamination, can be used to check water quality for disease-causing pathogens of humans (Abreu-Acosta and Vera 2011). Although there are numerous wastewater techniques and facilities available, such as adsorption methods, chemical techniques, precipitation, constructed wetlands, and other processes, the biological method is the most appropriate because it is the least expensive for developing countries in particular (Wei et al. 2008). A wastewater treatment method based on algae is a better option for areas where expensive conventional methods are not feasible (Zhang and Farahbakhsh 2007). Because algae play an important role in removing nutrients, pathogens, heavy metals, and other pollutants from sewage, this type of treatment process is simple, effective, and environmentally friendly. Another advantage of using algae is that nutrient extraction from sewage using algae produces useful biomass that can be used to produce biofuels (Oswald 1995). In most developing countries, wastewater treatment ponds are widely used for sewage treatment. And in this type of treatment system, algae are used (Awuah 2006).

According to Ahmad et al. (2014), sunlight affects the dissolved oxygen, pH, and sedimentation in algae wastewater ponds. It has the potential to play an important role in pathogen removal. It can be significant to overcome the bacterial population if there are large amounts of protozoa present. The following are some mechanisms for pathogen removal from wastewater by algae:

- Algae compete the coliform bacteria for the nutrients.
- Due to carbon dioxide consumption, pH increases which is lethal for pathogens.
- Production of long-chain fatty acids which kill pathogens.
- Aeration process enhances faecal coliform die-off rates.
- Attachment to the algal cells (Awuah 2006).

Ahmad claims that algal wastewater treatment is very effective for the reduction in coliform bacteria along with other parameters from municipal wastewater. It not only gives 100% removal efficiency but also brings pollutants into their permissible limits (Ahmad et al. 2014).

#### 23.2.6.2 Reduction in Chemical Oxygen Demand and Biochemical Oxygen Demand

Algae can fix carbon dioxide through photosynthesis and effectively remove excess nutrients at a low cost. Furthermore, photosynthetically produced oxygen can reduce the biochemical oxygen demand (BOD) in wastewater (Laliberte et al. 1994). Algal growth can help to clean the water and make natural waters more suitable for human consumption. The selection of microalgae for wastewater treatment is influenced by their resistance to wastewater as well as their ability to grow in and absorb nutrients from wastewater (Sen and Bhattacharyya 1994). Climax cultures that are not easily grazed in well-oxygenated high-rate ponds are primarily coccids green algae forms. *Chlorella, Scenedesmus, Synechocystis, Gloeocapsa, Chroococcus, Anabaena, Lyngbya, Oscillatoria, Spirulina,* and other algae are commonly used for wastewater treatment. Using an activated algal process to treat wastewater, Sahu discovered that the maximum per cent removal for COD was 72% and BOD was 74%. According to the author, *Chlorella vulgaris* is suitable for reducing nutrient content in wastewater in a significant amount and can help to achieve the government environment protection agency’s effluent quality directive; it is also a good option for biological tertiary treatment of domestic wastewater treatment plants (Sahu 2014).

#### 23.2.6.3 Removal of Nitrogen and Phosphorus

Wastewater contains an abundance of nutrients, most notably inorganic nitrogen. Nitrogen exists in a variety of forms, with ammonium ($NH_4$) and nitrate ($NO_3^-$) being the most common nitrogen compounds assimilated by microalgae (Larsdotter 2006). Excess nutrient loading has been identified as one of the primary causes of eutrophication.

According to Lau’s research, both carrageenan and alginate immobilized *Chlorella vulgaris* cells which removed more nutrients from primary settled wastewater than free cells. Within three days, carrageenan and alginate immobilized algal cells removed more than 95% of the N and P from the wastewater. The polyanionicity of the carrageenan gel increased $NH_4^+$-N adsorption to the gel for algal consumption, whereas the calcium associated with alginate gelling precipitated phosphate from the wastewater (Lau et al. 1997). Phosphorus is another macronutrient that is required for growth and is absorbed by algae as inorganic orthophosphate ($PO_4^{3-}$). The uptake of orthophosphate is an active process that necessitates the expenditure of energy. Phosphorus stored in cells is assimilated by microalgae in the form of polyphosphate granules (Larsdotter 2006). A study by Rasoul-Amini shows that *Chlorella* sp. and *Chlamydomonas* sp. remove the nutrients from urban wastewater. They are capable of reducing N and P, respectively, from wastewaters containing high concentrations of nitrate and phosphate and can achieve high biomass (Rasoul-Amini et al. 2014). A study by Farahdiba et al. evaluates the effects of high nitrate concentration on the kinetics of cell growth during nitrate and phosphate removal by a macroalgae *Cladophora glomerata*. The macroalgae *Cladophora glomerata* has high removal efficiency to reduce nitrate and phosphate concentration in high nitrate concentration (Farahdiba et al. 2020).

### 23.2.6.4 Removal of Heavy Metals from Wastewater

Heavy metal (HM) contamination in waterbodies may have increased as a result of urbanization, industrialization, and natural earth processes. These HMs can accumulate in food chains as a result of consuming contaminated water and food. Various clean-up technologies and conventional methods have been used to sequester HMs, including electrolytic technologies, chemical extraction, ion exchange, precipitation, hydrolysis, polymer microencapsulation, and leaching. However, most of these processes are prohibitively expensive for large-scale projects, require time-consuming control and constant monitoring, and have low efficiency for effective HM removal. Algae offer an alternative, sustainable, and environmentally friendly HM remediation approach (Salama et al. 2019). Phycoremediation is the use of algae in wastewater treatment (Jais et al. 2017). HMs such as $Mn^{2+}$, $Ni^{2+}$, $Cu^{2+}$, $Mo^{2+}$, $Fe^{2+}$, and $Zn^{2+}$ are required for algal growth and are referred to as “trace elements” that are desirable as micronutrients. Other HMs, on the other hand, such as $Sn^{2+}$, $Au^{3+}$, $Cd^{2+}$, $Pb^{2+}$, $Sr^{2+}$, $Ti^{3+}$, and $Hg^{2+}$, serve no biological purpose and are toxic to algae (Jais et al. 2017). For example, *Spirulina* sp. removed 91% and 98% of $Cu^{2+}$ and $Ca^{2+}$ after cultivation in municipal wastewater, respectively (Al-Homaidan et al. 2015; Anastopoulos and Kyzas 2015).

HM ions are removed from wastewater by microalgae via two mechanisms: biosorption and bioaccumulation. Biosorption is a self-contained metabolic process that occurs in both living and dying cells. HM ions are attached to functional groups on the cell surface as a result of ion exchange, complexation, chelation, and microprecipitation in this process (Kumar et al. 2005; Park et al. 2016). According to research, algal cell wall components with key functional groups, such as alginate and fucoidan, are primarily responsible for heavy metal ion biosorption (Anastopoulos and Kyzas 2015; Zeraatkar et al. 2016). There are various ways (active and passive transport systems) of the transportation of heavy metal ions across the living cells. In certain algae, the accumulation of the heavy metals inside the cell causes inhibition of photosynthesis activity and reduces growth. It also causes an irreversible increase in plasmalemma permeability, resulting in the loss of cell solutes, disruption of membrane integrity due to protein structure deterioration, enzyme inhibition due to displacement of essential metal ions, abnormal morphological development, and flagella loss. To overcome the heavy metal toxicity, many intracellular and extracellular metal-binding approaches (such as ion exchanges, chelation, physical adsorption, and complexation) have been implemented (Priyadarshini et al. 2019). These mechanisms are effective as they convert the toxic metal into non-toxic forms (Mantzorou et al. 2018) (Fig. 23.4).

There are many internal and external factors which affect the efficiency of HMs removal by algae: pH, temperature, ionic strength, and presence of counter ions (Salama et al. 2019).

### 23.2.6.5 Removal of Personal Care Products and Pharmaceuticals from the Wastewater

Global attention has been drawn in recent years to preserve freshwater resources due to the increased discharge of pollutants, especially emerging micropollutants

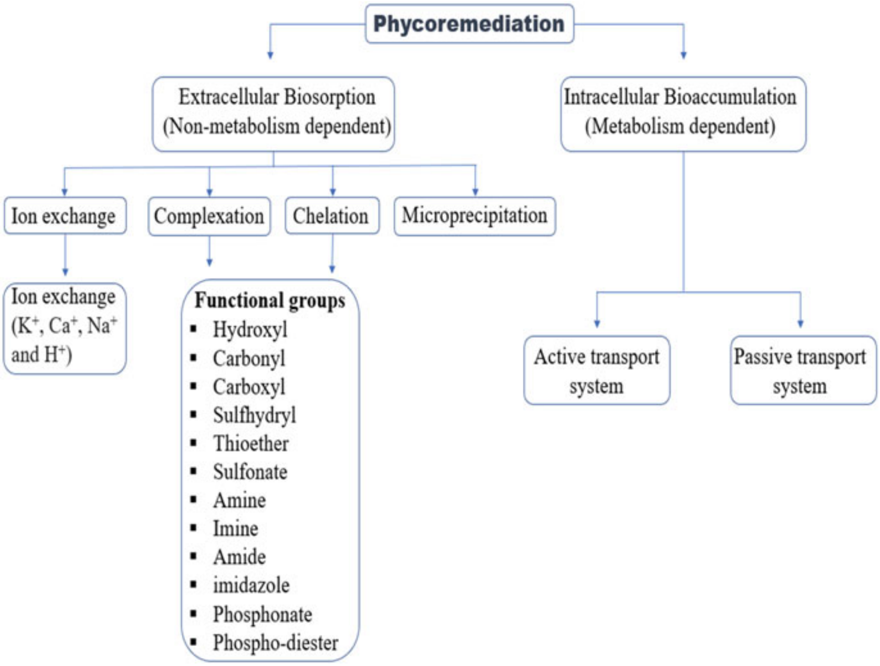

**Fig. 23.4** Heavy metals removal by the phycoremediation approaches

(EMPs) to the aquatic environment. Among different EMPs, the prevalence of pharmaceutical compounds/contaminants (PCs) has become a major threat to the environment. The commonly detected PCs in the aquatic environment include antibiotics, hormones, non-steroidal anti-inflammatory, analgesics, and antiretroviral (ARV) drugs (Reddy et al. 2021). The increased occurrence of ARV drugs in different aquatic ecosystems has become a grave concern due to their toxic effects on aquatic organisms, and the induction of microbial drug resistance. Pharmaceuticals and personal care products (PPCPs) are made up of a wide range of compounds that are widely used in the treatment of human and animal diseases, as well as for health and cosmetic purposes. Because of their widespread presence in the environment and high environmental risks, PPCPs are classified as emerging environmental contaminants.

The removal of PCs from wastewater can be challenging, depending on the complexity and hazardous nature of the compound. The universally employed biological treatment processes such as activated sludge and trickling filters are not theoretically designed to remove PCs from wastewater. Therefore, the likelihoods of these drugs surpassing the different stages of wastewater treatment processes are generally high. This is also evident from the recent studies where a substantial amount of PCs, including ARV drugs, have been detected from the effluents of different conventional WWTPs (Aminot et al. 2015; K'Oreje et al. 2016; Schoeman et al. 2015). In addition, chlorination, which is the most commonly employed tertiary treatment process, could facilitate the formation of disinfection by-products which might be more toxic than the parent compound (Wood et al. 2016). On the contrary, the advanced wastewater treatment processes such as reverse osmosis and ozonation have shown potential for the treatment of a wide range of compounds; however, they are not widely practised due to their high operational costs (Silva et al. 2017).

Many PPCPs cannot be efficiently removed in wastewater treatment plants using traditional activated sludge processes. As a result, there is a growing need for more effective and cost-effective methods of removing PPCPs while treating wastewater. Algae-based technologies have recently gained popularity due to their potential applications in wastewater treatment and hazardous contaminant removal, which have advantages in terms of lowering operating costs while also producing valuable products and sequestering greenhouse gases (Wang et al. 2017). Algae-based systems have been recognized as an alternate and sustainable polishing step to remove residual nutrients from wastewater at conventional wastewater treatment facilities (Reddy et al. 2021). This approach has dual advantages such as residual nutrient removal and microalgal biomass production, where wastewater grown biomass can be used for various applications such as biofuels, biofertilizers, pigments, and feed. Recent literature has also shown the capability of algae to remove a wide range of PCs, including anti-inflammatory drugs (De Wilt et al. 2016), antibiotics (Leng et al. 2020), steroids (Sami and Fatma 2019), and other organic pollutants (Nguyen et al. 2021) from the aqueous environment (Bai and Acharya 2017; Ding et al. 2020; Xiong et al. 2018, 2019).

The ability of algal species to remove PCs from wastewater is well known and it is also apparent that algae can tolerate high concentrations of ARV drugs in the aqueous environment which are higher than the reported concentrations in the aquatic environment. Although tolerance to ARV drugs cannot be a direct indicator of their potential for the removal and/or degradation of these contaminants, it can still form a basis to understand their capability to cope with stress and survive in the environment containing ARV drugs. Furthermore, algae have shown potential for removing a wide array of PCs in the aqueous environment and the active pharmaceutical ingredients found in ARV drugs are similar to the building blocks of commonly studied PCs (Reddy et al. 2021; Fortunak et al. 2014). Therefore, it can be proposed that the algae could be capable of removing ARV drugs either fully or partially from the aquatic environment depending on their chemical composition.

Therefore, the ARV removal mechanisms by algae could be explained based on the known mechanisms of PC removal, which include bioadsorption, bioaccumulation, and biodegradation. Algae can act as a biosorbent for different contaminants including PCs in the environment due to the chemical composition of their cell wall (cellulose, chitin, alginate, and glycan), which provide essential sites for sorption (De Wilt et al. 2016; Flemming and Wingender 2010; Gadd 2009; Gentili and Fick 2017; Wang et al. 2017). Therefore, it is highly possible that bioadsorption could be one of the potential mechanisms for ARV drug removal by algae through adsorption to suspended algal biomass similar to other PCs. The algal cell wall is negatively charged due to the dominant functional groups such as carboxyl, phosphoryl, and amine. They also have assemblages of polymers, which are similar to cellulose, hemicellulose, and lignin (Peng et al. 2014). Since adsorption is extracellular, the process could vary according to hydrophobicity and chemical structure of the drugs as well as the functional groups and the type of algal species (Ghernaout 2014). Bioaccumulation has also been recognized as a potential mechanism of PC removal by algae. Another possible mechanism of PCs removal by algae involves intracellular and extracellular biodegradation (Xiong et al. 2019). Biodegradation of PCs and the formation of different TPs have been reported in algae-based treatment processes. Simpler molecules are formed through catalysis of the complex parent compound by algae (Peng et al. 2014). Intracellular degradation is generally achieved through enzymatic reactions, while exopolymeric substances (EPS) play an important role in the extracellular degradation of PCs. Algae are reported to possess phase I and phase II enzyme families, which play an essential role in biodegradation and biotransformation of chemical compounds and coping with oxidative stress. The phase I family of enzymes (cytochrome P450) adds or exposes a hydroxyl group by hydrolysis, oxidation, or reduction reactions, which makes the compounds more hydrophobic (Torres et al. 2008). Different types of enzymatic reactions for algae-mediated biodegradation/biotransformation of chemical contaminants include hydroxylation, carboxylation, oxidation, hydrogenation, glycosylation, demethylation, ring cleavage, decarboxylation, dehydroxylation, and bromination (Xiong et al. 2018) (Fig. 23.5).

### 23.2.7 Algae/Bacteria Interactions for the Wastewater Treatment

Algae have gotten a lot of attention because of their useful properties in biotechnology processes like wastewater treatment and biofuel production (Menetrez 2012). Algae-associated bacteria have also been shown in studies to be capable of promoting algae growth and influencing process performance (De-Bashan et al. 2008). Bashan et al. discovered that the presence of *Azospirillum brasilense*, a bacterium that produces the plant hormone indole3-acetic acid, significantly increased the growth of *Chlorella vulgaris*, an alga commonly used for tertiary wastewater treatment (De-Bashan et al. 2008) (Fig. 23.6).

## 23.3 Phycoremediation of Different Wastewaters

### 23.3.1 Municipal and Animal Husbandry Wastewater

Nitrogen (N), phosphorus (P), carbon (C), and silicon (Si) are potential limiting nutrients in mass algal cultivation systems in the case of diatoms. On the other hand, wastewater treatment facilities are strictly regulated to limit the number of nutrients (N and P) discharged into the environment due to eutrophication concerns in the receiving waters. As a result, incorporating microalgae into a wastewater treatment process benefits from a low-cost source of nutrients for microalgae growth while also treating the wastewater. Effluents from confined animal feeding operations (CAFOs)

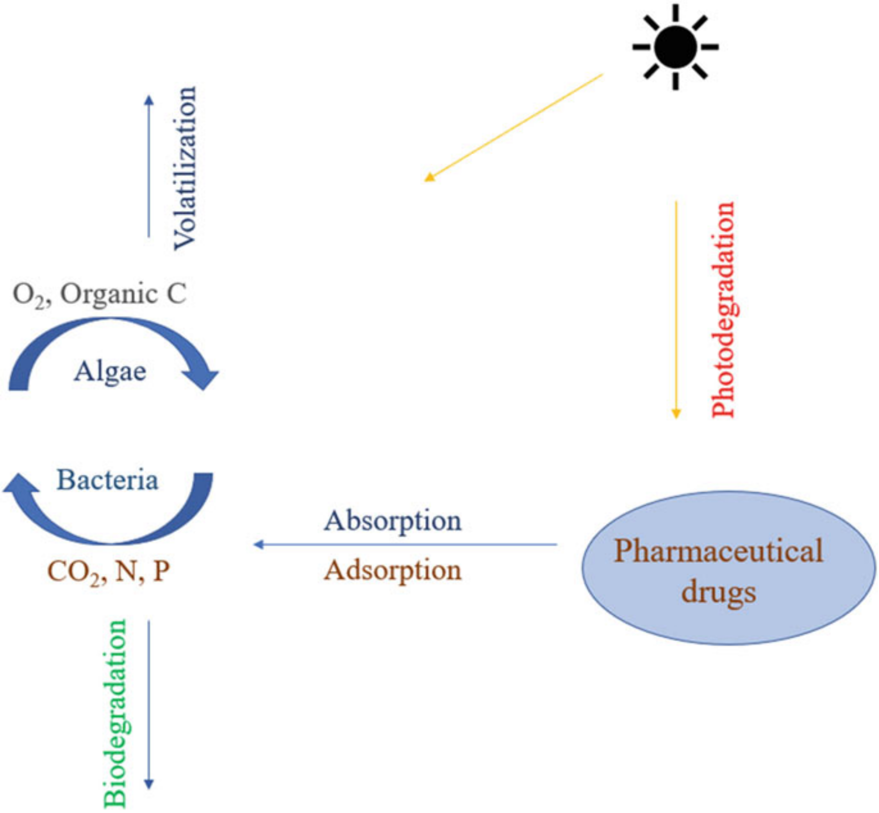

**Fig. 23.5** Processes and mechanisms involved in PPCP removal using algae-based technologies (Wang et al. 2017)

typically contain higher nutrient and solid concentrations than municipal wastewaters, making the use of an algal biofilm-based system to treat a wide range of wastewaters, including CAFOs, desirable. Experiments with different nutrient loading rates and environmental parameters such as light, temperature, nutrient forms such as ammonia, total Kjeldahl nitrogen, nitrate, and nitrite, and pH can be used to determine the system's robustness. Algal biofilm-based biotechnology for the treatment of wastewater can be categorized as follows:

- Assessing the wastewater stream's suitability as a nutrient source for algal biofilm growth.
- Assessment of the technology's ability to meet the required wastewater treatment goals.
- Identifying optimal growth conditions for biomass productivity and/or nutrient uptake.

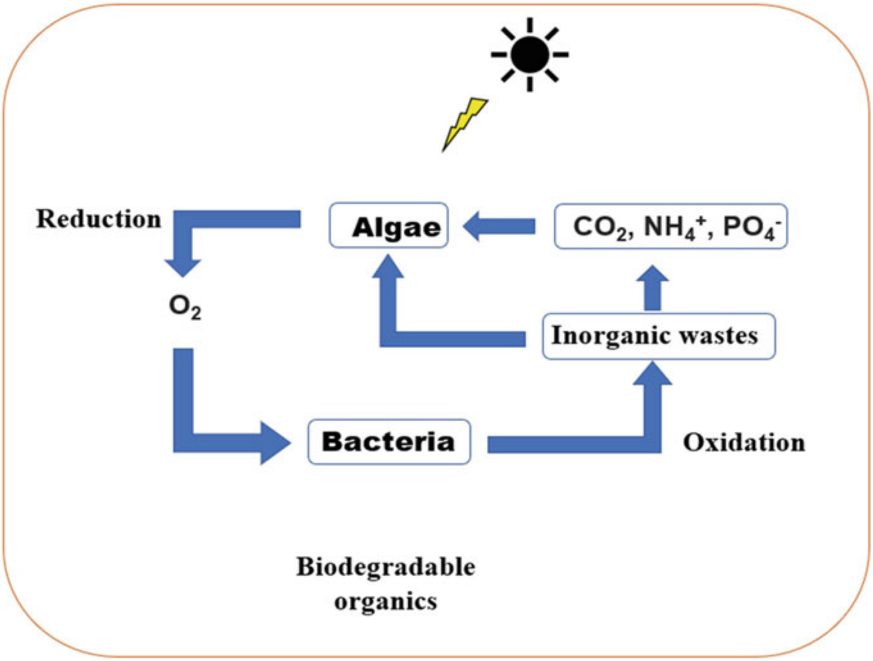

**Fig. 23.6** Interaction of algae and bacteria for wastewater treatment

- Identifying and optimizing system processes to promote the production of desired bioproducts.

A recent research by Saúco et al. (2021) compared a conventional wastewater treatment plant and a hybrid alternative treatment plant for the treatment of real urban wastewater from the city of Almería, Spain. The hybrid treatment plant combines a semi-extensive wastewater treatment system such as a high-rate algae pond (HRAP), a dissolved air flotation unit to separate algae biomass from treated wastewater, and an extensive wastewater treatment system such as vertical flow constructed wetland. The conventional wastewater treatment plant has a treatment capacity of 4000 $m^3$ $d^{-1}$. The experimental demo plant with a 3000 $m^2$ HRAP and 200 $m^2$ of wetlands surface has been operated throughout one year in parallel with a conventional activated sludge full-scale plant, obtaining a complete comparison of both systems. Regarding removal efficiencies, the new hybrid system achieved values of 96.6%, 76.6%, 89.8%, and 99.9% for COD, TN, TP, and TSS, respectively. The hybrid systems reach disinfection rates of 4 and 5 log units for total coliforms and *E. coli*, respectively. Energy demand required in the hybrid system was five times lower than a conventional activated sludge (0.1 kWh $m^{-3}$). The results here obtained demonstrated the capacity of using hybrid alternative treatment plant as an efficient way of treating wastewater in small populations.

### 23.3.2 Industrial Wastewater

There are many advantages of the use of algae for the treatment of industrial wastewaters such as metal removal, organic pollutant degradation, $CO_2$ utilization as a carbon source, and the potential for bioproduct generation (Saravanan and Vijayakumar 2012). Wastewater treatment using algae has the ability to generate revenue through generating bioproducts after the treatment of the wastewater. The combination of phycoremediation and the production of valuable bioproducts within a biorefinery framework may improve the economic viability of many proposed industrial waste remediation efforts. Algal strain selection is critical due to the diverse environments provided by industrial effluents. Geographic location, temperature, light, pH, nutrients, bioproducts of interest, and the potential effects of culture contamination are all factors to consider when choosing a unialgal or mixed culture system (Markou and Georgakakis 2011). Biofilms' natural resiliency lends itself to the often extreme and unstable nature of many industrial wastewaters. Organic chemicals, total dissolved solids, and heavy metals are frequently present in high concentrations. Cyanobacteria genera, particularly *Oscillatoria, Phormidium*, and *Lyngbya,* are frequently found in industrial effluents and are particularly well suited to thrive in harsh environments (Saravanan and Vijayakumar 2012). Metal concentrations in many industrial wastewaters, including acid mine drainage, oil and natural gas produced water, and metallurgical wastewaters, can be high. Metal bioremediation by algal biofilms has the potential to be a low-cost, high-efficiency recovery process. Metal-contaminated wastewaters are remedied by algae through uptake, precipitation, and biosorption mechanisms. Because of increased biosorption, immobilized algal biofilms have been shown to remove higher levels of metal contaminants than suspended cultures (Saravanan and Vijayakumar 2012). pH, temperature, competitive inhibition, and metals recovery are all important considerations for algal metal biosorption (Orandi and Lewis 2013).

Organic chemical contamination, such as hydrocarbons and aromatic compounds, is frequently discovered in surface waters exposed to oil and gas exploration. The largest waste stream generated by these hydrocarbon recovery activities is produced water from the oil and natural gas industries. The growth of algal biofilms and the degradation of organic chemicals in oil and gas-produced water represent a large area of potential for phototrophic biofilm research. Organic chemical degradation facilitated by algae has largely been attributed to and observed in *Cyanobacteria* microbial biofilms (Roeselers et al. 2008). This degradation is frequently initiated by associated bacteria and accelerated by algal biofilm oxic/anoxic diurnal shifts. Because of changes in the oxygen profile, multiple aerobic and anaerobic microbial communities can use the organic breakdown products. Hydrocarbon breakdown has been shown to improve with nitrogen supplementation. As a result, nitrogen-fixing *Cyanobacteria* that form heterocyst and non-heterocyst may benefit biofilm communities by supplying bioavailable nitrogen (Roeselers et al. 2008). This nitrogen-fixing capability could be especially useful in industrial wastewaters where nitrogen is scarce and/or ammonia volatilization occurs over time. Because of the localized area effect within a biofilm, Cyanobacterial biofilms have an advantage over suspended culture, where the degradation-enhancing benefits of *Cyanobacteria* co-culture with bacteria are dispersed more quickly.

## 23.4 Use of Algae Biomass for Bioproducts

Wastewater has high nitrogen and phosphorus concentration, so algae grown in this wastewater have high nutrients. This can be used as a cheap nutrient source for the biomass production ultimately, for the bioproducts (Abdel-Raouf et al. 2012). Many types of bioproducts such as biogas, compost, liquid fuels, food, and fine chemicals can be achieved. Microalgae produce many macromolecules such as carbohydrates, proteins, and lipids; hence, it has been used to manufacture many industrially valuable co-products (Olguín 2012) (see Fig. 23.7).

### 23.4.1 The Third-Generation Biofuel

The third-generation biofuel is derived from algal biomass so it can be called new direction of bioenergetics. Algal biomass contains high amount of lipid so this property allows it as a better source for biofuel production (Voloshin et al. 2016). Algal biomass can be produced from different methods such as open-air system, tubular photobioreactors, flat photobioreactors, and column photobioreactors (Voloshin et al. 2016). After the cultivation and production of algal biomass, the stage of conversion into biofuel comes. There are mainly four types of approaches, i.e. biochemical approaches, chemical approaches, thermochemical approaches, and direct combustion (Chisti 2007) (see Fig. 23.8). In present, there are many

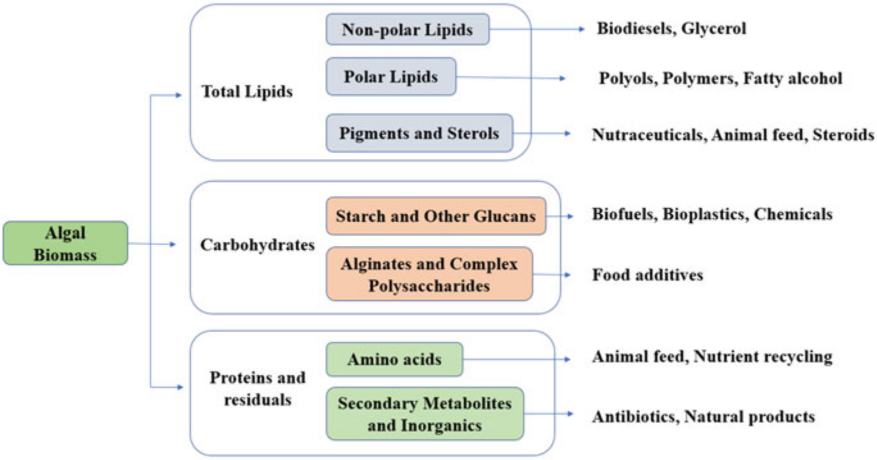

**Fig. 23.7** Products derived from algal biomass

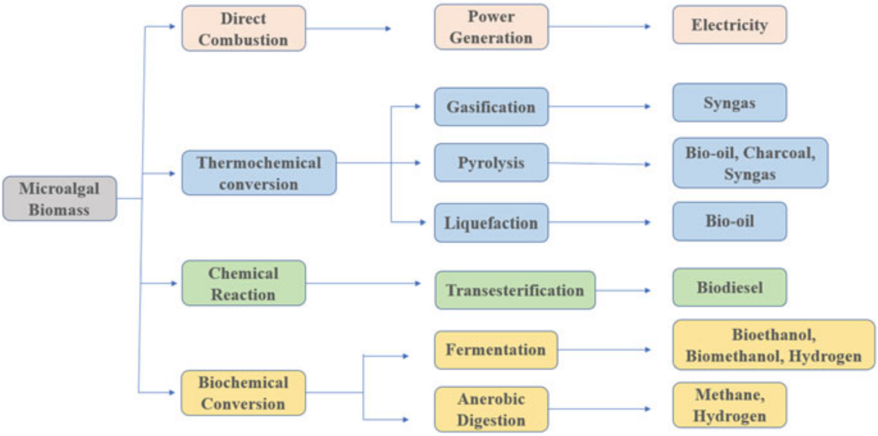

**Fig. 23.8** Different methods of processing algal biomass

companies which make biofuels by algae such as Algenol Biofuels, Solix Biofuels, Sapphire Energy, Solazyme, and Seambiotic.

## 23.5 Future Prospects and Challenges

Algae is a good candidate for wastewater bioremediation because of its low cost of cultivation, high metal ion uptake and selectivity, and suitable mechanical properties for large-scale production. With the advantages of low-cost raw material, big adsorbing capacity, and no secondary pollution, etc., algae can be a good option for wastewater treatment. Algae can remove heavy metals, dyes, and pharmaceuticals and can reduce the amount of BOD, COD, nitrate, phosphate, and coliform from the wastewater. Algae treat wastewater naturally, so it does not harm the environment. Along with the treated water, algae produce much biomass which has high nutrient amounts, so many food products, fertilizers, bioplastics, and biofuel can be generated with the algal bioremediation.

For a long time, wastewater from domestic, industrial, and agricultural processes has been regarded as a serious issue. However, as time passes, we can see a paradigm shift towards a new concept of wastewater, not as a problem, but as a source of energy and other valuable resources, including water itself. Researchers all over the world are working on technologies that will make wastewater-recovered products both socially and economically viable. Algae bioremediation is promising because, of all photosynthetic organisms, they have the fastest growth rates, can be grown on non-arable land, and can treat wastewater in a cost-effective manner. Biomass produced can be used to make biofuels, allowing society to have a more sustainable future. Algae treat water without the use of chemicals. The great advantage of this technology is that the majority of the algae species that have been shown to be effective are readily available. Because photosynthesis is the key feature of this technology, it is ideal for regions with high temperatures and sunlight exposure. However, despite the numerous benefits, there are numerous challenges in using algae for wastewater treatment, limiting its efficacy. The main challenges for the use of algae in wastewater treatment are algae harvesting due to settling characteristics and operational conditions; biomass composition control for the desired products; and micropollutant removal. Given the world's growing population, rising consumption, and scarcity of natural resources, we need to develop effective resource-recovery technologies to promote long-term progress. Algae-based technologies are excellent for treating wastewater and producing useful products at a low cost and with high efficiency. Further research should be encouraged in order to broaden the use of these alternatives around the world and improve current systems.

Wastewater contains substances such as organic matter and non-biodegradable polymers that cause stress and hampers the aquatic life and later on the abiotic and biotic factors. Algae are mainly used for the production of abundant valuable sources focused on pharmaceutical and industrial approaches. The genetically engineered algae have been used due to the fast-growing demands for algae-based biofuel production. Apart from that, microalgae and genetic engineering algae are also been used as a resource for the treatment of wastewater. However, to date, only a few genetic modifications of algal species are reported and investigated as a potential source of wastewater treatment. Thus, it is important to develop efficient algae-based wastewater treatment technologies. An ideal algal strain includes attributes such as fast-growth density, east to harvest, and low susceptibility. As an example, the microalgal strain *Scenedesmus* sp. is capable of mitigating and growing in all the types of wastewaters sourced from industrial, domestic, and agricultural, etc., through accumulating neutral lipids. Marine algae species are easy and have an inexpensive growth requirement which is presently the best choice for cell factories of recombinant protein production too (Singh and Ghosh 2022). To generate transgenic strain with enhanced profiles, the authors offer advances in genetically modified microalgae for wastewater treatment. The market is still developing for biotechnological applications of algae and future research is very promising. Thus, it is encouraging that the development of sustainable algal products through genetic engineering of algae is needed. This chapter investigates the pros and cons of the conventional methods and also the future algae hold for us.

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# The Use of Biopesticides for Sustainable Farming: Way Forward toward Sustainable Development Goals (SDGs)

## 24

Sonia Sethi, Saurabh Dhakad, and Sudipti Arora

**Abstract**

In this chapter, the potential benefits of employing biopesticides to achieve the United Nations Sustainable Development Goals (SDGs) are discussed. Various agroecological practices are proliferating nowadays in response to food safety and environmental concerns. The development of sustainable farming techniques shows how it facilitates most of the SDGs in various ways. However, the indiscriminate use of agrochemicals especially insecticides/pesticides contributed to an accumulation of toxic residues in food, soil, air, and water and caused the development of resistance among pests. Also, these chemicals have affected the soil enzymes, which are required for catalysis in soil. Therefore, to meet the food security for feeding the world population, it is requisite to produce more food, sustainably and harmlessly. So, the use of alternative substances that are ecofriendly and cost-effective for pest control should be employed. The use of compounds of botanical origin and strategies including encapsulation techniques contributes to sustainable agricultural practices. The major component for integrated pest management is the biological control "systems approach," against insecticide-resistant pests, which includes minimizing the usage of pesticides.

**Keywords**

Biological control · Botanical insecticides · Integrated pest management · Sustainable agriculture

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## 24.1 Introduction

According to the most recent UN population forecasts, the global population would grow by 34% between now and 2050, from 6.8 billion to 9.1 billion. It's a massive task to feed an ever-increasing population, especially when land productivity is falling by the day. In order to feed billions of people, agricultural productivity will need to double or triple in the next years. As a result, increasing yield in existing production regions should be prioritised. Higher agricultural yields have begun to emerge, most crops have stabilised (a phenomena known as yield stability), and pest and disease damage has been reduced as a result of the Green Revolution.

In the last century, modern agriculture, which relies heavily on the utilization of external inputs such as hybrid seeds, fertilizers, and pesticides for greater output, has aided much in the alleviation of hunger around the world. However, it has not gone well with contemporary agriculture, and it has resulted in the creation of various second-generation issues such as decreased factor productivity, increased multinutrient deficit in soil, and increased pollution. The use of hazardous and even fatal agrochemicals such as synthetic pesticides in the environment must be avoided. Because this pollution is mostly irreversible, and the chain of horrors it has triggered in the living world is also irreversible. Pesticide overuse, particularly in vegetables and fruits, has resulted in residual levels far beyond the acceptable threshold.

It took millennia for life to emerge on this planet, and time was a key factor in that process. Time must once again be regarded as a crucial component for preserving today's ecosystem and environment. In today's environment, however, no one has time and everyone wants a speedy reaction. When farmers observe their agricultural crops suffering from insect, pest, and disease infestations, as well as a drop in yield, they frequently anticipate a spectacular, miraculous cure to transform them back into lush, green, and healthy plants, ensuring increased production. As a result, they begin utilizing chemical pesticides without consideration for the long-term consequences. Insecticide resistance in pests, return of smaller pests, and excessive levels of pesticide residue in the environment and food have all resulted from this uncontrolled use of chemical pesticides. Pesticide poisoning and mortality are caused by high levels of pesticide residue in the food chain, which induce organ dysfunction, immunological suppression, neurotoxicity, reproductive function impairment, carcinogenicity, paralysis, and harm to beneficial fauna and flora. The negative impacts of chemical pesticides drove people to look for alternatives, which is where the notion of biopesticide was born.

Agrochemical residues are polluting the environment and destroying the natural resource base. To feed the world's growing population, the production system must remain sustainable. Sustainable agricultural systems are economically feasible and fulfil society's requirement for safe and nutritious food while preserving or improving natural resources and environmental quality for future generations. It is environmentally friendly while preserving vigor and dynamism in agricultural expansion in order to fulfil fundamental human requirements while protecting natural resources.

Its goal is to create food that is both healthy and safe for human consumption (Dhakal and Singh 2019).

Various studies have expressed pesticide conditional and measurable changes in the soil microbiota (Hartmann et al. 2015), nitrogen cycling (Damin and Trivelin 2011), soil enzymatic activity changes, and symbiosis disruption between mycorrhizae and root nodules causing changes in soil fertility and, finally, plant growth (Malik et al. 2017). The use of insecticides in all cropping systems for controlling pests or insects can harm non-target organisms and soil microbes and their activity, induce resistance in targeted pests, persist in the soil, and pollute water sources and human health (Hawkins et al. 2019). Management of pests based only on chemical insecticides is therefore unsustainable. The 17 Sustainable Development Goals (SDGs) adopted in 2015 by the United Nations offer a roadmap to attain a sustainable world by 2030. This roadmap is made of 169 specific targets. About sustainable pest management and aligning research on insecticide, SDG can be used as scaffoldings (Sachs et al. 2019).

The impact of insecticides on target and non-target organisms determines the contamination caused by insecticides, their degradation, and bioremediation in the environment. However, a contamination event is limited in time because these pesticide compounds will ultimately degrade naturally through both abiotic (e.g., volatilization, oxidation) and biotic (e.g., enzymatic degradation) pathways (Carvalho 2017). For the removal of contaminants (insecticides) from the soil, bioremediation has been developed (Sanchez-Hernandez et al. 2019). More research on insecticides and their degradation, along with their impacts on non-target organisms, will help to promote access to clean water free of chemicals (SDG6), reduce food and water contaminations to support good health (SDG3), encourage responsible consumption and production of insecticides (SDG12), and reduce the impact on both aquatic (SDG14) and terrestrial (SDG15) biodiversity.

In the last century, agriculture has developed rapidly in most parts of the world, especially in areas where food and fiber can be produced. This intensification increases the net primary production of agricultural land for the population. But this shows a negative impact on biodiversity, including birds, butterflies, and bees. A sustainable agricultural system is between the balance of production needs and the support on which the agricultural system depends (Bianchi et al. 2006), and pesticide risk management is an important aspect.

Increased awareness of the risk of pesticide addiction has led to the development of alternative control strategies and the return of integrated pest management. Recent laws such as the Food Quality Protection Act (DiFonzo 2020) and the Directive on the Sustainable Use of Plant Protection Products (EU 128/2009/EG) restrict the use of plant protection products, especially products with a wide range of activities. Control of pests biologically is an important part of many pest control programs in various ecosystems. Genz believes that in the backdrop of the IPM program, judicious chemicals used in combination with biocontrol agents can impart more extensive management than either alone (Gentz et al. 2010).

These SDGs focus on sustainable production and use, reliance on biodegradable feedstock, and environmental protection, all of which are aims of green chemistry

(GC) principles to some extent. Because their qualities fit the prerequisites of GC principles and the tripartite idea of sustainable development, biopesticides have a significant impact on sustainable agriculture as green agrochemicals. Furthermore, the interconnectivity of sustainable development and GC provides environmental protection, quantitative and qualitative production, safe and effective technology, and prudent and effective resource utilization through biopesticide-driven commercial farming.

Biological control in a changing world has brought more and more pests to new parts of the world and undermined established and stable IPM (integrated pest management) programs. Quickly adapting IPM methods to the new confronts posed by invasive pests is critical in maintaining the profitability of crops facing such problems, such as tomato leaf miner, *Tuta absoluta* (Meyrick). This has led to a significant augmentation of pesticide use to avert crop damage. Quick relay of insecticides to ensure safety from the complex natural enemies that can attack *Tuta absoluta* (Urbaneja et al. 2012). IPM is achieved through a mix of natural enemy protection and the timely application of selective growth-regulating pesticides. For example, broad-spectrum pesticides were frequently employed to destroy natural enemies and promote the quick development of resistance when the species were invaded; this problem improved when farmers shifted to growth regulators. The widespread use of insecticides is in response to the rise of new invasive pests, but a long-term transition to more selective and biological control measures can be made with the full support of research and education initiatives. These actions can be added into the existing IPM strategy (Naranjo and Ellsworth 2009). As a result, biological pest control may get the upper hand and contribute significantly to pest control. To protect T. absoluta from natural enemies, the need for alternative control methods is strengthened by the release of insecticides in the environment.

In order to meet the food production and security, in the area of decrease water supply, interest in the use of agrochemicals has been increased sustainably. Interest in the use of alternative substances to synthetic agrochemicals that present less risk to the environment and human health with increasing food safety has been increased. Promising results have been obtained using compounds derived from aromatic plants for the control of agricultural pests. Such compounds of botanical origin can be highly effective, with multiple mechanisms of action, while at the same time having low toxicity toward non-target organisms. However, the large-scale application of these substances for pest control is limited by their poor stability and other technological issues. The use of compounds of botanical origin, as well as strategies employing encapsulation techniques that can contribute to the development of systems for use in sustainable agricultural practices, has also been increased.

“Biopesticides are some forms of insecticides generated from natural elements such as animals, plants, microbes, and certain minerals,” according to the US Environmental Protection Agency. Biopesticides are divided into three categories by the EPA: Microbial pesticides, plant-incorporated protectants (PIP), and biochemical pesticides are the three types of pesticides. Biopesticides now include genetically modified (GM) plants, according to the EPA (Fig. 24.1). This use of agrochemicals using integrated pest management (IPM), the substitution of

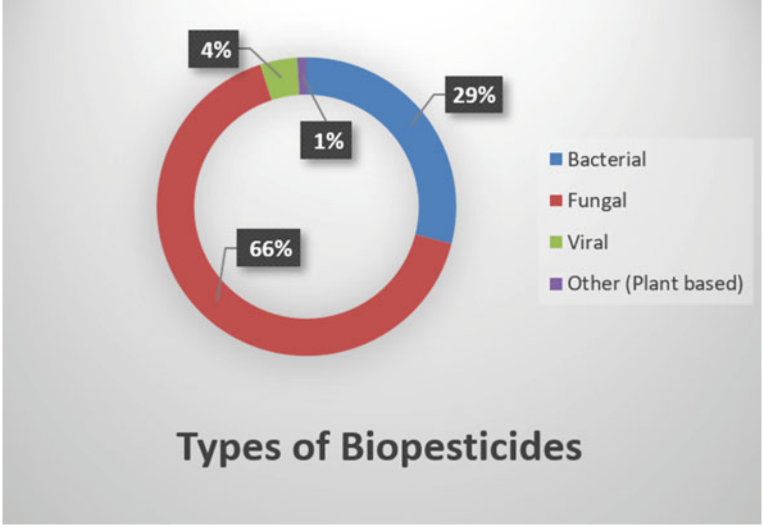

**Fig. 24.1** Types of biopesticides. (Source: DPPQS, DAC, Ministry of Agriculture & Farmers Welfare, Government of India)

materials, and the application of alternative technologies with low-energy consumption and plant/animal integration are major sustainable pathways (Alam et al. 2016). The use of botanical insecticides as secondary metabolites in plants plays an important role in the life cycles of plants, acting as defense agent against pathogen and herbivore attacks, or attracting pollinators and seed disseminators (Pavela and Benelli 2016). Botanical insecticides containing essential oils consist of complex mixtures of volatile and lipophilic compounds which are responsible for the different flavors and aromas.

Botanical insecticides exhibit a variety of biological activities, acting as repellents, insecticides, fungicides, nematicides, and bactericides. These disrupt the cell walls and cytoplasmic membranes of the organisms causing cell lysis and loss of intracellular components (Tian et al. 2012). Botanical insecticides act differently in insects and mammals especially in the nervous system, causing an effect on γ-aminobutyric acid (GABA)-gated chloride channels, acetylcholinesterase, nicotinic acetylcholine receptors (nAChR), octopamine and tyramine receptors, and sodium channels.

The neem tree (azadirachtin), the pyrethrum plant (pyrethrum), and the tobacco plant (nicotine) are all botanicals. These botanicals are natural items that are less expensive, more readily available, safer, and more environmentally friendly. There are concerns about efficacy and cost–benefit analysis when compared to choices, such as synthetic pesticides. As a result, authorities have pushed “sustainable” technology during development. With this aim, comes the risk of misunderstanding when it comes to assessing farmers’ adoption of such “sustainable agriculture” methods who relies on how obvious the advantages are and how easily they can be incorporated into existing methods from the farmers' perspective. Although nicotine and rotenone are effective insecticides, their dangers to non-target species (such as humans and other vertebrates) should prevent them from being used in large-scale or broadcast applications. Rotenone is a lipophilic insecticide that inhibits electron transport in mitochondrial complex I in a wide range of phyla.

## 24.2 Sustainable Agriculture Methods

Pesticides, chemical fertilizers, and other chemical enhancers are frequently used in industrial agriculture. The bulk of the food we ate in the last decade was farmed in this way. However, due to the negative characteristics of the approach, there has been a minor trend toward the employment of sustainable agriculture practices in the previous several years as shown in Fig. 24.2.

### 24.2.1 Genetic Engineering and IPM

The use of indirect (preventive) crop protection techniques, which rely on an understanding of the environment, crop, insect, and natural enemy biology, and the use of optimal agricultural practices to manage pests, is at the heart of an IPM strategy. Selection of appropriate crop cultivars for the region, soil, nutrient, and

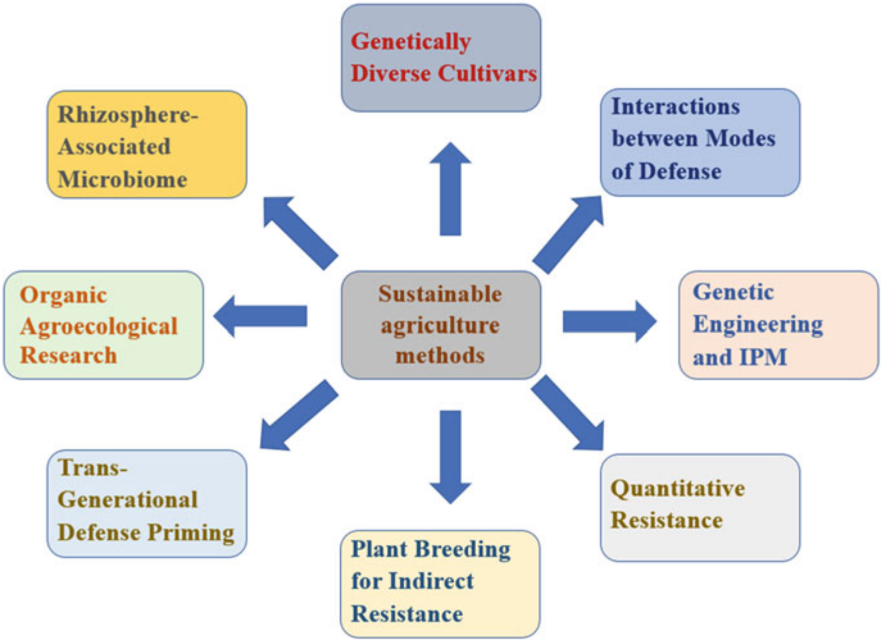

**Fig. 24.2** Different sustainable agricultural methods

water management, use of sustainable pest suppression practices, and implementation of practices that promote the abundance and diversity of beneficial species such as natural enemies, decomposers, and pollinators are all part of this (Owen 2016). In the "high-dose refuge" method, GE plants expressing insecticidal toxins, such as Cry proteins from *Bacillus thuringiensis*, have been used to successfully reduce the selection pressure for pesticide resistance. In reality, it appears that monitoring for pesticide resistance and secondary pest outbreaks demands more field attention to insect pests, indicating that the autopilot approach predicted by critics would result in a lack of monitoring never materialized (Frisvold and Reeves 2010). Bt (*Bacillus thuringiensis*) and other GE crops have been suggested for use by natural predators to conserve real enemies, particularly where insect pests have indeed established efficacy of current chemical insecticides or where a pest complex exists that necessitates population suppression using both biocontrol agents and conventional chemical insecticides. Newer mathematical models of GE agriculture incorporate changes in pesticide resistance, crop rotation, or rotation-resistant pest phenotypes (Ascough et al. 2008). Biopesticides include natural enemies, naturally occurring plant extracts or insect pheromones, and genetically modified plants.

Microbial control agents (MCAs) are natural enemies with the particular purpose of controlling insect pests. Bacteria, protozoa, fungi, and viruses are among them. GE MCAs are frontrunners for usage in IPM due to their benefits over present stringent specificity, and if regulatory modifications are implemented, application rates may be decreased even more. Newer pesticides can be used in IPM programs to limit the spread of insect vectors of plant diseases, as seen with the *Bemisia tabaci*-transmitted cucurbit yellow stunting disorder virus. Researchers have established and described the genes that contribute to the infestation in the entomopathogens *Metarhizium anisopliae* and *Beauveria bassiana* and also reconfigured them to enhance their efficiency as a biocontrol agent and transformed the entomopathogen to drive the development of a scorpion venom-derived insecticidal neurotoxin.

### 24.2.2 Organic Agroecological Research for Sustainable Pest Management

The organic agroecological agricultural movement's basic ideas broadly support sustainable pest management. Organic producers must develop or embrace agroecological-based approaches rather than relying on curative conventional pesticides. Soil health and biodiversity are improved through organic agroecological systems.

Plant breeding and selection, phenotyping, metagenomics, and chemical ecology (Raguso et al. 2015) have all made significant advancements recently, which can be used to promote organic agroecological sustainable pest control. Organic agricultural researchers can use these tools to advance the field of sustainable pest management by pursuing research on:

1. Programs to promote the healthy rhizosphere-associated microbiome nurtured in organic agroecological systems.
2. Raising the use of organic seeds by exploiting intergenerational transmission defense priming.
3. Plant breeding to combat pests through transgenerational defense priming.
4. Plant breeding for numeric resistance characteristics.
5. Creating heterogeneous cultivar mixes.
6. Boosting farmscape diversification.
7. Improving interactions between various types of pest defenses.

These techniques, when combined, have the potential to change pest control on and off organically managed land by reducing pesticide usage and enhancing agricultural sustainability; these are two of the most important areas to invest in to close the yield gap.

#### 24.2.2.1 Rhizosphere-Associated Microbiome

Soil nutrient status is usually the subject of soil management. The physical and biological state of the soil, on the other hand, is crucial for crop growth. Crops with a sufficient supply of nutrients are less stressed and can better defend themselves against pests. Soil structure, drainage, and pore space are all key factors in fostering good crop development. The rhizosphere-related microbial community is indeed an indicator of soil sustainability in organic systems.

When compared to traditional farms, the soil microbiome on organic farms can have more functional diversity and activity, greater evenness, and even larger taxonomic variety (Lupatini et al. 2017). Importantly, the soil-borne disease is less of a concern on organic farms for a wide range of row and horticultural crops, due to improved soil health. It is commonly acknowledged that the rhizosphere-associated microbiome supports healthier plants; as a result, we need to better understand and nurture these microbiomes through management approaches for sustainable agriculture (Philippot et al. 2013).

#### 24.2.2.2 Trans-Generational Defense Priming

Through transgenerational defensive priming or induction, seeds produced organically may be better prepared for future pest challenges. Priming refers to a condition in which a plant can respond to biotic stress more quickly and strongly, whereas induction refers to defenses that have already been engaged. Although the mechanisms of transgenerational induction and priming are unknown, evidence suggests that heritable epigenetic alterations are responsible (Conrath et al. 2015).

#### 24.2.2.3 Plant Breeding for Indirect Resistance

Plant breeding for indirect resistance is at the forefront of pest management innovation in organic agroecological systems. Plant characteristics that aid insect predators by giving a signal about prey position, habitat, or food supplies, or confuse herbivore host discovery are examples of traits that might increase indirect resistance. While plant breeders may be interested in breeding for beneficial plant volatile profiles, the genetic diversity for resistant volatile profiles seen in wild progenitors and landraces is generally missing in today's elite cultivars (Mutyambai et al. 2015), making introgression of these characteristics difficult.

#### 24.2.2.4 Quantitative Resistance

Agricultural insects keep showing a remarkable capacity to develop resistance to insecticides and genetically modified resistance characteristics (Sparks and Nauen 2015). Because it is foreseeable that a pest would overcome any resistance characteristic under strong selection pressure, the organic community should take the lead in establishing effective management techniques that reduce pest selecting pressure for long-term resistance through plant breeding. Plant breeders should aim for quantitative resistance, which is a partial degree of resistance imparted by several genes rather than qualitative resistance, which is produced by a single gene (Niks et al. 2015).

Pests are less likely to rapidly develop to overcome several small selection pressures at the same time when plants are bred for quantitative resistance. This increases the lifetime of plant resistance efficacy. Breeding for statistical resistance to pathogens and insect pests is exacerbated by a lack of knowledge of molecular pathways and difficulties with precise phenotyping, particularly in discrete components of plant–insect interactions, and the pest population ultimately determines the durability of resistance (Nelson et al. 2018).

#### 24.2.2.5 Genetically Diverse Cultivars

Intraspecific diversity is crucial for robust natural and agricultural systems, according to a large body of ecological literature. Cultivar combinations are generally recognized as an effective plant disease management strategy, and research in small grains and soybean has demonstrated that intraspecific variety can boost the number of natural enemies of insect pests (Pan and Qin 2014). These examples show how insect outbreaks can be slowed by using intraspecific plant genetic variation.

Plant breeding for mixing ability or population improvement can help increase the adoption of genetically diverse cultivars. According to a recent study (Barot et al. 2017), breeding strategies for crop mixes include screening large numbers of genotypes for final performance attributes or using ecological knowledge of functional features to shape mixtures. Plant breeders may be able to make quick progress if they use methods like genomic selection to choose just the most promising plant genotypes for extensive field trials.

#### 24.2.2.6 Interactions Between Modes of Defense

Finally, interactions between activities can have synergistic effectiveness, in addition to each practice in a sustainable pest control system working in conjunction with the farm agroecosystem. The significance of investigating interactions across techniques was the primary premise of a recent outstanding review on integrated pest control (Stenberg 2017); therefore, it is researched and used in the blooming agroecosystems of organic farms. In summary, direct plant defenses can impede the growth of insect pests, giving predators or parasitoids (indirect defense) a longer time window to discover and eat their victims, and on-farm biodiversity, as previously stated, can offer a vital habitat for these natural enemies. As the most fascinating example of pest control synergies for organic agroecological systems, we would like to bring attention to the link between soil health and plant defenses against biotic pests. Soil health has been linked to top-down insect pest management in different systems.

## 24.3 Promising Plant Species as Botanicals

Nearly more than 6000 species of various plants have been screened and more than 2500 plants belonging to 235 families were found to possess biomolecules against various types of pests. The most important plant families include *Apocynaceae, Asteraceae, Euphorbiaceae, Fabaceae, Meliaceae* (maximum), *Myrtaceae, Ranunculaceae,* and *Rosaceae*. The mode of action of major botanical pesticides and promising plant species are documented in Tables 24.1 and 24.2, respectively.

### 24.3.1 Types of Botanical Insecticides

Essential oils extracted from various aromatic plants are used in perfumes and as insecticides. They have repellent, antifeedants, growth inhibitors, oviposition inhibitors, ovicides, and growth-reducing effects on a variety of insects (Regnault-Roger et al. 2012). The most significant category of natural chemicals involved in insecticidal activity is alkaloids (Rattan 2010). The malaria vector *Anopheles gambiae* was shown to be resistant to pyridine alkaloids derived from *Ricinus communis*, according to Wachira et al. (2014). The larvicidal and antifeedant effects of furocoumarin and quinolone alkaloids isolated from *Ruta chalepensis* leaves were tested against *Spodoptera littoralis* larvae. Flavonoids are useful in protecting plants against insect pests and animals that graze on plants (Acheuk and Doumandji-Mitiche 2013). Cyanogenic glucosides present in plant species have an important role in plant defense against herbivores. Glycosides from the extract of A. hypogaea

**Table 24.1** Important botanicals and their mode of action

| Name | Source | Mode of action | Uses |
|---|---|---|---|
| Sabadilla | Seeds | Interferes with Ns and k ion movement in nerve axons | Control of squash bug and citrus thrips |
| Pyrethrum | Flowers | Interfere with Na and K ion movement in nerve axons | Aerosol bombs for mosquitoes |
| Rotenone | Roots | Disrupts energy metabolism in mitochondria in nerve axons | Beetles, fish poisoning |
| Ryania | Woody stem | Activates Ca ion release channels and causes paralysis in muscles | Control of caterpillars and thrips |
| Nicotine | Tobacco plants | Mimics the neurotransmitter acetylcholine | Control of aphids, thrips, and bugs |

**Table 24.2** Promising insecticidal plant species with their properties

| Plants | Active compounds | Activity |
|---|---|---|
| *Abies balsamea* | Juvabione | JH agonist |
| *Acorus calamus* | Asarone | Antifeedant |
| *Ageratum houstonianum* | Precocene | Anacyclin anti-JH |
| *Ajuga remota* | Ajugarin | Feeding deterrent |
| *Allium sativum* | Diallyl sulfide | Repellent |
| *Atlantia racemosa* | Luvangetin | Antifeedant |
| *Citrullus colocynthis* | Cucurbitacin-B | Antifeedant |
| *Citrus paradisi* | Isolimonic acid | Oviposition deterrent |
| *Clerodendron infortunatum* | Clerodin | Antifeedant |
| *Curcuma longa* | Termeron | Growth inhibitor |
| *Glycine max* | Glyceollin | Antifeedant activity |
| *Tagetes minuta* | E-ocimenone | Repellent |
| *Ricinus communis* | Ricinine | Oviposition deterrent |
| *Medicago sativa* | Butyric acid | Repellant |
| *Ocimum basillicum* | Juvocimene | JHA |
| *Piper nigrum* | Piperin | Oviposition deterrent |
| *Quassia amara* | Quassin | JHA |
| *Parthenium hysterophorus* | Parthenin | Growth inhibitor |
| *Pongamia pinnata* | Karanjin | Antifeedant |

**Table 24.3** Some plant extracts and their uses

| Extract | Plant | Use |
|---|---|---|
| Essential oils | *Mentha piperita* | Repels ants, flies, lice, moths including Callosobruchus maculatus and Tribolium castaneum (Kordali et al. 2005) |
| Essential oils | *Trachyspermum* sp. | Larvicidal property against Aedes aegypti and southern house mosquito (Culex quinquefasciatus) |
| Alkaloids (pyridine) | *Ricinus communisis* | Malaria vector *Anopheles gambiae* (Velu et al. 2015) |
| Alkaloids | *Arachis hypogaea* | Larvicidal activity against chikungunya and malarial vectors (Wachira et al. 2014) |
| Flavonoids | *Rice* | Nilaparvata lugens (Campos et al. 2016) |
| Anthrax quinones | *Cassia* species | Antimalarial and insecticidal activity |
| Glycosides | *A. hypogaea* | Larvicidal activity against chikungunya and malarial vectors |

show larvicidal action against chikungunya and malarial vectors (Velu et al. 2015). *Solanum lycocarpum* methyl esters have been found to have larvicidal action against the vector *C. quinquefasciatus* (Silva et al. 2015) (Table 24.3).

### 24.3.2 Essential Oil: Potential New Botanicals for Insect Pest Control

Investigation on the effects of essential oils on the larvae of Limantria dispar (Lepidoptera: Lymantriidae, gypsy moth), one of the most severe pests of cork oak woods, was carried out. The findings revealed that the oils tested (*Thymus herba-barona* Loisel*, Rosmarinus officinalis* L.*, Myrtus communis* L.*,* Eucalyptus globules Labill.*, Salvia officinalis* L.*, Helichrysum italicum* sub *microphyllum* G Don) have interesting larvicidal effects, making them suitable for use in integrated control strategies (Moretti et al. 2002). Ants, cockroaches, fleas, head lice, flies, and moths are all attracted to chemical compounds found in the roots of vetiver grass. Termites are repellant and poisonous to vetiver oil and several of its components. Furthermore, wood has been treated with nootkatone, a feeding deterrent for *Formosan subterranean* termites.

Sujatha (2010) discovered that the root extract of *Vetiveria zizanioides* (L.) had insecticidal properties. Eucalyptus essential oil has a wide range of biological activity, and according to Batish et al. 2008, extracts have antimicrobial, fungicidal, insecticidal/insect repellant, herbicidal, acaricidal, and nematicidal. Lemongrass (*Cymbopogon flexuosus*), eucalyptus (*Eucalyptus globules*), rosemary (*Rosmarinus officinalis*), vetiver (*Vetiveria zizanioides*), clove (*Eugenia caryophyllus*), and thyme (*Thymus vulgaris*) essential oils are recognized for their pest-control capabilities. Ants, flies, lice, and moths are all repelled by peppermint (*Mentha piperita*). Basil (*Ocimum basilicum*) and spearmint (*Mentha spicata*) are also effective against various insects like essential oils of *Artemisia vulgaris*, *Melaleuca leucadendron*, *Pelargonium roseum*, *Lavandula angustifolia*, *Mentha piperita*, and *Juniperus virginiana*. The volatile oil components of Mentha species, on the other hand, are extremely efficient against the common stored grain pests *Callosobruchus maculates* and *Tribolium castaneum*. Animal repellents, antifeedants, insecticides, miticides, and antibacterial treatments have all been proven to be effective with essential oils produced from eucalyptus and lemongrass.

Essential oils from *Cinnamomum zeylanicum*, *Cymbopogon citratus*, Lavandulaspiepr angustifoliasyn, and citronella (*Cymbopogon winterianus*) have long been used as an insect and animal repellent. Citronella oil's larvicidal action has been attributed mostly to its primary monoterpenic component citronellal.

When put in closets, drawers, and chests, vetiver (*Vetiveria zizanioides*) root essential oil is proven to protect clothing and other precious goods from insect assault. The essential oil of catnip (*Nepeta cataria*) is very efficient at repelling mosquitoes, bees, and other flying insects. Nepetalactone has been discovered as the main active component in catnip. It is ten times more effective than DEET (*N,N*-diethyl-meta-toluamide) in repelling mosquitoes. It is especially effective against the mosquito *Aedes aegypti*, which is a carrier for the yellow fever virus. *Trachyspermum* sp. oil is also larvicidal against *Aedes aegypti* and *Culex quinque fasciatus*, the southern house mosquito.

*Zingiber officinale* rhizomes (Zingiberaceae) and *Piper cubeba* berries (Piperaceae) essential oils were shown to have insecticidal and antifeeding properties against *T. Castaneum* and *S. oryzae*. Plant essential oils are a complex combination of terpenoids, primarily monoterpenes (C10) and sesquiterpenes (C15), as well as a range of aromatic phenols, oxides, ethers, alcohols, esters, aldehydes, and ketones, which determine the donor plant's unique fragrance and smell. Plants that contain volatile monoterpenes or essential oils have a significant defense mechanism against herbivorous insect pests and harmful fungus. These volatile terpenoids also play a vital role in plant–plant interactions and serve as attractants for a pollinator. They act as signaling molecules and depict evolutionary relationships with their functional roles.

Furthermore, the presence of essential oil provides eucalyptus leaves with a defense advantage against herbivory and insect assault (Brooker and Kleinig 2006). Plant secondary metabolites such as phenolics, tannins, and even monoterpenes are thought to have developed in tandem with herbivory. Components in eucalyptus oils such as 1,8-cineole, citronellal, citronellol, citronellyl acetate, p-cymene, eucamalol, limonene, linalool, -pinene, -terpinene, terpineol, alloocimene, and aromadendrene have been found to have pesticidal action. The different components of eucalyptus essential oil work together synergistically (rather than additively) to increase pesticidal effectiveness. The different components of eucalyptus essential oil work together synergistically (rather than additively) to provide pesticidal action. The most significant component of eucalyptus oil is 1,8-cineole, which is a distinctive chemical of the Eucalyptus genus and is primarily responsible for a range of pesticidal characteristics.

Eucalyptus oil may be used as a natural insect repellent against mosquitoes and other hazardous arthropods, or it can be used as an antifeedant against herbivores. Essential oils from *E. globules*, as well as its main monoterpene 1,8-cineole, were shown to be poisonous to human head lice, *Pediculus humanus capitis*. Essential oil and its primary component 1,8-cineole have higher pediculicidal action than commercially available pediculicides-delta-phenothrinor pyrethrum.

Ceferino et al. recently show that essential oils from *E. cinerea, E. viminalis,* and *E. saligna* had fumigant toxicity/repellent action against permethrin-resistant human head lice (Ceferino et al. 2006). As a result, essential oils might be employed in the creation of novel lice-control treatments. Eucalyptus oil has also been employed as an insecticide, especially against biting insects. Eucalyptus-based insect repellents can protect humans from biting insects. Lucia et al. found that *E. globulus* essential oil is harmful to *Aedes aegypti* larvae (Lucia et al. 2007). Burning the leaves of *E. citriodora* provides a cost-effective technique of mosquito prevention in Africa.

The CDC (Center for Illness Control and Prevention, USA) has recently suggested the use of lemon eucalyptus oil (including p-menthane-3,8-diol, PMD) for protection against West Nile virus, which causes neurological disease and even death and is carried by mosquitoes. Ticks and mites, both parasitic and free-living, can be successfully dispelled with essential oils and their components. *Varroa* mite, *Varroa jacobsoni*, an important honey bee parasite, *Tetranychus urticae,* and *Phytoseiulus persimilis* and *Dermatophagoides pteronyssinus* have all been found to be effective against eucalyptus oils high in cineole. Choi et al. concluded that eucalyptus essential oils might be utilized as a natural acaricide for the control of *T. urticae* based on their research (Choi et al. 2004).

Chagas et al. studied the biocidal activity of essential oils from *Eucalyptus citriodora*, *Eucalyptus globulus*, and *Eucalyptus staigeriana* against the tick *Boophilus microplus* and found that eucalyptus oils may be utilized as a more environmentally friendly acaricide (Chagas et al. 2002). Gardulf et al. found that Citriodiol, a commercially available product based on eucalyptus essential oil, decreased the number of tick bites in people and might be used to prevent tick-borne illnesses (Gardulf et al. 2004).

Another two species, *O. suave* and *O. canum,* have traditionally been employed to combat pests. To keep mosquitoes at bay, the *O. suave* plant was frequently put around windows and doors. The main component of basil essential oils was eugenol, which has been proven to have a significant mosquito repellent effect. Linalool, another terpenoid present in basil, is to blame for the bruchid *Zabrotes subfasciatus* and other storage pests' toxicity. In bioassays on maize weevils, *Sitophilus zeamais*, extracts from *O. suave,* showed good effects. The facility was supposed to be the subject of future village-level evaluations of botanical pesticides in stored grain.

Essential oils have been shown to impact a wide range of insect species in recent research. Essential oils from *Pinus brutia*, *Laurus nobilis, Liquidambar orientalis, Juniperus communis* subsp. *nana* (Willd.)*, Cupressus sempervirens, Lavandula stoechas, Lavandula angustifolia, Eucalyptus camaldulensis,* and *Thymus vulgaris* were found to have insecticidal effects against the larvae of the pine processionary moth, Thaum Schiff. Ben Jemba et al. discovered that the essential oil of *Laurus nobilis* was poisonous to *Rhyzopertha dominica* and *Tribolium castaneum* (Ben Jemba et al. 2012).

Papachristos et al. investigated the insecticidal activity of *Lavandula hybrida*, *Rosmarinus officinalis*, and *Eucalyptus globulus* oils, as well as their 16 main components, against Acanthoscelides obtectus adults. Except for linalyl and terpinyl acetate, all essential oils and monoterpenoids were active against both male and female *A. obtectus* adults, with LC50 values ranging from 0.8 to 47.1 mg $L^{-1}$ air, depending on the insect's sex and the monoterpenoid's composition. Terpinen4-ol, 1,8-cineol, verbenone, and camphor were the most effective monoterpenoids against *A. obtectus* adults. Ketones were more active than alcohols in general, and they were both more active than hydrocarbons (Papachristos et al. 2004).

### 24.3.3 Commercialized Botanical Pesticides in Agricultural Pest Management

#### 24.3.3.1 Neem-Based Insecticides

*Azadirachta indica* is the most common source of neem products (Ali et al. 2017). Azadirachtin, meliantriol, salannin, desacetylsalannin, Nimbin, desacetylnimbin, and nimbidin are the main active components in neem. Azadirachtin is a kind of actin that has a wide range of effects on insects, including repellents, antifeedants, insect growth regulators, and antiovipositional activities (Weinzierl 2000). *Dictyoptera, Orthoptera, Heteroptera, Isoptera, Lepidoptera, Diptera, Coleoptera, Homoptera, Siphonaptera,* and *Hemiptera* are the orders where Azadirachtin is most effective. The most efficient pesticide against sucking insect pests like whitefly, jassid, and mites was neem extract.

#### 24.3.3.2 Rotenone

Rotenone is a botanical insecticide derived from the roots and stems of tropical legumes Derris (*Derris elliptica*), Lonchocarpus (*Lonchocarpus utilis, Lonchocarpus urucu*), and *Tephrosia virginiana*. Rotenone is a kind of flavanoid chemically (Sola et al. 2014). Rotenone is the active component, and it functions as a contact and food poison, as well as a cellular respiratory enzyme inhibitor and a stomach poison.

#### 24.3.3.3 Pyrethrum

Pyrethrum, which is derived from the blooms of *Chrysanthemum cinerariaefolium,* is one of the most significant botanical pesticides used in India. Pyrethrum is made up of six active ingredients: pyrethrin I, pyrethrin II, cinerin I, cinerin II, jasmolin I, jasmolin II, and jasmolin III. The esters of chrysanthemic acid are pyrethrin I, cinerin I, and jasmolin I, whereas the esters of pyrethric acid are pyrethrin II, cinerin II, and jasmolin II (Grdiša and Gršić 2013).

#### 24.3.3.4 Sabadilla

Sabadilla is produced from the seeds of the Venezuelan shrub *Schoenocaulon officinale*. With a mammalian LD50 of 5000 mg/kg, Sabadilla is one of the least poisonous plant insecticides on the market. Sabadilla is a gastrointestinal toxin and a contact toxicant. Sabadilla causes loss of nerve function, paralysis, and death by damaging nerve cell membranes (Singh 2017). Caterpillars, leafhoppers, thrips, stink bugs, and squash bugs are all susceptible to it.

#### 24.3.3.5 Avermectins

The macrocyclic lactones avermectins are produced from the actinomycete *Streptomyces avermitilis*, with a fatal dose of 50% in the range of 10–11.3 mg/kg for rats. In insects and mites, avermectins inhibit the neurotransmitter GABA at the neuromuscular junction (Singh 2017).

#### 24.3.3.6 Spinosads

Spinosad is a combination of spinosyn A and spinosyn D that was first isolated from *Saccharopolyspora spinosa*, a soil actinomycete. Spinosad is effective against a wide variety of caterpillars, leaf miners, and foliage-feeding insects. Spinosyns have a unique mechanism of action, principally targeting binding sites on nicotinic acetylcholine receptors that are different from those targeted by conventional insecticides, resulting in acetylcholine neurotransmission disruption (Singh 2017).

#### 24.3.3.7 (Z) Asarone

(Z) Asarone is an insecticide derived from the *Acorus calamus L.* plant. This compound has fumigant and contact toxicity and is more effective against adults of *Sitophilus oryzae*, *Lasioderma serricorne*, and *Callosobruchus chinensis*.

## 24.4 Challenges to the Utilization of Botanical Pesticides

Challenges include

1. Lack of knowledge and understanding of the effectiveness of botanicals in pest management.
2. Genetic diversity of plant species across geographical areas.
3. Difficulties in registering and patenting natural goods, as well as a lack of standardization of botanical pesticide products.
4. Economic instability induced by seasonal seed supply, the perennial nature of most botanical trees, and variations in potency owing to geographical restrictions.
5. Problems in handling since there is no way to automate the process of collecting, storing, or managing seeds, leaves, or blooms from some perennial trees.
6. When exposed to direct sunshine, the active components become unstable.
7. Commercial-formulated botanicals compete with synthetic pesticides via aggressive promotion by commercial pesticide dealers.
8. Fast degradation, while desirable in some ways, necessitates more accurate timing or more frequent applications.
9. Data on the efficacy and long-term (chronic) mammalian toxicity of several botanicals are missing, and tolerances for some have yet to be developed.

## 24.5 Microbes as Bioinsecticides

Biopesticide is a potentially useful technique for environmental protection. The insect pests and humans have an uneasy alliance, which is referred to as “balance in nature.” This equilibrium is the product of two conflicting forces: the “biotic potential,” or the incredible ability of insects to reproduce and proliferate, and the environmental resistance that keeps their numbers from increasing. Desiccation, malnutrition, parasites, predators, diseases, and other adverse environmental conditions result in the death of adults and the mortality of eggs, larvae, or pupae of insects due to environmental resistance. The shift in “environment resistance” could be due to a variety of factors, both natural and imposed by various agencies. To popularize biopesticide as a significant input for safe and sustainable agriculture, more reasonable approaches are required (Kamble et al. 2016).

Manufacturers would need production and quality control training, as well as organizational training for extension workers and farmers, to help biopesticides gain traction. Because environmental safety is a global concern, we must raise awareness among the general public about the benefits of using biopesticides for pest control. Biopesticides are expected to deliver predictable results, and they must do so in a cost-effective manner to gain acceptance and adaption. Biopesticides must be effective enough in controlling the targeted pests to be widely accepted by end users. Biopesticides offer enormous potential to improve agricultural sustainability and environmental safety. However, bacterial pesticides have three different environmental benefits. They (a) have a limited target range and a highly precise mode of action; (b) are sluggish to act; (c) have short application times; (d) have a short shelf life and low field persistence; (e) are safer for persons and the environment than conventional pesticides; and (f) leave no residue.

They derive from bacteria, fungus, algae, viruses, and protozoans that are naturally occurring or genetically changed. Chemical insecticides can be replaced by microbial control agents, which are more effective. A biological toxin material generated from a microbe, such as a bacterium or a fungus, is known as a microbial toxin. Those bacteria' pathogenic effects on the target pests are highly species-specific. The effect of microbial entomopathogens is caused by their invasion of the insect's integument or gut, followed by pathogen proliferation and death of the host, such as insects. An insecticidal toxin produced by pathogens is crucial in pathogenesis, according to studies. The majority of the toxins produced by microbial infections that have been found are peptides, but their structure, toxicity, and specificity vary widely.

ICM (integrated crop management) currently considers the many actions of biopesticides. For example, the *S. entomophila* AB2 strain found in epizootic *Heliothis* sp. (Lepidoptera) was fungicidal as well as nutrition solubilizing. Bacterial insecticides can also lessen the danger posed by chemical pesticides in agricultural fields since they are (Acheuk and Doumandji-Mitiche 2013) less toxic, (Alam et al. 2016) only impact the target pest, (Ali et al. 2017) effective in very tiny amounts, (Ascough et al. 2008) disintegrate fast, and (Ballhorn et al. 2013) reduce chemical pesticide residues in soil.

Major holders of significant stacks, *Bacillus popilliae*, *B. sphaericus, B. thuringiensis*, *Clostridium bifermentans, Pseudomonas alcaligenes*, *Pseudomonas aureofaciens*, *Saccharopolyspora spinosa*, *Serratia entomophila*, and *Streptomyces avermitilis*, are commercially exploited bacteria. As seen by the great success of Bacillus thuringiensis crops (Bt crops: crops genetically engineered to express crystal (Cry) proteins of B. thuringiensis), many of these restrictions can be alleviated when the identification of a BCA's biocidal activity and method of action are understood (Carzoli et al. 2018).

*B. thuringiensis* is a biocontrol agent for Lepidoptera (*Helicoverpa armigera*, *Spodoptera exempta*, *Cydia pomonella*, etc.), Diptera (*Aedes aegypti*, *Anopheles albimanus*, *Culex obscures*, etc.), Coleoptera (*Leptinotarsa decemlineata*, *Popillia japonica*, *Tribolium confusum*, etc.), and Hymenoptera (*Megastigmus spermotrophus*, *Megachile frontalis*, *Xylocopa aruana*, etc.) (Bravo et al. 2007). Throughout the world, certain endotoxin-producing *B. thuringiensis* var. *israelensis* and *B. sphaericus* strains have been employed to restrict or eliminate mosquito larval stages, particularly in malaria and filariasis endemic zones. In addition, *B. thuringiensis* var. *israelensis* is efficient against the larval stages of the Simulium black fly, a vector of river blindness in humans (onchocerciasis) in tropical Africa. There are over 40 Bt treatments on the market for managing caterpillars, beetles, and blood-feeding flies like mosquitoes (LARVECT 50®, Mosquito Dunks®, Monterey B.t.®). It accounts for 1% of the global insecticide market when taken as a whole.

Strains of *S. entomophila*, on the other hand, are an efficient natural biocontrol agent for the grass grub *Costelytra zealandica* (Coleoptera: Scarabaeidae), a severe pasture pest in New Zealand. *S. entomophila* was first identified as a natural biocontrol agent against C. zealandica. Later, a Mexican strain (*S. entomophila* Mor4.1) was discovered to be potent against *Phyllophaga blanchardi*, another white grub (Nuñez-Valdez et al. 2008). *S. entomophila AB2 has also been identified as a biocontrol agent for Lepidopteran pests* (Chattopadhyay and Sen 2013; Chattopadhyay et al. 2014). Several researchers have also shown *S. entomophila*'s biocontrolling activity against several insect genera (Anomala, Costelytra, and Phyllophaga).

Insecticide genes from bacteria can be utilized in plants to create transgenic crops that are resistant to insect pests. The use of genetic combinations to improve the efficacy of bacterial pesticides could be beneficial. Plant-colonizing pseudomonads, for example, can be utilized to deliver Bt genes to boost efficacy. The combined impact of a Bt enhancer and Bt protein against the hardest *Lepidopteran* caterpillar Manker et al. (2002).

## 24.6 Types of Microbial Insecticides

### 24.6.1 Entomopathogenic Fungi

Entomopathogenic fungi are key natural regulators of insect populations and could be used as mycoinsecticides against a variety of agricultural pests. To circumvent insect immune reactions, these fungi infect their hosts by penetrating through the cuticle, getting access to the hemolymph, creating poisons, and growing by utilizing nutrients available in the hemocoel. Entomopathogenic fungi can be applied as conidia or mycelium, which sporulates after being applied. Pesticide resistance management could benefit from the use of fungal entomopathogens as an alternative to insecticides or a combination of insecticide and fungal entomopathogens. To control second-generation outbreaks of *Cydia pomonella* L., researchers utilized the commercial mycoinsecticide "Boverin," which is based on B. bassiana and contains lower dosages of trichlorfon. When *B. bassiana* and sublethal pesticide doses were used to suppress the Colorado potato beetle (*Leptinotarsa decemlineata*), it increased insect death, attributing this to higher rates of synergism between two agents. The insect-pathogenic fungus Metarhizium anisopliae has also been used to kill adult *Aedes aegypti a*nd *Aedes albopictus* mosquitos.

### 24.6.2 Viral Pesticides

There are around 1600 distinct viruses that infect 1100 different insect and mite species. Baculovirus, a type of virus that affects roughly 100 insect species, accounting for more than 10% of all insect pathogenic viruses. Baculoviruses are DNA-containing rod-shaped particles. A virus inclusion body is made up of most viruses that are wrapped in a protein coat. The protein covering is dissolved by the alkaline environment of the insect's midgut, and the viral particles are freed from the inclusion body. These particles bind to the epithelial cells of the midgut, grow quickly, and finally kill the host. Viral pesticides, on the other hand, are more expensive than chemical pesticides. In addition, several baculoviruses have host specificity. As a result, they cannot be employed to control multiple pests. Baculoviruses' influence on insect larvae is too sluggish for farmers to be satisfied. These viral preparations are not stable when exposed to the sun's UV radiation. To provide a longer field life, efforts are being made to encapsulate baculoviruses with UV protectants.

Although NPVs and GVs are utilized as pesticides, the nucleopolyhedrosis virus group is substantially broader. Sandoz Inc. released the first viral pesticide, ElcarTM, in 1975. ElcarTM was a *Heliothis zea* NPV preparation, which is a broad-range baculovirus that infects a variety of Helicoverpa and Heliothis species. Cotton bollworms, as well as pests from these genera that attack soybean, sorghum, maize, tomato, and beans, were all controlled with HzSNPV. Sandoz decided to stop producing the product in 1982. The resistance to a variety of chemical pesticides, including pyrethroids, reignited interest in HzSNPV, and the virus was renamed GemStarTM. HzSNPV is the product of choice for *Helicoverpa armigera* biocontrol]. Countries with significant regions of crops such as cotton, pigeonpea, tomato, pepper, and maize, such as India and China, have implemented unique biological pest control programs. *H. armigera* was traditionally eliminated from Central India by shaking pigeonpea. The case of *Anticarsia gemmatalis* nucleopolyhedrovirus (AgMNPV), which was used to suppress the velvetbean caterpillar in soybean, is a well-known success of using baculovirus as a biopesticide. The case of *Anticarsia gemmatalis* nucleopolyhedro virus (AgMNPV), which was used to suppress the velvet been caterpillar in soybean, is a well-known success of using baculovirus as a biopesticide.

### 24.6.3 Protozoa

Protozoan pathogens infect a wide variety of insect hosts in the wild. Even though these diseases can kill their insect hosts, many of them are more important for their persistent, incapacitating effects. A reduction in the number of progenies generated by infected insects is one of the most prevalent and major consequences of protozoan infection. Although protozoan diseases play an important role in natural insect population control, few appear to be suitable for use as insecticides. Microsporidia, for example, contains species that may be useful in biological control. Insect microsporidian infections are assumed to be prevalent and responsible for low to moderate insect mortality in the wild.

### 24.6.4 Microbial Semiochemicals

Insects use chemical communication to change their behavior in reaction to their surroundings. Foraging, mating, hazard avoidance, kin recognition, and social interactions are among these behaviors. Semiochemicals are substances that transmit messages between organisms and cause recipient organisms to change their behavior. Using semiochemicals to alter insect behavior is a sustainable way to pest management. Insect pheromones (semiochemicals that mediate interspecific interactions) account for a significant portion of the market for plant and urban pest attractants. A range of agricultural, forestry, and urban pests, such as the cotton boll weevil Anthonomus grandis, the gypsy moth *Lymantria dispar*, and the house fly *Musca domestica,* are monitored and controlled using pheromone-baited traps (Witzgall et al. 2010).

Multiple Lepidopteran pests, such as the codling moth *Cydia pomonella*, the pink bollworm, *Pectinophora gossypiella*, and the diamondback moth, have been demonstrated to be effectively controlled in the field by restricting their capacity to locate mates, a technique known as mating disruption. Many commercially utilized insect semiochemicals are plant or microbial volatile organic compounds (VOCs), not insect pheromones. VOCs are a vast family of low-molecular-weight chemicals with low vapor pressure and high volatility. Fermentation, amino acid catabolism, sulfur reduction, and terpenoid biosynthesis are some of the metabolic mechanisms that can synthesize VOCs (Choudoir et al. 2019). Many VOCs are semiochemicals that only require minute amounts to elicit responses from insects, who have highly sensitive chemosensory (particularly olfactory) systems. By moving through the air, VOCs enable long-distance communication and serve as crucial environmental sensors.

Microbial VOCs (mVOCs) have recently gained popularity as pest management strategies due to their discovery and implementation. This pattern has been influenced by several causes. First, only 10% of known mVOCs are investigated or ascribed specific functions, according to mVOC 2.0, an online database of over 2000 microbial volatiles from approximately 1000 microbial species. Second, there is an increasing demand for natural products in agricultural pest management, as these products are generally better perceived by the public in terms of safety and environmental sustainability than synthetic chemicals, given that an estimated 1018 microbial species are expected to exist on earth.

Microbes have been around for nearly three billion years, and their metabolism has evolved to be extraordinarily diverse and flexible. Microbiomes have metabolic capacities that are typically significantly greater than those of their eukaryotic hosts. Additionally, genetic engineering techniques such as recombinant and synthetic approaches can be used to enhance microbial metabolism.

In a variety of ways, advances in “-omics” tools and computational biology for studying microbial metabolism can help to speed up the discovery of mVOCs. High-throughput sequencing, in particular, is shedding light on the metabolic potential of microbiomes that range from cultured to uncultured bacteria found in plants, soils, and other natural resources. Assembling complicated metagenomes and meta transcriptomes, as well as predicting biosynthetic gene clusters from large data sets of DNA/RNA sequences, are all becoming more computationally feasible. Biologically active natural products can be obtained from uncultured bacteria by cloning environmental DNA into plasmid vectors and expressing in recombinant systems such as *E. coli*, yeast, or baculovirus (Hofer 2018).

In recent years, this strategy has been extensively pursued in the search for new antibiotics and medicinal medications, and it can theoretically be used in the discovery of microbial-based agricultural compounds, such as novel semiochemicals and biopesticides. Microbes can influence insect behavioral interactions with plants via modifying plant VOC emission profiles, in addition, to directly acting on insects. In response to stimulation by jasmonic acid (JA), a key plant hormone involved in antipest defense, growth, and development, Ballhorn et al. (2013) found that lima bean plants colonized with Rhizobia generated a distinct VOC profile than non-colonized plants. Increased indole secretion was ascribed to the unique VOC profile from colonized plants, which corresponds to considerably stronger *Epilachna varivestis* (Mexican bean beetle) repellency. Several symbiotic fungi, such as endophytic fungi and arbuscular mycorrhizas, have also been found to alter plant volatile emissions and, as a result, plant susceptibility to certain insect pests. These findings point to indirect benefits of symbiotic bacteria in plant defense that could be transformed into plant probiotics.

## 24.7 Combining Microbial-Based Biopesticides with Nanotechnologies

Nanotechnology advancements are supporting the successful and long-term usage of biopesticides in field applications. When entomopathogenic bacteria, such as Bt and *Photorhabdus luminescens*, were administered to insect pests in the form of nanoparticles, their efficacy rose dramatically. Murthy et al. (2014) found that larvae exposed to Bt nanoparticles died faster and more frequently than larvae exposed to unhomogenized Bt powder, owing to greater Cry toxin solubility in the alkaline midguts. Furthermore, the development of nanoformulation delivery technologies such as nanoemulsion, nanocapsule, and nanosuspension has improved the longevity and stability of biopesticides in a variety of environments, including UV radiation and humidity (Vassilev et al. 2020).

Smart nanopesticides are an emerging theme stemming from nanoformulation research, in which active chemicals are contained in stimuli-responsive carriers and their release is controlled by stimuli like light, temperature, humidity, or pH. The stimuli-responsive administration strategy allows for more precise spatial delivery while reducing pesticide dosage and frequency, resulting in a less environmental effect. In the case of VOCs, nanoformulations have the potential to fine-tune the compounds' thermal stability, resulting in optimal semiochemical release and lifetime (Camara et al. 2019).

The application of nanotechnology in agrochemicals is still in its early stages. Commercial products in this category, on the other hand, are starting to appear on the market. Seltima®, a fungicide introduced by BASF in 2016 to protect rice crops, is an example. Seltima encapsulates pyraclostrobin, a fungicide agent that is very poisonous to aquatic organisms, using humidity-responsive polymers. This encapsulation is water-resistant, enabling for regulated delivery of the fungicide to the rice leaf surface while reducing pollution of the aquatic habitats nearby. The combination of microbial products and nanotechnology offers several options for improving efficacy and stability while also regulating environmental dispersal. Microbiome mining and nanotechnology, when coupled, have a lot of promise for developing breakthrough bioinsecticide applications shortly.

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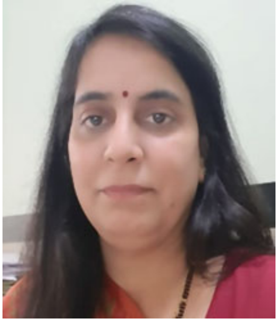

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# Part III

# Environmental Safety, Health and Risk Assessments

# Endocrine Disruptor Compounds: Human Health and Diseases

# 25

Mahantesh M. Kurjogi, Gulamnabi L. Vanti, and Ram S. Kaulgud

## Abstract

Endocrine disruptor compounds are the chemicals that interfere with the normal function of the endocrine system by mimicking the natural hormones, thus having adverse effect on human health. Several studies across the world showed adverse effect of endocrine disruptor compounds on human and environmental health. Researchers from India also identified several endocrine disruptor compounds in consumer products available in the market and attempted to show the direct effect of endocrine disruptor compounds on environmental health through their animal model experiments. Alike hormones endocrine disruptor compounds even at a very low concentration have impact on functioning of the body. A combination of several endocrine disruptor compounds persists in body due to prolonged environmental exposure. Therefore, extensive usage of endocrine disruptor compounds in consumer products has increased threat to global population. Furthermore, this chapter aimed to shed more light on impact of endocrine disruptor compounds on human and environmental health.

## Keywords

Endocrine disruptor compounds (EDCs) · Toxicology · Hormones · Pesticides · Human diseases

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## 25.1 Introduction

The endocrine system (ES) of human is a network of glands and organs that produce, store, and secrete hormones in the human body. When ES functions normally, it works with other body parts to regulate healthy development and function throughout life span. However, Endocrine-disrupting chemicals (EDCs) are substances in the environment such as air, soil, water, food sources, personal care products that directly or indirectly interfere with the normal functioning of the ES. In a report by World Health Organization (WHO), public health and environment, 2016, it was estimated that 13.7 million (globally 24%) people died as a result of living or working in an unhealthy environment (https://www.who.int/data/gho/data/themes/public-health-and-environment). Moreover, this report also estimated that contamination of environmental abiotic factors such as air, water, and soil by chemical exposer may increase ultraviolet radiation which contributes more than 100 diseases and injuries in underdeveloped or developing countries. According to the U.-S. Environmental Protection Agency (EPA), an EDC was defined as "an exogenous agent like natural, synthetic chemicals or mixtures of synthetic chemicals by-product that act as enzymatic competitor with the body's hormones network which directly involved in synthesis, secretion, transport, metabolism, binding action, or elimination of natural blood-borne hormones that are present in the body and are responsible for homeostasis, reproduction, and developmental process" (Diamanti-Kandarakis et al. 2009). Of the thousands of human-made synthetic chemicals, it is estimated that more than 1000 chemicals and/or chemical by-products have properties similar to hormones which act as EDCs (Hormone Health Network n.d.). In the present chapter, we will mainly emphasize on sources of EDCs, mechanism, and mode of actions and how EDCs related to human diseases (Fig. 25.1).

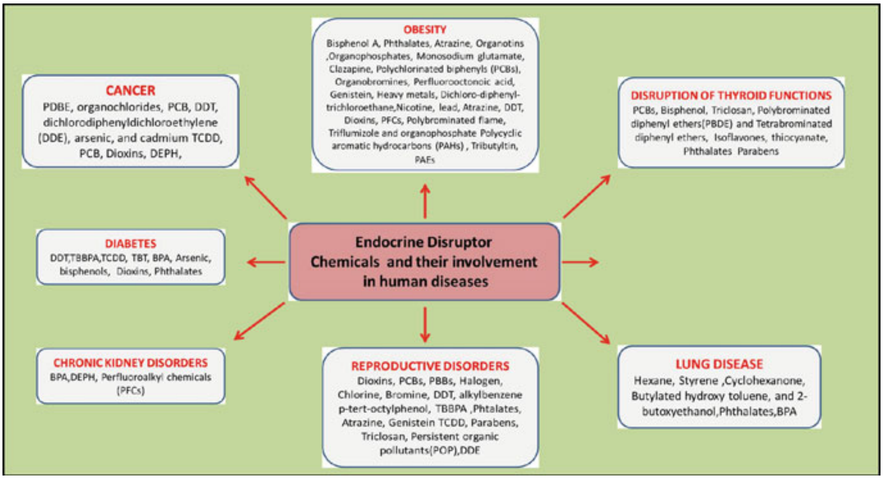

**Fig. 25.1** Effect of endocrine disrupting compounds on human health

## 25.2 EDCs Sources in the Environment

The prevalence of EDCs in our environment and in our physiological system represents a significant global public health challenge in the coming years. As we know, ES system plays a central protagonist in all vertebrates and regulates important biological function in metabolism, development, and reproduction (Kumar et al. 2020). In the recent years, many epidemiological studies carried out by the researcher revealed the link of EDCs to many non-communicable diseases which effect on reproductive system, neurobehavioral neurodevelopmental changes, metabolic syndrome, bone disorders, immune disorders, and cancers in humans (Genoa and Jodi 2018; Adegoke et al. 2021). However, animal studies also emphasized the role of EDCs on human health such as asthma, learning and behavioral problems, early puberty, infertility, breast and prostate cancer, Parkinson's disease, obesity, and other diseases (Bergman et al. 2013; Diamanti-Kandarakis et al. 2009; Schug et al. 2011). The well-studied EDCs include bisphenols such as bisphenol A, and phthalates are key chemical ingredients in plastic industries, diethylstilbestrol (DES), dioxins, and other chlorinated hydrocarbons such as dichloro-diphenyl-trichloroethane (DDT). Some of the EDCs have long half-lives for industrial purposes which are known as persistent organic pollutants (POPs), some of examples include polychlorinated biphenyls, dichloro-diphenyl-dichloroethylene (DDE), dioxin, organochlorine pesticides, and hexa-chlorobenzene (HCB) (Kumar et al. 2020). Some of these EDCs are fat-soluble chemicals which tend to accumulate in the food chain and undergo biomagnification (Calafat and Needham 2007). Table 25.1 summarizes the commonly used EDCs and their exposure sources from previous research articles.

## 25.3 Endocrine-Related Health Disorders

EDCs have notable effects on biological systems. EDCs alter the endocrine system of the body and cause serious metabolic, neurological, cardiovascular, and immunological effects in humans (Schug et al. 2011).

### 25.3.1 Obesity

A subset of EDCs, called "obesogens," these promote adiposity by altering programming of fat cell development, increasing energy storage in fat tissue, and interfering with neuroendocrine control of appetite and satiety (Adegoke et al. 2021). Obesity is generally associated with other morbidities such as metabolic disorders such as type 2 diabetes, cardiovascular diseases, pulmonary complications, and liver disease (Kumar et al. 2020). Various drugs such as thiazolidinediones, tricyclic antidepressants, 5-hydroxytryptamine, and also typical antipsychotic drugs might have role in obesity (Serretti and Mandelli 2010). Some studies indicate that EDCs such as tributyltin, estrogenic chemicals such as bisphenol A (BPA), and chemicals

**Table 25.1** Commonly used endocrine-disrupting chemicals (EDCs) and their common sources

| Sl. no | Chemical name | Sources |
|---|---|---|
| 1 | Biphenyls | Transformers, capacitors, electrical equipment's (regulators, switches, bushings, electromagnets), motors and hydraulic oil, fluorescent light ballasts cable insulation, thermal insulation, fiber glass, foam, adhesives, tapes, paint, caulking plastics, copy papers, floor finish, and so on |
| 2 | Perfluoroalkyl | Food packaging, outdoor clothing (gloves, ski waxes, baking papers), water-proofing agents, carpets, leather goods, fire-fighting foams, paints, wood glue, cleaning agents, and so on |
| 3 | Dioxins | Waste incineration (coal, petroleum, and building), forest fires, paper and chemical manufactures, transformers, capacitors, lighting fixtures, volcanic eruptions, metals smelting, and so on |
| 4 | Bisphenols | Polycarbonate plastics, epoxy resins, food packaging, food cans, food cans lining, plastic dinnerware, dental sealants, thermal receipts, glass frames, plastic mobile case, compact disks, DVDs, medical devices, electronic equipment, and so on |
| 5 | Benzo(a)pyrene | Car exhaust fumes, wood burning, tobacco smoke, oil burning, coal tar, gas product, grilled foods, combustion of organic material (cigarette smoke), charbroiled food, sources, and so on |
| 6 | Triclosan | Soap, dental (mouthwash, toothbrush), cosmetics, deodorant, first aid, kitchenware, clothes (undergarments), ear plugs, helmet, foot warmer, air filters, fitness mats, and so on |
| 7 | Perchlorate | Missile fuels, fireworks, fertilizers, vehicle airbags, nitrate salts, military operations, propellant disposal, lightning, volcanic activity, seaweed, marine algae, railroad signal flares, medicinal applications, oxidizing agent, and so on |
| 8 | Parabens | Food preservatives, cosmetics (makeup, moisturizers, hair care, shaving goods), foods (cloudberry, white and natural wines, bourbon vanilla), and so on |
| 9 | Phthalates | Polyvinyl chloride (PVC) plastics, food packaging, coating, personal care product, building materials, car interiors, drinking straws, swimming pools, adhesive, insecticides, and so on |
| 10 | Arsenic | Rodent poisons, wood preservatives, pesticides and insecticides, medical applications (cure-all tonic, arsphenamine), car batteries, bullets, glassmaking, doping agent, laser technology, and so on |
| 11 | DDT, chlorpyrifos, atrazine and 2,4-D, glyphosate | Pesticides |
| 12 | Lead and cadmium | Children's products |

(continued)

**Table 25.1** (continued)

| Sl. no | Chemical name | Sources |
|---|---|---|
| 13 | Polychlorinated biphenyls (PCBs) | Industrial solvents or lubricants and their by-products |
| 14 | Phenol | Plastics and food storage materials |
| 15 | Brominated flame retardants | Electronics and building materials |
| 16 | UV filters | Personal care products, medical tubing, sunscreen |
| 17 | Lead, cadmium BPA | Children's product |
| 18 | Ethinyl estradiol (synthetic steroid) | Contraceptive |

such as lead, perfluorooctanoic acid, phthalates, polychlorinated biphenyls (PCBs) energy homeostasis and the basal metabolic rate which further leads to weight gain (Thayer et al. 2012; Adegoke et al. 2021). PAHs which occur in oil and coal are produced as by-products of fuel burning; cigarette smoke can result in low birthweight and preterm birth. Results from the previous studies have told that maternal smoking leads to 50–64% increase in obesity in children by meta-analyses (Ino 2010). The molecular mechanism of obesogens illustrates that EDCs act as competitors to transcriptional regulators, and those are involved to control lipid flux and/or adipocyte proliferation/differentiation, especially the peroxisome proliferator-activated receptors (PPAR-α, PPAR-, and PPAR-ϒ) and steroid hormone receptors (Grun and Blumberg 2009). It is estimated that 40% of world population will be overweight or obese by 2030 [Kelly et al., 2008]. Obesity act as important cofactor for several health conditions such as hypertension, diabetes, and cardiovascular disease, and recent studies have shown a positive correlation between Alzheimer's disease (Kelly et al. 2008; Kandimalla et al. 2017). Thus, control of obesity is always supreme to overcome heath comorbidities.

### 25.3.2 Diabetes

Studies have been carried out in the recent past to show to the effect of EDC compounds on well-known metabolic disorder diabetics. Exposure to EDCs such as bisphenols, pesticides, and dioxins is reason for development and progression of diabetes. (Alonso-Magdalena et al. 2010; Hectors et al. 2011). In addition, persistent organic pollutants mainly BPA, dichlorodiphenyltrichloroethane (DDT), dioxins, organo-chlorinated pesticides, and phthalates have all been documented to play a role in diabetic (Marroqui et al. 2018). These EDCs alter metabolic balance through multiple mechanisms including alterations in peroxisome proliferator-modulated pathways adipogenesis, pancreatic β-cell function, and hypothalamic neuropeptides (Walker et al. 2014). EDCs also in mimicking the estrogen receptors α and β and thereby acting like estrogen and sometimes lead to insulin resistance/glucose intolerance disease which leads to diabetics (Alonso-Magdalena et al. 2010). It was observed that EDCs play a crucial rule to lead to type 1 diabetes mellitus, especially if exposer occurs during the development stage (Howard 2018).

### 25.3.3 Hypertension

Hypertension (high blood pressure) is well known because of its prevalence worldwide. Hypertension related to stress and hormone imbalance is common among humans all over the world. The main cause for hypertension could be vasodilation promoted by estrogen via both genomic and non-genomic pathways. The studies have confirmed that the EDCs can act like estrogen and which could be one of the risk factors contributing to the onset of hypertension (Yan et al. 2011). The survey conducted on elderly citizens showed that the BPA in urinary was reported to be correlated with increased diastolic blood pressure and increased blood pressure could lead to cardiovascular diseases (Bae et al. 2012; Aekplakorn et al. 2015). One of the epidemiological studies have showed that urinary BPA in humans has positive association with kidney disease, which further leads to elevated risk of albuminuria (Li et al. 2012; Gowder 2013). Kidney disease prevalence worldwide is estimated to be 10–15% and the fact that EDCs may also contribute to its prevalence (Hill et al. 2016; Lerma and Koch 2017). BPA and phthalate derivatives mimic the estrogen hormones; the receptors for estrogen binding are present on the myocardial cells which are involved in the vasodilation. Further, activation of K+ channel due to EDCs leads to cell hyperpolarization, increases aortic stiffness, potentiates endothelial vasodilator function, and blocks and activation of $Ca^{2+}$ channels, resulting in decreased intracellular $Ca^{2+}$ concentration (Adegoke et al. 2021).

### 25.3.4 Lung Disease

Several studies have demonstrated that EDCs are involved in lung diseases. Day-to-day activities in building construction, furnishing materials, cleaning agents, air fresheners, and spraying insecticides may carry EDCs to the lungs through respiration. One of the recent cross-sectional studies has reported that exposer to mixture of EDCs such as hexane, styrene, cyclohexanone, butylated hydroxyl-toluene, and 2-butoxyethanol may lead to the development of asthma (Paciencia et al. 2019). It has been revealed that exposer to BPA promoted the proliferation of human lung cancer cells (Zhang et al. 2014). The mechanism of EDC effect of lung cells has been studied by several group of researchers; it was shown that exposure to EDCs can activate extracellular signaling pathway ERK1/2 via GPER/EGFR. Subsequently, GPER/ERFR/ERK1/2 sends the signal to nucleus to upregulate metalloproteinases, which are generally crucial in inflammatory, infectious pathogenesis, neoplastic diseases, and movement of carcinogenic lung cells (Nowak et al. 2019).

### 25.3.5 Neurodegenerative Disorder

Neurodegenerative disorder is type of disease in which cells of the central nervous system working get worse over a time and occurs in peoples who are exposed to certain toxins or viruses or people who drink large amount of alcohol. Examples of neurodegenerative disorders include Alzheimer's disease and Parkinson's disease. These neurodegenerative diseases affect people worldwide, especially older age group. It is estimated that 70% of Alzheimer's disease cases is due to genetics, whereas 30% of cause of disease is due to environmental factors including EDC exposures (Yegambaram et al. 2015). Regular use of insecticides, pesticides in agricultural fields, and bisphenols, phthalates in plastic industry, and parabens group of EDCs is neurotoxic to humans (Zaganas et al. 2013; Adegoke et al. 2021). A study carried out by Steenland et al. (2016) group showed that PCB level in the blood of industry workers was 10 times higher compared with control group, and also, other neurological diseases such as dementia and Parkinson's disease were reported among highly exposed women populations. Another observational study revealed that the cases of neurological disease were higher in high pesticide use areas (Parron et al. 2011). A pregnant woman exposed to BPA is thought to effect the brain development of the fetus that leads to poor social behavior, higher anxiety, and depressive behaviors of the child especially in boys (Braun et al. 2011; Lim et al. 2017). It was also observed that uterus exposed to phthalates was shown to be associated with lower IQ, attention deficit, hyperactivity, and poorer social communication (Ejaredar et al. 2015; Kim et al. 2018).

### 25.3.6 Cancer

Carcinogen in the environment is a result of an extended process that depends on different mechanisms such as genetic, exposers to toxins, change in lifestyle, industrialization, and some environmental EDCs are thought to be responsible (Gore et al. 2015). It was observed that large fraction of EDCs acts through estrogenic and androgenic receptors. EDC dioxins, DEHP, and BPA can stimulate estrogen receptors which further involved in the promotion of estrogen-dependent prostate and breast cancers. Estrogen-regulated onco-miR-21 has been shown to play a vital role in the development of breast cancer (Tilghman et al. 2012). Recent review articles have emphasized that EDCs can bind to nuclear receptors, such as estrogen receptors (ERα and β), binding of EDCs to receptors enhance the proliferation of cancer cells through Stat3 and ERK1/2 pathway (Soto and Sonnenschein 2010; Del Pup et al. 2016). One of the studies by Sprague group showed positive relationship between serum EDC (BPA and mono-ethyl phthalate) levels and mammographic images of breast density, which is an indicator of breast cancer risk (Sprague et al. 2013). On the contrary, meta-analysis studies did not find any association between organochlorine pesticides and breast cancer (Ingber et al. 2013). However, only handful reports explored the association between EDCs and cancer, and most of the studies carried out in animal models.

### 25.3.7 Role of EDCs on Male and Female Reproduction

It is well known that sexual differentiation is largely mediated by estrogen and androgen; thus, EDCs might produce different disorders in males and females. During early stage of girl, the levels of hormone in blood are very low, it is possible that estrogenic EDC phthalates, BPA, and organo-halogens binds to sex steroid receptors and bring early puberty (Vasiliu et al. 2004; Deng et al. 2012; Bulus et al. 2016). On the contrary, few studies reported no such correlation between phthalates, BPA, and early puberty, and this might be due to the result of different environmental exposer or may be due to exposer to EDCs during parental and perinatal period (Axmon 2006; Lomenick et al. 2010). However, most of the information on direct effect of EDCs on fertility is available from in vitro and animal studies; only limited studies have been carried out on women, with variable results (Kumar et al. 2020). Recent investigations have demonstrated that BPA is found to be associated with reduced ovarian reserve, polycystic ovary syndrome, implantation failure, and lower antral follicle count in infertile women (Kandaraki et al. 2011; Ehrlich et al. 2012; Souter et al. 2013). One of the cross-sectional studies in more than 30,000 American women population showed effect of EDCs on early menopause; indeed, detailed study is required to claim adverse effect of EDCs on ovarian function (Grindler et al. 2015). EDCs may also act on male reproductive system; it was observed that healthy sperm count was affected by reactive oxygen species generated might be during metabolism of EDCs (organophosphate pesticides, BPA, perfluorinated compounds, phthalates, and organochlorines) which lead to infertility (Sidorkiewicz et al. 2010; Kumar et al. 2020).

## 25.4 Conclusion

The study of EDCs in the literature is best described in different fields as an interdisciplinary approach to determining how environmental factors influence the biology of human through interfering endocrine system. Indeed, sudden increase in the rate of these non-communicable metabolic disorders might be directly associated with global industrialization, which is ultimately involved in the production and release of EDCs to the food chain through environment. Despite research evidence in recent years, there are no proper studies available about role of endocrine disruptor on group of population, level of toxicity exposed, and the damage that has already been done. Some of these challenges can be addressed through technological advances and proper study design, because EDCs are generally present at low concentration in the environment. However, detailed comprehensive studies need to be carried out to establish threshold concentrations of EDCs in the environment to avoid the biomagnification of EDCs. Also, epidemiology studies in large scale with different locations need to be carried out to understand the mechanism of action of EDCs.

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# Monitoring of Paralytic Shellfish Toxins Using Biological Assays

# 26

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## Abstract

The enrichment of nutrients in aquatic ecosystems is an important factor that leads to eutrophication and to accelerated growth of cyanobacteria. Some species produce toxins as secondary metabolites, which can impact ecosystems, animal, and human health. In this chapter, we presented some tools to assess paralytic shellfish toxins (PSTs) and their bioaccumulate effects in fish to bioassay and in field studies. An in vitro method to evaluate the effects in fish primary neuron culture is also described. Besides the analysis of the presence of the PSTs, biological assays in vivo and in vitro studies are important tools to assess the mechanism of actions in cellular and tissue target.

## Keywords

Saxitoxins · Bioassays · In vitro studies · Biomarkers · Eutrophication · Cyanobacteria bloom

## 26.1 Introduction

Eutrophication can occur naturally but has been exacerbated by anthropogenic actions and can result in cyanobacterial blooms (Yan et al. 2017). This process compromises water quality (O'Neil et al. 2012) and increases the cost of treatment to

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make it potable (Chorus and Bartram 1999). This phenomenon is caused by the excessive enrichment of organic matter and nutrients, especially phosphorus and nitrogen that are limiting factors for the growth of algae and aquatic plants. The proliferation of algae and plants contributes to the reduction in dissolved oxygen, death of aquatic biota, and decrease in species richness (Misra and Chaturvedi 2016). Among comprising the phytoplankton algae, cyanobacteria are causing increased environmental and economic problems mainly due to the production of toxins. These toxins, called cyanotoxins, are classified according to their toxicity and can be harmful to aquatic organisms and human beings.

Fish can bioaccumulate these toxins when present in the aquatic environment, causing loss of balance and even death of the animal. Calado et al. (2017) reported poisonings in humans cause burning sensations, salivation, vomiting, diarrhea, or even lethal reactions dependent on the amount ingested (Carmichael 2012). These toxins can cross the blood–brain barrier and cause effects to the brain and alteration in behavior and neural development (O'Neill et al. 2016).

Biological analyses should be added to water monitoring, allowing an assessment that integrates water quality and its toxic effects on human population and aquatic communities (Altenburger et al. 2019). The implementation of more effective strategies in program evaluation of water quality in reservoirs is important, as some methodologies in the water treatment system have been ineffective in removing some contaminants such as drugs and heavy metals and may even increase genotoxicity of these compounds (Palma et al. 2010).

The use of this type of strategy allows an overall assessment of the effects of substances in the water, in addition to their synergistic and/or antagonistic effects. Therefore, monitoring of water bodies from biological analysis can determine the real risks to human health and the environment (Calado et al. 2018).

### 26.1.1 Saxitoxin (PSTs)

The saxitoxins (STXs) paralytic shellfish toxins called—PSTs—are neurotoxins that block sodium channels, preventing the transmission of nerve impulse (Carmichael 2012). Moreover, the blockade of sodium channels can damage the permeability of the membrane, altering cellular homeostasis (Stevens et al. 2011). There are over 20 analogs described STX divided into four groups of magnitude and toxicity (Oshima et al. 1993). This grouping will depend on the group linked to its chain, being decarbamoyl, hydroxyl, and sulfate (Oshima et al. 1993; Chorus and Bartram 1999). The first group is considered the most toxic and understands the saxitoxins (decarbomoilsaxitixona, saxitoxin, dercabamoilneosaxitoxina, and neosaxitoxin). The second group consists of sulfated toxins; these are considered less powerful than saxitoxins (gonyautoxins 1-6). Therefore, they are the most abundant toxins in shellfish. The epimers and GTX2 GTX3 are highly absorbed by the gut epithelium; in humans, this absorption occurs by simple diffusion, facilitating the contamination when these toxins are present in the environment. The third group consists of toxins doubly sulfated; they are less powerful than the previous two groups (C-toxins 1-4).

The last group consists of varieties of American Lyngbya strains wollei 1-6. The LD50 in mice via intraperitoneal to saxitoxin is 10 μg $kg^{-1}$ and orally is 260 μg $kg^{-1}$ (Chorus and Bartram 1999). A study carried out in rats at the concentration of 3 μg $L^{-1}$ (equiv.STX) showed a significant effect on chronic antioxidant defenses and oxidative stress induction (Ramos et al. 2014).

The *Raphidiopsis raciborskii* (also called *Cylindrospermopsis raciborskii*) is a planktonic cyanobacteria in inland waters that can produce of some of these variants (Calado et al. 2019). This is a filamentous cyanobacteria alga, highly invasive, and, in addition, cosmopolitan, occurring in tropical, subtropical, and temperate regions; this causes water contamination by the presence of this species which is a major problem for global health (Zanchett and Oliveira-Filho 2013).

*Raphidiopsis raciborskii* can produce various toxic secondary metabolites, including the hepatotoxic alkaloid represented by cylindrospermopsin (CYL), the neurotoxic represented by saxitoxins (STX, neoSTX, gonyautoxins, and C-toxins) (Lagos et al. 1999) and toxoids (ANA-a) (Chorus and Bartram 1999) and unidentified analogs.

The most severe case of poisoning in humans by these cyanotoxins was in 1979 "Palm Island Mystery Disease" affecting 138 children and 10 adults. The intoxicated people showed hepatoenterite, renal dysfunction, and diarrhea (Byth 1980). Another case of public health involving *Raphidiopsis raciborskii* occurred in 1995 at a reservoir in Queensland and caused the interruption of the public water supply of the city of Brisbane (Hawkins et al. 1997). In Brazil in 1991, in Lake Paranoá, Brasilia, flowering non-toxic strains have also caused great damage in water use for recreation and public supply (Branco and Senna 1991).

#### 26.1.1.1 Reservoir

The reservoir has been widely used by human for power generation, public water supply, recreation, and landscaping. Therefore, these water bodies receive the input of various toxic substances due to agricultural practices, sewage discharge, and leaching. Alagados Reservoir located in the state of Paraná, Brazil, has been detected cyanobacterial blooms, such as *Raphidiopsis raciborskii* and *Dolichospermum* spp. (Table 26.1).

**Table 26.1** Physical and phytoplankton analysis of water samples from Alagados Reservoir, Brazil, in 2015–2016

| Sampling | Temperature (°C) | pH | DO (mg/L) | *C. raciborskii* (cell/mL) | Total phytoplankton (cell/mL) |
|---|---|---|---|---|---|
| Aug S1 | 17.9 | 6.8 | 4 | 33,570[a] | 458,417 |
| Aug S2 | 18.3 | 6.7 | 3 | 1020 | 34,389 |
| Aug S3 | 18.3 | 6.6 | 3 | 31,540[a] | 153,622 |
| Feb S1 | 25.0 | 7.4 | 5 | 111,095[a] | 518,443 |
| Feb S2 | 25.4 | 6.7 | 5 | 0 | 29,401 |
| Feb S3 | 22.1 | 6.5 | 6 | 172,940[a] | 439,385 |

Adapted by Calado et al. (2019)

[a] Above to the limit recommended by Brazilian law (20.000 cells/mL)

**Table 26.2** Total concentrations of PSTs and saxitoxin equivalent (Eq. STX) in the Alagados Reservoir, Brazil, during winter 2013 until Fall 2014

| | Total PSTs (μg/L) | Eq. STX (μg/L) |
|---|---|---|
| Winter | <LQ | — |
| Spring | <LQ | — |
| Summer | 24.21 | 13.18 |
| Fall | 6.66 | 3.62 |

Adapted by Calado et al. (2017)
*<LQ* under the quantification limit of the method

These cyanobacteria are potentially producing neurotoxins, and a study reported concentrations of these toxins in the water and in fish muscle (Table 26.2). The reservoir is heavily used for recreation, and such human activities have caused the degradation of this water body.

As a result of eutrophication since 2002 are reported intense blooms of potentially toxic cyanobacteria in this reservoir, especially persistent blooms of *Cylindrospermopsis raciborskii*. The results of studies (Clemente et al. 2010; Wojciechowski et al. 2017; Calado et al. 2017) show the need for effective monitoring of water quality.

In light of the problems caused by cyanobacteria, it is necessary to detect these organisms in the environment and the monitoring of its abundance. To detect the presence of cyanobacteria is the technique used to measure the number of photosynthetic pigments and make the recognition and counting cells under a microscope. However, none of these techniques allows whether the cyanobacteria are producing toxins; thus, chemical water analysis should be performed (Merel et al. 2013).

The cyanobacteria density established by the Brazil Ministry of Health (No. 2914/2011) is 20,000 cells mL$^{-1}$ for primary contact waters and established as acceptable limit in water sampling point for human consumption. When the blooms are above, this value is needed for the weekly monitoring of cyanobacteria and cyanotoxins.

### 26.1.2 Bioaccumulation, Biomagnification, and Biotransformation of the Cyanotoxins

The toxic compounds such as cyanotoxins in aquatic ecosystems can remain for a long time in the environment. Therefore, these compounds are called persistent and may have a high potential for toxicity to aquatic biota. According to the persistence of these substances contributes to their adsorption in the sediment, bioaccumulation in aquatic biota, and biomagnification in the food chain.

The bioaccumulation is the process in which a substance is absorbed and accumulated in the tissues of organisms, and this process may occur through water or food intake. However, the bioaccumulation occurs when the assimilation rate exceeds the rate of elimination of the compounds in the body. Therefore, this process can result in biomagnification, which is the increasing concentration along the food chain; i.e., the chain top organisms can accumulate very high concentrations (Van

Der Oost et al. 2003). The biotransformation of these compounds occurs primarily in the liver with chemical reactions generally mediated by enzymes in order to yield water-soluble metabolites that are more readily eliminated. The biotransformation can decrease the toxicity of compounds and may also convert for more toxic substances. Calado et al. (2018) found PSTs in water and fish samples, and they were estimated as a potential risk to humans, mainly for children. In addition, toxins were accumulated, biotransformed to other analogs, and excreted by the fish. After 90 days, the toxins were still present in the water and fish muscle. Therefore, PSTs can remain for a long period in water, and fish can be a carrier of these neurotoxins.

### 26.1.3 Bioindicator and Biomonitor

The organisms that respond to environmental changes are called bioindicator or biomonitor. They provide information on ecosystem health and assist in environmental impact assessments. Being constantly exposed, the physical and chemical conditions of the environment the biota of aquatic ecosystems can be considered a good bioindicator of environmental quality. Fish are widely used and considered good biological indicators of water quality because they are in direct contact with the aquatic environment, present wide distribution, body size suitable for the analysis, and be distributed in different trophic levels (Yamamoto et al. 2017). They are constantly exposed to environmental conditions and can carry contaminants over the food web (Aguilar-Betancourt et al. 2016; Cerveny et al. 2016; Guiloski et al. 2017; Silva et al. 2018).

### 26.1.4 Biomarkers

The biomarkers can describe the effects of environmental stress on different biological levels. The biomarkers are often used to identify changes caused by sublethal contaminants. The use of them is considered advantageous for allowing the presence of contaminants to be detected before they can cause adverse effects on organisms, thus enabling preventive action to conserve ecosystems.

There are several types of biomarkers, such as biochemical, genetics, morphological, and hematological, which have been used as important tools to assess effects of exposure of organisms to pollutants (Guiloski et al. 2013; Silva de Assis et al. 2013).

#### 26.1.4.1 Biochemical Biomarkers

Acetylcholinesterase is a biochemical biomarker which can be used to evaluate neurotoxicity, because this enzyme acts on nerve synapses hydrolyzing acetylcholine (ACh) and aiding in the transmission of nerve impulses. Many studies using fish have shown inhibition of this enzyme when exposed to organophosphate pesticides, even in small concentrations (Guiloski et al. 2013). Another contaminant that can also inhibit this enzyme is the cyanotoxin anatoxin (Rodríguez et al. 2012).

The biotransformation mechanism is mediated by enzymes and the cytochrome P450 enzymes responsible for the major components phase I. The main subfamily responsible for the biotransformation of xenobiotics is CYP1A. The catalytic activity of this subfamily can be measured by ethoxyresorufin-*O*-deethylase (EROD). Many studies have shown the induction of CYP1A activity by exposure to organic pollutants as polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), and polychlorinated dibenzofurans (PCDF) (Van Der Oost et al. 2003). The proteins of the cytochrome P450 family are known to be involved in the biotransformation of various xenobiotics in aquatic invertebrates, such as bivalve species (Ruiwen et al. 2018).

The GST enzyme is essential in the phase II biotransformation mechanism performing the conjugation with the involvement of GSH in hydrophilic lipophilic compounds, facilitating elimination by the cell (Kurutas 2016).

The metabolism in normal conditions or when the body is exposed to some stress produces the so-called ROS or reactive oxygen species. These include hydrogen peroxide ($H_2O_2$), superoxide anion ($O_2^-$), and hydroxyl radical (HO). Biomarkers involved in metabolism control the production and degradation of ROS in order to avoid oxidative stress causing damage by molecules such as DNA. Some of these biomarkers are the enzymes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), and non-enzymatic compound reduced glutathione (GSH).

SOD is a metalloenzyme engaged in degradation of $O_2$, forming $H_2O_{2,}$ and oxygen ($O_2$). This result requires CAT and GPx enzymes involved in degradation of $H_2O_2$ into $H_2O$ and $O_2$. The CAT is present in peroxisomes of most cells (Kurutas 2016).

GPx degrades other types of peroxides besides $H_2O_2$. This is a selenium-dependent enzyme and the main peroxidase in fish. It is important in the degradation of lipid peroxides avoiding the reactions involved in lipoperoxidation. The cofactor for their activity is the GSH (Van Der Oost et al. 2003).

GSH is a non-enzymatic compound cofactor of the GPx activity and also takes part in reactions in phase II metabolism through the activity of GST. Changes in GSH levels are related to detoxification mechanisms of xenobiotics by organisms (Van Der Oost et al. 2003).

The biotransformation mechanism is divided into two phases. Phase I is one where oxidation, reduction, and hydrolysis reactions will occur. The products formed by these reactions may be more reactive than previous compounds. In phase II, conjugation reactions occur which aim the elimination of these compounds (Mels et al. 2011).

The lipid peroxidation (LPO) is a major damage caused by oxidative stress. This damage occurs in the phospholipids of cell membranes which are very susceptible regions to oxidation reactions. When ROS react with the lipid membrane, it affects the function of cell membranes causing the increased permeability reaching total breakage and cell death (Kurutas 2016).

The protein carbonylation (PCO) is also molecules in damage caused by oxidative stress. The damage consists in protein modification to ketone and aldehyde groups and the loss of some function enzymes such as pyruvate dehydrogenase (Suzuki et al. 2010).

#### 26.1.4.2 Genetics Biomarkers

The toxic components can also cause genetic damage as breaks in the DNA molecule resulting in biological effects in organisms in the population and in the community. Genetic biomarkers are those that detect DNA damage and some of the methods used to identify such damage is the micronucleus test and analysis of morphological changes as well comet assay (Carrasco et al. 1990; Speit and Hartmann 2005).

The concern of contamination of water bodies by cyanotoxins is growing requiring the use of tools to identify the real damage they cause in the ecosystem. The use of different biomarkers is important to understand the effects of these toxins and they area a useful tool in environmental monitoring programs.

## 26.2 Biomonitoring: Since Field Assessment to Bioassay

Field study is very important to evaluate the environmental quality such as the water and its aquatic ecosystems. Several authors have been showed that PSPs are frequently found in mussel, oysters, and other filter-aquatic organisms and marine fishes (Ben-Gigirey et al. 2012; Xie et al. 2013). Furthermore, it was reported that freshwater fish present in public supply reservoirs with microalgae blooms may contain PSPs and that they can endanger human health when consumed (Clemente et al. 2010; Calado et al. 2020).

However, the field research little quantity elucidates the mechanisms of action of these xenobiotics and their toxic effects on exposed organisms and thus making a simple indicator of presence, or absence, inside, or outside acceptable limits. In this way, how to measure the problem to animal health? How to assess the real effects on the biota directly exposed to PSPs or through the trophic chain?

The answer to these questions can be the bioassay for PSP animal exposure. In field study, the organisms suffer environmental influences and the action and toxic effects to many xenobiotics over PSPs. The results found in the field study not revealed only PSP effects, but the synergism of several substances presents in the aquatic ecosystem. The bioassay is performed in controlled conditions at laboratory and thus the results can be considered trustworthy of PSPs effects. It is possible to measure the capacity of bioaccumulation, the toxic effects on gill, kidneys, liver, brain, blood, and others.

Fish's bioassay can be regarded as an indispensable tool to evaluate the health of ecological and public health. For example, a bioassay realized with fishes on saxitoxins extracts from *Raphidiopsis raciborskii* strain revealed that the liver that until then was not considered target this xenobiotic has damaged several enzymatic level and tissue, suggesting that PSPs are able to provoke hepatological changes (Silva de Assis et al. 2013).

Similar results were found in fish bioassay by Silva et al. (2011) in brain, suggesting that PSPs are not only able to block the sodium channel but damaged the tissue and leading the neuron cells to death and provoke genotoxic effects.

Bioassays can be performed of different methods such as direct exposure to PSPs extract or purified substances into water or through flow trophic chain.

The PSP concentration and the time exposure depend on the aims of the study. The animal's exposure to PSPs dissolved in water has the disadvantage that the substances present can undergo oxidation and change their toxicities, leading to variation in results. To mitigate these variable effects due to molecular instability of substances it is suggested to perform a dynamic bioassay with constant water changes and measuring the periodic PSPs concentration. It is also possible to evaluate the effects of water from site contained PSPs since be realized chemical water analysis. The water test should be carried out in aquarium and performed the exposure with fish or other aquatic organisms that be sensitive to PSPs. The choice of bioindicator or biological model depends on the experimental design.

To assess the PSPs bioaccumulation recommends chronic or subchronic PSPs exposure considering the lifelong time of the model organism. Low concentration of PSPs test can make the bioaccumulation not detectable or a false absence result.

The flow trophic chain has the vantage of ensuring that the organism is exposed to the PSPs and the flux of this substance is metabolized and its effects should be measured during the bioassay. This type of bioassay can be performed using feed animal with PSPs included when the animal is not predominantly carnivorous or directly applied in small specie and offered as food to the top of the chain specie.

Flow trophic experimental design recommends this method:

1. Choose a top of the chain specie.
2. Choose small specie that serves food to the top–base trophic chain.
3. Define the condition of the experiment: time of exposure, PSP concentration, doses, temperature water, and others.
4. Define the experimental groups according to concentration or dilution of PSPs extract or water containing PSPs.
5. Introduce intraperitoneal PSPs in small specie.
6. Food to the top with the small specie contained PSPs.
7. Monitor the experiment during the time previous defined.
8. Sacrifice the animals exposed.
9. Analyze the kidneys, liver, brain, blood, and others using chemical and biochemical analysis such as biomarkers.
10. Analyze the presence of PSPs and their analogs in the muscle.

It is recommended that the bioassay is performed using top and basic native's biological models and the doses should be according to the lapse's temporal food to the top chain, example: one dose every four days. The total time of the experiment should be defined according to the number of doses.

Uses of biochemical, genotoxic, histopathological, morphological, and hematological biomarkers with chemical analysis are recommended to get a better interpretation of results and clarification.

### 26.2.1 New Perspective to Monitoring PSPs: In Vitro Bioassay

Although safety limits for PSPs are reported in the literature (Chorus and Bartram 1999), the direct effects of toxins on target cells still require elucidation and particularly to define and search antidotes and treatments.

On the other hand, in vivo studies considered the environmental influences such as the field assessment which can lead to false diagnosis for animal life and ultimately to public health.

In this way, how to ensure that the limits on exposure to PSPs are safe for animals and their ecological relationships?

The safety limits for biota should be not only based on human health because the aquatic toxins are able to danger the ecological equilibrium and result in several damages to ecosystem aquatic with consequences to support of quality of water and organism's life.

Studies in vitro can provide knowledge about the mechanisms of the damages and detoxification of saxitoxin and other PSPs on brain tissue, provided that a purification of the cells is performed in order to avoid conflicting results due to various cell types which may be nonspecific responses to the test substance.

Several types of animal organisms have been used to cellular donor to primary culture (Martins et al. 2011; Southam et al. 2013; Ribas et al. 2014; Schnell et al. 2015; Oliveira-Mello et al. 2021). However, it is important that the in vitro bioassay be carried in a specie that has evolved exposure to environmental toxins containing and, in this case, rats are not good biological models for assessing the harmful effects of PSPs on neuronal cells as never had contact natural with these substances, despite this specie can be used to understand the mechanisms of toxins in mammals, but do not express the real damage caused by PSPs in aquatic organisms.

Fish is reported as a good biological model to elucidate the effects of many aquatic pollutants and the water quality (Mela et al. 2010; Katsumiti et al. 2013; Calado et al. 2018, 2020). In vitro, freshwater specie fish such as *Hoplias malabaricus* has cited in several types of in vitro studies (Bussolaro et al. 2010; Liebel et al. 2011; Ribas et al. 2014; Silva et al. 2014) and can be to monitor PSPs on biological assay using cells cultivated.

The problems found in fish primary culture is the microbial contamination, but this can be mitigated by pre-washing with 70% ethanol throughout the body of the fish having been anesthetized (benzocaine 0.02%) in laminar flow sterile hood.

For the brain, for example, after being sacrificed by medullar section and decapitated the fish head should be again rinsed with chlorhexidine 2%. Then, the brain can be removed of cranium and transferred to Hank's balanced salt solution (HBSS) supplemented with 2 mM glucose and antibiotic penicillin/streptomycin (ATB; 50 U $mL^{-1}$ Pen, 50 μg $mL^{-1}$ Strept) and the meninges have been cleaned before slices the brain at 0.5 mm using a scalpel to avoid the cell excess impurities, following the mechanical dissociation using a sequence flamed Pasteur pipette.

Many types of proteases can be used for cellular dissociation. To fish brain is recommended Papain 30 U $\mathrm{mL}^{-1}$ at 30 °C for 30 min with occasional gently mixing in Neurobasal with B27 supplement (B27; 1:50) medium for digestion. The tissue was triturated with a flame-polished Pasteur pipette for 1 min followed by another digestion for 15 min at 30 °C. This procedure should be repeated twice followed by a new triturating step with a new flame-polished Pasteur of a minor diameter.

A segregation cellular is important to choose the cells type which will be used in the bioassay such as the density gradient prepared with Optiprep and Neurobasal/B27 medium (Brewer and Torricelli 2007) and centrifuged at 800 × *g* for 15 min at 22 °C. To remove the debris and other cells types that such as oligodendrocytes the fractions should be aspirated and discarded. Then, the fraction contained neurons will be transferred to a new tube and added Dulbecco's modified Eagle's medium (DMEM/F12) and cells were centrifuged at 800 × *g* for 5 min at 22 °C. The supernatant, which contained debris, was discarded and pelleted cells should be washed in DMEM/F12 and collected by a new centrifugation at 800 × *g* for 5 min at 22 °C. Then, cells should be re-suspended in culture medium appropriated and total and viable cells counted by Trypan blue exclusion in Neubauer chamber (Hu and Fakahany 1994) and diluted before seeded in Petri dishes.

To increase the adherence and development and cell differentiation, in vitro studies with fish neurons is necessary several types of nutrients into culture medium. Silva et al. (2014), for example, recommend the use of DMEM/F12 medium supplemented with B27 + 5% fetal bovine serum (FBS) + glutamine (0.29 mg $\mathrm{mL}^{-1}$) + ATB + fibroblast growth factor (bFGF) (from bovine pituitary glands; 3 ng $\mathrm{mL}^{-1}$) and seeded for experimental condition (recommended: 24 °C and 1.7% $CO_2$ at humidified atmosphere) in plate coating substrate for the cellular adhesion with poly-L-lysine (15 μg $\mathrm{mL}^{-1}$) for overnight. Before use, plates should be washed three times with phosphate-buffered saline (PBS). The whole procedure is summarized in the flow diagram shown in Fig. 26.1.

But how to monitor PSPs using in vitro bioassay? See next section how to apply this innovative method to assess these substances in fishes

#### 26.2.1.1 Evaluating PSP Effects by In Vitro Bioassay

In order to evaluate the toxic effects of PSPs substances, the cells after counted by Trypan blue exclusion in Neubauer chamber should be seeded at $2 \times 10^5$ cells at 96-well dish treated with poly-L-lysine coating substrate and added DMEM/F12 medium supplemented with B27 + FBS + glutamine + ATB + bFGF according to section above should be incubated for 24 h for adhesion.

After incubation of neuronal cells from fish, the medium should be removed and added new medium contain PSPs interest test such as saxitoxins (STX) or its analogs, or cyanobacterium extracts, or water test for 1 day at experimental condition 24 °C and 1.7% $CO_2$ at humidified atmosphere.

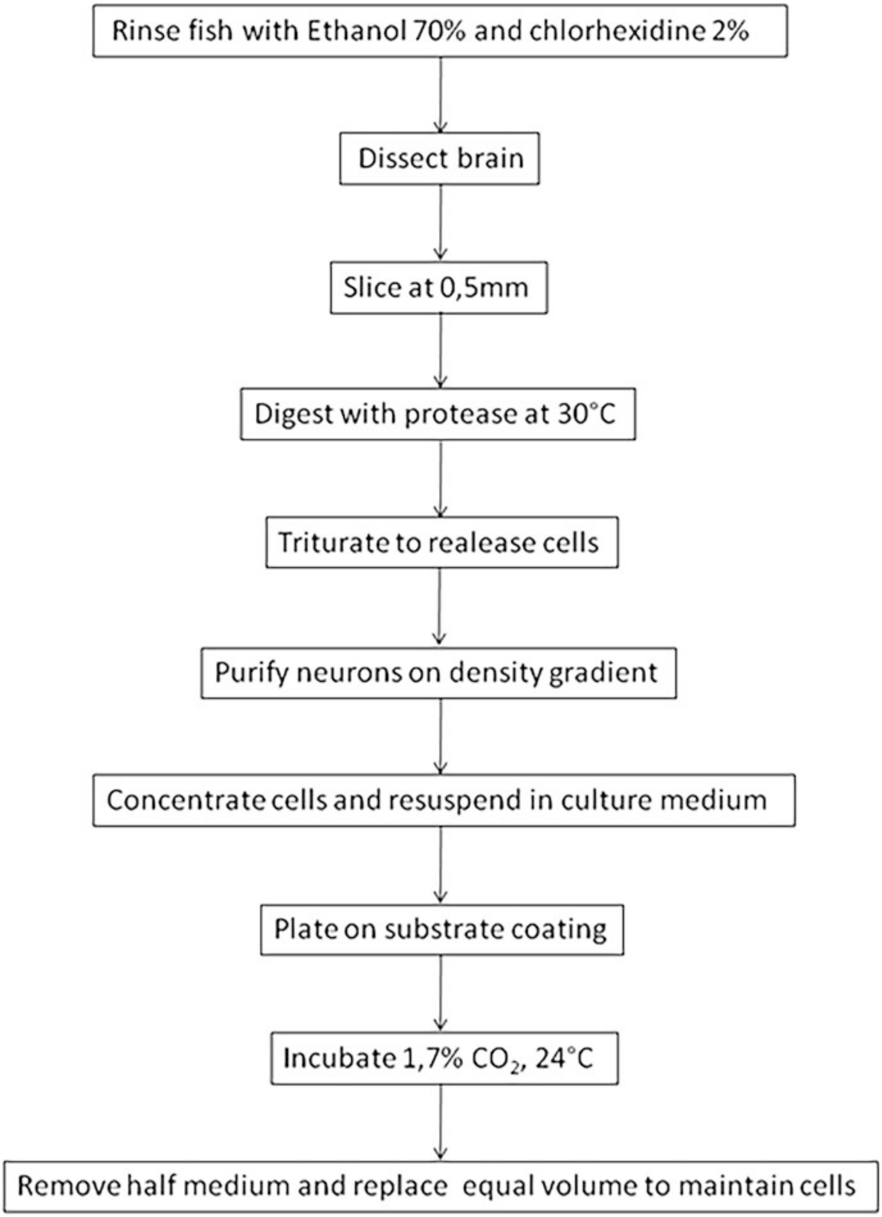

**Fig. 26.1** Diagram for brain cellular obtention. (Source: authors' collection)

The PSP concentrations for experimental design depend on the degree of toxicity from substances to be tested and which tests are carried out. It is suggested that they carry a pilot experiment before of the bioassay.

After 24 h incubated in conditions described above, it is possible to assess the effects of the PSPs in target cells using several tests and analysis such as biochemical and genotoxic biomarkers. In this case, the plates should be washed three times with PBS before starting the carry out the analysis. It suggests five repetitions for each experiment.

It is recommended that the viability of cultured cells be determined by MTT method. This assay is based on the conversion of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, soluble form) into dark blue formazan crystals by mitochondrial dehydrogenases. The accumulation of formazan reflects directly the activity of mitochondria and indirectly the number of viable cells can be taken expressed as optical density (OD) at 570 nm, according to the method described by Sarmento et al. (2004).

Indeed, it is necessary to quantify the neuron cells in primary culture using immunocytochemistry test. For this trial, it is suggested to use III β-tubulin antibody (1:200) and anti-mouse Ig Texas Red to mark neuron protein specific.

To the biochemical biomarkers, the cells were plated after washing with PBS; the plate should be frozen at −80 °C for lysis cellular.

Then, the cells lysed can be homogenized in phosphate buffer (0.1 M, pH 7.0) and centrifuged at 3400 × *g* for 30 min at 4 °C using rotor plate. The supernatants should be carried out to biochemical biomarkers such as superoxide dismutase, glutathione peroxidase, caspases, and other enzyme activities. In addition, supernatants can be used to assess the concentration of hydroperoxides and protein carbonylation. It is recommended to use the Bradford protein test to normalize the results of the tests applied.

Beyond that, the potential genotoxic actions of PSPs can be evaluated by comet assay detaching cells of the plate rising PBS/EDTA and carrying out the cells to procedure described by Singh et al. (1988) modified by Ferraro et al. (2004). For each brain slide, 100 cells should be visually analyzed and scored as belonging to one of five classes from undamaged (0) to maximally damaged (4) pre-defined with reference to the tail intensity.

The diagram shown in Fig. 26.2 summarizes the exposure PSPs procedure for bioassay in vitro.

### 26.2.2 Advantage and Disadvantage of In Vivo Versus In Vitro Studies

Monitoring PSPs by bioassay allows identifying risks to animal and public health and establishing safe limits according to the results observed in the exposure organisms. However, what kind of bioassay chooses: in vivo or in vitro methods?

Both have advantages and disadvantages. Bioassay in vivo, for example, enables checking the animal behavior when exposed to high or low concentration of PSPs. Moreover, it is possible to evaluate the bioaccumulation and metabolites and biodegradation or detoxification and their toxic effects.

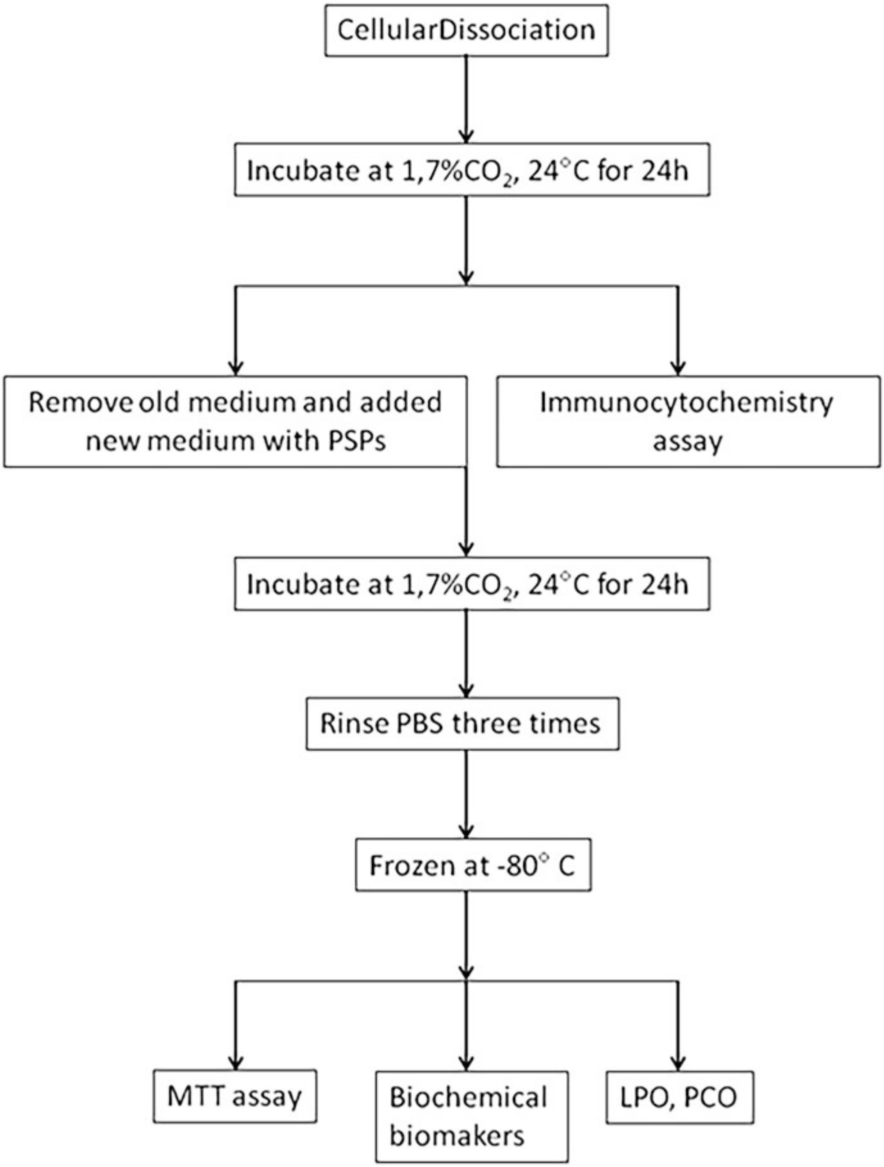

**Fig. 26.2** In vitro bioassays to assess the PSP exposure. (Source: authors' collection)

The exposure time can also be controlled in accordance with lethal or sublethal concentration in acute, subchronic, or chronic assay, making possible a valuable observation of the damage caused by exposure and its respective bioaccumulation.

Allow yet the observation of the trophic flow of PSPs by direct exposure to water through the tegument and gills of the animal or due to application in food offered to the organism.

On other hand, the PSPs metabolizing during in vivo bioassay can mask the mechanism of action of the PSPs testing leading to false clarification about the starting and endpoint effects.

In addition, for in vivo studies is needed several types of chemical analyzes in the test water during the exposing and use high concentration of PSPs strain toxic extracts to expose the animals. Apart from that, the number of individuals is an important factor to consider to in vivo bioassay.

Ethics committees have denied authorization depending on the manner and the number of individuals used in the tests which can make unfeasible in vivo bioassays.

Additionally, depending on the quantity of experimental groups will be necessary lot of space for aquariums, especially if working with a top species the trophic chain that sometimes they can have territorial behavior and will need individual aquariums.

In contrast, the in vitro bioassay provides several types of results using a bit number of individuals considering the independents experiments. It is possible to perform the test using the total number of cellular from the tissue selected, and thus, it is a population of cells and not a sample like an in vivo bioassay. One individual can be donator of many millions of cells (Silva et al. 2014).

The environmental interference can be controlled and monitored. Because the target cells are cultivated from a tissue and applying PSPs directly over them, the effects are evidenced with no changes in molecular test substance.

In vitro studies allow to elucidate the mechanisms of the action of PSPs such as expression of proteins.

By the fact that there are no digestive metabolic processes of the organism donor cells due to exposure to xenobiotic, the time of exposure can be reduced and the toxic effects observed in a short period as some hours or minutes. In addition, the concentration can also be abridged because of the direct action on the cellular structures.

However, these studies are more expensive than in vivo bioassay and do not provide assessing the bioaccumulation in tissues or organisms.

Finally, the primary culture does not provide information about the interactions between tissues and organs from animals, and this method should be avoided for clarification about the interaction’s mechanisms.

Table 26.3 shows the main characteristics of the in vivo and in vitro bioassays for the best choice between the two methods.

**Table 26.3** Principal characteristics of the in vivo and in vitro studies

| | In vivo bioassay | In vitro bioassay |
|---|---|---|
| PSP concentration | High concentration is required | Response to low concentration |
| Bioaccumulation test | Yes | No |
| Number of individuals | High. It is necessary about 20 individuals per group | One individual can be enough |
| Statistical test | Sample | Cell population |
| Death during test | It is probable death of individuals in acute or chronic bioassay | No. Under optimal conditions, the cells can survive up to 30 days |
| Ecosystem clarification | Yes. It is possible to estimate results observed in bioassay to aquatic ecosystems | No |
| Ecological interaction | Yes. It is possible to elucidate some interaction about ecological interaction | No |
| Tissues and organ interaction | Yes. It is possible to elucidate interaction between tissues and organs from animal exposure and toxic effects | No. The toxic effects are measured for only tissue or organ donor of cells |
| Mechanism of PSP metabolism | Yes. The bioassay allows clarification about the PSP metabolism in organs and tissues | No. It is only possible to estimate the PSP influence about cellular metabolism |
| Mechanism of biodegradation or detoxification | Yes. It is possible to estimate the biodegradation in tissues and organs | Yes. Biodegradation and detoxification in cellular and molecular levels |
| Expression of specific proteins and molecular | Yes. However, the analytical methods are expensive and difficult | Yes. A simple method can elucidate the expression of proteins |
| Mechanism of action of PSPs | Yes. It is possible to estimate the action mechanism to tissue and organs | Yes. It is possible to clarify the mechanisms about target cellular and receptors |
| Apoptosis clarification | Yes. It is possible to estimate the probability of the tissue, organ, or cells which are dying by apoptosis or necrosis | Yes. It is possible to clarify the mechanism of cellular death, including the caspase cascade |
| Time of bioassay exposure | High | Low. Some minutes or hours |
| Staff for bioassay | Recommends trained staff | Trained staff is required |
| Cost | Low or medium | Expensive or medium |

Source: authors' collection

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# 27

# Bioinformatics Toward Improving Bioremediation

Surbhi Khanna and Ashwani Kumar

## Abstract

Environmental pollution has become a major issue of concern. With the rapid growth of industrialization, agricultural practices, and energy generation processes, the exploitation of natural resources has occurred. The result of which is the pollution of air and soil. To get rid of it, several practices are applied one of which is the bioremediation. This requires microbes, which have potential and enzymatic capability to undergo complete transformation or mineralization into harmless end products. To aid this process at a faster rate, bioinformatics has emerged as an advantageous approach. It helps in diversification and implementation of bioremediation in a productive way by employing the computational tools and software packages. This in silico approach of studying the bioremediation is very efficient by giving knowledge and understanding of the pathways and structural and functional aspects of microorganisms involved in biodegradation. Thus, this chapter gives the detailed complete idea of tools and software that bioinformatics provide toward improvement of bioremediation.

## Keywords

Bioinformatics · Computational tools · Metabolic pathways · Chemical toxicity · Omic approaches · Databases

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## 27.1 Background

With the rapid industrialization, thousands of chemical compounds are produced causing air and soil pollution. Out of which, some are very toxic in nature and remain in the environment possessing a major threat to life of living organisms. Hence, therefore it is important to look for the techniques that can be employed for either the removal of these contaminants or to convert them into nonhazardous products that are eco-friendly to the environment. This is achieved by the use of enzymatic capabilities of microorganisms that break down toxic chemical compounds into end products or metabolites, which are not toxic anymore, and this whole process is known as bioremediation. Thus, such degradation is carried out by particular microbes, and to know more about it, the knowledge about the properties of these toxic chemicals such as classification, identification, environmental properties, toxicity, and distribution can enhance the biodegradation process. This technique has potential to restore the contaminated environment effectively with low cost and labor. But the information for the factors that control the growth and metabolism is still not known completely, making the implementation of it bit restricted.

Bioinformatics, which has now become the essential part of every life science-related research, has given new direction in view of bioremediation technique also. With the development of software packages and tools with the help of computational biology, bioinformatics has revolutionized the integration of it with bioremediation. In last few decades, branches of bioinformatics like genomics, proteomic, transcriptomics, or metabolomics have given a lot of contribution in exploration of bioremediation process.

Hence, bioinformatics with its multidisciplinary approach has assisted in understanding the bioremediation by unveiling the pathways, chemistry of toxic chemicals that are undisclosed for making it a process for control of environmental contamination. The aim of this chapter is to provide a complete overview of the bioinformatic approaches and its applications present in relation to bioremediation.

## 27.2 Introduction

Bioremediation is the deliberate use of microorganisms, which act as biological catalysts for removing pollutants from the environment. In general, we can say that it is an environmental science approach where natural biological actions are used to remediate the polluted groundwater and contaminated soil. A variety of pollutants like xenobiotics, polycyclic aromatic hydrocarbons known as PAHs, and chlorinated and nitro-aromatic compounds are present, which can be cancer-causing and mutagenic to all the present life forms (Zhang and Bennett 2005; Samanta et al. 2002). With the use of these microbes for biodegradation, the natural environmental conditions can be maintained efficiently. So, the role of these microorganisms (bacteria, fungi, insects, worms, etc.) in bioremediation technique has proven to maintain our planet with its greenery.

The general microbial mode of action to perform bioremediation activity is done by metabolization of a compound to another metabolite, which is not harmful to the environment. The basic principle involved in biodegradation of pollutants is either biotic or abiotic conditions. It can be done by number of known processes, such as bioventing, biopiles, bioaugmentation, biostimulation, and bioattenuation. So, the bioremediation can be effective only where environmental conditions permit microbial growth and activity, and its application often involves the manipulation of environmental parameters to allow microbial growth and degradation to proceed at a faster rate (Kumar et al. 2011; Abatenh et al. 2017).

Being the natural process, it is cheap, harmless to the ecosystem, needs less labor requirement, eco-friendly, and sustainable (Dell Anno et al. 2012).

Thus, the use of bioremediation technique is an environmental-friendly approach for restoring and sustaining the contamination-free environment for future generations.

### 27.2.1 Introduction to Bioinformatics

Bioinformatics is the combination of biology and information technology. It involves the knowledge of both. The field of bioinformatics does the computer-based analysis of biological datasets followed by its interpretation. This is done by using statistical tools and algorithms.

In understanding the bioinformatics and its applications, it is important to know about the various approaches used for performing analysis. This includes genomics, proteomics, data mining, biological databases, phylogenetic analysis, and (trancriptomics, metabolomics) system biology. All of it together plays a significant role. Figure 27.1 below shows its various branches.

### 27.2.2 Integrating Bioinformatics with Bioremediation

The role of microbes in soil and water-based biodegradation and cleaning of the environment has shown us the way to maintain and sustain a greener earth. The use of bioinformatic domain for the study of bioremediation has shown in the past the suggested promising results. With the help of bioinformatic-based applications only, it has been made possible to perform the in silico studies and analyzation of data. For uplifting the technique of bioremediation and the study of specific microbes at the molecular level including the gene-to-gene interactions, pre-requirement of conditions needed to be used for the changes at genetic level can be done only with bioinformatic strategies. Also, the bioremediation process can be enhanced using databases for gene identification and microbial degradation pathways of compounds (Ellis et al. 2001).

Thus, bioinformatics along with its branches is revolutionizing and will continue to do so in its future prospects. The pictorial representation above in Fig. 27.2 is depicting the use of bioinformatic approach for the improvement of bioremediation process.

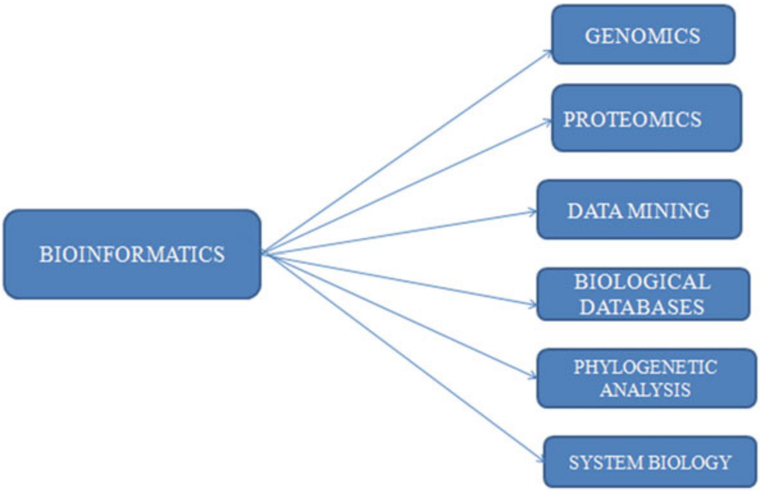


**Fig. 27.1** Branches of bioinformatics

## 27.3 Bioinformatics in Improving Bioremediation

Although microbes are known for their potential to perform biodegradation, still the process has its own limitation. And this is because of scarcity of data for factors, which control the growth and metabolism of microbes with bioremediation potential (Dua et al. 2002). Therefore, bioinformatics aids in using microarray data by enhancing the structural characterization of microbial proteins with contamination degradable capabilities (Singh 2006).

Hence, by understanding the microbial process at the molecular level with use of bioinformatic analyses we can learn about the following below mentioned aspects of bioremediation in more depth.

1. Prediction of Degradation Pathways
2. Omic-Based Approaches
3. Prediction of Toxicity of Chemicals
4. Databases

### 27.3.1 Prediction of Degradation Pathways

For the bioremediation process, a microbe undergoes enzymatic reactions to change the pollutant into a metabolite, which is not harmful. For all this, the study of enzymatic kinetic aspect is important. This includes the physical and chemical characteristics of the degradation pathway (Okoh 2006).

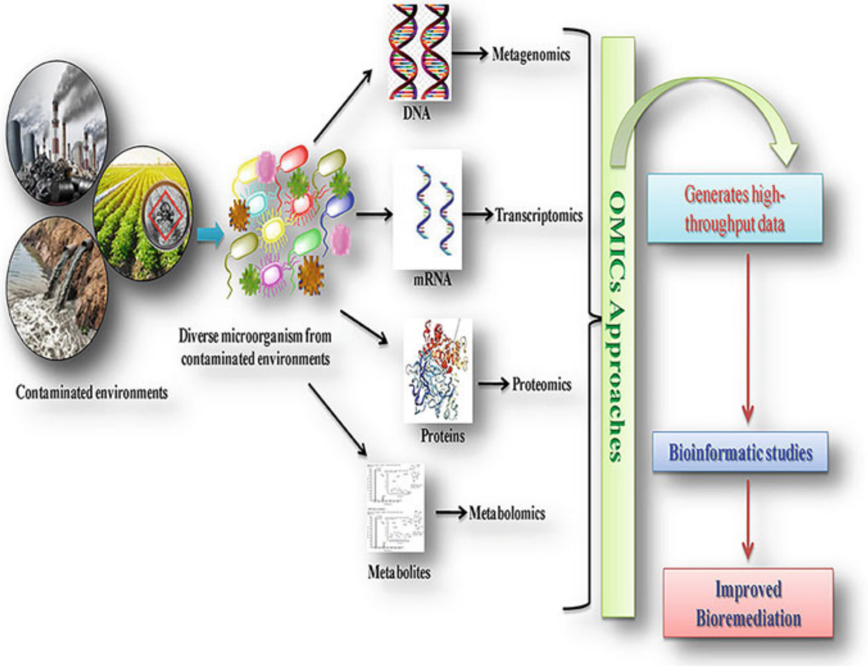


**Fig. 27.2** Pictorial representation of integrated approach of advanced technologies in biodegradation of xenobiotic compounds. (Source: Mishra, S., Lin, Z., Pang, S., Zhang, W., Bhatt, P., & Chen, S. (2021). Recent Advanced Technologies for the Characterization of Xenobiotic-Degrading Microorganisms and Microbial Communities. Frontiers in bioengineering and biotechnology, 9, 632059. https://doi.org/10.3389/fbioe.2021.632059. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC-BY). The use, distribution, or reproduction in other forums is permitted, provided the original author(s) and the copyright owner (s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice)

But for the prediction of products and pathways associated with microbial degradation by in silico methods, classification approach is required (Wicker et al. 2010). This classification can be done as knowledge-based and machine learning-based approach. Both of which have some limitations and strengths. Taking into account the *machine learning approach*:

Firstly, this approach does prediction for a biotransformation when it has a quite a general class (Gomez et al. 2007) or whether it is the substrate of some broad reaction class, e.g., oxidoreductase catalyzed reactions (Mu et al. 2006).

Next is knowledge-*based approach*:

**META:** META is a knowledge-based expert system that simulates the biotransformation of xenobiotics. It operates with the help of a dictionary (knowledge base) to seek target fragments in a compound and transform them to products (Klopman et al. 1997).

**METEOR:** It is a knowledge-based expert system for prediction of metabolism (Marchant et al. 2008).

**CATALOGIC:** It is a platform for models targeting environmental fate of chemicals. It explicitly aims at probability estimates (Dimitrov et al. 2010).

**UM-PPS:** It stands for the University of Minnesota Pathway Prediction System (UM-PPS) and comes under the UM-BBD (University of Minnesota Biocatalysis/Biodegradation Database). It is available at http://umbbd.msi.umn.edu/predict/. Presently, it contains information on almost 1200 compounds, over 800 enzymes, almost 1300 reactions, and almost 500 microorganism entries (Gao et al. 2011).

The UM-PPS predicts plausible biodegradation pathways for organic compounds on the basis of sets of biotransformation rules derived from the UM-BBD database or from the scientific literature (Fenner et al. 2008). The user can predict both aerobic and anaerobic degradation pathways of chemicals and can select whether they will view all or only the more likely aerobic transformations. Users can also obtain the most accurate prediction for those compounds similar to compounds with biodegradation pathways that have been reported in the scientific literature (Gao et al. 2011; Arora and Bae 2014).

**Usage**

1. Prediction can be made both for aerobic and anaerobic degradation pathways of chemicals, and it can be selected that whether the user will view all or only the more likely aerobic transformations.
2. Also, we can obtain the most accurate prediction for those compounds similar to those biodegradation pathways that have been reported in the scientific literature.
3. For the prediction, users may enter a compound into the system by either drawing the structure and generating SMILES or entering SMILES directly.
4. For example, the degradation pathways of 4-nitrophenol have been thoroughly investigated, while those of 2-fluro-4-nitrophenol and 2-bromo-4-nitrophenol have not. However, the structures of 2-fluro-4-nitrophenol and 2-bromo-4-nitrophenol are similar to 4-nitophenol. Therefore, PPS can provide very accurate predictions for degradation of 2-flouro-4-nitrophenol and 2-bromo-4-nitrophenol (Arora and Bae 2014).

#### 27.3.1.1 PathPred

It is a knowledge-based prediction system, which uses data derived from the Kyoto Encyclopedia of Genes and Genomes (KEGG) in the form of KEGG REACTION and KEGG RPAIR database. The KEGG RPAIR database has collection of biochemical structure transformation patterns, called RDM patterns, and chemical structure alignments of substrate–product pairs (reactant pairs) in all known enzyme-catalyzed reactions taken from the enzyme nomenclature and the KEGG PATHWAY database (Moriya et al. 2010).

It is a web-based server available at http://www.genome.jp/tools/pathpred/. It predicts plausible pathways of multi-step reactions starting from a query compound, based on the local RDM pattern match and the global chemical structure alignment against the reactant pair library. The server provides transformed compounds and reference transformation patterns in each predicted reaction and displays all predicted multi-step reaction pathways in a tree-shaped graph (Moriya et al. 2010). It basically aims at predicting pathway for microbial biodegradation of environmental compounds and biosynthesis of plant secondary metabolites.

#### Usage

The PathPred server can be used for predicting microbial biodegradation pathways of xenobiotics in bacteria and biosynthesis pathways of secondary metabolites in plants. This can be done by

1. Selecting Reference Pathway—the user is requested to choose the reference pathway for either of biosynthesis and biodegradation, which determines the subset of RDM patterns to be utilized.
2. Query Format—the query can be inputted as a query compound in the MDL mol file format, in the SMILES representation, or by the KEGG compound/drug identifier (C/D number). This compound, termed initial compound, corresponds to the compound to be degraded or the compound to be synthesized.
3. Output—The output of the PathPred server shows the prediction results as tree-shaped graph. For example, the biodegradation prediction of glycolate (C00160) from 1,2,3,4-tetrachlorobenzene. The output tree graph predicts the other possible pathways including biodegradations through known compounds such as 3,4,6-trichlorocatechol (C12831), 6-chlorobenzene-1,2,4-triol(C06328), and 1,2,4-trichlorobenzene (C06594) (Fig. 27.3).

### 27.3.1.2 BNICE

It stands for Biochemical Network Integrated Computational Explorer, a computational approach developed to generate every possible biochemical reaction based on a set of enzyme reaction rules of the enzyme commission (EC) and starting compounds (Finley et al. 2009). In general, it predicts whether a particular compound is biodegradable and whether alternate routes can be engineered for compounds already known to be biodegradable.

The BNICE screens out all possible pathways for thermodynamic feasibility based on the Gibbs free energies of the reaction and selects feasible novel thermodynamic pathways (Soh and Hatzimanikatis 2010). Hence, it is used to (1) study the combinatorial nature of polyketide synthesis (Gonzalez-Lergier et al. 2005), (2) to provide systematic framework for linking of enzymatic chemistry and reactive sites of metabolic compounds (Hatzimanikatis et al. 2004), and (3) for the prediction of biodegradation pathways of compounds, which represent various classes of xenobiotics.

Further, it has been also suggested by Soh and Hatzimanikatis et al. that the pathways generated by BNICE can be further evaluated using established pathway analysis approaches, such as thermodynamic-based flux balance analysis (FBA) Grow match allows investigation of the overall effects of these novel pathways on metabolic network performance in host organisms. FBA can help predict maximum yield, phenotypic changes, effects of gene knockouts, changes in bioenergetics of the

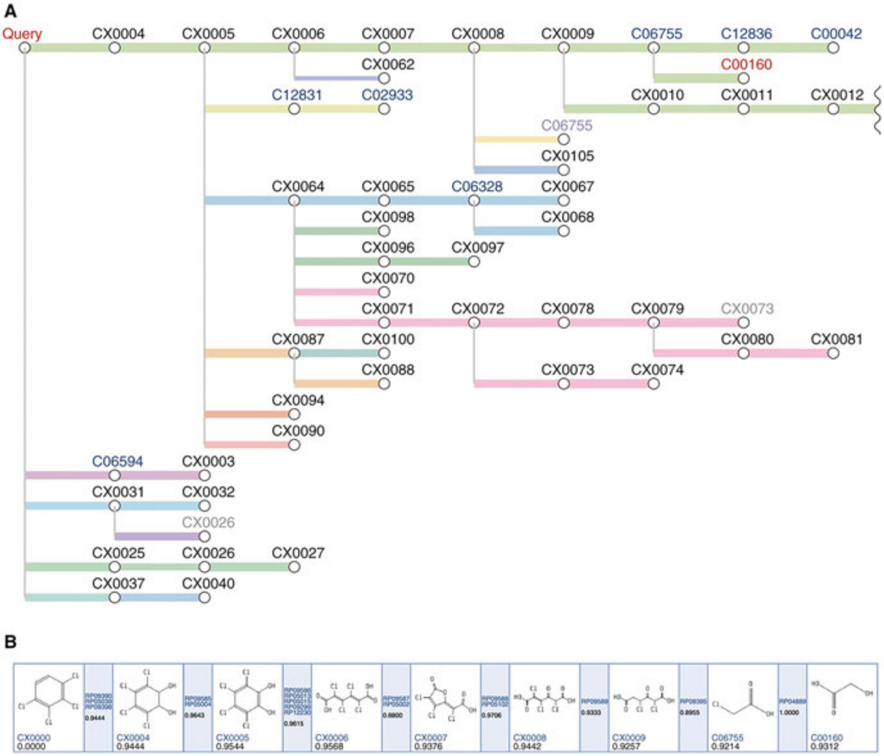


**Fig. 27.3** Example of the predicted pathway tree of tetrachlorobenzene biodegradation (**a**) and the detail of the top green pathway from the query compound (query) to the final compound (C00160) (**b**). Structure images popup when the mouse is moved over nodes and edges in the tree if JavaScript is enabled in the web browser. (Source: Moriya Y, D. Shigemizu, M. Hattori, T. Tokimatsu, M. Kotera, S. Goto, Minoru Kanehisa, PathPred: an enzyme-catalyzed metabolic pathway prediction server, *Nucleic Acids Research*, Volume 38, Issue suppl_2, 1 July 2010, Pages W138–W143, https://doi.org/10.1093/nar/gkq318. Reused with Licence Number 5125780225626 dated August 11, 2021)

system for metabolic engineering, and synthetic biology (Soh and Hatzimanikatis 2010).

## Usage

The BNICE framework searches for pathways by considering the starting compound and/or products, the requested length of the pathway, and the range of reactions to search over (Henry et al. 2010; Medema et al. 2012).

The user can also choose to search for a number of possibilities, either by searching for a pathway using enzyme reactions from known pathways, by combination of multiple pathways, or the whole metabolic network (Henry et al. 2010; Hatzimanikatis et al. 2005). A set of molecules is given as an input and every molecule is evaluated to determine whether it has the appropriate functionality to undergo reactions corresponding to the specified reaction classes (Bashir Sajo and Mohd 2015).

While predicting the possible pathways the BNICE predicts more than 10,000 different pathways for the biosynthesis and degradation of the compound of interest, due to the fact that the system relies on few criteria. However, Henry et al. had pioneered a prioritization approach in this framework, in which generated pathways are ranked according to four criteria: pathway length, thermodynamic feasibility, maximum achievable yield, and maximum achievable activity.

Output: The output of the BNICE is a graph-theoretic matrix representation of biochemical compound, enzyme reaction rules, and molecules. It is represented using the bond-electron matrix (BEM) where each atom in a molecule is represented by a row and column. The BEM is characterized by diagonal elements, which denote the non-bonded valence electrons and non-diagonal elements, which give the connectivity via bonding between different atoms and the bond order between atoms (Hatzimanikatis et al. 2005).

#### 27.3.1.3 DESHARKY

It is a Monte Carlo algorithm, which finds a metabolic pathway from a target compound by exploring database of enzymatic reactions. It predicts a possible route connecting the specified target metabolism with the host metabolism, instead of using pathway selection by enumeration of possible metabolic routes. It finds pathway within shortest possible time by computing its associated genetic burden. Also, it can be used also in distributed computing to sample most of the solution spaces (Rodrigo et al. 2008).

##### Usage

The algorithm is implemented in C/C+ +, and it is easily compiled and runs in UNIX environment (e.g., in Linux or in Windows using Cygwin). The algorithm calculates thermodynamic favorability and energy loss in transcription and translation.

The input of the algorithm is usually the target compound, while its output is the designed metabolic pathway together with quantification of the transcriptional, translational, and metabolic load (Rodrigo et al. 2008). This framework also provides the sequence of amino acids of the enzyme involved in the pathway.

Output: The output is the designed metabolic pathway together with the quantification of the transcription, translation, and metabolic load. It provides the sequence of amino acids of the enzymes involved in the pathway. These amino acid sequences provided are usually the closest phylogenetically to Escherichia coli according to KEGG classification of organisms (Rodrigo et al. 2008).

#### 27.3.1.4 FMM

It stands for from metabolite to metabolite, a web server. It is available freely at http://FMM.mbc.nctu.edu.tw/. It can reconstruct metabolic pathways from one metabolite to another metabolite among different species, based mainly on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database and other integrated biological databases (Chou et al. 2009). Even though KEGG maps utilized in many metabolic tools, none of them can connect metabolites from different KEGG maps. FMM supports the connection of different KEGG maps.

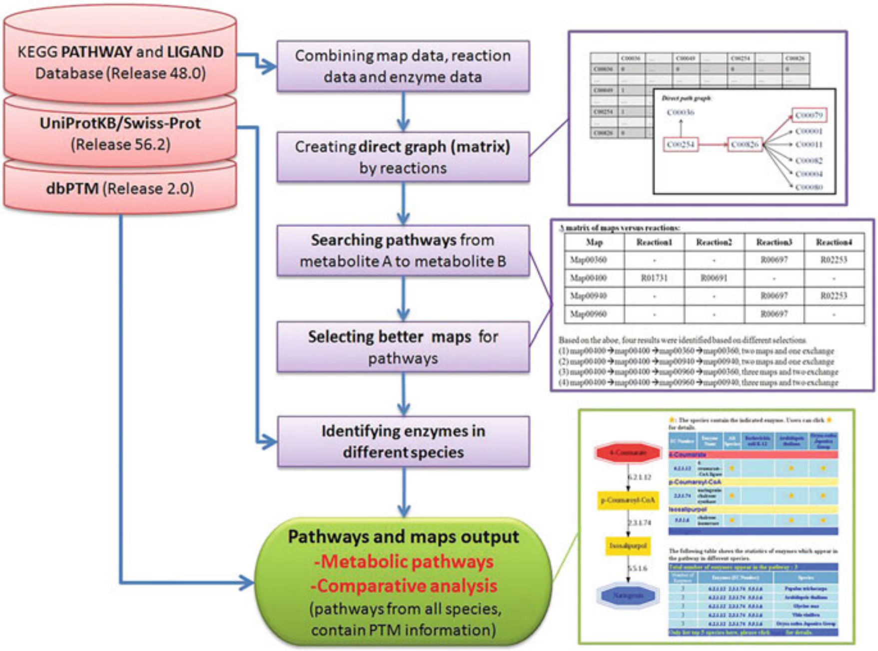
FMM has many applications in synthetic biology and metabolic engineering. For example, the reconstruction of metabolic pathways to produce valuable metabolites or secondary metabolites in bacteria or yeast is a promising strategy for drug production. FMM provides a highly effective way to elucidate the genes from which species should be cloned into those microorganisms based on FMM pathway comparative analysis (Chou et al. 2009).

#### Usage

1. Data collection and Integration:
   Reaction definitions, species-specific reactions, reaction maps, and enzyme list can be obtained from KEGG/LIGAND and KEGG/PATHWAY databases recent releases. Information such as gene names, enzyme commission numbers, and species-specific enzymes can be retrieved from UniProtKB/Swiss-Prot and NCBI taxonomy databases. Additionally, the data in FMM are usually updated on a regular basis.
2. Construction of reaction matrix information on reactions and enzymes can be obtained from KEGG maps and the equation of each reaction can be determined. Therefore, reaction matrices can be constructed based on maps, reactions, and enzyme data.
   The workflow of FMM in above Fig. 27.4 shows the reaction matrix, which was developed to identify numerous reaction processes from one metabolite to another. Enzyme annotations from UniProtKB/Swiss-Prot (Boutet et al. 2007) were employed to identify enzymes from different species in comparative analysis.
3. Reconstruction of metabolic pathway from various KEGG pathway maps: After all possible reaction paths were identified, the number of pathway maps was calculated. Usually, found paths occurred not in only a single pathway map, but also in a complicated fashion in several maps. Pathway maps that contain the most paths are selected and the one pathway map that has only one reaction is avoided. A matrix of maps versus reactions was employed to reconstruct metabolic pathway from different KEGG maps.
4. Comparative Analysis: Comparative analysis provided in FMM is useful in synthetic biology. Comparative analysis provides an easy way to elucidate which genes from which species should be cloned into those microorganisms. First, the enzymes identified in the reconstructed pathway were processed to search for orthologous encoding genes from various species. Then, the presence or absence of the pathway in a particular species can be known.

### 27.3.1.5 RetroPath

It is a server, which applies a retrosynthetic approach, a concept originally proposed for synthetic chemistry, which uses reverse chemical transformations (reverse enzyme-catalyzed reactions in the metabolic space) starting from the desired target compound to identify the reactants (precursors) that are indigenous to the selected



A matrix of maps versus reactions:

| Map | Reaction1 | Reaction2 | Reaction3 | Reaction4 |
|---|---|---|---|---|
| Map00360 | - | - | R00697 | R02253 |
| Map00400 | R01731 | R00691 | - | - |
| Map00940 | - | - | R00697 | R02253 |
| Map00960 | - | - | R00697 | - |

Based on the aboe, four results were identified based on different selections.
(1) map00400 →map00400 →map00360 →map00360, two maps and one exchange
(2) map00400 →map00400 →map00940 →map00940, two maps and one exchange
(3) map00400 →map00400 →map00960 →map00360, three maps and two exchange
(4) map00400 →map00400 →map00960 →map00940, three maps and two exchange

**Fig. 27.4** Workflow of FMM. (Source: Chou, C. H., Chang, W. C., Chiu, C. M., Huang, C. C., & Huang, H. D. (2009). FMM: a web server for metabolic pathway reconstruction and comparative analysis. *Nucleic acids research*, *37*(Web Server issue), W129–W134. https://doi.org/10.1093/nar/gkp264. Reproduced with License no 5125960782113 dated August 11, 2021)

host (Carbonell et al. 2012). It is available at http://www.issb.genopole.fr/~faulon/retropath.php.

This method of metabolic pathway design is unique because it addresses the complexity problem by coding substrates, products, and reactions into molecular signatures. The approach used by RetroPath is characterized by metabolic maps, which are represented in hypergraphs. The complexity involved in the reactions is controlled by varying the specificity of the molecular signature. Each signature has different "heights," *h*, that correspond to levels of structural detail. The height can be varied, which reduces the number of reactions that can be generated (Carbonell et al. 2011).

The proliferation of several metabolic databases with rich information is considered to be a significant breakthrough. KEGG that is a database resource integrated with chemical and systematic functional information and genomics is linked to RetroPath, where information on the reactions predicted using this framework can be found in KEGG. BRENDA (Schomburg et al. 2013) is another database that contains one of the largest collections functional enzyme data. Incomplete knowledge or gaps still exist in many cases, especially when looking for novel ways to synthesize a target compound of interest (Carbonell et al. 2013).

To successfully achieve a heterologous pathway design, the process needs to be rationalized by following the principles of synthetic biology: modeling of the biological system of interest, modular design through standardization, goal-oriented optimization, and experimental validation (Carbonell et al. 2013).

#### Usage

In the research study done by Carbonell et al. (2013), they have suggested that for retrosynthetic design of heterologous pathways, the following steps will be required: (1) host chassis selection, (2) in silico model selection for the chassis from BiGG (Schellenberger et al. 2010) or biomodels (Le Novere et al. 2006), (3) definition of the metabolic space, (4) pathway enumeration, (5) gene selection, (6) estimation of yields by metabolic analysis software, e.g., COBRA, OptFlux (Rocha et al. 2010), and COPASI (Hoops et al. 2006; Schaber 2012), (7) toxicity prediction of pathway metabolite, (8) definition of an objective function to select the best pathway to engineer, and (9) pathway implementation and validation (Fehér et al. 2014).

### 27.3.1.6 Metabolic Tinker

It is a web tool used to design synthetic metabolic pathways between user-defined target and source compounds. The interface is available at http://osslab.ex.ac.uk/tinker.aspx. It uses a tailored heuristic search strategy to search for thermodynamically feasible paths in the entire known metabolic universe. The program contains a directed graph known as universal reaction network (URN), which represents the entire set of known reactions and compounds from the Rhea database (McClymont and Soyer 2013). Nodes and edges on this graph represent metabolites and reactions, respectively, and thus, the entire graph represents the current known metabolic universe. This tool searches possible biochemical paths between two compounds within this URN using standard search algorithms developed in computer science and graph theory (McClymont and Soyer 2013). To complete the search, the Rhea/CHEBI identification codes of both the source and target compounds are needed.

### 27.3.1.7 Carbon Search

It is an algorithm-based approach, which identifies pathways within existing metabolic networks by tracking the conservation of atoms moving through them. On the basis of this approach, two algorithms are developed that find metabolic pathways by using atom mapping data to track the movement of atoms through metabolic networks. One algorithm finds linear pathways, and the other algorithm finds branched pathways. They both take as input atom as mapping data, a start compound, a target compound, and a minimum number of atoms to conserve and a maximum number of pathways to return (Heath et al. 2010). In the output, a set of metabolic pathways, which conserve at least given number of atoms from the start compound to the target compound, are returned. They have also demonstrated that this carbon search tool based on the algorithms has efficiently identified both linear and branched metabolic pathways, in which a certain threshold of atoms is conserved. The resulting metabolic pathways are validated on known functional pathways. The algorithms are having the potential to find novel or alternative pathways that may span multiple organisms (Heath et al. 2010).

Using this atom tracking approach, earlier Pitkänen et al. in 2009 have also enabled graph theoretical-based method for finding biologically meaningful linear and branched metabolic pathways in genome-scale metabolic networks.

#### 27.3.1.8 The Furusawa Platform

It is an in silico platform that uses a developed algorithm for finding feasible heterologous pathways by which non-native target metabolites are produced by microorganisms, using Escherichia coli, *Corynebacterium glutamicum,* and *Saccharomyces cerevisiae* as templates (Chatsurachai et al. 2012).

##### Usage

The usage of this platform for heterologous pathway design comprises of following four steps:

1. Construction of an in-house database of metabolic reactions—This is done by considering known metabolic reactions from KEGG ligand section database and BRENDA. These metabolic reactions are considered as candidate heterologous reactions that could be added to the host metabolic networks (Chatsurachai et al. 2012). All metabolic reaction information regarding genes, enzymes, pathways, and organism in the KEGG database can be collected into the database. The information collected the information in a constructed database using PostgresQL. The enzymatic information employed can be retrieved from BRENDA, and python script can be used to access the constructed in-house database (Chatsurachai et al. 2012).
2. Genome-scale metabolic models of host microorganisms—The microorganisms that are widely used in industry were adopted as chassis templates to demonstrate the viability of it on in silico platform. This includes *Escherichia coli*, *C. glutanicum,* and *S. cerevisiae*, which were selected based on a number of criteria such as having high growth activity under various conditions, ease of genetic manipulation, and hence are considered as ideal hosts for bioengineered products (Chatsurachai et al. 2012).
3. Heterologous pathway identification for target production—The developed platform can be used to screen all producible target metabolites listed in the database by adding heterologous reactions to host microorganisms. For all producible target metabolites, the user can estimate the production yields using FBA, assuming steady-state conditions and the maximum biomass production rate (Chatsurachai et al. 2012). The entire list of producible target metabolites in different hosts can be analyzed, and a set of rational heterologous pathways and hosts can be selected that will likely produce the desired targets.
4. Flux balance analysis (FBA)—FBA is based on a genome-scale metabolic model and optimization of a specific objective flux by linear programming. One can use FBA to estimate the metabolic flux profile of metabolic networks expanded with heterologous reactions. All FBA simulations in this framework can be performed under the MATLAB interface (Chatsurachai et al. 2012).

## 27.3.2 Omic-Based Approaches

### 27.3.2.1 Proteomics

According to Keller and Hettich (2009) and Aslam et al. (2017), proteomics has emerged as an interesting and fruitful technology for the study of protein expression (it includes post-translational modifications, protein turnover, proteolysis, and changes in the corresponding gene expression) of the microbial world. Proteomics has been used to identify microbial communities/microorganisms in various ecosystems including soil and sediment, activated sludge, marine and groundwater sediment, acid mine biofilms, and wastewater plants (Williams et al. 2013; Colatriano et al. 2015; Grob et al. 2015; Bastida et al. 2016; Jagadeesh et al. 2017). Thus, the inclusion of a proteomic approach helps to identify related enzymes and their metabolic pathways in the bioremediation of xenobiotics from various contaminated sites (Liu et al. 2017; Wei et al. 2017). Studies also revealed important and hidden information related to protein synthesis, gene expression stability, mRNA turnover, and protein–protein interaction networks in microbial communities in stress environments Aslam et al. (2017). Hence, the studies related to proteomic analysis plays important role for bioremediation process.

*Protein Analysis:* Generally, there are four primary steps that involve proteomic analysis of microbial communities:

1. preparation of a biological sample;
2. extraction and separation of proteins by using two-dimensional gel electrophoresis (2D-GE);
3. protein gel images are examined by means of image analysis software such as ImageMaster 2D or PDQuest; and
4. proteins are identified by using mass spectroscopy (MS)/MALDI-TOF/MS or LC-MS (Yates et al. 2009; Chakka et al. 2015; Velmurgan et al. 2017).

The workflow of proteomic analysis is shown below in Fig. 27.5.

#### Applications of Proteomics in Bioremediation

1. The bioremediation of compounds done by microorganisms has shown involvement of several proteins. This is demonstrated by the study done by Vandera et al. (2015). In their study, they have done comparative proteomic analysis of *Arthrobacter phenanivorans* Sphe3 on aromatic compounds phenanthrene and phthalates. The proteomic approach confirmed the involvement of several proteins in aromatic substrate degradation by identifying those mediating the initial ring hydroxylation and ring cleavage of phenanthrene to phthalate. This study also revealed the presence of both the ortho- and meta-cleavage pathways for the degradation of these aromatic compounds, and it also identified all proteins that take part in these pathways and are highly upregulated upon phthalate growth in comparison with phenanthrene growth.
2. The proteomic analysis of pyrene-degrading bacterium *Achromobacter xylosoxidans* PY4 done by Nzila et al. (2018) has identified a total of 1094

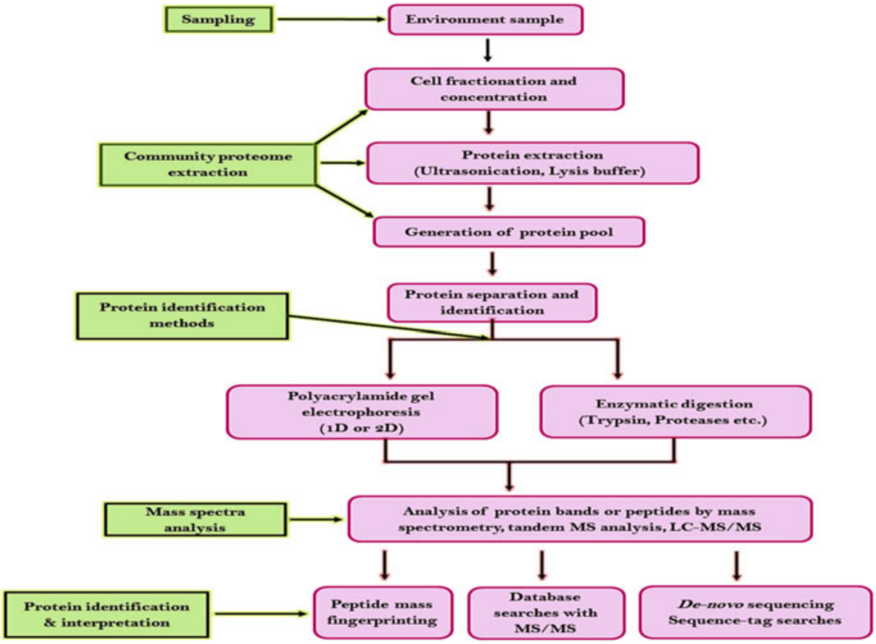


**Fig. 27.5** Workflow of proteomic analysis. (Source: Chandran, H., Meena, M., & Sharma, K. (2020). Microbial Biodiversity and Bioremediation Assessment Through Omics Approaches. *Frontiers in Environmental Chemistry*, *1*, 9. https://doi.org/10.3389/fenvc.2020.570326. Frontiers is fully compliant with open-access mandates, by publishing its articles under the Creative Commons Attribution Licence (CC-BY))

proteins. Out of which, 95 proteins were detected in glucose supplementation, and 612 proteins were detected in the presence of pyrene. Furthermore, they have found 25 upregulated proteins to be involved in stress response and the progression of genetic information. Two upregulated proteins, 4-hydroxyphenylpyruvate dioxygenase and homogentisate 1,2-dioxygenase, are implicated in the lower degradation pathway of pyrene. Enzyme 4-hydroxyphenylpyruvate dioxygenase may catalyze the conversion of 2-hydroxybenzalpyruvic acid (metabolite of pyrene) to homogentisate. Homogentisate 1,2-dioxygenase is involved in the incorporation of 2 oxygen atoms to produce 4-maleyacetoacetate, which is an intermediate in several metabolic pathways (Nzila et al. 2018).

3. Lee et al. (2016) have performed proteomic analysis of PAH-degrading bacterial isolate *Sphingobium chungbukense* DJ77. This strain exhibited outstanding degradation capability for various aromatic compounds. With this study, it was demonstrated that the degradation of three xenobiotic compounds, i.e., phenanthrene, naphthalene, and biphenyls (PNB), and their associated proteins was analyzed by 2-DE and MALDI-TOF/MS analysis. During PNB biodegradation by bacterial cells, an alteration was observed in protein expression to cope with the stress condition.

4. In year 2019, Chen et al. have investigated a biodegradation mechanism of tetrabromobis-phenol (TBBPA) in *Phanerochaete chrysosporium* by using a proteomic approach. With aid of this approach, they have found that compared to control TBBPA, stress caused 148 differentially expressed proteins in *P. chrysosporium*, among which 90 proteins were upregulated and 58 proteins were downregulated. The upregulation of cytochrome p450 monooxygenase, glutathione-*S*-transferase, *O*-methyltransferase, and other oxidoreductases is responsible for the biotransformation of TBBPA via oxidative hydroxylation and reductive debromination.
5. Another bioremediation study with proteomic analysis was performed by Yu et al. (2019). It was of decabromodiphenyl ether (BDE-209). It was explored in *Microbacterium Y2* in a polluted water-sediment system. The results of study have shown that the overexpression of haloacid dehalogenases, glutathione *S*-transferase, and ATP-binding cassette (ABC) transporter might occupy important roles in BDE-209 biotransformation. Moreover, heat-shock proteins (HSPs), ribonuclease E, oligoribonuclease (Orn), and ribosomal proteins were activated to counter the BDE-209 toxicity. Thus, it is suggested that these proteins are implicated in microbial degradation, antioxidative stress, and glycolysis.
6. Another application of proteomics in bioremediation is researched by Gregson et al. (2020). It was reported that LC–MS/MS shotgun proteomics is used to determine variations in the proteome of hydrocarbon-degrading psychrophile *Oleispira antarctica RB-8* when grown on *n*-alkanes in cold temperatures.

#### 27.3.2.2 Genomics and Metagenomics

Genomics is the powerful computer technology used to understand the structure and function of all genes in an organism based on knowing the organism’s entire DNA sequence. This field includes intensive efforts to determine the entire DNA sequence of organisms and in-depth genetic mapping efforts (Fulekar and Sharma 2008).

Whereas metagenomic studies unblock the traditional ways of uncultured microorganisms and explore their genetic advantages in the process of bioremediation (Rahimi et al. 2018; Nascimento et al. 2020). It uses the pool of environmental genomes of microorganisms, which increases the probability to discover unique genes and diverse pathways with new enzymes containing highly specific catalytic properties (Scholz et al. 2012; Yergeau et al. 2017; Awasthi et al. 2020). This technology gives a new parade to microbiologists for understanding unculturable microbiota with a genetic variability of microbial communities (Devarapalli and Kumavnath 2015; Zhu et al. 2018; Awasthi et al. 2020). Hence, metagenomic information will enable researchers to integrate pure culture study with genomics (Hodkinson and Grice 2015). Current metagenomic practices allow for identifying the whole-genome structure of microorganisms and specifying particular genes that are attributed to encode degradative enzymes for the mineralization of xenobiotics (Zafra et al. 2016; Zhu et al. 2020). This clearly highlights the crucial role of novel genes in connecting the entire microbial population with functional diversity and structural identity. Based on it, the metagenomics involves the manufacturing of metagenomic libraries. With the help of these, biological information can be retrieved from these metagenomic libraries by two types of analysis:

1. Sequence-Driven Analysis: This analysis is based on the sequencing of clones with phylogenetic anchors or conserved DNA sequences, which is the plausible origin of the DNA fragment (Wu et al. 2010; Felczykowska et al. 2015; Wong 2018). This type of analysis is increasingly used owing to the availability of several software packages for data analysis and the ease to assess metagenomic sequencing data. This approach is predominantly influenced by the precision of genome annotation, the integrity of the available data, algorithms, and facts in databases to ascertain the function of novel genes (Ferrer et al. 2009).

   The complete genome analysis or sequence analysis is progressed through three technical transformations:

   (a) *First-Generation Sequencing*—Frederick Sanger and Allen Maxam Walter Gilbert sequencing techniques were categorized as the first-generation DNA sequencing methods. Sanger sequencing uses denatured DNA template, radioactively labeled primer, DNA polymerase, and chemically modified nucleotides called di-deoxynucleotides to generate DNA fragments with various lengths.

   (b) *Next-Generation Sequencing*—It is also called high-throughput sequencing. Next-generation sequencing involves library preparation, sequencing, base calling, alignment to the established genome, and assorted annotation. Library preparation begins with the fragmentation of DNA into multiple fragments by sonication, enzymatic digestion, or transposase followed by ligation with adaptor sequences. The prepared library is then amplified using clonal amplification and PCR methods to generate DNA replicas. DNA replicas are then sequenced using different approaches (Samorodnitsky et al. 2015). The major platforms used for microbiome studies in next-generation sequencing are pyrosequencing (Roche/454 sequencing), Illumina, SOLiD, Ion Torrent, PacBio RS, etc.

   These are high-throughput sequencing techniques of ribosomal genes that quantify community structures and functions at a higher resolution, e.g., 16S rRNA in prokaryotes, and 5S or 18S rRNA genes, or the internal-transcribe-spacer (ITS) region in eukaryotes (Luo et al. 2012). The effectiveness of such NGS technologies in analyzing microbial communities from diverse environments was elucidated, validated, and documented in many studies (Brown et al. 2013; Shokralla et al. 2014; Zhou et al. 2015; Niu et al. 2016; Scholer et al. 2017).

   (c) *Third-Generation Sequencing*—It is also called single-molecule long-read sequencing. It offers lower sequencing charge and contented sample preparation without PCR amplification. The two most widely used sequencing platforms in third-generation sequencing are Pacific Biosciences, Oxford Nanopore Technology, and Helioscope technology.

   The competitive analysis of platforms used in second and third-generation sequencing is discussed below in Table 27.1.

**Table 27.1** Comparative analysis of different platforms used for second- and third-generation Sequencing. Source: Chandran, H., Meena, M., & Sharma, K. (2020). Microbial Biodiversity and Bioremediation Assessment Through Omics Approaches. *Frontiers in Environmental Chemistry*, *1*, 9. https://doi.org/10.3389/fenvc.2020.570326. Frontiers is fully compliant with open-access mandates, by publishing its articles under the Creative Commons Attribution Licence (CC-BY)

| S No: | Omics technologies | Principle | Advantages | Disadvantages | Applications |
|---|---|---|---|---|---|
| I | Second generation sequencing platforms | | | | |
| 1. | Pyrosequencing | Sequencing by synthesis established on the identification of pyrophosphate discharged after nucleotide amalgamation in the newly synthesized DNA strand | Fast and firm method with real-time read out appropriate for sequencing short fragments of DNA, low cost, nucleotide dispensation easily programmable, alterations in the pyrogram pattern reveal mutations, deletions and insertions | Generation of long homopolymers generating sequencing errors, difficulty in deciding the quantity of integrated nucleotides in homopolymeric areas | Identification of microbes, whole genome sequencing |
| 2. | Illumina | Sequence-by-synthesis method | Cells not required, highest throughout, produce relatively short reads with length up to 300 bp, lowest cost per base output compatible with most applications | Slow, short reads, high cost of reagents, aberrant incorporation of incorrect dNTPs by polymerases | Gene expression studies to identify isoforms, novel transcripts, gene fusions, exocrine sequencing, whole genome sequencing |
| 3. | Ion Torrent | Sequencing established through the recognition of hydrogen ions released during polymerization of DNA | Produces reads up to 400 bp length, less run time, reliable and cost-effective tool, high accuracy and short run time | High error rates for specific regions | To study microbial diversity in complex ecosystems, targeted, exome, transcriptome, *de novo*, small RNA sequencing |
| 4. | ABI SOLiD system | Sequencing technology based on ligation of DNA fragments | High accuracy since each base is read twice | relatively short reads and log run times | Whole genome sequencing, targeted sequencing, transcriptome research, epigenome analysis |

| | | | | | |
|---|---|---|---|---|---|
| II | Third generation sequencing platforms | | | | |
| 1. | Pacific biosciences SMRT sequencing | Sequencing through synthesis method and real time detection of integrated fluorescently labelled nucleotides | Long reads, high accuracy, uniform coverage, single-molecule resolution | Longer reads make create scaffolds in repeat regions | Whole genome sequencing targeted sequencing, RNA sequencing, epigenetic studies, study complex populations |
| 2. | Oxford nanopore sequencing | Measurement of physical changes when DNA sequence translocates through nanometer size pores under influence of electric domain | Cheap, fast and accurate DNA sequencing, longer reads, better resolution, small size | Based errors, high cost per read | DNA, RNA or protein analyses |
| 3 | HeliScope | Single-molecule sequencing platform using a highly sensitive fluorescence detection system | Large number of single molecules read, to reduce high error rates | Short reading sequence, the sequencing process deteriorate from numerous biases due to inadequate clonel amplification and DNA extension devastation | Re sequencing, transcript counting |

***Shotgun Sequencing***

It is also called shotgun metagenomic sequencing. It is a powerful technique in microbial ecology because it provides a vigorous and reliable evaluation of microbial diversity (Hillmann et al. 2018). It does not depend on PCR amplification and is used to examine the functional potential and microbial composition of the community.

Importance of shot-gun sequencing in bioremediation

(a) It is the only way to study the microbial community with no markers like viruses (Quince et al. 2017; Vermote et al. 2018).
(b) It allows strain-level remodeling in the taxonomic analysis and pathway predictions for the functional annotation of the microbiome under study (Han et al. 2020).
(c) It is an emerging molecular method to bridge the gap amid community structure and functional competence.
(d) It also helps in understanding the strategies adopted by microorganisms to thrive in adverse conditions (Sharpton 2014; Peabody et al. 2015; Ranjan et al. 2016).
(e) This techniques workflow for taxonomy analysis consists of quality pruning and evaluation of a reference database involving whole genomes or specifically designed marker genes to create a taxonomy profile. Since it contains all genetic information in a sample, the information can be used for supplementary analyses like metagenomic assembly and binning, metabolic function profiling, and antibiotic-resistant gene profiling (Chandran et al. 2020).
(f) Shotgun metagenomic analysis of microbial communities from deep seabed petroleum seeps in the Eastern Gulf of Mexico revealed the presence of diverse communities of chemoheterotrophs and chemolithotrophs (Dong et al. 2019).
(g) Whole-genome shotgun sequencing was engaged to identify the taxonomic diversity and gene repertoire of bacteria isolated from tannery effluents and petrol-polluted soil samples for degradation of persistent organic pollutants like naphthalene, toluene, petrol, and xylene (Muccee and Ejaz 2020).

2. Function-Driven Analysis: The function-driven analysis is based on the identification of clones that express their functional activity. If the sequence analogy does not complement to a functional association or the original gene has less analogy to some genes whose products have been investigated biochemically or a specific gene is capable to accomplish diverse tasks in the cell (Hallin et al. 2008), then in such cases, function-driven screening is preferred to discover genes with novel functions or to explore the sequence variation of protein families (Singh et al. 2009; Meena et al. 2016). The workflow below is showing the general methodology used for metagenomic research in Fig. 27.6.

### Applications of Metagenomics

1. With metagenomic analysis, research area has increased to analyze microbial communities, their genetic diversity, and metabolic pathways. It has provided opportunities to discover microbial consortia and genes involved in the

**Fig. 27.6** Workflow of Metagenomic Research. (Source: Chandran, H., Meena, M., & Sharma, K. (2020). Microbial Biodiversity and Bioremediation Assessment Through Omics Approaches. *Frontiers in Environmental Chemistry*, *1*, 9. https://doi.org/10.3389/fenvc.2020.570326. Frontiers is fully compliant with open-access mandates, by publishing its articles under the Creative Commons Attribution Licence (CC-BY))

bioremediation of xenobiotic compounds. For example, phenol-degrading pathways of uncultivated bacteria in activated sludge were studied using metagenomics (Sueoka et al. 2009).

2. The metagenomic approach was used by Silva et al. (2013) to characterize genes and metabolic pathways associated with the degradation of phenol and other aromatic compounds in sludge from a petroleum refinery wastewater treatment system.
3. Also, Jeffries et al. in 2018 have employed metagenomic analysis to outline the functional potential and taxonomic community composition, and to predict the

breakdown of chemical compounds of soils with organophosphorus pesticide exposure.

4. A combined physical and chemical analysis along with metagenomics was done by Gaytán et al. (2020) to explicate probable metabolic pathways associated with polyurethane-degrading to alleviate plastics and xenobiotic pollution.
5. Studies are done by Aubé et al. (2020), using metagenome and enriched mRNA metatranscriptome sequencing on the persistent impact of petroleum pollutants on the taxonomic and metabolic structure of microbial mats.
6. Auti et al. (2019) have demonstrated that 16S rRNA gene sequencing analysis is a highly recommended cost-effective technique for the phylogenetic resolution and taxonomic profiling of microbial communities. As 16S rRNA gene sequence similarity between two strains provides a simple yet robust criterion for the identification of newly isolated strains, whereas phylogenetic analyses can be used to elucidate the overall evolutionary relationship between related taxa (Johnson et al. 2019).
7. Using metagenomic approach, Zhu et al. (2020) have explored microbial assemblage and functional genes potentially involved in upstream and downstream phthalate degradation in soil. Results of which indicate that bacterial taxon Actinobacteria (*Pimelobacter*, *Nocardioides*, *Gordonia*, *Nocardia*, *Rhodococcus*, and *Mycobacterium*) was a major degrader under aerobic conditions, and bacterial taxa Proteobacteria (*Ramlibacter* and *Burkholderia*), Acidobacteria, and Bacteroidetes were involved under anaerobic conditions.
8. By metagenomic analysis, Hidalgo et al. (2020) in their research have exposed that the members of *Geobacteraceae* and *Peptococcaceae* microbiota present in the jet-fuel-contaminated site could be exploited for their remarkable metabolic potential for the mitigation of toluene and benzene.

#### 27.3.2.3 Transcriptomics

Transcriptomics is the study of an organism's transcriptome, i.e., the sum of all of its RNA transcripts. The information content of an organism is recorded in the DNA of its genome and expressed through transcription. Here, mRNA serves as a transient intermediary molecule in the information network, while noncoding RNAs perform additional diverse functions. A transcriptome captures a snapshot in time of the total transcripts present in a cell (Lowe et al. 2017).

It is also called gene expression profiling because it provides the understanding of up- or downregulation of genes under various environments in microbial communities. mRNA analysis provides a direct vision of cell and tissue-specific gene manifestation like (1) the existence, nonexistence, and assessment of transcript, (2) assessment of alternative splicing to foresee protein isoforms, and (3) quantitative evaluation of genotype impact on gene expression via expression assessable trait loci analysis or allele-specific expression (Chandran et al. 2020). Thus, transcriptomic analysis provides a large amount of gene information about the potential function of microbial communities in adaptation and survival in extreme environments (Singh et al. 2018).

There are a number of techniques in transcriptomics that supports in reviewing and evaluating mRNA expression of an organism. This includes the following:

1. Microarrays: DNA microarray is a powerful technique in transcriptomics that supports in reviewing and evaluating mRNA expression of every single gene existing in an organism. The technique has been employed to evaluate variance in metabolic and catabolic gene expressions, to analyze the microbial community physiology from diverse environments, identify new bacterial species, etc. (Dennis et al. 2003; Greene and Voordouw 2003).
2. RNA Sequencing: RNA sequencing uses next-generation sequencing to determine the amount of RNA in a sample. It is very extensive as it facilitates different types of RNA at a much-advanced coverage and broad discovery studies (Shendure 2008; Nagalakshmi et al. 2010).
   The generation of raw transcriptome data involves purification of fine RNA of interest followed by transformation of RNA to complementary DNA (cDNA), fragmenting cDNA to build a library using sequence by synthesis (RNA sequencing), running the microarray or sequence through superior software platform and carrying out ad hoc QC (Chandran et al. 2020). Thus, it a better approach to understand the basic nature and mechanism of differently expressed genes in the host and symbiotic microbes at a time (Kaur and Kaur 2016).
3. GeoChip: It is a high-throughput tool, which analyzes microbial community composition, structure, and functional activity. It uses key enzymes or genes to spot various microbe-mediated mechanisms for biogeochemical cycles, resistance mechanism for heavy metals, and degradation pathways of xenobiotics (He et al. 2010; Xiong et al. 2010; Xie et al. 2011).
4. DNA and RNA-SIP: These are both stable isotope probing technologies. They are used for probing hydrocarbon degraders. They are also valuable to uncover the microbial taxa and catabolic genes that are important for the bioremediation of polluted environments (Lueders 2015).
5. microRNAs: The regulation of gene expression can be studied also by the collective analysis of mRNA and microRNA levels. MicroRNAs (mRNAs) are short, noncoding RNA molecules that control transcription of mRNA. The precise binding of mRNAs to a target mRNA (by sequence homology) either impedes mRNA binding to the ribosome or targets it for degradation. mRNA profiling along with miRNA expression can be used to explore variations in the transcriptome profile, particularly to identify the miRNA transcripts that are subjected to regulation, emphasizing the probable molecular pathways supporting a particular trait or condition (Chandran et al. 2020).

### Applications of Transcriptomics

1. Comparative transcriptomics have been used to reveal highly upregulated degradation pathways and putative transporters for phenol to improve phenol tolerance and utilization by lipid-accumulating *Rhodococcus opacus PD630* (Yoneda et al. 2016).

2. Hong et al. in year 2016 have studied hydrocarbon-degrading bacterium *Achromobacter* sp. using transcriptomics. The species was isolated from seawater and indicated that the upregulation of enzymes such as dehydrogenases, monooxygenases, and novel genes associated with fatty acid metabolism is responsible for its enormous capability for hydrocarbon degradation and survival.
3. The investigation done by Lima-Morales et al. in year 2016 using transcriptomic approach on the microbial organization and catabolic gene diversity. They have worked on three types of contaminated soil under continuous long-term pollutant stress with benzene and benzene/toluene/ethylene/xylene (BTEX). The results obtained have shown shifts in community structure and the prevalence of key genes for catabolic pathways. Moreover, de novo transcriptome synthesis gives new insights into and reveals basic information about nonmodel species without a genome reference.
4. Metatranscriptomic analysis of the wheat rhizosphere identified dominant bacterial communities of diverse taxonomic phyla, including *Acidobacteria*, *Cyanobacteria*, *Bacteroidetes*, *Streptophyta*, *Ascomycota*, and *Firmicutes*, having functional roles in the degradation of various xenobiotic pollutants (Singh et al. 2018).
5. Transcriptomic along with genomic approaches was used by Sengupta et al. in year 2019 for studying mechanistic insights of 4-nitrophenol (4-NP)-degrading bacterium *Rhodococcus* sp. strain BUPNP1. The study identified a catabolic 43 gene cluster named *nph* that harbors not only mandatory genes for the breakdown of 4-NP into acetyl co-A and succinate by nitrocatechol, but also for other diverse aromatic compounds.
6. Transcriptome analysis of activated sludge microbiomes decoded the role of the nitrifying organisms in heavy oil degradation (Sato et al. 2019).
7. Also, studies done by Das et al. in year 2020 using transcriptome analyses of crude oil-degrading Pseudomonas aeruginosa strains revealed the significance of differentially expressed genes implicated in crude oil degradation.

#### 27.3.2.4 Metabolomics

A metabolome is the total metabolites in an organism, and the study of the metabolite profile of a cell within a given condition is called metabolomics (Beale et al. 2017). Metabolomics explores the relationships between organisms and the environment, such as organismal responses to abiotic stressors, including both natural factors such as temperature, and anthropogenic factors such as pollution, to investigate biotic–biotic interactions such as infections, and metabolic responses (Lindon et al. 2006; Griffiths 2007; Mallick et al. 2019).

Metabolomics analyzes the metabolites produced by the cell in response to changing environmental conditions, which in turn provide information about the regulatory events in a cell (Krumsiek et al. 2015). A metabolomic analysis workflow starts with sample acquisition and preparation followed by separation and detection of analytes. Detection and quantification of metabolites are normally accomplished through an amalgamation of chromatography techniques (liquid chromatography and gas chromatography) and detection systems like mass spectrometry and nuclear magnetic resonance (Aldridge and Rhee 2014).

### Applications of Metabolomics

1. Seo et al. in 2013 have investigated the degradation mechanism of carbaryl and other *N*-methyl carbamates pesticides in *Burkholderia* sp. strain C3 by using metabolomic approach. The result of this study has shown that the metabolic adaptation of *Burkholderia* sp. C3 to carbaryl in comparison with glucose and nutrient broth. The metabolic changes were notably associated with the biosynthesis and metabolism of amino acids, sugars, PAH lipids, and cofactors. Thus, this metabolomic study could provide detailed insights into bacterial adaptation to different metabolic networks and the metabolism of toxic pesticides and chemicals.
2. Wang et al. in 2019 have applied comparative metabolic approach for studying the microbial degradation of cyfluthrin by *Photobacterium ganghwense*. This approach has explored the biotransformation pathway of cyfluthrin with the identification of 156 metabolites during the biodegradation process.
3. In 2018, Li et al. on the basis of interactions of indigenous soil microorganisms to PAH-contaminated soil have that the majority of microbial metabolic functions were adversely affected to cope with PAH pollution. This study includes the combined study of enzyme activity and sequencing analysis with metabolomics, which further exposed the specific inhibition of soil metabolic pathways associated with carbohydrates, amino acids, and fatty acids due to microbial community shifting under PAH stress.
4. High-throughput sequencing and soil metabolomics were used by Song et al. in 2020 for investigating the differential structures and functions of soil bacterial communities in the pepper rhizosphere and bulk soil under plastic greenhouse vegetable cultivation (PGVC). In the study, a total of 245 metabolites were identified, among which 11 differential metabolites were detected between rhizosphere and bulk soil, including organic acids and sugars that were positively and negatively correlated with the relative abundances of the differential bacteria. A starch and sucrose metabolic pathway was the most differentially expressed pathway in rhizospheric soil. The main functional genes participating in this pathway were predicted to be downregulated in rhizosphere soil.
5. Wright et al. in 2020 also evaluated the metabolomic characterization of two potent marine bacterial isolates, *Mycobacterium* sp. *DBP42* and *Halomonas* sp. ATBC28, capable of the degradation of phthalate and plasticizers such as ATBC, DBP, and DEHP. This research study presented the molecular analysis of metabolites generated during biodegradation. It also confirmed that DBP and ATBC were degraded through the sequential removal of ester side chains and generated monobutyl phthalate and phthalate in the case of DBP degradation and citrate in the case of ATBC degradation in Mycobacterium species.
6. Metabolite pathway databases and repositories are there, which can be used to supervise and investigate the information about metabolites and their pathways. They provide a databank on metabolic information and help in the unification of

complex data into metabolic pathways. These databases and repositories also help in modeling metabolic pathways that can be investigated and prompted using mathematical modeling techniques (Chandran et al. 2020).

### 27.3.3 Prediction of Chemical Toxicity

Determination of chemical toxicity level, which is lethal for the survival of the degrading microbes, is very important. Several tools and computational models are present, which can predict the toxicity of chemicals involved.

QSAR-Based Models: It stands for quantitative structure regulatory activity relationship. This calculates toxicity based on the physical characteristics of the structure of chemicals such as the molecular weight or the number of benzene rings (molecular descriptors) using mathematical algorithms (Eriksson et al. 2003). There is number of tools based on QSAR:

1. VirtualToxLab—It is for prediction of the toxic potential of drugs, chemicals, and natural products. This includes endocrine and metabolic disruption, and some aspects of carcinogenicity and cardiotoxicity (Vedani et al. 2009).
2. Toxicity Estimation Software Tool (TEST)—This tool is for prediction of the acute toxicity of organic chemicals based on their molecular structures. It allows a user to estimate toxicity without requiring any external programs. Users input a chemical to evaluate by drawing it in an included chemical sketcher window, entering a structure text file, or importing it from an included database of structures. Once entered, the toxicity is estimated using one of several advanced QSAR methodologies (http://www.epa.gov/nrmrl/std/qsar/qsar.html).
3. Sarah Nexus—It is a statistical-based model used for prediction of the mutagenicity of chemicals (Barber et al. 2016).
4. TOPKAT—It is for prediction of the ecotoxicity, mutagenicity, and reproductive or developmental toxicity of chemicals (Prival 2001).
5. Ecological Structure–Activity Relationships (ECOSAR)—The Ecological Structure–Activity Relationships (ECOSAR) Class Program is a computerized predictive system that estimates aquatic toxicity. The program estimates a chemical's acute (short term) toxicity and chronic (long term or delayed) toxicity to aquatic organisms, such as fish, aquatic invertebrates, and aquatic plants, by using computerized structure–activity relationships (SAR) (http://www.epa.gov/oppt/newchems/tools/21ecosar.htm). This software is available for free.
6. Estimation Programs Interface (EPI)—The Estimation Programs Interface (EPI) Suite is a Windows-based suite of physical/chemical property and environmental fate estimation programs. It is a screening-level tool. It uses a single input to run the following estimation programs: KOWWIN, AOPWIN, HENRYWIN, MPBPWIN, BIOWIN, BioHCwin, KOCWIN, WSKOWWIN, WATERNT, BCFBAF, HYDROWIN, KOAWIN, and AEROWIN, and the fate models WVOLWIN, STPWIN, LEV3EPI, and ECOSAR (http://www.epa.gov/opptintr/exposure/pubs/episuite.htm).

7. CAESAR—The CAESAR QSAR model is developed for assessment of chemical toxicity under the REACH (Cassano et al. 2010).
8. ToxinPred—It is a web server available for prediction of aqueous toxicity of small chemical molecules in *Tetrahymena pyriformis*. It is available at http://crdd.osdd.net/raghava/toxipred. It is used for environmental risk assessment of small chemical compounds based on quantitative structure–toxicity relationship (QSTR) model (Mishra et al. 2014).
9. ACD/TOx suite—It is a tool for potential bacterial system to be employed in textile dye decolorization and degradation studies (Srinivasan et al. 2017).

### 27.3.4 Databases

In relation to bioremediation, the number of databases has been developed to provide information regarding chemicals and their biodegradation. Given below is the list of chemical databases:

1. TOXNET—It is developed by the National Library of Medicine (NLM), is a Web-based system of databases providing information on toxicology, hazardous chemicals, and the environment. Databases fall under the general headings of Toxicology Data, Toxicology Literature, Toxic Releases, and Chemical Identification/Nomenclature (Wexler 2001). There are various databases under it, and this includes:
    (a) CCRIS—It stands for Chemical Carcinogenesis Research Information System. The database contains chemical records with carcinogenicity, mutagenicity, tumor inhibition test results. It was developed by the National Cancer Institute (NCI). Data are derived from studies cited in primary journals, current awareness tools, NCI reports, and other sources. Test results have been reviewed by experts in carcinogenesis and mutagenesis (http://toxnet.nlm.nih.gov/cgibin/sis/htmlgen?CCRIS).
    (b) Developmental and Reproductive Toxicology Database (DART)—It provides references related to developmental and reproductive toxicology literature (http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?DARTETIC).
    (c) Genetic Toxicology Data Bank (GENE-TOX)—It provides genetic toxicology (mutagenicity) test data from expert peer review of open scientific literature for more than 3000 chemicals from the United States Environmental Protection Agency (EPA). It was established to select assay systems for evaluation, review data in the scientific literature, and recommend proper testing protocols and evaluation procedures for these systems (http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?GENETOX).
    (d) Integrated Risk Information System (IRIS)—This program supports the mission by identifying and characterizing the health hazards of chemicals found in the environment. Each IRIS assessment can cover a chemical, a group of related chemicals, or a complex mixture. IRIS assessments are an important source of toxicity information used by EPA, state and local health

agencies, other federal agencies, and international health organizations (http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?IRIS).

2. Biodegradative Strain Database (BSD)—It is a Web-based database that provides detailed information about biodegradative bacteria and the hazardous chemicals that they degrade (Urbance et al. 2003). It is available at http://www.bsd.cme.msu.edu/.
3. MetaRouter—It maintains varied information regarding biodegradation networks, predicting biodegradative pathways for chemical compounds (Pazos et al. 2005). It is available at http://pdg.cnb.uam.es/MetaRouter.
4. ECHA Classification & Labeling Inventory—It gives the information about the classification and labeling of substances reported and registered by manufacturers and importers (Schöning 2011).
5. N-CLASS—It stands for the Nordic N-Class Database on Environmental Hazard Classification. It provides information describing chemicals that have been or are currently being considered by the European Commission on classification and labeling for environmental effects (http://apps.kemi.se/nclass/default.asp).
6. International Toxicity Estimates for Risk (ITER)—It provides risk information for 600 chemicals from authoritative groups worldwide (Wullenweber et al. 2008).
7. ProteoWizard—It is used for rapid proteomic analysis (Kessner et al. 2008). It is available at http://proteowizard.sourceforge.net/
8. SuperToxic—It is a Web database having collection of about 60,000 toxic compounds and their structures. With the aid of implemented similarity searches, it can provide information about possible biological interactions. Also, connections to the Protein Data Bank, UniProt, and the KEGG database are available, to allow the identification of targets and the pathways, the searched compounds that are involved in Ref. Schmidt et al. (2009). This database is available online at http://bioinformatics.charite.de/supertoxic.
9. Acutoxbase—It aims to optimize and prevalidate an in vitro testing strategy for predicting acute human toxicity. The database consists of two principal parts for archiving in vitro and in vivo data, respectively. The in vitro part, designed following the principles of Good Cell Culture Practice (GCCP), provides a standard format for collection of in vitro data, together with detailed descriptions of methodologies (Standard Operating Procedures, SOPs), generated by research laboratories participating in the project (Kinsner-Ovaskainen et al. 2009).
10. Biodegradation Network-Molecular Biology (Bionemo)—The Bionemo database is available at http://bionemo.bioinfo.cnio.es. It was developed by the structural Computational Biology Group at the Spanish National Cancer Research Center. Bionemo is a manually curated database that provides information regarding proteins and genes involved in biodegradation metabolism. The protein information involves sequences, domains, and structures for proteins, whereas the genomic information involves sequences, regulatory elements, and transcription units for genes (Carbajosa et al. 2009). It complements UM-BBD, which focuses on the biochemical aspects of

biodegradation. Bionemo has been developed by manually associating sequence database entries to biodegradation reactions based on the information extracted from published articles.

11. OxDBase—It is an enzymatic database that contains all literature-cited information related to oxygenases (Arora et al. 2009). It is available at www.imtech.res.in/raghava/oxdbase/.
12. PAHbase—The PAH database contains significant information on PAH-degrading bacteria, their occurrence phylogeny, metabolic pathways, and the genetic basis of their biodegradation capability (Surani et al. 2011). It is available at http://www.pahbase.in.
13. BioRadBase—It is a comprehensive knowledge database that provides detailed information about the bioremediation of radioactive waste through microorganisms (Reena et al. 2012). It is available at http://biorad.igib.res.in.
14. BiofOmics—It is a novel, systematic, and large-scale database for the management and analysis of biofilm data from high-throughput experiment studies of microorganisms (Lourenco et al. 2012). It is available at www.biofomics.org.
15. Kyoto Encyclopedia of Genes and Genomes (KEGG)—It provides information regarding genetic, metabolic, enzymatic, and cellular progressions of microorganisms (Kanehisa et al. 2017). It is available at http://genome.ad.jp/kegg/
16. Proteomics Identifications (PRIDE)—It is a world's largest database for analysis of mass spectrometry-based proteomic data. It includes generic standard-based format that can be annotated to capture data generated using any proteomic pipeline (Vizcaino et al. 2016). It is available at http://www.ebi.ac.uk/pride/.
17. MetaboLights—It is a database for metabolomic studies that provide primary research data and metadata for cross-platform and species metabolomic studies (Kale et al. 2016). It is available at http://www.ebi.ac.uk.
18. MetaCyc—It is a database of metabolic pathways derived from the scientific experimental literature that comprises more than 2097 experimentally determined metabolic pathways from more than 2460 different organisms. This is the largest curated database of metabolic pathways of all domains of life. This database provides information regarding the metabolic pathways involved in primary and secondary metabolism with associated compounds, enzymes, and genes (Capsi et al. 2016). This database is freely available at http://metacyc.org/. It provides multiple scientific applications:
    (a) provide reference data for computational prediction of the metabolic pathways of organisms from their sequenced genomes,
    (b) support metabolic engineering,
    (c) facilitate comparison of biochemical networks, and
    (d) serve as an encyclopedia of metabolism.
19. BioCyc—This database was developed and curated by the BioCyc group at SRI international. It is available at BioCyc (http://biocyc.org/). It is a collection of more than 2988 organism-specific Pathway/Genome Databases (PGDBs). Each PGDB contains the full genome and predicted metabolic pathway of a single

organism. The pathway tool software predicts pathways using MetaCyc as a reference database.

The BioCyc PGDBs contain information about predicted operons, transport systems, and pathway hole fillers. BioCyc pathway tool-based websites offer multiple tools for querying and analysis of PGDBs, including analysis of gene expression, metabolomics, and other large-scale datasets (Capsi et al. 2016).

20. Molecular Evolutionary Genetic Analysis (MEGA 7.0)—It is used for sequence alignment, hierarchical classification, and constructing phylogenetic trees (Kumar et al. 2016). It is available at www.megasoftware.net.

## 27.4 Conclusion and Future Prospective

With the advent of bioinformatics, the application area of bioremediation has increased. The progressive increase in research from last few decades to present has changed the scenario a lot. The applications of genomics, proteomics, transcriptomics, and metabolomics have given in-depth knowledge of genes, proteins, and enzymes with which the ability to understand the cellular mechanism of microbes has widened. Hence, it can be concluded that this interdisciplinary approach would be supporting the bioremediation by providing distinctive and comprehensive knowledge to build new biodegradative pathways at the molecular level, new hypotheses, postulations, and paradigm for the bioremediation of contaminated living habitat. But in view of future prospective, still research is required for recognition of specific genes and protein sequences of microbes for efficaciously eliminating contamination. Also, studies related to homogeneity shared by genes and proteins involved in bioremediation practice.

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# Role of Environmental Factors in Increased Cancer Incidences and Health Impacts

# 28

Anita Khokhar and Priyanka Sharma

## Abstract

Cancer incidence and mortality are rapidly growing worldwide. The International Agency for Research on Cancer (IARC) estimates that one in five men and one in six women worldwide will develop cancer over the course of their lifetime and that one in eight men and one in 11 women will die from their disease. However, countries are facing an overall increase in the absolute number of cancer cases. Asia accounts for nearly half of the new cancer cases and more than half of cancer deaths. There are more than 100 types of cancers. Cancers are a large group of diseases in which there is unregulated, abnormal growth of cells with invasion of the nearby organs and potential to spread to distant parts of the body as well. Some of the changes in the DNA which occur in cancers are caused naturally, whereas some others are caused due to damage caused by exposure to various environmental factors. These exposures may be alcohol, tobacco, diet, physical activity cancer-causing substances, radiation exposure, sunlight, obesity, infectious agents, pollution, radon, workplace and household exposure, ultraviolet light, etc. The current chapter intends to cover the global burden of cancers, etiology of cancers, epidemiology of various cancers, evidence from research, role of different environmental exposures in cancers, and strategies for prevention and control of these factors. Although we may not be able to control and prevent many of the environmental exposures, better understanding of these will help us prepare better strategies to intervene in our fight against cancers.

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**Keywords**

Environmental carcinogens · Cancer etiology · Cancer impact · Cancer prevention

## 28.1 Global Burden of Cancers

As per Global Cancer Observatory (GLOBOCAN), there were an estimated 18 million new cases and 9.5 million deaths due to cancer worldwide in 2018 which is expected to rise to 29 million new cases and 16.3 million deaths by the year 2040 (International Agency for Research on Cancer. World Health Organization 2018a). Incidence rates and mortality rates for all cancers combined worldwide are higher by 20% and 50%, respectively, among men as compared to women. The largest share to cancer incidence and mortality is contributed by Asia which can partly be attributed to its substantial population burden. The proportion of cancer deaths is comparatively higher than incidence in Asian and African continents. However, almost all the countries are experiencing an overall increase in the absolute number of cancer cases (International Agency for Research on Cancer. World Health Organization 2018b).

The cumulative risk of developing cancer and dying from it before the age of 75 years is 20% and 10.6%, respectively. One in five men and one in six women worldwide will develop cancer and one in eight men and one in 11 women will die from their disease over the course of their lifetime. The countries with higher Human Development Index (HDI) have greater cumulative risks, both for cancer incidence and mortality, than low or medium HDI nations. Top five leading cancers in terms of incidence are lung (11.6%), breast (11.6%), colorectum (10.2%), prostate (7.1%), and stomach (5.7%) for both sexes. However, lung (18.4%), colorectum (9.2%), stomach (8.2%), liver (8.2%), and breast (6.6%) are the leading cancers in terms of mortality. The most commonly occurring cancer and cause of cancer death are lung among males and breast among females (International Agency for Research on Cancer. World Health Organization 2018b) (Fig. 28.1).

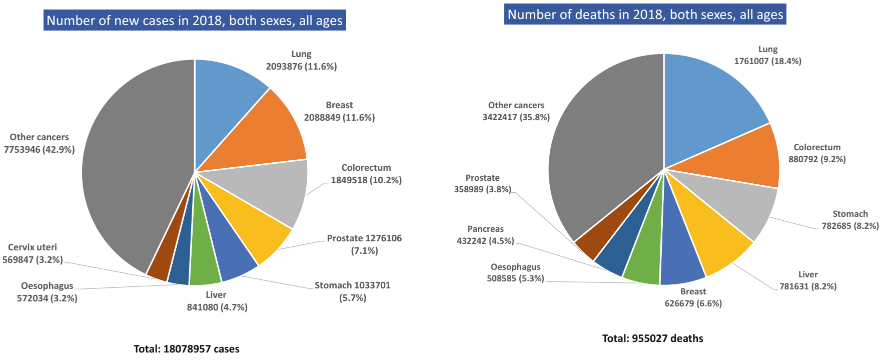

**Fig. 28.1** Incidence and mortality due to different types of cancers in both sexes and all ages. (Source: IARC, WHO, GLOBOCAN 2018a)

## 28.2 Impact of Cancer

Cancer, whether in early stages or during terminal advanced disease state, impacts not only an individual suffering from the disease, but also the people involved in care including spouse, other family members and friends and the society as whole. The impact is multidimensional as it envisages physical, psychosocial, economic, and legal impact. Physical impact in patients manifests as symptoms like pain, sleep disturbances, fatigue, cachexia, neuropathy, and cognitive problems. It affects their overall quality of life and mental well-being (Naughton and Weaver 2014). One's general sense of orderliness and security is disrupted by cancer diagnosis. The negative stigma associated with the disease and fear and uncertainty about future, fear of pain, suffering and abandonment, loss of control, identity, loved ones, and hope leads to plethora of emotional and psychological turmoil (Gorman 2018). Depression and anxiety are common presentations not only in cancer patients but also among cancer survivors (Naughton and Weaver 2014). The family members and friends do not remain untouched as cancer diagnosis and treatment of a loved one invade their sense of security and disrupt family structure, and a feeling of helplessness, distress, anxiety, and uncertainty engulfs them. Family fears loss of relationship, loss of control, sorrow, pain, and suffering of the patient. Recurrence and periods of remissions followed by exacerbations add to the misery. The patient progressively becomes weaker, and family members may react with detachment with fear of reinvestment required at their level for patient care with meager possibility of positive outcome (Gorman 2018).

The diagnosis, treatment, and palliative care of the cancer has a significant impact on finances of the patient and his/her family, both for the insured and uninsured ones; however, impact is higher among uninsured ones due to their sociodemographic vulnerability and late-stage presentation at the time of diagnosis which requires extensive and more expensive therapeutic care (American Cancer Society 2020). Loss of wages, unemployment, poor socioeconomic status, travel costs, requirement of special nutrition and supplements, etc., are ancillary costs adding further to the financial expenditure related to cancer (The University of Texas. MD Anderson Cancer Center 2020; Fenn et al. 2014). The financial burden aggravates the psychosocial hardship of the patients and their families and makes many of them impoverished with time. For a society, cancers cause huge economic impact and the costs are increasing with time. As per International Agency for Research on Cancer (IARC) report, the total annual economic cost of cancer in 2010 was about US$1.16 trillion (International Agency for Research on Cancer. World Health Organization 2014a).

## 28.3 Etiology of Cancer

Cancer is not a single disease, rather constituted by a large number of diseases, and it begins when anomalous cells start growing uncontrollably, invade other parts of the body, and/or spread to other organs, by going beyond their usual boundaries. The process of spread of cancer from one organ to another is called metastasis which is among the leading causes of cancer deaths. Cancers can start in virtually any organ or tissue of the body (World Health Organization 2020b). Malignancy and neoplasm are other words used for cancers. Usually, benign lesions progress to malignant tumors through a multistage process which involves sequential agglomeration of mutations modifying the DNA sequence in genes which control proliferation and death of cells and maintain genetic integrity. These genetic mutations can either lead to loss of function of tumor suppressor genes like BRCA1 and BRCA2 in breast cancer or activation of certain genes converting them into oncogenes like KRAS and LMYC in lung cancer (International Agency for Research on Cancer n.d., pp. 84–125). Besides these genetic mutations, another mechanism for cancer development involves epigenetic changes in which chromatin conformation is modified. Chromatin is the cellular structure that wraps up the DNA and regulates its access (International Agency for Research on Cancer 2008).

Genetic and epigenetic changes affect each other and work in parallel to generate sequential changes. Accumulation of multiple genetic and epigenetic changes occurring during the course of evolution of cell from normal to malignant, and their cumulative effect results in emergence of malignant cell population (International Agency for Research on Cancer n.d., pp. 84–125). There are more than 23,000 genes in human genome, but genetic mutation or epigenetic changes mainly target few hundreds of them which are important regulators of cell division, proliferation, and death, and this leads to creation of conglomeration of dysregulated cells which do not follow normal cell growth and behavior rules, hence rightly called as “outlaw” cells and, if they survive, lead to development of malignancy (International Agency for Research on Cancer 2008).

There are innumerable ways that can lead to such genetic and epigenetic changes. Many chemical agents, radiations, viruses, and other environmental factors have been found to mutate or damage DNA which on accumulation progress to become cancerous. Chemical carcinogens can be classified as genotoxic or non-genotoxic. Most common mechanism of action of genotoxic chemical carcinogens is binding to nucleophilic sites of DNA resulting in formation of adducts, and replication of such DNAs can either lead to mutations evident from sequence changes or chromosomal aberrations by rearrangements of DNA. This becomes an irreversible genetic damage ensuing either in permanent DNA change, which is transmissible or in altered chromosomes resulting in loss of information. This is the “initiation” step of carcinogenesis (International Agency for Research on Cancer n.d., pp. 84–125). On the other hand, there are certain chemicals that are non-genotoxic carcinogens. They do not cause DNA structural modifications or genetic changes directly, but induce carcinogenesis by cytotoxicity, cellular proliferation, hormonal effects, or epigenetic mechanisms. An important way to distinguish genotoxic and non-genotoxic carcinogens is the use of gene mutation assays and cytogenetic assays (Nohmi 2018). Similar to chemical carcinogens, ultraviolet (UV) radiations cause double mutations in DNA as they bridge together two adjacent cytosines, an amino acid in DNA structure, and lead to the formation of dipyrimidine dimer. Ionizing radiations, on the other hand, break single or double strands of DNA. Infection with human papilloma virus (HPV) leads to production of viral genes mainly E6 and E7 through host cell machinery. These viral genes bind to and deactivate tumor suppressor proteins, namely p53 and pRB and enzymes, thereby deregulating host cell growth cycle, and final outcome is cellular proliferation and delayed differentiation of host cell (Burd 2003).

IARC has classified about hundreds of agents found in environment in different categories of carcinogens based on assessment of risk of carcinogenicity in humans. The list has about 120 agents listed as confirmed carcinogens, 88 as probable carcinogens, 313 agents as possible carcinogens, and 499 agents as non-carcinogen to humans (International Agency for Research on Cancer. World Health Organization 2020). The next section provides description about most of the confirmed carcinogens, their routes of exposure, and cancer sites associated with them; however, the description of other categories of carcinogens is beyond the scope of this chapter.

## 28.4 Confirmed Carcinogens

### 28.4.1 Diet-Related Factors

#### 28.4.1.1 Salted Fish: Chinese-Styled

Salted fish is prepared by a variety of methods including treatment of fish with dry salt or aqueous salt solution which is followed by sun drying. When this salted fish is partially decomposed for softening before or during the process of salting, this is called as Chinese-styled or Cantonese marine salted fish. Salted fishes are found to have high levels of sodium and micro-organisms including those producing aflatoxins, *N*-nitrosodimethylamines (NDMA), polynuclear aromatic hydrocarbons (PAHs), and many other contaminants which are known carcinogens. Chinese-styled salted fish causes nasopharyngeal carcinoma among consumers, and the risk significantly increases with increase in amount of consumption with stronger association found for consumption during childhood (International Agency for Research on Cancer. World Health Organization 2012h).

#### 28.4.1.2 Processed Meat

Meat that is transformed through fermentation, curing, salting, smoking, or any other process for increasing the shelf life or enhancing flavor is called processed meat. This includes cured meat pieces, fresh industrial processed meat products, fermented sausages, pre-cooked ready to eat products, and dried meat (Fig. 28.2). Processed meat also contains many chemical additives, namely salt, nitrate and nitrite, ascorbic acid, phosphates, antioxidants, preservatives, monosodium glutamate, and food colors. Procedures involving direct contact with hot surface or flame at high temperatures like barbecuing, pan frying, and grilling produce high levels of carcinogens like heterocyclic aromatic amines, nitrosoamines, and PAHs in processed meat. Heme iron present in meat can cause cytotoxicity and mediates formation of nitrosamines and lipid oxidation products. Environmental carcinogens like dioxins, lead, heavy metals, and brominated flame retardents can also

8.3.3 FROZEN PROCESSED COMMINUTED MEAT AND POULTRY PRODUCTS.

PLEASE DO NOT THAW, COOK FROZEN. CONSUME AFTER PROPER COOKING.

8.2.2 HEAT-TREATED PROCESSED MEAT AND POULTRY PRODUCTS IN WHOLE PIECES OR CUTS.

PLEASE DO NOT RE-FREEZE ONCE IT THAWS. CONSUME AFTER PROPER COOKING.

**Fig. 28.2** Processed meat

contaminate meat through air, water, and animal feed. Processed meat causes colorectum cancer. Stomach cancer and processed meat consumption are also found to be positively associated (International Agency for Research on Cancer. World Health Organization 2018e).

## 28.4.2 Tobacco

### 28.4.2.1 Smoked Tobacco

Smoked tobacco products include numerous types of cigarettes, cigars, pipes, bidis, and water pipes (Fig. 28.3). More than 5300 compounds are identified in tobacco smoke out of which about 70 are carcinogenic in either humans or animals which include PAHs, nitrosamines, aldehyde, phenols, and volatile hydrocarbons. Levels of carcinogens are higher in cigars compared to cigarettes, while bidis contain comparable levels of nitrosamines. These cause cancers by many complex mechanisms, and most of them are not fully understood. Some of the mechanisms include in formation of DNA adducts, oxidative damage, inflammation, tumor promotion, co-carcinogenesis, and activation of cellular proliferation. Tobacco smoke causes cancers of the lung, naso-, oro-, and hypopharynx, nasal cavity and accessory sinuses, oral cavity, larynx, esophagus, stomach, colorectum, pancreas, kidney, ureter, urinary bladder, liver, uterine cervix, ovary, and myeloid leukemia. Female breast cancer is also associated with tobacco smoking. Parental smoking on the other hand causes hepatoblastoma and is found to be associated with childhood leukemia (International Agency for Research on Cancer. World Health Organization 2012ao).

### 28.4.2.2 Secondhand Smoke (SHS)

Smoke released from burning tip of a cigarette or other burned tobacco product between puffs, also known as sidestream smoke (SS), and smoke exhaled by the

**Fig. 28.3** Cigarette packet

smoker, known as mainstream smoke (MS), together constitute secondhand smoke. It is also named as environmental tobacco smoke, involuntary smoking, or passive smoking. The constituents of SS and MS differ only quantitatively. SHS gets rapidly diluted and dispersed into indoor environment, and its constituents' composition varies with time and environmental conditions. The carcinogens present in it are acrolein, benzopyrene, and nitrosamines. SHS is present in all the environments where smoking takes place, and the exposure setting differs between populations. About one-fifth of males and one-third of females were estimated to be exposed to SHS globally in 2016 (Drope et al. 2018). Secondhand smoke causes lung cancer and is positively associated with larynx and pharynx cancer (International Agency for Research on Cancer. World Health Organization 2012aq).

#### 28.4.2.3 Smokeless Tobacco

Smokeless tobacco means usage of unburned tobacco products in finished products and used either orally by chewing, sucking, gargling, and applying to gum and teeth or through inhalation of fine mixtures by nostrils (Fig. 28.4). Chemical composition of tobacco varies with tobacco species, growing, curing, processing, and storage. Highest prevalence of tobacco use among men and women was present in Southeast Asian Region (SEAR) (World Health Organization 2019a). Carcinogenic compounds present in smokeless tobacco include tobacco-specific *N*-nitrosamines, *N*-nitrosamine acids, volatiles nitrosamines, PAHs, aldehydes, acrolein, and

**Fig. 28.4** Smokeless tobacco products

**Fig. 28.5** Betel quid

uranium. Smokeless tobacco causes cancer of oral cavity, esophagus, and pancreas (International Agency for Research on Cancer. World Health Organization 2012an).

### 28.4.3 Betel Quid and Areca Nut

Betel quid, commonly called as pan, is a quid wrapped in betel leaf in which quid indicates a substance or mix of substances, placed and retained in mouth and commonly swallowed. Common constituents of pan are betel leaf, areca nut, slaked lime, and catechu with or without tobacco which can be supplemented with sweetening agents, condiments, etc. (Fig. 28.5). Pan masala is ready to chew dry powdered mixture of areca nut, lime, catechu, unspecified spices without betel leaf, and tobacco and is sold commercially in India (Ramchandani et al. 1998). When tobacco is added to it, it is called gutka (Nair et al. 2004). Many alkaloids like arecoline, arecaidine, and tannins are present in areca nut. Reactive oxygen species and nitrosamines are produced due to betel quid chewing with areca nut. It causes continuous local irritation of buccal epithelium, resulting in chronic inflammation, cytokine production, and oxidative stress. Betel quid with or without tobacco is a carcinogen on its own. Tobacco containing betel quid causes oral cavity, pharynx, and esophageal cancer, while betel quid without tobacco causes oral cavity and esophageal cancer and is associated with liver cancer (International Agency for Research on Cancer. World Health Organization 2012ak).

### 28.4.4 Alcohol

Mainly consumed beverage types are beer, spirits, and wine which can be prepared commercially or at home at local levels. Alcohol content in beverages differs based on type and country and is indicated by percentage by volume. It can range from 2.3% to 10% in beer, 8–15% in wine, and 20–40% in spirit and is mainly composed of ethanol and water, but some volatile and non-volatile compounds may also be present (Fig. 28.6). Certain contaminants or toxins may be present in alcoholic beverages including pesticides, benzenes, nitrosamines, lead, cadmium, arsenic, and chromium and acetaldehyde is produced in body as first product of ethanol metabolism. About 2.3 billion people were current drinkers in 2016. In Europe, Americas, and Western Pacific regions, more than half of the population consumes alcohol (World Health Organization 2018). Alcohol consumption causes cancers of oral cavity, pharynx, larynx, esophagus, colorectum, liver, and female breast. Pancreas cancer is also found to be associated with alcohol consumption. Its metabolite acetaldehyde causes cancer of esophagus and upper aerodigestive tract (International Agency for Research on Cancer. World Health Organization 2012al).

### 28.4.5 Outdoor Air Pollution

Air pollution is defined as the presence of one or more substances at concentrations or durations exceeding the natural levels, with potential to cause adverse effects. Exposure to outdoor air pollutants occurs continuously including periods of indoor stay, though composition and levels of pollutants may differ. These pollutants can be in gaseous form or consists of particulate matter (PM). PM is a suspension of liquid or solid matter miniscule particles of size of few micrometers to nanometers. Gaseous pollutants include ozone, nitrogen oxides, sulfur oxide, and volatile organic compounds like aldehydes and aromatics. PM is classified on basis of size and chemical composition. The pollutants can be primary, if emitted directly or secondary, if formed in atmosphere. Fossil fuel and biomass combustion, automobile emissions, chemical plants, and industries are some of the man-made contributions to air pollution. Natural sources include wildfires, lightning, sea sprays, volcanoes, wind and dust storms, plants, and microbial activities. Spatial variations exist in levels and composition of pollutants with higher levels in urban areas and places near

**Fig. 28.6** Alcohol

roads and industries. Seasonal and diurnal variations also exist. Outdoor air pollution causes lung cancer and is positively associated with cancer of urinary bladder. Particulate matter in outdoor air pollution also causes lung cancer (International Agency for Research on Cancer. World Health Organization 2016b).

## 28.4.6 Coal Combustion Indoor

When using small and simple combustion devices such as household cooking and heating stoves, coals are difficult to burn without substantial emission of pollutants and a substantial fraction of the fuel carbon is converted to products of incomplete combustion. The products that are formed can be present in the gas phase, the particle phase, or both, depending on their volatility and include carbon monoxide, nitrogen dioxide, benzene, formaldehyde, PAHs, PM, and large number of compounds that are precursor components of photochemical smog, such as aldehydes. Coal also contains contaminants from its mineral deposits, such as sulfur, arsenic, silica, fluorine, lead, or mercury. During combustion, these contaminants are released into the air in their original or oxidized form. In households that use sulfur-rich coals, sulpfur dioxide is present at elevated levels. Indoor emissions from household combustion of coal cause cancer of the lung (International Agency for Research on Cancer. World Health Organization 2012am).

## 28.4.7 Biological Agents

### 28.4.7.1 Epstein–Barr Virus (EBV)

EBV belongs to Lymphocryptovirus genus, gammaherpesvirus subfamily, and is a human herpesvirus with double-stranded DNA and linear genome. EBV-1 and EBV-2 are the two main types of EBV identified in humans (Sample et al. 1990). Humans are the only natural hosts for the virus, and route of transmission is oral via saliva. Transmission through transfusion has also been documented (de-Thé et al. 1975). Exposure is associated with socioeconomic factors (Evans 1971) like overcrowding, poor sanitation (de-Thé et al. 1975), and hygiene, low socioeconomic status, and larger family size. Age at primary infection differs worldwide and is responsible for resulting clinical manifestation. Primary infection during early childhood, common in developing countries due to overcrowding and practice of pre-chewing food for infants, is either subclinical or presents as respiratory illness, while if acquired during late childhood or adolescence, can manifest as infectious mononucleosis which is common in developed countries (Rickinson and Kieff 1996), where likely mode of transmission is intimate oral exposure (Hjalgrim et al. 2007). Infection with EBV causes Burkitt lymphoma, immunosuppression-related non-Hodgkin lymphoma, extranodal NK/T-cell lymphoma (nasal type), Hodgkin lymphoma, and cancer of the nasopharynx. Also, a positive association has been observed between exposure to EBV and lymphoepithelioma-like carcinoma (International Agency for Research on Cancer. World Health Organization 2012ae).

### 28.4.7.2 Helicobacter pylori (H. pylori)

Helicobacter belongs to Helicobacteraceae family and Helicobacter genus with about 23 validated species. Human species is H. pylori, gram-negative bacilli, which are spiral or slightly curved and have 2–6 unipolar flagella. It targets gastric mucus-secreting cells. Blood group antigen A is the main cell receptor responsible for its adherence to target cells. Once infection has occurred, it persists for life in the absence of treatment, and at the present, no vaccine is available. Global prevalence of H. Pylori infection is more than 50% but inter-country variations exist (Hooi et al. 2017), with prevalence being higher in developing countries with an exception of eastern European countries. Infection rates are also higher in older age groups (The

EUROGAST Study Group 1993) and differ by race/ethnicity and socioeconomic factors. Low socioeconomic status associated with unhygienic conditions, overcrowding, and large number of young children in home during childhood increases the risk of infection (Malaty and Graham 1994; Goodman et al. 1996). Humans are only significant reservoir of the bacteria (Oderda 1999). Primary route of transmission is person-to-person contact. Other possible routes are oral–oral, feco-oral, waterborne, and iatrogenic transmission. Chronic infection causes non-cardia gastric carcinoma and low-grade B-cell MALT gastric lymphoma. Esophageal adenocarcinoma is also linked with chronic infection; however, data is insufficient to confirm this association (International Agency for Research on Cancer. World Health Organization 2012af).

#### 28.4.7.3 Human Immunodeficiency Virus (HIV) Type 1

It is a retrovirus, contains two copies of single-stranded RNA, belonging to the family Retroviridae, and is strongly associated with acquired immunodeficiency syndrome (AIDS). There were an estimated 38 million people living with HIV all over the world, with a prevalence of 0.7 among adults aged 15–49 years in the year 2019 (World Health Organization 2020c, d). The prevalence varies from 3.7 in African region to 0.1 in Eastern Mediterranean and Western Pacific region (World Health Organization 2020d). Natural hosts for virus are humans. Main routes of transmission are blood contact, sexual intercourse, and mother to child, and the probability of transmission is different for all these routes. Highest probability is for blood transfusion and mother-to-child transmission and lowest for heterosexual route. The use of condoms, mode of sexual exposure, viral load, stage of HIV infection, penile circumcision, co-existing other sexually transmitted diseases (STDs), and mucosal inflammation are all related to varying risk of sexual transmission with high viral load and genital ulceration being the major determinants. Blood contact can occur during blood transfusion, occupational exposure by needle stick injury or splash exposure to mucus membranes, and through intravenous (IV) drug use by sharing the needles. Regarding mother-to-child transmission, it has been observed that in the absence of any intervention, the transmission risk during in utero, intra-partum or breastfeeding, from HIV-positive mother to infant, ranges from 15% to 45% (World Health Organization 2020e). HIV-1 infection causes cancers of cervix, anus, conjunctiva, non-Hodgkin and Hodgkin lymphoma, and Kaposi sarcoma. Cancers of vulva, vagina, penis, hepatocellular carcinoma, and non-melanoma skin cancer are found to be associated with HIV-1 infection (International Agency for Research on Cancer. World Health Organization 2012aj).

#### 28.4.7.4 Hepatitis B Virus (HBV)

This is a DNA virus belonging to family Hepadnaviridae, which consists of ten genotypes, which differ in virological characteristics and geographic distribution and infects only humans (Cao 2009).

It is highly infectious and gets transmitted through percutaneous and permucosal exposure to infected blood, semen, and vaginal fluids. Highest concentrations are found in blood and wound secretions and lowest in saliva (Margolis et al. 1997). It does not spread by air, water, or food. Modes or transmission is similar to those for HIV-1, including mother-to-child, child-to-child, blood transfusion, unsafe injection practices, and sexual contact. Perinatal transmission mainly occurs during time of birth, in utero transmission is rare, and it does not spread by breastfeeding (Beasley et al. 1975). Child-to-child transmission occurs through contact of skin sores, breaks in skin or mucus membranes with blood or skin sore secretions (Margolis et al. 1997), through saliva by bites, skin breaks, or during pre-mastication of food (Scott et al. 1980; MacQuarrie et al. 1974; Williams et al. 1997; Beasley and Hwang 1983). Spread can also take place from fomites like towels and toothbrushes (Department of Health and Human Services. Centers for Disease Control and Prevention. Division of Viral Hepatitis 2010). Multiple sexual partners, prostitution, and unprotected intercourse are risk factors for sexual transmission. Infections can be acute symptomatic or asymptomatic and can progress to chronic infection. In 2015, an estimated 257 million people were living with chronic HBV infection, and more than half of the proportion of infections was contributed by African and Western Pacific regions (World Health Organization 2017). Less than 5% of people who are infected in adulthood develop chronic infections, and 25–30% chronically infected adults develop cirrhosis and/or liver cancer (World Health Organization 2020f). Around 45% world population lives in high endemicity areas for HBV chronic infection, 43% in moderately endemic, and 12% in low endemic areas (Mahoney 1999). Infection endemicity varies due to age at infection and modes of transmission. In high endemicity areas, infections are acquired perinatally or child-to-child, prevalence of liver cancer and cirrhosis is very high and acute infections are not common. In intermediate endemicity areas, pattern of transmission is mixed and infant, child, and adult transmissions co-exist, while in areas with low endemicity, infection is acquired during adolescence or young adulthood and is mainly confined to high-risk groups of IV drug users, homosexual men, healthcare workers, and patients having regular blood transfusions or dialysis (Hou et al. 2005). Chronic infection with HBV causes hepatocellular cancer either directly or indirectly through chronic necroinflammation known as cirrhosis and is observed to be associated with cholangiocarcinoma and non-Hodgkin lymphoma (International agency for Research on Cancer. World Health Organization 2012q).

#### 28.4.7.5 Hepatitis C

Hepatitis C virus belongs to Flaviviridae family, hepacivirus genus, and contains six genotypes, which have different geographical distributions. Man, higher primates, and members of tree shrew genus are susceptible to infection (Shimizu et al. 1998; Sung et al. 2003; Pachiadakis et al. 2005). Transmission occurs through blood and blood products transfusion, organ transplants, unsafe injection practices, IV drug abuse, occupational exposures, and perinatal and sexual route, although the rate of transmission differs. Co-existing HIV infection increases the risk of perinatal transmission. The role of cosmetic procedures like tattooing and body piercing in transmission is not confirmed (Alter 2002; Hwang et al. 2006). About 15–27% of chronically infected people develop cirrhosis, and accelerated progression to severe liver disease is observed among males, late age at infection, alcohol intake, and co-infection with HBV or HIV (Lauer and Walker 2001; Perz et al. 2006; Alter 2007). About 71 million people across the world were estimated to be living with chronic HCV infection in 2019 with highest prevalence of HCV being reported from Eastern Mediterranean and European region (World Health Organization 2017). It is responsible for hepatocellular carcinoma and non-Hodgkin lymphoma and is found to be associated with cholangiocarcinoma (International Agency for Research on Cancer. World Health Organization 2012ag).

#### 28.4.7.6 Human Papillomavirus (HPV)

It belongs to Papillomaviridae family, double-stranded DNA virus, which infects only humans and mainly targets stratified epithelium of mucosa or skin. HPV 16 is the most common type out of all the HPV types. Infection peaks among women age less than 25 years and more than 45 years. Main mode of transmission is skin-to-skin or skin-to-mucosa contact, and major route is sexual transmission, while other routes include perinatal transmission and may be fomites or medical procedures. The use of condoms and circumcision is found to be protective, while having multiple sex partners elevates the risk (Burchell et al. 2006; Dunne et al. 2006). The role of smoking, oral contraceptive pills, age at first intercourse, other sexually transmitted infections (STIs), and parity is unclear. Persistent infection with carcinogenic types and re-infection with HPV-16 increases risk of cancer. HPV-16 causes cancer of cervix, vagina, vulva, anus, penis, oral cavity, tonsil, and oropharynx and is associated with laryngeal cancer. HPV-18 is responsible for cervical cancer and is found to be associated with cancer of penis, vulva, anus, larynx, and oral cavity. HPV 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59 cause cervical cancer and are observed to be linked to vulvar and anal cancer (International agency for Research on Cancer. World Health Organization 2012r).

#### 28.4.7.7 Human T-Cell Lymphotropic Virus Type 1 (HTLV-1)

It is a member of delta-type retrovirus group, and natural infection is only found in humans. Close to 20 million people were estimated to be infected with HTLV-1 (de Thé and Kazanji 1996). Prevalence is higher in women, in low socioeconomic strata, among people with history of blood transfusion and populations with low geographic mobility, and it increases with age. Main modes of transmission are vertical through breastfeeding, parenteral, and sexual. Breastfeeding for longer durations, unprotected intercourse, sexually transmitted diseases (STDs), multiple sexual partners, injecting drug use, and blood transfusions increase the risk. It causes adult T-cell leukemia and lymphoma with lifetime risk of development being 2–4% among carriers of infection, and time period for the development of cancer from initial infection is 40–60 years (International Agency for Research on Cancer. World Health Organization 2012e).

#### 28.4.7.8 Opisthorchis viverrini (O. viverrini) and Clonorchis sinensis (C. sinensis)

Both of them belong to same genus Opisthorchis but different species. Humans and other fish-eating mammals like dogs and cats are definite hosts (Wang 1983;

Harinasuta 1984; Rim 2005; Lun et al. 2005). Freshwater snails are first intermediate hosts, and fishes are secondary intermediate hosts. Humans and other mammals are infected by eating raw or undercooked freshwater fish containing infective form of fluke. Adult flukes reside in biliary system. Poor educational status, lack of sanitation resulting in sewage drainage into water bodies, use of animal or human feces as fertilizer, contamination of dinner sets from infected fish, and non-existent control measures are the factors associated with transmission. C. sinensis can survive up to 26 years (Attwood and Chou 1978) and O. viverrini up to 10 years in humans (Harinasuta 1984). Globally about 45 million people are infected with these flukes combined with 35 million from C. sinensis and rest ten million from O. viverrini. Chronic infection with both of these flukes causes cholangiocarcinoma (International Agency for Research on Cancer. World Health Organization 2012f).

#### 28.4.7.9 Schistosoma Hematobium

These are parasitic flukes of genus Schistosoma of which six species are of main pathological importance to humans (Webster et al. 2006). The intermediate host is freshwater snail, and definite host is human. When human host comes in contact with water-containing larva, the larva penetrates the skin, reaches liver, and matures into adult worms which produce eggs. Half of the produced eggs get trapped in tissues of urinary bladder. Life span of adult worm can range from 3 to 40 years (Warren et al. 1974; Chabasse et al. 1985). Infection subsides with death of adult worm, but in endemic areas re-infection occurs frequently. It is mostly found in rural areas in natural ponds, streams, and lakes; however, due to population growth, migration, and creation of man-made reservoirs and irrigation systems, urban infection is also on rise (Mott et al. 1990; McManus and Loukas 2008). Man–water contact behavior and snail population can affect focal distribution. Infection peaks in age group of 5–15 years, and there are gender differences due to behavioral, cultural, religious, and professional factors (Jordan and Webbe 1993). Schoolage children, fishermen, irrigation workers, farmers, women, and other groups using infested water for domestic use are the main risk groups (World Health Organization 2020g). Co-infection with hepatitis, climatic conditions, and household clustering increases the risk. Chronic infection with this fluke causes cancer of the urinary bladder (International Agency for Research on Cancer. World Health Organization 2012ai).

#### 28.4.7.10 Kaposi Sarcoma Herpes Virus (KSHV)

This is a gamma 2 herpes virus with double-stranded DNA genome, and natural hosts are humans. Primary route of transmission is through saliva, and other reported routes are blood transfusion, organ transplantation, and IV drug use. Infection peaks at age of 6–10 years (Whitby et al. 2000; Mbulaiteye et al. 2004). Risk factors are infection in mothers or other family members, HIV infection, number of sexual partners, source of water, and insect bites (Plancoulaine et al. 2000; Coluzzi et al. 2003; Dedicoat et al. 2004; Mbulaiteye et al. 2005; Malope et al. 2007; Minhas et al. 2008). Many co-factors act along with KSHV to cause cancer including HIV co-infection and other immunodeficient states. Other suggested co-factors are malaria and other parasitic infections, exposure to onco-weeds, and fine volcanic soils (Ziegler 1993; Serraino et al. 2003; Whitby et al. 2007; Lin et al. 2008). It causes Kaposi sarcoma and primary effusion lymphoma (International Agency for Research on Cancer. World Health Organization 2012ah).

## 28.4.8 Radiation

### 28.4.8.1 X-Ray and Gamma Radiation

X-rays and gamma rays are electromagnetic radiations, which differ in their origin and energies with gamma rays having highest energy. Exposure to these rays can be through naturally via terrestrial sources including soil and building materials (UNSCEAR), medical uses, nuclear explosions and accidents, nuclear weapon production, nuclear power production, and occupational exposures during industrial operations, military activities, flying and medical procedures and nuclear power production and recycling of fuel (UNSCEAR). In medical field, these are used for diagnosis and radiotherapy. Even though dose for diagnostic procedures is lower than therapeutic dose, diagnostic procedures are main source for exposure in medical use. With increasing use of computed tomography (CT), interventional radiology, and other diagnostics, the level of exposure due to medical use is increasing (Fig. 28.7). Fluoroscopy delivers higher doses than radiography. These radiations cause cancer of esophagus, salivary glands, stomach, colon, lung, kidney, female breast, basal cell of skin, bone, urinary bladder, brain and CNS, thyroid, and leukemia except chronic lymphocyte leukemia (CLL). In utero exposure is also carcinogenic. Cancer of rectum, pancreas, liver, ovary, prostate, non-Hodgkin lymphoma, and multiple myeloma are associated with their exposure (International Agency for Research on Cancer. World Health Organization 2012n).

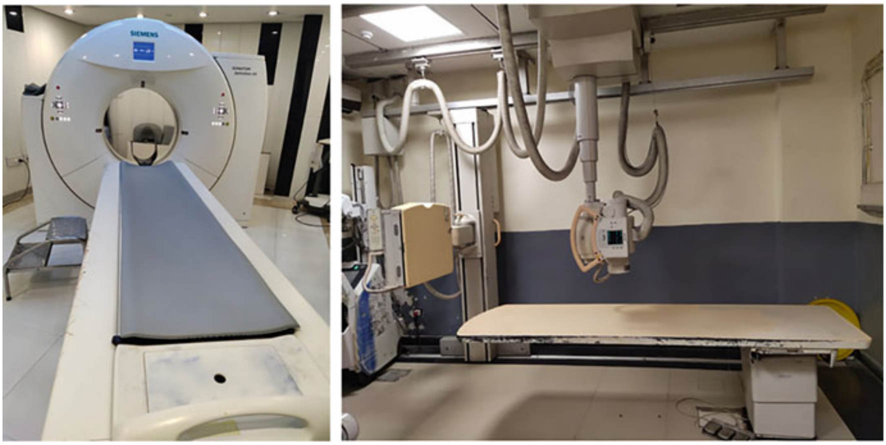

**Fig. 28.7** X-ray and CT machines

#### 28.4.8.2 Solar Radiation and Ultraviolet Radiation (UVR)

Solar radiation is mainly optical radiation and consists of infrared, visible light, and ultraviolet radiations. About 5% of terrestrial radiation is UV radiation. UV component of terrestrial radiation from midday sun comprises 95% UVA and 5% UVB. Major part of UVB and UVC from extra-terrestrial radiation is removed by ozone in stratosphere. Air pollutants and tropospheric ozone can decrease UVR. Clouds also attenuate solar radiation. During summers, about 75% of total daily amount of UVR is received between 9:00 and 15:00 sun time and about 20–30% between 11:00 and 13:00 sun time. Seasonal variation occurs in temperate regions, and annual exposure dose of UVR decreases with increasing distance from equator. Total solar irradiance and contribution by different wavelength radiations vary with altitude. With increasing altitude, sun burning effectiveness of sunlight increases. Two-thirds of sun's energy entering into atmosphere penetrates the ground, and reflection from certain ground surfaces may contribute significantly to total amount of scattered UVR. Contribution from surface reflection varies with type of surface with grass lawn scattering 2–5%, sand reflecting 10–15%, fresh snow 80–90%, and white foam in sea about 30%.

Artificial sources of exposure can increase annual cumulative exposure. Indoor tanning devices emit both UVA and UVB. UVB phototherapy is used in the treatment of many dermal conditions. Hospital staff working with unenclosed phototherapy equipment have potential exposure risk if protective measures are not taken. Electric arc welders may get acute ocular overexposures.

Solar radiation causes squamous cell carcinoma of skin, basal cell carcinoma of skin, and cutaneous malignant melanoma. Positive association has been found with lip cancer, ocular melanoma, conjunctival squamous cell carcinoma. UV-emitting tanning devices cause ocular melanoma and cutaneous malignant melanoma. UV-emitting tanning devices cause ocular melanoma and cutaneous malignant melanoma and positive association has been found with squamous cell carcinoma of skin (International Agency for Research on Cancer. World Health Organization 2012m). Other radioactive carcinogens are mentioned in Table 28.1.

### 28.4.9 Toxins: Aflatoxins

Aflatoxin B, G, mycotoxin cyclopiazonic acid and M1, a metabolite of aflatoxin B1, are the important toxins responsible for carcinogenesis. Aspergillus flavus and A. parasiticus are the major fungus that produce largest proportion of aflatoxins found in foodstuffs across the globe. Another fungus A. australis also produces aflatoxins, but is limited in its geographic distribution to southern hemisphere and Australian peanut soils. Maize, peanuts, and cottonseeds are the chief hosts for A. flavus, and spices and tree nuts are less commonly affected. However, small amounts of aflatoxins may be detected in other foods. A. parasiticus has similar food hosts except maize. Aspergillus invades plants and developing seed or nut of maize, cottonseed, and peanuts before harvest which results in high levels of toxins in them which are difficult to eliminate. Other crops can be protected from aflatoxin

**Table 28.1** Other radioactive agents, their exposure, and cancers caused by them (International Agency for Research on Cancer. World Health Organization 2012l)

| Carcinogen | Use and exposure | Cancer |
|---|---|---|
| Plutonium-239 | Used in production of nuclear weapons and mixed oxide fuels. Most commonly exposed group is people working in production of these weapons and fuels. Other people get exposed due to accidents. | Bone, lung, and liver |
| Thorium-232 and its decay products | Used as a radiographic contrast agent in medical practice in form of thorotrast. Miners can also be exposed. | Gallbladder, liver, extrahepatic bile ducts, leukemia except CLL. Associated with pancreatic and prostatic cancers. |
| Radium-224/226/228 and its decay products | Major exposure is found in radium watch-dial painters due to practice of pointing the tip of paintbrush by lips. Other exposed groups include patients suffering from tuberculosis, ankylosing spondylitis, and other diseases treated with high doses of injected Radium 224. Radium has been found in drinking water also. | Bone sarcomas. Radium 226 additionally causes cancer of paranasal sinuses and mastoid process. |
| Radon-222 and its decay products | Natural radioactive gas present in small quantities in rocks and soils. Underground miners are at elevated risk for exposure. Indoor radon exposure is also observed. | Lung cancer. Positive association with leukemia. |
| Fission products including Strontium-90 | Gets deposited in environment and food products due to release from nuclear facilities and in water due to discharge of nuclear waste into rivers. | Leukemia, solid cancers. |
| Radioiodine including Iodine-131 | Atmospheric nuclear weapon tests, routine, or accidental releases from nuclear power plants, and nuclear weapon production facilities are the sources. Radioactive iodine is used in medical settings for treatment of thyrotoxicosis and thyroid cancer. | Exposure to short-lived radioisotopes of iodine including I-131 during childhood and adolescence causes thyroid cancer, and risk enhances if children are iodine deficient at time of exposure. Positive association is found with cancer of salivary gland, digestive tract, bone, and soft tissue sarcoma and leukemia. |
| Phosphorous-32 as Phosphate | Pure beta particle emitter. Used for polycythemia vera. | Acute leukemia in patients with polycythemia vera. |
| Neutron radiation | Exposure group includes atomic bomb survivors, who were exposed to fission neutrons, nuclear workers, and airline crew. It is also used in treatment for many cancers. | NA<br>Inadequate evidence among humans. |

production by eluding the post-harvest contamination through drying and good storage practices. Aflatoxin M1 is found in milk of animals who feed on foods containing aflatoxin B. Exposure occurs through diet and occupational sources. Consumption of maize and peanuts as dietary staples can result in daily intakes ranging from nanograms to micrograms. Levels of aflatoxins in food products mainly depend on food type, source, storage conditions, and climate. Occupational exposure occurs while handling and processing of contaminated grains and animal feed, and toxins are found to be present in workplace air samples also. These toxins cause hepatocellular carcinoma in humans (International Agency for Research on Cancer. World Health Organization 2012g).

## 28.4.10 Hormones and Chemotherapeutic Agents

### 28.4.10.1 Oral Contraceptive Pills (OCPs)

Combined OCPs contain estrogen, along with progestogen to prevent pregnancy. Other therapeutic purposes include treatment for acne and premenstrual disorders. They are usually given in a monthly cycle in which pill is taken for about 20–22 days, followed by pill-free interval of 7 days to allow for withdrawal bleeding. Emergency contraceptives contain a comparatively high dose of estrogen (Fig. 28.8). Apart from oral preparations, transdermal formulations and intravaginal contraceptive rings are also available. There were about 151 million users of OCPs containing either estrogen and progestogen combination or progestogen alone for contraception in the year 2019, and the number of users varies within countries (Department of Economic and Social Affairs. United Nations 2019). The use of OCPs, especially those containing high doses of estrogens, causes hepatocellular carcinoma, breast cancer, and in situ and invasive cancer of uterine cervix. However, a protective effect against ovarian, colorectal, and endometrial cancer has also been observed. Progestogen-only contraceptives are possible human carcinogen as evidence is insufficient at present (International Agency for Research on Cancer. World Health Organization 2012ap).

**Fig. 28.8** Progesterone only and combined OCPs

#### 28.4.10.2 Estrogen Menopausal Therapy (EMT)

The use of estrogen alone for women during time around menopause to treat menopausal symptoms and prevent conditions like osteoporosis and ischemic heart disease, which are common in postmenopausal period, is classified as post-menopausal estrogen therapy. It can be given orally, through injections, transdermally, by implants, or can be applied locally and topically to relieve urogenital symptoms. Combinations with an androgen or an anxiolytic are also commercially available. Dose of estrogens varies according to indication and route of administration. Treatment for climacteric symptoms usually starts in peri-menopausal period and commonly lasts from 2 to 5 years. Treatment for other conditions can last for 10 or more years. This causes cancer of endometrium and ovary and is found to be associated with breast cancer. However, a protective effect against colorectal cancer is also observed (International Agency for Research on Cancer. World Health Organization 2012k).

#### 28.4.10.3 Estrogen–Progestogen Menopausal Therapy

Progestogen along with estrogen is commonly used globally for conditions mentioned in section J.2. It was initiated because of two main reasons, firstly due to increasing incidences of endometrial cancer and secondly to better control uterine bleeding, among postmenopausal women with intact uterus on EMT. Treatment regimen can consist of daily administration of estrogen with intermittent, periodic, or daily progestogen administration. Indications are similar as for EMT. Combined therapy causes cancer of breast and endometrium (International Agency for Research on Cancer. World Health Organization 2012j). Other chemotherapeutic carcinogens are mentioned in Table 28.2.

### 28.4.11 Dusts and Fibers

#### 28.4.11.1 Asbestos (All Forms)

Asbestos is a naturally occurring mineral silicate fiber. It is used in flooring, roofing, friction materials, gaskets, cement pipes and sheets, electrical and thermal insulation, paper, plastics, textiles, mastics, thread, fiber jointing, coating and compounds and millboard. It can be found in nature as large natural deposits or as contaminants in other minerals. Mining, milling of ore, screening, crushing, manufacture and use of asbestos products, demolition of buildings containing asbestos products and transport and disposal of asbestos-containing waste lead to release into air. Water contamination occurs through rock erosion, asbestos-containing waste pile erosion, wastewater run-off from industries, corrosion of asbestos cement pipes, and annihilation of roofing made up of asbestos. In similar ways, soil can also be contaminated through rock erosion and dumping asbestos-containing waste into landfills. Exposure to humans occurs through ingestion and inhalation. Occupational exposures are common among mine workers and asbestos-manufacturing industries' workers. Fibers on hair or clothing can be carried to home and expose family members of these workers. Asbestos causes mesothelioma and cancer of lung, larynx, and ovary

**Table 28.2** Other chemotherapeutic agents, their use, and cancers caused by them (International Agency for Research on Cancer. World Health Organization 2012i)

| Carcinogen | Uses | Cancer |
|---|---|---|
| Diethylstilbestrol (non-steroidal synthetic estrogen) | Prevention of abortions, treatment of menopausal symptoms, atrophic vaginitis, vulvar dystrophy, female hypogonadism, dysfunctional menstrual cycles, emergency contraceptive, prevention of postpartum breast engorgement, and as a livestock growth stimulant. Rarely in breast and prostate cancer. | Positive association with endometrial cancer. Exposure during pregnancy causes breast cancer women. In utero exposure causes vaginal cancer and cervical clear cell adenocarcinoma and is associated with squamous cell cancer of cervix and testicular cancer. |
| Tamoxifen | Prevention and treatment of breast cancer among women, treatment of breast cancer among males and for anovulatory infertility. | Endometrium. Protective against breast cancer among women. |
| Cyclosporine | Used in organ and tissue transplantation, for prevention of graft rejection, prophylaxis, and treatment of graft-versus-host disease, treatment of chronic allograft rejection, management of severe rheumatoid arthritis, psoriasis, atopic dermatitis, to increase tear production. | Skin (squamous cell carcinoma), cancer at multiple other sites, and non-Hodgkin lymphoma |
| Mechlorethamine, oncovin, procarbazine, prednisone (MOPP) | For treatment of Hodgkin lymphoma | Lung, AML |
| Semustine [1-(2-Chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea, Methyl-CCNU | Treatment of primary and metastatic brain tumors, Lewis lung tumor, L1210 leukemia, cancers of digestive tract, Hodgkin lymphoma, malignant melanoma, and epidermoid carcinoma of lung. | Acute myeloid leukemia |
| Chlorambucil | Treatment of CLL, Waldenstrom macroglobulinemia, indolent non-Hodgkin lymphoma, Hodgkin lymphoma, polycythemia vera. | Acute myeloid leukemia |
| Treosulfan | Treatment of ovarian cancer, malignant melanoma, and breast cancer. Used for bone marrow ablation before stem cell transplantation. | Acute myeloid leukemia |

(continued)

**Table 28.2** (continued)

| Carcinogen | Uses | Cancer |
|---|---|---|
| Methoxsalen (8-methoxypsoralen) plus ultraviolet A radiation | Produced naturally by various plants (celery, figs, limes, parsnips). Used in psoriasis, cutaneous T-cell lymphoma, idiopathic vitiligo, chronic graft-versus-host disease, treatment of rejection after solid organ transplantation. | Skin (squamous cell carcinoma) |
| Melphalan | Treatment of multiple myeloma, advanced ovarian adenocarcinoma, childhood neuroblastoma, early and advanced breast cancer, polycythemia vera, regional arterial perfusion in localized malignant melanoma, and localized soft tissue sarcoma of extremities. | Acute myeloid leukemia |
| Busulfan | For palliative treatment of chronic myelogenous leukemia. polycythemia vera, in myelofibrosis, primary thrombocythemia and in conditioning regimen to prepare patients for stem cell transplantation. | Acute myeloid leukemia. |
| Thiotepa | Treatment of residual tumors, as adjuvant therapy for prophylaxis of superficial bladder cancer, palliative treatment of adenocarcinoma of the breast and ovary, to control pleural, pericardial, or peritoneal effusions caused by metastatic tumors. | Leukemia |
| Cyclophosphamide e | In treatment of several malignant diseases, CLL, Lymphoma, soft tissue, estrogenic sarcoma, solid tumors. | Bladder and acute myeloid leukemia |
| Chlornaphazine | Treatment of Hodgkin lymphoma, control of polycythemia vera. | Urinary bladder |
| Azathioprine | Prevention of rejection of kidney allografts, management of rheumatoid arthritis, acute lymphocytic leukemia. | Skin cancer (squamous cell carcinoma) and non-Hodgkin lymphoma |

(continued)

**Table 28.2** (continued)

| Carcinogen | Uses | Cancer |
|---|---|---|
| Etoposide + cisplatin + bleomycin | Treatment of tumors of the testis, small cell cancer of the lung, and in acute leukemias. | Acute myeloid leukemia. |
| | Bleomycin malignant disease particularly squamous cell carcinomas, including those of the cervix and external genitalia, esophagus, skin, and head and neck; Hodgkin lymphoma and other lymphomas; malignant neoplasms of the testis and malignant effusions. In carcinoma of the bladder, lung, and thyroid, and some sarcomas, including Kaposi sarcoma. | |
| | Cisplatin treat metastatic ovarian tumors, cervical tumors, lung cancer, advanced bladder cancer, and squamous cell carcinoma of the head and neck. | |
| Phenacetin | As an analgesic and antipyretic for humans and animals, stabilizer for hydrogen peroxide in hair-bleaching preparations. | Renal pelvis, Ureter. |

and has positive associations with cancer of stomach, pharynx, and colorectum (International Agency for Research on Cancer. World Health Organization 2012b).

#### 28.4.11.2 Silica Dust

It is available commercially in three categories, namely sand and gravel, diatomites, and quartz crystals. Silica is used in glass manufacture, foundry, ceramics, hydraulic fracturing applications and abrasives, as a gemstone jeweler, in electronics and optical components industry, in pesticides, paints and polishes, in insulators, packing material, and in filtration. Silica is present in rocks and soil and gets released into environment by natural and anthropogenic activities. Exposure route for humans is through inhalation from quartz-containing products like cosmetics, cleansers, talcum powder, mortar, paint, putty, caulk, art clays, and glazes. Ingestion of quartz particle-containing potable water can also lead to exposure. Workers working in mining, construction, farming, quarrying, demolition of masonry and concrete, production of ceramics, glass, foundry processes, etc., get occupational exposure to silica dust. This dust causes lung cancer (International Agency for Research on Cancer. World

**Fig. 28.9** A carpenter being exposed to wood dust

**Table 28.3** Dust and fiber agents, their exposure, and cancers caused by them (International Agency for Research on Cancer. World Health Organization 2012a)

| Carcinogen | Exposure | Cancer |
|---|---|---|
| Erionite | Naturally occurring, found in sediments of volcanic tuffs which contributes to non-occupational exposure. Occupational exposure through mining and production. | Mesothelioma |
| Leather dust | Occupational exposure occurs in footwear industry and leather tanning and processing industry. | Nasal cavity and paranasal sinuses |
| Wood dust | Occupational exposure among workers involved in various wood-related industries like furniture, plywood, logging, sawmills, and particleboard manufacture (Fig. 28.9). Highest exposure in construction sector and furniture industry. General population gets exposed by woodworking during repair and building works in homes but duration of exposure is usually short. | Nasal cavity, paranasal sinuses, and nasopharynx |

Health Organization 2012c). Other dust and fiber carcinogens are mentioned in Table 28.3.

### 28.4.12 Metals

Many metals have been classified as human carcinogens by IARC. Metals naturally occur in environment in mineral rocks, soils, volcanic eruptions or are produced in industries for various uses. Natural activities like winds, volcanic activity, weathering of rocks, leaching into soil, and water usually takes place and contaminates water, air, and vegetation. Anthropogenic activities like mining, burning of fossil fuels, smelting, waste incineration, and effluents from industries are other major sources of environmental contamination. Usual route of exposure for general public is ingestion and inhalation. Occupational workers employed in industries producing and using these metals get exposed through inhalation, ingestion, and dermal route.

#### 28.4.12.1 Arsenic

Arsenic is commonly being used in wood preservatives, pharmaceuticals, glass-making industry, agricultural chemicals, and mining, semiconductor, and metallurgical industries. In medicine, it was used for treatment of leukemia, chronic bronchial asthma, psoriasis, and in some antibiotics. Agricultural pesticides, herbicides, defoliants, cotton desiccants, insecticides, feed additives for swine and poultry, etc., contain arsenic. Contamination of soil, air, and water occurs naturally by volcanic activity, windblown dusts, exudates from vegetation, low-temperature volatilization and by anthropogenic sources through burning of fossil fuels, mining and smelting of non-ferrous metals, and use of arsenic-based pesticides. It is found in air in industrial, urban, and suburban areas, in deep well, and other sources of oxygenated water, as contamination in groundwater and soil. Highest arsenic content has been found in seafood, meats, poultry, cereals, dairy products, fruits, and vegetables. Arsenic compounds cause cancer of the lung, urinary bladder, and skin (International Agency for Research on Cancer. World Health Organization 2012d).

#### 28.4.12.2 Chromium

It is used as pigment for textile dyes, paints, inks, and plastics, in corrosion inhibitors, wood preservatives, metal finishing, chrome plating, leather tanning, and as an impurity in Portland cement. It can be generated during casting, welding, and cutting operations. Anthropogenic activities and leaching of wastewater cause contamination of the drinking water. Tobacco smoke also contains chromium. The general population residing in the vicinity of anthropogenic sources of chromium (VI) may be exposed through inhalation of ambient air or ingestion of contaminated drinking water. Its compounds cause lung cancer, and positive associations have been observed with cancer of nose and nasal sinuses (International agency for Research on Cancer. World Health Organization 2012p). Other carcinogenic metals are shown in Table 28.4.

**Table 28.4** Carcinogenic metals, their uses, and cancer caused by them (International Agency for Research on Cancer. World Health Organization 2012a)

| Carcinogen | Uses | Cancer |
|---|---|---|
| Beryllium | Used in industries of aerospace, automotive, defense, biomedical, fire prevention, energy and electrical, sporting goods, telecommunications, manufacturing, consumer products, scrap recovery, and recycling. | Lung |
| Cadmium | Used in batteries, pigments, coatings, and plating and stabilizer in plastic. | Lung. Positive association with cancer of kidney and prostate. |
| Nickel | Used in electroplating, ceramics, pigments, in manufacture of alloy steel and stainless steel, manufacture of nickel–cadmium and nickel–metal hydride batteries, in petrochemical and metallurgical industry. | Lung, nasal cavity and paranasal sinuses. |

## 28.4.13 Occupational Exposures

### 28.4.13.1 Painting

Paint contains finely divided pigment particles, dyes, fillers, a binder or resin, a volatile solvent or water, and some additives. The solvents include xylene, toluene, alcohols, ketones, aliphatic compounds, esters, and glycol ethers. Pigments can be chromate, azo-based, or lead-based, and the most common pigment is white titanium dioxide and carbon black. Azo-based pigments were found to have 2-naphthylamine, benzidine, 4-aminobiphenyl, and 4-chloro-ortho-toluidene. Resins include cellulose-based, vinyl, acrylic, alkyd, phenolic, polyester, polyurethane, silicone oils, styrene-butadiene, and chlorinated rubber derivatives. Solvents encompass a wide range of petroleum and coal tar distillates, esters, glycols, alcohols, glycols, and a broad range of chemical mixtures. Additives include plasticizers, rheological agents, driers, biocides, surfactants, anti-skinning agents, corrosion inhibitors, defoamers, and UV stabilizers. Workers employed in painting industry get exposure to all the abovementioned chemicals through inhalation, dermal route, and ingestion during their production, application, removal, stripping from wood and metal surfaces. Exposure to silica, asbestos, and diisocyanate is also a probability as painters may get exposed by being a bystander to construction and demolition activities. Occupational exposure as a painter causes mesothelioma, lung, and urinary bladder cancer. A positive association has been observed with maternal exposure during pre-conception and pregnancy with childhood leukemia in the child (International agency for Research on Cancer. World Health Organization 2012z).

### 28.4.13.2 Welding

The process of joining metals through coalescence is called welding which can be classified as arc or gas welding, depending on the method used to generate arc. Arc welding uses electricity, while gas welding uses acetylene or hydrogen. Other processes which are closely related to welding and usually performed by welders

**Fig. 28.10** Welding process

include flame cutting, brazing, gouging, soldering, and plasma arc or carbon arc cutting. Sheet metal workers, blacksmiths, mechanics, toolmakers, firefighters, etc., are also exposed to welding fumes. It has been estimated that number of people exposed to welding fumes might be 10 times more than the number of occupationally designated welders. The welding process involves exposure to gases, fumes, ionizing and non-ionizing radiation, and co-exposures to asbestos and solvents (Fig. 28.10). Gases encountered during welding comprise argon, carbon dioxide, helium, acetylene, propane, butane, ozone, carbon monoxide, hydrogen fluoride, and nitrogen oxides. Fumes consist of silicates, metal oxides, chromium, nickel, and fluorides. Concentration of welding fumes varies according to type of welding process, presence of coatings, metal welded, arc time, and personal and workplace characteristics. Inhalation is the main route of exposure. Welding fumes cause lung cancer and are associated with kidney cancer, while UV radiation from welding is causative for ocular melanoma (International Agency for Research on Cancer. World Health Organization 2018d). Other occupational exposures and cancers caused by them are mentioned in Table 28.5.

### 28.4.14 Chemicals

#### 28.4.14.1 Benzene

Benzene is used in manufacture of organic chemicals like phenol, styrene, aniline, alkylbenzenes, etc. It is naturally present in petroleum products and added to unleaded gasoline. It is also used as an ink component in printing industry, in chemical and drug industries, rubber, plastics, dye, detergent, lubricants, and pesticides manufacture. Occupational exposure occurs while working in these industries and while crude-oil refining. Workers employed at service stations involved in transport and dispensing of gasoline, street workers, taxi drivers, etc.,

**Table 28.5** Occupations, implicated exposures, and cancers caused by them (International agency for Research on Cancer. World Health Organization 2012s)

| Occupation | Exposed to | Cancer |
|---|---|---|
| Aluminum production | PAHs, sulfur dioxide and fluorides, aluminum fluoride, fibrous sodium aluminum tetrafluoride particles, fluorspar, alumina, carbon monoxide, carbon dioxide, various trace metals, such as vanadium, chromium and nickel, asbestos, extreme heat, and high static magnetic fields. | Bladder and lung |
| Auramine production | Dimethyl-aniline, formaldehyde, sulfur, ammonium chloride, ammonia, benzidine, 1-naphthylamine, 2-naphthylamine, magenta, aniline. | Urinary bladder |
| Hematite mining (International agency for Research on Cancer. World Health Organization 2012o) | Radon | Lung |
| Coke production | PAHs, asbestos, silica, amines, arsenic, cadmium, lead, nickel, vanadium, hydrocarbons, sulfur dioxide, sulfuric acid, and aldehydes. | Lung |
| Isopropyl alcohol production | Propylene, sulfuric acid, isopropanol, diisopropyl and isopropyl hydrogen sulfates, diisopropyl ether, propanal, acetone, sulfur oxides, polymeric oils, and residues. | Nasal cavity |
| Magenta production | Aniline, ortho- and para-toluidine arsenic acid, benzidine, 1-naphthylamine, 2-naphthylamine, auramine. | Urinary bladder |
| Graphite and silicon carbide synthesis work with Acheson process (International Agency for Research on Cancer. World Health Organization 2017c) | Fibrous silicon carbide | Lung |
| Coal gasification | PAHs, asbestos, silica, amines, arsenic, cadmium, lead, nickel, vanadium, hydrocarbons, sulfur dioxide, sulfuric acid, and aldehydes. | Lung |

(continued)

**Table 28.5** (continued)

| Occupation | Exposed to | Cancer |
|---|---|---|
| Coal tar distillation | Benzene, toluene and xylene isomers, tri- and tetra-methylbenzenes, indene, hydrindene, coumarone, phenol, cresols, pyridine, picolines, methylpyridines, dimethylpyridines, naphthalene, thionaphthene, indene, methylnaphthalene isomers, biphenyl, acenaphthene, fluorine, anthracene, phenanthrene, pyrene, fluoranthene, PAH. | Skin (including, but not limited to, cancer of the scrotum). |
| Iron and steel founding | Silica, carbon monoxide, PAHs, chromium, nickel, phenol, formaldehyde, isocyanates, and various amines | Lung |
| Rubber production | *N*-nitrosamines, polycyclic aromatic hydrocarbons, solvents, and phthalates, cyclohexane-soluble compounds. | Leukemia, lymphoma, cancer of urinary bladder, lung, and stomach. Positive association with cancer of prostate, esophagus, and larynx. |
| Chimney sweep workers | Soot, sulfur dioxide, arsenic, organic solvents, PAHs and their derivatives, oxides, salts, metals, sulfur and nitrogen compounds, water, and other adsorbed liquids and gases. | Skin (observed in the scrotum), and lung. |

**Fig. 28.11** Petrol filling stations

also get exposed. Benzene contaminates environment mainly through release from industries, gasoline filling stations, and automobile exhaust (Fig. 28.11). General population get exposed through air having tobacco smoke or contaminated with benzene in heavy traffic areas and near gasoline filling stations, through contaminated water or eating contaminated food. Benzene causes acute myeloid leukemia and acute non-lymphocytic leukemia and is found to be associated with multiple myeloma, acute and chronic lymphocytic leukemia, and non-Hodgkin lymphoma (International agency for Research on Cancer. World Health Organization 2012u).

### 28.4.14.2 Formaldehyde

It is mainly used in production of resins that are further used in wood production, synthetic fiber industries, pulp and paper industry, production of plastics and coatings, paints, varnishes, and in textile finishing. It is also used in manufacture of many other chemicals. Formalin, an aqueous solution of formaldehyde, is used as a disinfectant and preservative. Formaldehyde naturally occurs in environment, fruits, and some foods and is produced in humans and other mammals. It is produced from motor vehicle emissions, tobacco smoke, and combustion processes in incineration plants, power plants, wood stoves, refineries, and kerosene heaters. Formaldehyde can be absorbed from skin from cosmetics and other consumer products. These together constitute occupational and non-occupational exposure to this chemical. It causes cancer of nasopharynx and leukemia and has positive association with sinonasal cancer.

### 28.4.14.3 Vinyl Chloride

Its main use is in manufacture of polyvinyl chloride for plastic piping. Vinyl chloride is also utilized in many consumer goods, transport sector, floor coverings, electrical applications, cosmetics, drugs, manufacture of solvents, and resins. Environmental sources include emissions and effluents from plastic industry, accidental releases, mainstream tobacco smoke, and in landfill gas and groundwater due to degradation of solvents deposited in landfills. It causes hepatocellular carcinoma and angiosarcoma of liver (International agency for Research on Cancer. World Health Organization 2012x).

### 28.4.14.4 Sulfur Mustard

This compound exists in gaseous form and is used in biological studies, in medicine for psoriasis treatment and in wars. It was used in past as antineoplastic agent, but was a failure. Occupational exposure occurs during its storage and destruction, construction work on soils having mustard gas deposits as contaminants in military bases, in excavated amunition dumps, during fishing due to marine dumping of the gas in past, in laboratories, and during armed conflicts. It can contaminate water and air also, thereby exposing general population. Sulfur mustard causes lung cancer and is positively correlated with laryngeal cancer (International agency for Research on Cancer. World Health Organization 2012ab).

### 28.4.14.5 Trichloroethylene

It is mainly used as a cleaning and degreasing agent for metal parts in industries of furniture and fixture, fabricated metal products, transport equipment, electric and electronic equipment, and miscellaneous manufacturing industries. Trichloroethylene is also used as an anesthetic, production of chlorofluorocarbons and other chemicals, in spotting agents to remove spots from garments, in plastics, paper, printing and glass, textiles, in waterless dyeing, as an extraction agent for fats and oils, paint-related products and lubricants, etc. Industrial emissions are the main source of environmental contamination. Occupational exposure is primarily through degreasing. It causes cancer of kidney and has been found to be associated with liver cancer and non-Hodgkin lymphoma (International Agency for Research on Cancer. World Health Organization 2014c).

### 28.4.14.6 Ethylene Oxide

Ethylene oxide is used for production of mono-ethylene glycol, as a sterilizing agent for drugs, hospital equipment, disposable and reusable medical items, foods, packaging materials, museum artifacts, books, furs, clothing, scientific equipment, aircraft, railcars, beehives, etc., and as an insecticide and fumigant. It is also found in mainstream tobacco smoke, fossil fuel combustion smoke, skincare products and as a residue in spices, and other food products which are main exposure sources for general population. Hospital patients get exposed through medical equipment during dialysis. Evidence establishing causal association of its exposure with lymphatic and hematopoietic cancers and breast cancer is limited among humans (International agency for Research on Cancer. World Health Organization 2012w).

### 28.4.14.7 1,3 Butadiene

This chemical is used in synthetic rubber and polymer production, which are used in endless products like clothing, automobiles, computers, packaging, etc. It is also used in production of many petrochemicals. Exposure occurs among workers employed in these operations. Although butadiene is present widely in ambient air, elevated levels occur in proximity to wood and bush fires, municipal structural fires, vehicle emissions, cigarette smoking, and gasoline volatilization. It causes cancer of hematolymphatic organs (International agency for Research on Cancer. World Health Organization 2012t).

### 28.4.14.8 Benzo[a]pyrene and Other Polycyclic Aromatic Hydrocarbons (PAHs)

These are the contaminants present ubiquitously in environment including air, water, soil, and food. Major sources include incomplete combustion or pyrolysis of organic material, tobacco smoke, coal tar-based pharmaceutical products, motor vehicle exhaust, residential and commercial heating with coal, wood, or other biomass, industrial emissions, and forest fires. Barbecued/boiled/grilled and smoke-cured meats, baked, fried and roasted foods, cereals, grains, bread, and vegetables grown in contaminated soil lead to food-borne exposure to PAHs (Fig. 28.12). Occupational exposure occurs in coal and coke industries, power plants, during aluminum production, roofing and paving, chimney sweeping, wood impregnation with creosote, and carbon electrode manufacture. It is a confirmed human carcinogen (International agency for Research on Cancer. World Health Organization 2012v).

**Fig. 28.12** Barbequed food and biomass fuel combustion

#### 28.4.14.9 Mineral Oils, Untreated or Mildly Treated

These oils are refined from crude petroleum oils and contain aromatic hydrocarbons. They can be used as lubricant in engine oils, gear oils, transmission fluids, metal-working fluids, hydraulic fluids and as non-lubricant in printing inks, agricultural spray oils, and tyre oils. Both these applications are potential generators of oil aerosols, leading to occupational exposure. These are also used in food and pharmaceutical preparations, which expose general population to mineral oils. Untreated or mildly treated mineral oils cause cancer of the skin (observed in the scrotum) (International agency for Research on Cancer. World Health Organization 2012y).

#### 28.4.14.10 Fluoro-Edenite

These are the fibers, existing naturally in quarries in volcanic areas and thereby contaminating environment naturally. They are used in building materials and unpaved roads which lead to their release into air and water. General population living near quarries is exposed through inhalation. Quarry and construction workers' exposure contributes to occupation sources of exposure. It causes mesothelioma (International Agency for Research on Cancer. World Health Organization 2017b).

#### 28.4.14.11 Shale Oils

These have been used in paraffin waxes, burning oil for lamps, for medicinal purposes, to prepare gasoline, diesel oils, lubricants, kerosene, coke, gas turbine fuel oil, high sulfur petroleum fuel oil, to generate electric power, and as a refinery feedstock. They cause skin cancer (observed in scrotum) (International agency for Research on Cancer. World Health Organization 2012aa).

#### 28.4.14.12 Engine Exhaust: Diesel

Burning of fuel leads to formation of PAH, elemental carbon, carbon monoxide, nitrogen oxides, soot, hydrocarbons and unburned/partially burned oil and ash. The exhaust from engines also contains partially burned lubricating oil and ash from metallic additives in wear metals and lubricating oil. Occupational exposure occurs in workers employed in mining, construction, railroad and transportation industries, heavy goods vehicle's drivers, mechanics, bus garage and other bus workers, and firefighters. General population gets exposed through traffic contribution to air pollution, diesel generators, at seaports, etc. Traffic contaminants enter indoor air also, thereby exposing people there. Diesel engine exhaust causes lung cancer and is found to be positively associated with urinary bladder cancer (International Agency for Research on Cancer. World Health Organization 2014b).

#### 28.4.14.13 2,3,7,8-Tetrachlorodibenzo-Para-Dioxin (TCDD), 2,3,4,7,8-Pentachloro-Dibenzofuran (PeCDF) and 3,3′,4,4′,5-Pentachlorobiphenyl (PCB)

TCDD has no known commercial application. It occurs as a contaminant in some herbicides and is used as a research chemical, pesticide, in incineration of sewage sludge and municipal, hospital, and hazardous waste, for metal processing, and paper pulp bleaching. It is also released from combustion of fuels, vehicle exhaust, and forest fires. These sources lead to contamination of air, sediments, surface, and groundwater and soil. Apart from accidental and occupational exposures, general population gets exposure through eating milk, fish, meat, eggs, and related products. TCDD is carcinogenic for all cancers combined and is positively associated with soft tissue sarcoma, non-Hodgkin lymphoma, and cancer of the lung.

PeCDF is manufactured commercially only for scientific research purposes. Combustion and incineration from cement kilns and hazardous waste, metal smelting, refining and processing, pulp bleaching, chemical manufacture, and existing reservoirs are sources of environmental release. Contaminated food is the main route of human exposure, followed by inhalation and dermal absorption.

PCB was used in electrical industry, in hydraulic fluids, paints, plastics, and in manufacture of organic chemicals. The use, combustion, and disposal of these products lead to release into all environmental compartments including soil, air, water, sediments, and biota (International agency for Research on Cancer. World Health Organization 2012ac). Other chemical carcinogens are mentioned in Table 28.6.

## 28.5 Prevention Measures for Cancers

Prevention measures for any disease can be categorized into primary, secondary, and tertiary prevention measures. For cancers, the control spectrum is more or less similar including primary prevention, early diagnosis and screening, multimodal treatment, survivorship, and palliative care. For each of these domains, the availability of highly effective interventions mostly in high-income countries has reduced the burden of cancers in them. About one-third to half of the total cancers globally can be prevented with currently available knowledge and technology (World Health Organization 2020a). In further sections, the individual preventive measures are discussed in detail.

### 28.5.1 Primary Prevention

The measures aimed to prevent a disease from occurring and target a susceptible population or specific individuals are categorized under primary level preventive measures. These measures either increase immunity of individuals or decrease the risk exposure (Kisling and Das 2020). For cancers, primary prevention includes all actions, which lower the risk of cancer development (International Agency for Research on Cancer. World Health Organization 2019a).

Specific protection measures to increase the immunity of the individual against a biological carcinogen include immunization. Vaccines for human papillomavirus and hepatitis B are available which provide protection against them, thereby reducing the chances of cancer development. For HPV, three vaccines including a bivalent, a quadrivalent, and a nonavalent vaccine depending on number of strains it provides protection against are currently being marketed in various countries. These are highly potent for prevention against infection with two main virus types 16 and 18 which cause about two-thirds of cervical cancers globally. They also prevent precancerous cervical lesions associated with these virus types. In most of the countries which recommend HPV vaccination, young adolescent girls in age group of 9–14 years are the primary target group and immunization schedule varies with recipient's age. A two dose schedule at 0 and 6 months is recommended for females less than 15 years of age, and three dose schedule at 0, 2, and 6 months is

**Table 28.6** Chemical carcinogens, their use in industries, and cancers caused by them (International Agency for Research on Cancer. World Health Organization 2012i)

| Chemical | Industries | Cancer |
|---|---|---|
| Strong inorganic acid mists | Manufacture of phosphate fertilizer, lead batteries, soap and detergents, cellulose fibers and films, inorganic pigments and paints, applications in petroleum refining, mining, metallurgy, and ore processing, in the synthesis of inorganic and organic chemicals, synthetic rubber and plastics, in processing of pulp and paper, and in water treatment. Exposure also occurs during copper smelting, and pickling and other acid treatment of metals. | Larynx. Positive association with lung cancer. |
| Aristolochic acid (International agency for Research on Cancer. World Health Organization 2012ad) | Extract of Aristolochia species. Used in traditional Chinese medicine as diuretics, anti-inflammatory and edema treatment, slimming agent. | Urinary bladder. |
| 4,4′-Methylenebis (2-chloroaniline) (MOCA) | Manufacture of castable urethane rubber products (e.g., shock absorption pads and conveyor belting). In the laboratory, as a model compound for studying carcinogens. | Urinary bladder |
| Ortho-toluidine | Synthesis of herbicides, in manufacture of more than 90 dyes and pigments, synthetic rubber and rubber-vulcanizing chemicals, pharmaceuticals, pesticides, and other chemicals, in clinical laboratory as an ingredient in a reagent for glucose analysis, for tissue staining. Significant non-occupational exposures to ortho-toluidine may result from the use of some hair dyes, the local anesthetic prilocaine, or tobacco smoke. | Urinary bladder |
| Pentachlorophenol (International Agency for Research on Cancer. World Health Organization 2019b) | Used as wood preservative, herbicide, algicide, defoliant, germicide, fungicide, molluscicide and could be found in ropes, paints, | Non-Hodgkin lymphoma. |

(continued)

**Table 28.6** (continued)

| Chemical | Industries | Cancer |
|---|---|---|
| | adhesives, canvas, leather, insulation, and brick walls, in tanneries. Also used in textiles and other fabrics. It is a persistent organic pollutant. | |
| Coal tar pitch | Used in electrode manufacture, roofing, and paving. Roofers are primarily exposed to PAHs. Other exposures include silica, diesel exhaust, asbestos, and organic solvents. | Lung. Positive association with cancer of the bladder. |
| 1,2 Dichloropropane (International Agency for Research on Cancer. World Health Organization 2017a) | In production of propylene, carbon tetrachloride, and tetrachloroethylene, as textile stain remover, paint and stain removers, oil and paraffin extractant, scouring compound, metal cleaner, metal degreaser, as grain and soil fumigant, in insecticides, solvent or diluent in coatings, inks, glues, and adhesives. | Biliary tract (confirmed as cholangiocarcinoma). |
| Lindane (International Agency for Research on Cancer. World Health Organization 2018c) | As insecticides to treat wood and wooden structures, seed, crops, and livestock, in baits and seed treatments for rodent control, in treatment for control of head lice and scabies. | Non-Hodgkin lymphoma |
| 2-Naphthylamine | Manufacture of dyes, in rubber industry, to produce 2-chloronaphthalene, in laboratory research. Indirect exposure through gases produced during incomplete combustion of organic material. 2-Naphthylamine has been detected in fumes of heated cooking oils. | Urinary bladder |
| Polychlorinated biphenyls (International Agency for Research on Cancer. World Health Organization 2016a) | Used in dielectric fluids in capacitors and transformers, paint additive, permanent elastic sealants, flame-retardant coatings, in inks, adhesives, dyes for carbonless duplicating paper, conveyor belts, rubber products, pesticide fillers, plasticizers, | Malignant melanoma. Positive associations with non-Hodgkin lymphoma and cancer of the breast. |

(continued)

**Table 28.6** (continued)

| Chemical | Industries | Cancer |
|---|---|---|
| | immersion oil for microscopes, cutting and lubricating oils, wire insulators, metal coatings, in fluorescent lights. Environmental contamination through improper handling of electronic waste (e-waste) and dismantling of ships. | |
| 4-Aminobiphenyl | Used as rubber antioxidant, dye intermediate, in detection of sulfates, food colorant. Occupational exposure occurs with exposure to benzidine and benzidine-based dyes, from which 4-aminobiphenyl can be metabolically released. It has been detected in fume from cooking oils. | Urinary bladder. |
| Benzidine and dyes metabolized to benzidine | In production of large number of dyes, particularly azo dyes for wool, cotton, and leather, in clinical laboratories for detection of blood, as rubber compounding agent, in manufacture of plastic films, for detection of hydrogen peroxide in milk, and for quantitative determination of nicotine. Some food colorants such as tartrazine and sunset yellow FCF also contain it. | Urinary bladder. |
| Bis(chloromethyl)ether; chloromethyl methyl ether (technical grade) | Used to manufacture dodecylbenzyl chloride, water repellants, plastics, ion-exchange resins, polymers, flame-retardant fabrics, as a chloromethylating reagent, as a laboratory reagent, for surface treatment of vulcanized rubber. | Lung |

advocated for females receiving first dose at or after the age of 15 years and for immunocompromised and HIV infected individuals (World Health Organization n.d.-c). Hepatitis B vaccination is now a part of immunization programs of almost all the countries (Fig. 28.13). It is recommended to vaccinate all infants within 24 h of birth with first dose of vaccine which is to be followed by 2 or 3 doses to complete the primary schedule. Protection is possibly lifelong (World Health

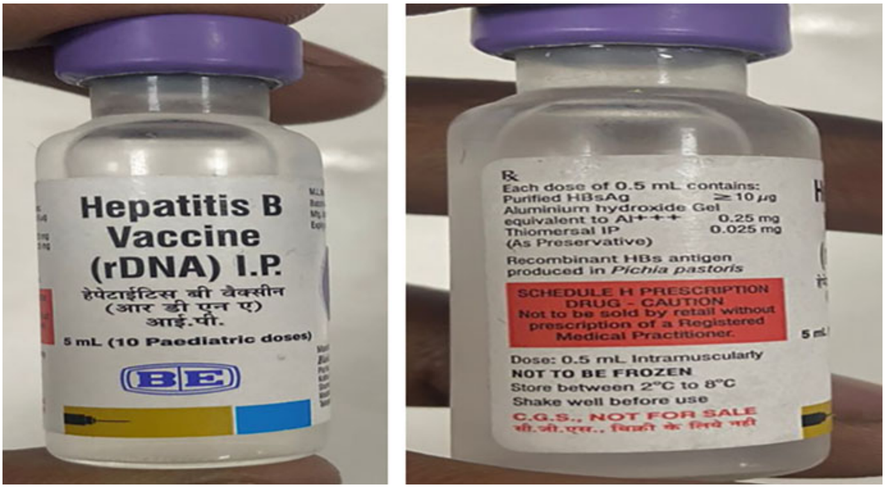

**Fig. 28.13** Hepatitis B vaccine

Organization n.d.-b). Full vaccination schedule consists of three vaccine doses, with first two doses given at least 4 weeks apart and third dose 1–12 months later (World Health Organization n.d.-d).

The second approach to primary prevention is interventions, which decrease the risk exposure. Health education aiming at altering the risky behaviors among general population or high-risk groups is a part of primary prevention. It is a vital component of programs for cancer control and is found to be effective in modifying lifestyle behaviors (Van Parijs 1986). It should generate awareness about common carcinogens, their effects, and resulting cancers. People should be sensitized about consequences of tobacco and alcohol use, poor genital hygiene, indulging in unsafe sexual practices, use of infected needles, and occupational exposures. They should be encouraged to alter such behaviors and practices to lessen the exposure to incriminated carcinogens and development of cancer. Tobacco use in any form including second-hand smoke needs to be avoided. Many infections that lead to cancer have specific transmission routes and can be prevented by modifying risk-enhancing behaviors. People should keep a check on unnecessary radiation exposures. As the evidence is growing in favor of obesity and inadequate physical activity as potential carcinogen and it has been found that obesity contributes to approximately 12 types of cancers, importance of maintaining a healthy weight and regular physical activity as per international recommendations should also be stressed upon simultaneously. Breastfeeding should be promoted as it is found to be protective against breast cancer among mothers and overweight and obesity among breastfed children, thereby protecting against cancers associated with overweight and obesity. Certain dietary exposures increase the risk of cancer development by contributing to obesity and overweight. This includes sugar and sweetened drinks, red meat, and fast food (Research n.d.). They should be consumed in limited quantities, if cannot be excluded from the diet. In place of them, people should be encouraged to include wholegrain, fiber, fruits, and vegetables in their diet which have shown protective effect against certain cancers and help in weight management (Research n.d.). Many nutritional supplements are marketed as providing protection against cancers. Such food myths should be busted with focused health education strategies. Supplements such as beta-carotene can increase the risk of lung cancer, and none of the other supplements except calcium for colorectal cancer have been proven to be protective against any type of cancer (Research n.d.).

Legislations, regulations, and public health policies which regulate the limits of chemicals and pollutants in environment and occupational settings (United States Department of Labor. Occupational Safety and Health Administration n.d.-b) safe disposal of industrial wastes, prohibition of discharge of industrial effluents into rivers are also an example of primary prevention measure. Other legislation and policies which regulate the public behavior like prohibition of smoking in public places, placing age restrictions on purchase of alcohol and tobacco, raising taxes, banning the use of pesticides containing carcinogens, and banning unsupervised tanning beds are also employed by many countries (World Health Organization n.d.-e).

For occupational cancers, complete elimination of the carcinogen and substituting it with a non-carcinogenic material in industries is the topmost control measure (Fig. 28.14). This approach can be used in few cases where risk of using a substance exceeds its benefits (US Department of Health and Human Services. Public Health Service. Centres for Disease Control. National Institute for Occupational Safety and Health 1986). It can be achieved with help of setting occupational standards and implementing strict enforcement of labor laws. However, this is not always feasible due to non-availability of any substituent agent. Engineering controls which include

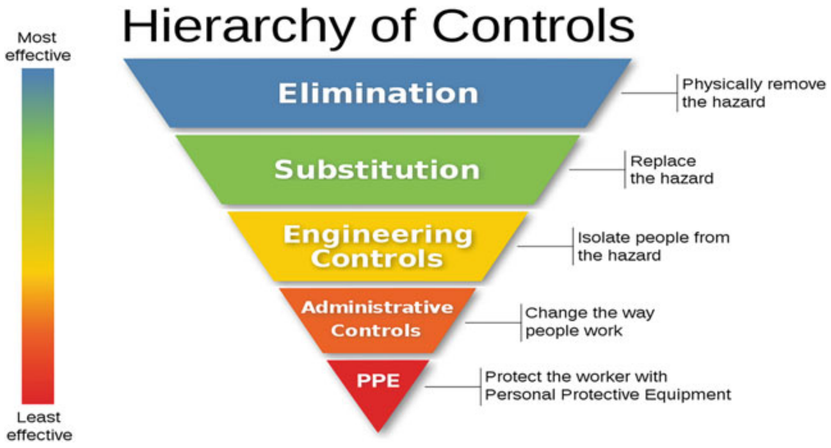

**Fig. 28.14** Hierarchy of control measures for occupational hazards by National Institute for Occupational Safety and Health (United States Department of Labor. Occupational Safety and Health Administration n.d.-a)

ventilation and enclosure of such environment where a carcinogen is being used or generated are next in hierarchy for control of exposures. When all of these measures are not possible, the last and least effective approach is use of personal protective equipment which reduces the exposure to a certain limit only (United States Department of Labor. Occupational Safety and Health Administration n.d.-a). Continuous and periodic evaluation of exposure, hazard recognition and identification and surveillance activities should supplement these control measures. Workers should be made aware about their exposures and associated risks and should be empowered to access the required information.

### 28.5.2 Secondary Prevention

Apparently, healthy individuals, who might have subclinical disease, are targets for secondary prevention, and it employs measures for early disease detection when a person usually is asymptomatic or in early phases with minimum signs and symptoms (Kisling and Das 2020). This can help identify and mitigate precancerous lesions and early identification of cancers, thereby aiding in more successful treatment (International Agency for Research on Cancer. World Health Organization 2019a). In low resource settings and among vulnerable populations, late-stage presentations due to delay in accessing cancer care are common resulting in poor prognosis, decreased likelihood of survival, more painful and costly treatment, increased incidence of avoidable cancer deaths, and disability. This can be done thorough screening and early detection. Screening is, therefore, an important public health strategy in all settings (World Health Organization n.d.-a). Periodic examination in certain occupations and industries serves the similar purpose as screening and early detection by identifying cancers in initial stages. Although most of the occupational cancers follow a long latent period to develop after initial exposure, nonetheless, association of certain cancers with specific occupations and industries can be helpful in designing periodic examinations in such settings.

Screening aims to presumptively identify unrecognized disease or defect by systematic application of a screening test. The test should be such that which can be applied rapidly in a presumably asymptomatic population and can distinguish probably diseased people from probably healthy individuals (Porta and Sander Greenland 2008). Screening is followed by diagnostic tests to confirm the presence or absence of disease. Some of the cancers may be detected sufficiently early through screening resulting in decrease in cancer risk, reduced incidence of more invasive caners, and prevention of cancer deaths though cure. Screening can be organized or opportunistic and can be for general population or only for high-risk groups. However, screening is not completely harmless and potential risk of over-diagnosis should be considered before employing it. Provision of screening services should be complemented by awareness generation strategies under early detection programs among general population and healthcare professionals about early signs and symptoms of the disease to increase the utilization of such services (World Health Organization 2007).

Few of the countries have integrated screening of common cancers in their ongoing healthcare programs. For example, Centers for Disease Control and Prevention (CDC) in USA supports screening for breast, lung, cervical, and colon cancer. For breast cancer, women in age 50–74 years are recommended to get a mammogram every 2 years as regular mammograms can reduce the risk of dying from breast cancer. MRI, clinical breast examination, and breast self-awareness can also be used (Division of Cancer Prevention and Control. Centres for Disease Control and Prevention 2020a). For cervical cancer, for women in age group of 21–29 years, only PAP test is recommended and screening should start at age of 21 years. For women 30–65 years of age, either PAP test or HPV test or a combination of both can be used to screen. If PAP test result is normal, next test will be done after 3 years and if HPV or co-test results are normal, next test will be done after 5 years (Division of Cancer Prevention and Control. Centres for Disease Control and Prevention 2019). Colorectal cancer screening is usually done after age of 50 years by stool tests, colonoscopy, flexible sigmoidoscopy, and CT colonography (Division of Cancer Prevention and Control. Centres for Disease Control and Prevention 2020b). Screening for lung cancer is done for people with history of heavy smoking, who are current smokers or have quit within last 15 years and are in age group of 55–80 years. Low-dose computed tomography is recommended for screening (Table 28.7) (Division of Cancer Prevention and Control. Centres for Disease Control and Prevention 2020c).

In India, opportunistic and population-based screening is advocated under the NPCDCS, for breast, cervical, and oral cavity cancer which are to be screened once in 5 years in population of age 30 years or more. For cervical cancer, VIA is recommended, and for oral cavity cancer, oral visual examination is to be conducted at primary healthcare levels (Ministry of Health and Family Welfare 2016).

Under National Health Service (NHS) in UK, screening for breast, cervical, and bowel cancer, is offered to general population. Cervical cancer screening is done among women aged 25–64 years of age with once in 3 years for 26–49 years age group and once in 5 years for 50–64 years age group. Breast cancer screening is offered to women of age 50–70 years and women above age of 70 years can self-refer themselves. For bowel cancer, home testing kit is given to population aged

**Table 28.7** Screening recommendations by CDC for US population

| Cancer | Screening methods available | Age group | Population |
|---|---|---|---|
| Breast | Mammography, MRI, clinical breast examination, breast self-examination | 50–74 years | Women |
| Cervical | Pap smear, HPV, VIA, VILI | 21 years or more | Women |
| Colorectal | Stool tests, colonoscopy, flexible sigmoidoscopy, CT colonography | 50–75 years | All |
| Lung | Low-dose computed tomography | 55–80 years | Heavy smokers at present or within last 15 years |

between 60 and 74 years and bowel scope screening is offered to population above 55 years of age (NHS 2018).

The screening tests are usually followed by diagnostic tests which are in general more invasive and confirmatory. Laboratory tests using blood or tissue sample for tumor markers, imaging modalities including X-rays, ultrasonography, computerized tomography (CT) scan with or without contrast, magnetic resonance imaging (MRI), nuclear scans, bone scans, and positron emission tomography (PET), and biopsy are commonly employed diagnostic tests for cancers which also help in staging the disease (National Institutes of Health. National Cancer Institute 2019a).

### 28.5.3 Tertiary Prevention

Tertiary prevention measures come into play once the disease has been diagnosed as it aims to either stop or slow the disease progression and reduce the disease severity and associated sequelae. It includes various measures, namely chemotherapy, radiotherapy, rehabilitation, and screening for complications (Kisling and Das 2020). In cancers, tertiary prevention implies measures used to reduce the impact of long-term disease and disability which occur due to cancer or its treatment (International Agency for Research on Cancer. World Health Organization 2019a).

The treatment modalities for various types of cancers include surgery, chemotherapy, radiation therapy, hormone therapy, immunotherapy, targeted therapy, and stem cell transplant. They can either be used alone or in combination depending on type of cancer, the site involved, stage of the disease, and potential side effects. Precision medicine, a relatively novel concept, is an approach that is based on genetic understanding of the disease in an individual and helps doctors to choose treatments best suited for the person. It is, therefore, aptly called as personalized medicine (National Institutes of Health. National Cancer Institute n.d.).

Palliative care, a part of tertiary prevention, does not cure the disease but makes the patient feel better and improves their quality of life continuing through the treatment and beyond (Fig. 28.15). This is applicable in early course of the disease and should traverse through the disease progression till the terminal phase (palliative care module). It encompasses treating or preventing the symptoms and side effects of cancer treatment including pain management, helping them with emotional and spiritual problems, and addressing the concerns of the patient and family members (National Institutes of Health. National Cancer Institute 2020). Palliative care prepares patients and their loved ones for physical changes that may occur during terminal stages, helps them cope with emotional issues and different thoughts arising during the disease course, and provides support to family members. Research has shown beneficial effects of palliative care on patients and their family members' health and well-being (National Institutes of Health. National Cancer Institute 2017). Once the disease is advanced and is no more amenable to treatment, the treating physician may advise the patient to be shifted to a hospice or end-of-life care (National Institutes of Health. National Cancer Institute 2019b).

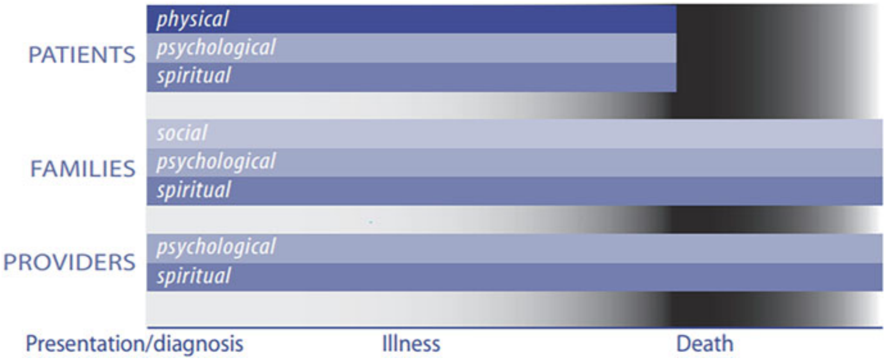

**Fig. 28.15** Quality of life dimensions of palliative care (World Health Organization 2002)

Rehabilitation, on the other hand, is a group of interventions that are required when an individual is likely to experience or is currently experiencing restrictions in everyday functioning due to certain conditions including chronic diseases such as cancers. It is a person-centered health strategy which helps them to maintain or restore their daily living activities, accomplish meaningful life roles, and maximize well-being. It can be delivered though other health programs or specialized rehabilitation programs (World Health Organization 2019b).

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# 29

# Wastewater-Based Epidemiology (WBE): An Emerging Nexus Between Environment and Human Health

Aakanksha Kalra, Akansha Mathur, Tamanna Pamnani, and Sudipti Arora

## Abstract

Water, an integral component of life on earth, is being hampered or exploited to a great extent owing to both natural and anthropogenic activities. The overexploitation of water resulting in the rise in the generation of wastewater not only results in water scarcity but also results in water pollution across. This creates an avenue to focus on technologies to deal with wastewater. Numerous wastewater treatment technologies exist, having the potential to treat wastewater and convert into useful resource. These wastewater treatment plants (WWTPs) serve as reservoirs of providing information on water and environmental health, as well as treat contaminants, especially numerous disease-causing pathogens. The applicability of wastewater as a diagnostic tool referred to as wastewater-based epidemiology (WBE), began in late 1990s when the presence of illicit drugs was detected and correlated with the drug consumption in the associated community. Since then, the technology has been exploited for various purposes such as poliovirus in 2003 and SARS-CoV-2 detection as the recent one. Meanwhile, antimicrobial resistance has been on the rise owing to uncontrolled and unregulated use of antibiotics, especially from environmental sources such as soil, water, and WWTPs. In this line, a pool of 6 Gram-positive and Gram-negative microorganisms referred to as "ESKAPE" have been identified as global priority pathogens by WHO and special emphasis has been given to understanding and control of emerging drug resistance toward them. This chapter therefore focuses on the applicability of WBE in the detection and diagnosis of these pathogens in

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wastewater. Also, the chapter briefly describes the molecular methods and tools employed for the detection of these resistant microorganisms in the wastewater samples, along with current national and international status of ESKAPE pathogens.

**Keywords**

Antibiotic resistance · ESKAPE · Environment and human health · Wastewater · Wastewater epidemiology · WWTPs

## 29.1 Environment and Health

Of the eight planets present in the solar system, earth is the only planet that supports life and this is possible due to the presence of favorable environment. However, over the years, the technological advancements on one hand have improved the quality of human life, while on the other have deteriorated the environment to a great extent. This in turn has affected both the plant and animal species in the ecosystem leading to their endangerment and extinction ultimately reducing the biodiversity. The studies over the years eventually made us realize that this harm to the environment has encircled back to human life and is one of the major factors impacting us in the present scenario. The exploitation of the environment particularly in the last 4–5 decades has been very serious and distinct leading to serious endangerment to life. The 50th anniversary celebration of Earth Day on April 22, 2020, has marked a reminder for the replacement of unsustainable activities and technologies with that of the eco-friendly green and biological approaches. Some of these technologies include the use of biofertilizers and biopesticides in agriculture, biofuel counterparts for energy generation, bioremediation for cleaning up of environment, and use of bioplastics and biofilters to reduce pollution. Since for more than last 5 decades have focused on the development of human life, which has affected the environment to a great extent, the focus for next decades should consist of upliftment of the environment and thus the planet. The sustainable development goals (SDGs) that have been set up by United Nations General Assembly in 2015 consists of 17 interlinked goals for the improvement of both human life and environment with the aim to be achieved by 2030 are one of the crucial steps in this direction (Arora 2019).

The impact of environment on human health was well focused and evident in the last few decades clearly stating it to be the major factor affecting human health. Surprisingly, as per WHO (World Health Organization) statistics 20% of deaths have been attributed to environmental-related factors consisting of diet, sanitation, socioeconomic status, literacy, and lifestyle. The factors affecting rise in the life expectancy between 1970 and 1992 were found to be better working conditions, increased gross development product (GDP), and health expenditure per capita suggesting crucial role of technological advancements (Or 2000). On the contrary, during the similar time frame, we started observing the negative impacts of environment, particularly air pollution caused by these anthropogenic activities on human health

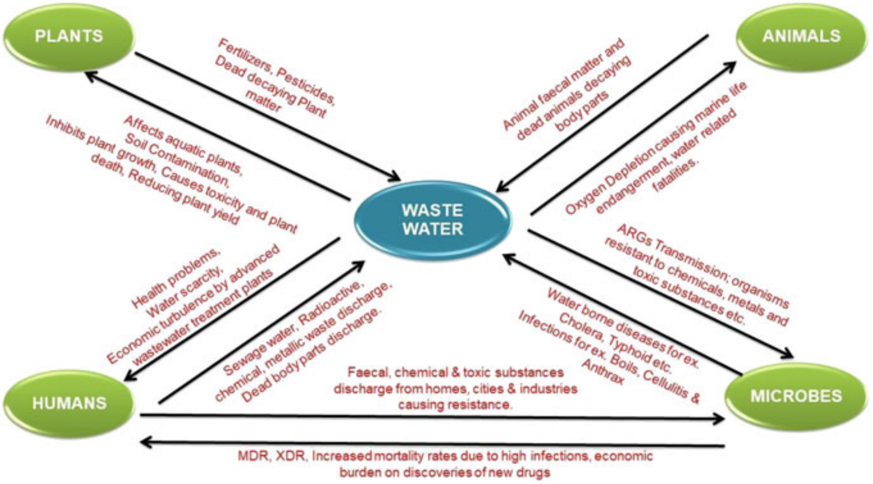

**Fig. 29.1** Impact of wastewater on different life forms and vice versa and nexus between humans and microorganisms

(Or 2000). This brought the attention of multiple groups to envision the role of environment in affecting human health and the modifications needed to combat the same (Blashki et al. 2007; Podein and Hernke 2010; Richardson et al. 2009). Ironically, the infrastructure and the technology needed to focus on well-being of humans including healthcare facilities and food-based industries are the ones that are the most energy-intensive facilities requiring technological advancements and excessive use of fossil fuels impacting the environment, which in turn affects human health. This vicious circle needs to be broken down at some point in order to protect both the environment and the human health. It is a well-known fact that excess of anything is as harmful as the lack of it. It would not be an exaggeration to state that in the race of improvements and technological advancements we have come a long way in excess of everything including diagnosis and treatment.

It is quite contrasting that the interlink between human activities and their impact on environment with the microorganisms is not a general point of consideration, while it is one of the major factors impacting both the environment and the human health. The role of microorganisms in human health is a well-known fact and does not need much description. With the amendments that have been taking place in the environment over these years, the proportion and the categories of microorganisms have also been changing and in turn impacting human lives. On the other hand, water is one of the major targets of environmental factors getting affected owing to human activities leading to the production of great deal of wastewater and has been studied in great detail. The impacts of wastewater on various life forms and vice versa have been described in Fig. 29.1. Besides, it also shows the impact that the microorganisms and humans share between each other owing to wastewater as a nexus. The earlier sections of this book clearly describe the impact that has been caused to the water resources including generation of wastewater, types of wastewaters, characterization of the same, the treatment strategies employed, and the role of microorganisms in remediation of wastewater. In this chapter, we will be focusing briefly on the wastewater generated owing to human activities followed by the introduction to wastewater-based epidemiology (WBE), an emerging tool for the prediction or diagnosis of disease spread in a particular geographical region. This will be followed by the emergence of drug resistance in the current scenario with special emphasis on ESKAPE pathogens and their current impact on the healthcare sector. The chapter also focuses on exploitation of WBE in reference to these ESKAPE pathogens and molecular tools employed for prediction purposes. The last section of the chapter would focus on the current status of ESKAPE pathogens and WBE in this regard both at the international and national levels.

## 29.2 Wastewater: An Introduction

Needless to say, water is one of the most essential elements for survival of life be it humans or any other flora or fauna. Considering the fact that almost 71% of the planet is covered with water, this should be one of the least concerns for a happy and healthy existence. However, of all the water available, only 0.3% is available for use owing to majority of the same frozen in glaciers or present with high salt content as sea water. This lack of water is further aggravated by the conversion of usable water to wastewater rendering the major proportion of the available water, useless. The simplest way to define wastewater is the water that cannot be used for basic amenities of life. However, this definition has much more depth considering the level of impurities and the extent of remedial processes required to make it useful. All this has led to a severe crisis of clean water and has forced us to identify novel strategies for the treatment of this wastewater to meet the rising demand. Extensive work has been done and is still undergoing in this regard, which is described elsewhere and is not in the scope of this chapter.

Before we discuss WBE and the antibiotic-resistant microorganisms and the role of WBE in prediction of infectious disease spread, we will focus on the common pollutants present in the wastewater and the types of wastewaters that need attention in the present scenario. The most common pollutants in wastewater include microorganisms, which may or may not be pathogenic, organic, and inorganic chemicals, heavy metals, sediments, radioactive substances, and many more such molecules. Depending on the source of the generation of wastewater and the type of pollutants present, wastewaters are majorly categorized as household and industrial. Household wastewater also referred to as sewage waste or sanitary sewage majorly consists of sewage, which includes fecal matter that has oxygen-requiring molecules and huge proportion of microorganism, while industrial wastewater consists of organic and inorganic chemicals, heavy metals, and complex compounds produced during the manufacturing or chemical processes, depending on the source industry. Apart from these two, agriculture and farming are another major cause of the wastewater generation leading to accumulation of a large proportion of sediments, fertilizers, insecticides, and pesticides in the water bodies. All these molecules are responsible for damaging the aquatic life in addition to rendering water unfit for use.

In addition to these molecules, based on the presence of microorganisms in the water samples owing to sewage, wastewaters particularly domestic wastewater has been further segregated into three different categories including white, gray, and black waters. White water is the useable water and free from all microorganisms, while graywater includes the water samples obtained from human activities such as washbasin, bathing, or washing with very low proportion of microorganism, especially nonpathogenic ones. On the other hand, water consisting of fecal matter and sewage with large proportion of microorganism is referred to as the black water. It is a well-known fact that wastewater is a potential source of transmission of these microorganisms leading to the spread of diseases such as cholera, diarrhea, dysentery, hepatitis A, typhoid, and polio infections (WHO). It has also been reported that about 1.5 and 12 million people die each year from waterborne diseases and diarrheal diseases have been listed in the top 15 leading causes of death worldwide (Xagoraraki and O'Brien 2020). This is further evident from the presence of SARS-CoV-2 viral RNA in the stool samples suggesting wastewater to be an indirect pathway for the re-infection process during the COVID-19 pandemic. In addition, this infected wastewater can be disseminated in surface and groundwater, in irrigated agriculture, or as aerosols. The situation is further worsened by the emergence of antibiotic-resistant bacteria owing to inappropriate and uncontrolled use of antibiotics. The emergence of these bacteria, the mechanism behind the resistance, and categorization of high-priority ESKAPE pathogens are discussed in detail in the upcoming section. The accumulation of these resistant microorganisms in wastewater had further led to their spread suggesting the severity of the situation. Thus, this wastewater consisting of antibiotic-resistant microorganisms and further causing its transmission is referred to as "the Black box" and is one of the highest priority wastewaters in the current scenario, which needs to be addressed.

## 29.3 Wastewater-Based Epidemiology (WBE): An Introduction

According to Center for Disease Control and Prevention (CDC), *epidemiology is defined as the method used to identify the causes of health outcomes and diseases in populations wherein the distribution, determinants, and events are analyzed in specified populations to control health problems* (https://www.cdc.gov/csels/dsepd/ss1978/lesson1/section1.html).

The previous sections of this chapter have clearly suggested the nexus between the human activities and the environment and water being one of the crucial links in this nexus. Also, it has been very clear that generation of wastewater and its impact on human health are a vicious circle, which needs to be broken at the earliest. In this section, role of wastewater in epidemiological analysis will be discussed. So far, it has been very clear that wastewater is a major source of disease spread, particularly for infectious pathogens, and thus, this can also be employed as an epidemiological tool for the prediction of disease spread. The analysis of wastewater is a prediction tool initiated in 1990s for monitoring real-time data on geographical and temporal trends in illicit drug use. After the introduction of the model in 2001 for the very first time by Daughton, it was successfully employed for the determination of cocaine residues in 2005 by Zuccato et al. (2005), which was followed by its application worldwide. The efficacy of the technology can also be assessed by the fact that it is used for policymaking and actions related to drug control activity in China. For this, the influent samples of the wastewater treatment plants (WWTPs) were analyzed for quantitative measurement of drugs or their metabolites that could have been excreted in the urine samples of people in a community. The advantages of this technology included noninvasive and near real-time analysis. The technology used for the identification of drugs in the given samples is subdivided into the following steps:

- Collection of representative composite samples of influent wastewater.
- Qualitative and quantitative analysis of particular substances in the samples.
- The observed concentrations are multiplied by daily sewage flow rate to get daily sewer loads.
- This was used for the estimation of the daily consumption using a specific correction factor to the daily load. The specific correction factor is dependent on the average excretion rate of the drug molecule and the molecular mass ratio of the parent drug to its metabolite.
- This daily consumption is then divided by total number of people served by treatment plant to get per capita value.

Though the process had been standardized even for quantitative estimation, it had certain limitations such as deciding the sampling method, identification of the appropriate biomarkers, the tools used for the analysis, and establishment of average excretion rates. All of these issues were considered and analyzed to determine solutions for each. This led to the development of WBE as an important prediction and diagnosis tool not just in case of drug usage determination but also in terms of other pollutants such as pesticides (Rousis et al. 2017) or heavy metals (Du et al. 2020).

Thus, WBE in terms of healthcare sector has now been identified as an integrated technique involving extraction, analysis, and data processing and interpretation of biomarkers obtained from the feces and/or urine samples in order to determine the overall community health of a given population. The choice of appropriate biomarkers is of utmost importance for the analysis of true health status of the community. Besides, for the rapid intervention and control of any infectious disease outbreaks, an effective surveillance system is the need of the hour. It can therefore be claimed that the identification of appropriate biomarkers can be utilized for surveillance and early warning of infectious disease outbreaks. This has very well been proven in case of pandemic in different researches done on SARS-CoV-2 (Medema et al. 2020; Arora et al. 2020, 2021a, b). For instance, a study in Guangzhou, China, has suggested fecal aerosol transmission of SARS-CoV-2 in a high rise building, thereby suggesting the importance of wastewater epidemiology in SARS-CoV-2 detection (Kang et al. 2020). This chapter therefore focuses on the application of

WBE in terms of antibiotic resistance, particularly in reference to ESKAPE pathogens.

## 29.4 Antimicrobial Resistance

It has been almost a century since the first antibiotic, penicillin was discovered in 1928 by Alexander Fleming. This discovery had revolutionized the treatment strategies toward bacterial infections. The better understanding of the life cycle of microorganisms and their infection cycles along with developments in the scientific technologies led to the identification of numerous antibiotics, each with its own advantages and limitations. This discovery was further enhanced tremendously with the advent of bioinformatics and computational biology when drug repurposing came into play. While the identification of novel antibiotics was useful on one hand, it created a drawback on the other when the target microorganisms began developing resistance toward these drug molecules. Needless to say, we have come a long way since then in terms of discovery and use of antibiotics for the treatment of bacterial infections. However, for common bacterial infections, including urinary tract infections, sepsis, sexually transmitted infections, and some forms of diarrhea, high rates of resistance, have been observed worldwide against antibiotics that are frequently used indicating that we are running out of effective antibiotics. The discovery of novel antibiotics and the emergence of resistant species in the last two decades have been very well compiled in the review by Ma et al. (2020). With the technological advancements and emergence of resistant isolates, we have already reached the fifth generation of antibiotics along with emergence of resistance against them. In 2019, WHO identified 32 antibiotics in clinical development that address the WHO list of priority pathogens, of which only six were classified as innovative. Furthermore, a lack of access to quality antimicrobials remains a major issue. Another issue is the development of novel effective antibiotics toward these infections. Also, it is also believed that if these antibiotic usages are not managed appropriately, these new antibiotics will suffer the same fate as the current ones and will become ineffective. It is also clear that the pace of emerging drug-resistant isolates is getting way faster than the discovery of novel drug molecules in spite of the scientific and technological advancements. Besides, there has been a constant disparity between the identification of novel antibiotics and emergence of resistance since time immemorial. Also, the prevalence of antibiotic-resistant isolates is escalating globally, especially in developing countries due to compromised drug administration monitoring along with overuse or misuse of drugs.

However, it is surprising to note that even with the plethora of microorganisms each affecting the human body differently, the drug resistance that emerged relies only on a few mechanisms. These mechanisms include antimicrobial inactivation, persistence, cell wall alterations, and porin alterations, which have been very well discussed in Santajit and Indrawattana (2016).

Therefore, antimicrobial resistance (AMR) is now a global health concern in the present scenario threatening global progress in various aspects including striking

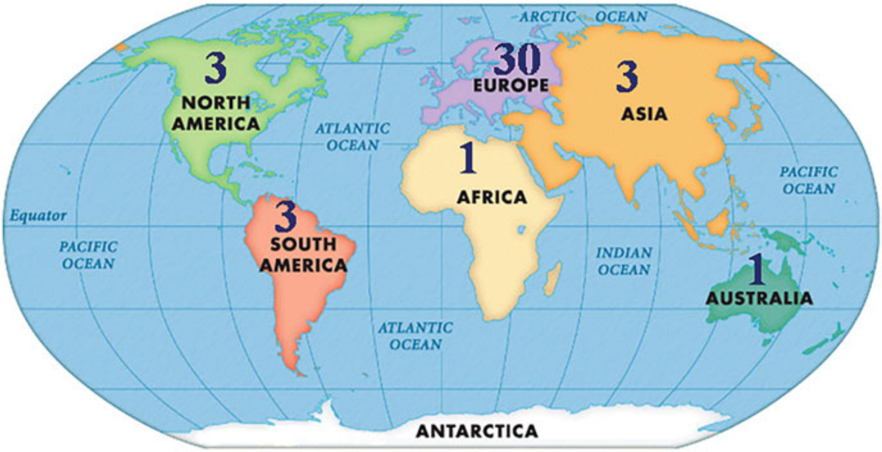

**Fig. 29.2** Continent-wise segregation of antibiotic-resistant microbes observed as per Center for disease dynamics, economics, and policy (CDDEP) stats 2021

hardest on the poor, untreatable infections even in the animals, thereby affecting sustainable food production, affecting healthcare systems being an integral part, contaminating water and land with residues, and many more. The continent-wise segregation of antibiotic-resistant microbes observed as per center for disease dynamics, economics, and policy (CDDEP) stats 2021 are shown in Fig. 29.2. According to World Bank Group Report on Drug-Resistant Infections (March 2017), the economic burden of AMR is expected to rise to about US $120 by 2050 suggesting a dire and urgent need to focus on the issue. The worldwide attempt to keep a check on the rising AMR was made in 2015 by WHO with the objective to create awareness, understanding, strengthening knowledge and evidence toward AMR, reducing the infection rates, and optimizing antibiotic use of the antimicrobial agents. However, lack of funds has been the major cause of the huge difference in the national action planning vs. implementation. India is one of the countries enrolled in the Global Antimicrobial Resistance Surveillance System (GLASS program) to create awareness and keep a check on AMR. One of the major reasons for this resistance is the overuse and inappropriate antibiotic use, ineffective drug delivery system being the second and genetic variations in the microorganisms being the third. There has been preliminary analysis on the isolates obtained from wastewater samples of Jawahar Circle (in Jaipur, Rajasthan) via VITEK and 16S rRNA sequencing, which suggests the presence of resistant pathogens (Shekhawat et al. 2021 under review). This emerging issue of antimicrobial resistance rose even higher during the COVID-19 pandemic when the fear and confusion led to uncontrolled and inappropriate use of antibiotics. Owing to the continuously rising AMR, ideally these antibiotics should have been under strict regulation and prescription, while these are being used as over-the-counter drugs as of now. Although COVID-19 is the major health problem for the world now, the continued use of antibiotics will be accelerating the AMR problem and will create major issues once the COVID-19 chaos is over. The antibiotics that are being consumed are not only causing AMR in the pathogens present in the human body but are also increasing the load of antibiotic-resistant bacteria (ARBs) and antibiotic-resistant genes (ARGs) in the environment specifically wastewaters. Evidences suggest that resistant bacteria and genes discharged by humans are circulated through the community wastewater and thus get accumulated as well (Larsson 2014).

The current understanding suggests that we need to analyze the bacteria at its genetic level to understand the defense mechanisms since only 4–5 defense mechanisms are sufficient enough to alter or inhibit our drug delivery or antibiotic delivery, even after the drugs are specific to each infection. Although separate sporadic studies in resistance of gram-positive and gram-negative bacteria have reported certain genes, which turn out to be the same, no systematic and targeted study has been done especially in context of Indian population where common molecular players in gram-positive and gram-negative bacteria have been searched. Arguably, it is highly possible that the same genes are linked to mechanisms, which commonly affect resistance in both types of bacteria. Studies have also demonstrated a direct relationship exists between antibiotic consumption and the emergence of resistant bacterial strains. Horizontal gene transfer (HGT) is majorly responsible for the transfer of antibiotic-resistant genes among different species of bacteria.

Currently, new line of drugs along with few effective previous generation drugs is in use for treatment, but they lack adequate specificity and efficiency and also lack a sufficiently good delivery system. For more than a decade now, we are still unsuccessful in finding either the drug effective enough for inhibiting these pathogens nor are we successful in identifying the specific gene or protein that is responsible for the resistance in bacterial strains.

## 29.5 ESKAPE Pathogens: An Introduction

The previous section clearly describes the current status of antibiotics and the efforts employed in this regard. In spite of numerous efforts, bacterial infections could not be contained till now and have been on the rise. In this context, ESKAPE pathogens, which are a group of six highly virulent and antibiotic-resistant (gram-positive and gram-negative) bacterial pathogens including *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp., have been analyzed. These six pathogens have emerged as a major cause of life-threatening nosocomial or community-acquired infections worldwide. ESKAPE pathogens have been considered to be the global priority pathogens (WHO reports 2017) owing to their emerging resistance toward available antibiotics.

ESKAPE pathogens are responsible for majority of nosocomial infections and are capable of “escaping” the biocidal action of antimicrobial agents (Navidinia 2016).

World Health Organization (WHO) has also recently listed ESKAPE pathogens in the list of 12 bacteria against, which new antibiotics are urgently needed (Tacconelli et al. 2018). They have described the three categories of pathogens including critical, high, and medium priority, as per the urgency of need for new antibiotics. Carbapenem-resistant *A. baumannii* and *P. aeruginosa* along with extended spectrum β-lactamase (ESBL) or carbapenem-resistant *K. pneumoniae* and *Enterobacter* spp. have been listed in the critical priority list of pathogens, whereas vancomycin-resistant *E. faecium (VRE)* and methicillin and vancomycin-resistant *S. aureus* (MRSA and VRSA) have been included in the list of high-priority group. Rates of antimicrobial resistance among Enterococci are particularly concerning, especially the incidence of vancomycin-resistant *Enterococcus* (VRE), which is mainly associated with *E. faecium* (Santajit and Indrawattana 2016). Pathologically, Enterococcus infections cause meningitis or endocarditis affecting gastrointestinal tract in humans and animals. *S. aureus* are present in the normal skin flora, especially nose and perineum of humans and animals, which can transmit via direct contact or airborne routes. Species of the genus *Klebsiella* are the bacterial pathogens most often found associated with infections in healthcare settings and infections may be endogenous or acquired through direct contact with an infected host (Santajit and Indrawattana 2016). The most important human pathogen is *A. baumannii*, which has a relatively long survival time on human hands, which can lead to high rates of cross-contamination in nosocomial infections (Houang et al. 2001), causing infections at a variety of sites, including the respiratory and urinary tracts. Strains are frequently antibiotic-resistant, which is a particular problem in surgical wards and intensive care units. *Pseudomonas aeruginosa* is part of normal gut flora; infections are mostly caused through exogenous source, but endogenous source is also possible. Enterobacter spp. can cause opportunistic infections in immunocompromised, usually hospitalized, patients and contain a wide range of antibiotic-resistant mechanisms. To sum up, ESKAPE pathogens can be regarded as the leading cause of bacterial infections throughout the globe, and they have the ability to affect almost all the major systems of the human body including respiratory, urinary systems, and skin. The emerging resistance to these pathogens is further worsening the situation since World's existing drug pipeline is drying and identification of novel drugs is still a long way ahead.

Figure 29.3 represents the number of publications relevant to ESKAPE pathogens in the last two decades suggesting the severity of the AMR caused by these pathogens as per Google Scholar.

## 29.6 Molecular Tools for Integrated Monitoring of Pathogens and Antimicrobial Resistance in Wastewater

Pathogens and AMR are emerging as major global threats to public health. Wastewater, as the unique interface between environments and humans receiving and spreading pathogens and AMR, is playing a more important role globally. It is important to understand the important molecular tools available for wastewater

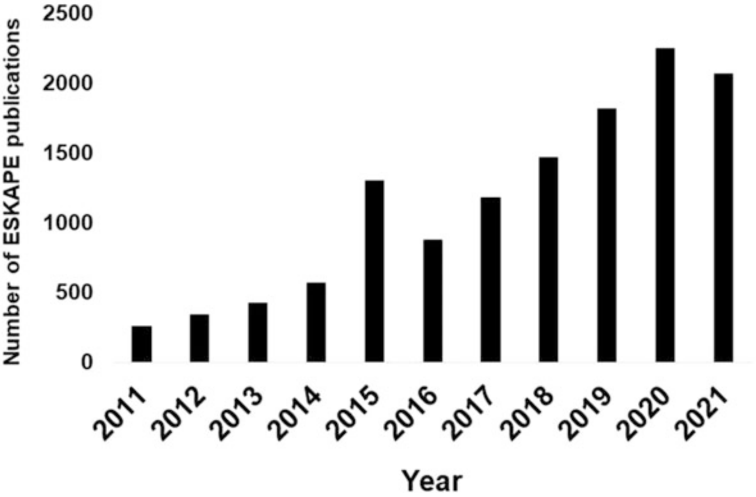

**Fig. 29.3** Number of publications relevant to ESKAPE pathogens in the last two decades suggesting the severity of the AMR caused by these pathogens

surveillance of the global pathogens, thus promoting early detection and providing mitigation measures. The analysis of different biomarkers present in wastewater collection networks can inform policymaking decisions and emergency responses to public health crises and governance, such as the propagation of infectious agents and the prevalence of drug use in a community, along with spread of AMR. WBE has been used as a powerful tool for real-time monitoring and analysis of a variety of biomarkers in wastewater. For example, the presence of viral (e.g., SARS-CoV-2) genomes in wastewater provides promise for better understanding of the spread of infectious disease within a population (Arora et al. 2020, 2021a, b; Ahmed et al. 2020). The monitoring of phthalate metabolites in wastewater can be used as an economic alternative for estimating human exposure to phthalates (González-Marino et al. 2017). Recently, monitoring of caffeine as an important biomarker was performed by Chakraborty et al. (2021) to understand the dynamics of wastewater and drug use.

There are various molecular tools employed to understand the AMR patterns, in laboratory research. These include traditional methods such as culturing microbes in the laboratory in growth media, which is a classical cultivation method for identifying pathogens (e.g., bacteria, viruses, fungi, and protozoa) and their antibiotic resistance. The pure culture of microorganisms is known as the foundation of research on infectious disease. However, the drawbacks of this method include time-consuming, lengthy, and laborious process. This is mainly due to the necessity of enriching and isolating pathogens on selective media from complex environmental samples. Although there may be some pathogenic microorganisms, which can pose a high risk of spread and infection, due to its low abundance in the environment, it increases the difficulties to enrich and isolate. Also, there are some pathogens, which can be also in a viable but nonculturable stage (VBNC) after exposure to a hostile environment for a long time (Cenciarini-Borde et al. 2009). Therefore, cultivation technique has many limitations for monitoring pathogens and AMR.

Recently, culture-independent molecular tools, such as PCR, loop-mediated isothermal amplification (LAMP), high-throughput qPCR, metagenomic sequencing, and single-cell genomic sequencing, have been applied to detect pathogens and their antibiotic resistance in environments (Guajardo-Leiva et al. 2020; Jahne et al. 2020; Liu et al. 2016). These culture-independent molecular tools are efficient at detecting genes of microorganisms that cannot grow in the laboratory or are in VBNC state. They are also operated in a high-throughput manner allowing capturing many different types of genetic information from taxonomy to functional genes such as ARGs, MGEs, and virulence factor. These molecular tools and methods have been recruited as a standard approach in the GLASS launched by WHO 2015, and are thus of great potential for wastewater surveillance to interpret pathogen and AMR profiles and understand their prevalence.

Polymerase chain reaction (PCR), referred as a gold standard technique of gene amplification, is the commonly used method for detecting the specific segments of DNA/RNA genomes of pathogen. Multiplex PCR assays with many different primers can be designed to detect multiple pathogens and/or resistance genes at the same time.

However, PCR required agarose gel electrophoresis for interpretations and qualitative analysis. Currently, this traditional agarose gel-based detection of PCR amplification is no longer been able to meet the high demand of accurate quantification and rapid detection of target genes. Therefore, real-time quantitative PCR (qPCR) is found to be more suitable and can rapidly detect target genes without electrophoresis and monitor DNA amplification in real time (Calderón-Franco et al. 2021; Rudko et al. 2017). SYBR Green and TaqMan are the two commonly used methods in qPCR to quantify the concentration of target genes by monitoring the real-time changes in fluorescence during thermal cycling (Bustin 2009).

Reverse-transcriptase quantitative PCR (RT-qPCR) is an extension of qPCR technology and has been demonstrated as an excellent method of choice for detecting RNA viruses (e.g., SARS-CoV-2 and norovirus) and pollution in wastewater (Achak et al. 2021; Flannery et al. 2013). During the COVID-19 pandemic, RT-qPCR has been widely used to detect SARS-CoV-2 in both human and environmental samples such as wastewater (Arora et al. 2021a, b; Medema et al. 2020; Bivins et al. 2020). Medema et al. found that the RNA signals of SARS-CoV-2 detected by RT-qPCR in wastewater correlated significantly with the prevalence of COVID-19 cases in the community (Medema et al. 2020). The surveillance of SARS-CoV-2 RNA concentrations can serve to early warn virus circulation in the population for early epidemic prediction. Due to the low concentration of viruses in the environment, concentration steps such as ultrafiltration, polyethylene glycol precipitation, and electronegative membrane adsorption are utmost necessary prior to direct RNA extraction and SARS-CoV-2 gene detection.

Droplet digital PCR (ddPCR) is another new sensitive method for nucleic acid detection based on the Poisson distribution statistics by fractionating DNA/RNA samples into tens of thousands of nanodroplets for PCR amplification within each individual droplet. Compared to traditional qPCR, the benefits of ddPCR include absolute quantification of target DNA copies without the requirement of running standard curves, reduced PCR bias by removing the amplification efficiency reliance of qPCR, increased signal-to-noise ratio allowing for sensitive and precise detection of rare targets due to the highly diluted nucleic acid templates and effective amplification in droplet (Hayden et al. 2013). ddPCR is a sensitive tool for target DNA measurement, microbial quantification, and viral load analysis.

In addition to PCR-based DNA measurement technology, loop-mediated isothermal amplification (LAMP) is also a simple, rapid, specific, and cost-effective nucleic acid measurement technology. Different from PCR, LAMP can complete amplification at a constant temperature (60–65 °C) without thermal cycler (Tomita et al. 2008). This advantage endows it great ability for field testing by using a paper-based device or microfluidic chip. LAMP has been widely used for the rapid detection of bacterial DNA of foodborne, airborne, and waterborne pathogens, such as *Pseudomonas aeruginosa*, *Legionella pneumophila*, *Vibrio parahaemolyticus*, and *Vibrio cholerae* (Yuan et al. 2018; Xu et al. 2019).

The recently developed high-throughput qPCR (HT-qPCR), which is capable of detecting PCRs of hundreds of different primers targeting almost all major classes of ARGs and pathogens in one chip, is becoming more and more popular for environmental surveillance (An et al. 2020; Ramírez-Castillo et al. 2015). HT-qPCR has been used to quantify the antibiotic resistome of a wide variety of environmental matrices, including soil, sediment, wastewater, drinking water, fresh water, air, snow, plant, and food.

Genomic sequencing, especially metagenomic sequencing, is being increasingly used for pathogens and AMR surveillance. Sequencing is different from PCR, which only targets selected genes, metagenome sequencing offers a vast information and highest resolution of all known genes by sequencing the whole genome of all microbes inhabiting in environments (Tringe and Rubin 2005; Hugenholtz and Tyson 2008; Aarestrup and Woolhouse 2020). It thus can not only reveal microbial community structure but also its functional genes such as ARGs, MGEs, virulence factor, and their microbial hosts in complete environments, such as wastewater. Metagenomic analysis can be also combined with meta-transcriptomic analysis to comprehensively reveal the prevalence an expression of ARGs.

Despite extensive sequencing-based characterization of microbiomes, there remains a dramatic lack of understanding of microbial functions, because most of the functional genes and whether they can be expressed are unknown. For example, only 30–60% of genes in human microbiome gene catalog can be annotated (Heintz-Buschart and Wilmes 2018). The lack of understanding of bacterial functions and the underlying genetic mechanisms calls for the integration of phenotypic with genotypic analysis. Stable isotope probing (SIP) is a powerful tool for exploiting the ecophysiology and functions of microbiomes based on the assimilation of isotopes (C, N, O, H, S) into newly synthesized biomass. It not only allows in situ study of specific physiological activities of microorganisms without prior knowledge of genetic basis, but also provides a way to distinguish functional bacteria incorporating isotopes without knowing the bacterial metabolic process.

For genotypic surveillance, molecular methods such as metagenome sequencing, amplicon sequencing, and polymerase chain reaction (PCR) have produced extensive and immense DNA sequence-based information on pathogens (species, virulence factor) and AMR (ARGs, MGEs). Such large information enables the construction of local, regional, and even global-scale distribution of pathogens and AMR. The high resolution also allows quantitative and comparative studies of the correlation among human, animal, and environmental samples, thereby providing an approach to surveil pathogens and AMR under the One Health framework (Gerner-Smidt et al. 2019). It is also possible to illustrate the microbial hosts, genetic locations, and context of ARGs and virulence factors. More importantly, molecular methods allow reconstruction of putative transmission networks to determine the source and evolution of pathogens/AMR that lead to diseases outbreaks (WHO 2020). However, molecular methods are not the choice under all circumstances.

Gene sequences only provide the potential or inferred pathogenicity and AMR instead of phenotypic features. In addition, genotypic analysis cannot distinguish the origins of genes from extracellular DNA, dead, nonactive, and active cells which risks are highly different. In contrast, phenotypic analysis indicates practical microbial infections or disease outbreaks. It can also be used to quantify the level of phenotypic AMR, so it is actionable to guide the clinical treatments of bacterial infections and promote research and development of new drugs to combat the growing global AMR. Phenotypic characterization is also an indispensable way to reveal the function of unknown or novel ARGs and virulence factors. However, compared with the rapid development of genotypic characterization, methods for phenotypic studies largely lag behind and have obvious drawbacks in implementation; i.e., they are time- and labor-consuming, have low throughput, and provide limited information to only cultivable bacteria, but preclude studies on uncultured cells, which represent a large fraction in diverse microbial communities (including wastewater) and may play a great role in the occurrence and spread of pathogens and AMR.

New phenotypic methods are highly demanded. Presently, there is still discrepancy and knowledge gap between genotypic and phenotypic analyses. For example, there are cases where ARGs are present, but resistant phenotypes are not found because ARGs are not always expressed. There are also cases where no known ARGs are present, but resistant phenotypes are observed due to the presence of novel ARGs or mechanisms. It is also possible that the same genotypic determinants confer varied phenotypic profiles. To bridge the gap and achieve a comprehensive understanding of the threat of pathogens and AMR to public health, there is a great need to integrate genotypic and phenotypic analysis for a complementary surveillance. When phenotypes are determined, genotypic analysis will add valuable information on genomic determinants underlying the pathogens with specific phenotypes. For example, genomic data can clarify ARGs conferring phenotypic antibiotic resistance, determine genomic mutations or acquired genes accounting for increased expression of phenotypic resistance or pathogenicity, and strengthen the identification of infection-causing pathogens more accurately. Therefore, an integrated approach will be invaluable for enhancing surveillance capacity and guiding strategy to tackle pathogens and AMR.

There is a great necessity for multiplex phenotypic and genotypic surveillance. As such, not only health risks but also the spread risks and the underlying mechanisms can be illustrated. The resulting comprehensive information is highly valuable for a more realistic and in-depth risk evaluation and predication. For example, some bacteria-harboring multiple ARGs may not be a superbug, but these ARGs, especially those located on MGEs (mobilome), still hold great potentials to disseminate to other species, which may become a superbug phenotypically resistant to nearly all antibiotics. In this case, it is important to know the mechanism accounting for the species with expression of ARGs (transcriptome). This is an urgent need because bacteria/virus and AMR are disseminating at an unprecedented speed and scale with the increasing waste disposal and more frequent global travel and trades.

Rapid and real-time monitoring is particularly important to signal the occurrence for early warning and track the dissemination trend to guide timely intervention strategy. Despite the advantages of Raman spectroscopy for a rapid cultivation-free identification of pathogens and AMR, combination with automatic sampling, detection, advanced data processing, and output such as deep learning are all needed to achieve real-time monitoring.

For molecular methods, laboratory-based assays are not enough for rapid and online surveillance, instead, miniaturized and portable devices, such as nanopore sequencing and loop-mediated isothermal amplification-based paper biosensors, show great promise for rapid, on-site, and sensitive monitoring pathogen and AMR. Moreover, they are low-cost and easily operated, endowing them great potentials to be popularized in more countries including low- and middle-income countries. Different laboratories are using their own methods (protocols, bioinformatic algorithms) and database to identify pathogens and AMR. To obtain comparable results, it is important to set standardization for both phenotypic and genotypic methods for pathogens and AMR monitoring. For this to become possible, methods and database should be better curated to improve the consistence, quality, and availability of surveillance data. This should need international organization such as WHO to coordinate. The current surveillance of pathogens is usually confined to an extremely limited number of microbial indicators. It is not enough to cope with the rising health risks. It is suggested to extend the range to more medically or health-relevant species, such as the antibiotic-resistant priority pathogens listed by WHO, and infectious diseases-causing virus, especially those without efficient vaccine. For AMR monitoring, it is suggested to extend from commonly consumed antibiotics to last resort or newly introduced antibiotics to determine the spread speed of this less abundant resistance.

## 29.7 Current Status of Drug-Resistant ESKAPE Pathogens and WBE Prediction Technology

### 29.7.1 International Status

A study conducted on ESKAPE pathogens on different landscapes by *Ria Benkő, Márió Gajdács, and Zoltán Pető et al.* (2014–2019) in Hungary along with data analysis from different countries showed the prevalence of different ESKAPE pathogens in different regions all over the globe (Benkő et al. 2020). In another study conducted in Kwazulu-Natal South Africa, which was also a 5-year retrospective analysis by *Yogandree Ramsamy, Sabiha Y. Essack, and Koleka P. Mlisana et al.* (2011–2015), the prevalence of *Staphylococcus* and *Klebsiella* is noted (Ramsamy et al. 2018). *Klebsiella pneumoniae* are common intestinal bacteria that can cause life-threatening infections. In addition, *K. pneumoniae* is a major cause of hospital-acquired infections such as pneumonia, bloodstream infections, and infections in newborns and intensive care unit patients. Resistant *K. pneumoniae* to last resort treatment (carbapenem antibiotics) has spread to all regions of the world. In some countries, carbapenem antibiotics have been rendered ineffective in more than half of the patients treated for *K. pneumoniae* infections due to emerging resistance. Similarly, *E. coli* species resistant to fluoroquinolone, an antibiotic used for the treatment of urinary tract infections, have become widespread. Colistin is the only last resort treatment for life-threatening infections caused by carbapenem-resistant *Enterobacteriaceae* (i.e., *E. coli* and *Klebsiella*). Bacteria resistant to colistin have also been detected in several countries and regions, causing infections for which there is no effective antibiotic treatment at present. Another of the ESKAPE pathogens, *Staphylococcus aureus*, is a part of our normal flora present on the skin and is also a common cause of infections both in the community and in healthcare-acquired infections. People with methicillin-resistant *Staphylococcus aureus (MRSA)* infections are 64% more likely to die than people with drug-sensitive infections.

Measures are being taken to combat and control these infections. In 2019, a new AMR indicator was included in the SDG monitoring framework to monitor the frequency of bloodstream infections due to two specific drug-resistant pathogens: methicillin-resistant *Staphylococcus aureus (MRSA)* and *E. coli* resistant to third-generation cephalosporins (3GC). In response to which 25 countries, territories, and areas provided data to GLASS on bloodstream infections due to MRSA and 49 countries provided data on bloodstream infections due to *E. coli*. While the data are still not nationally representative, the median rate observed for methicillin-resistant *S. aureus* was 12.11% (IQR 6.4–26.4) and that for *E. coli* resistant to third-generation cephalosporins was 36.0% (IQR 15.2–63.0).

Detection and identification of drugs and antibiotics in wastewaters and water bodies have been studied since long for a variety of reasons that have already been discussed. The detection of these molecules provides insights on their effects on the water bodies and aquatic animals (Yang and Carlson 2004). The wastewaters for the same have also been evaluated to monitor the use of illegal drugs in Eastern Europe region (Sulej-Suchomska et al. 2020). Thus, the detection of antibiotics in the wastewaters would be useful in surveillance of their use and also in identifying their role in the spread of resistance.

The role of wastewater in the spread of infectious diseases has been studied in great depth over the years and wastewater can be considered as an effective measure of the same. One of the reasons for the same is inadequate sanitation, while others include ineffective or partially effective treatment strategies. Also, owing to the existence of multiple microbial species in wastewaters in spite of the presence of toxic substances suggests that these can be one of the most exciting niches for the analysis of ARBs and ARGs and their roles. Though the majority of the studies have shown a marked decrease in the presence of ARBs in the effluents as compared to influent samples, there are evidences to claim an increase in the ARBs in the former, which is a big concern. In addition to playing a role in analysis of emerging resistance to bacterial species, WBE has also gained importance for the prediction and identification of COVID-19 infections owing to the correlation observed in the wastewater with infection rate in humans (Arora et al. 2020). With the release of Global Priority Pathogen List by WHO in 2017 considering the emerging resistance, a 5-year National Action Plan was prepared from 2017 to 2022 by WHA (World Health Assembly) in Geneva, for the surveillance of spreading antimicrobial resistance (AMR), antimicrobial use (ARU), and antimicrobial consumption (ARC). ESKAPE pathogens, six of the deadliest ones, have been highlighted in this list and need special and urgent attention. It is clearly known that the complexity of the wastewater system affects the fate of ARBs/ARGs. Analysis of treatment strategies has revealed the presence of about $10^{12}$ to $10^{18}$ ARBs/day in the secondary effluents (Manaia et al. 2016; Vaz-Moreira et al. 2014), which is a concerning situation. A similar study done in two different seasons in Ireland revealed the presence of ESBLs and multi-drug-resistant coliforms (resistant to majority of commonly used drugs such as amoxicillin, tetracycline, and colistin) in the effluent samples of WWTPs (Popa et al. 2020). Also, spread of carbapenem-resistant bacteria has been observed via contaminated food and water or via poor domestic hygiene in developing countries such as South Africa and has also stressed on proper treatment of wastewater effluent before discharge in water bodies (Ebomah and Okoh 2020). A comparison of the hospital wastewater and urban wastewater in France clearly suggested wastewater as a crucial source for spread of AMR and pollutants including antibiotics (Buelow et al. 2020). The study also clearly stated that machine learning along with multivariate analysis can be a good predictor of the resistome (Buelow et al. 2020). It is important to note that these pathogens not only enter the wastewaters via human sources, but animal sources such as livestock-associated wastewater are a huge reservoir of zoonotic bacteria (Savin et al. 2020). The insights in understanding the mechanism of emerging resistance suggest that the resistance can be classified as intrinsic and acquired. The resistance observed owing to genes present on the chromosomal DNA of the pathogens are referred to as intrinsic, while the ones encoded by transmissible genetic elements (TGE), which includes plasmids, transposons, or integrons, is referred to as acquired resistance. Since multiple evidences have suggested the presence and persistence of genetic elements in wastewaters, analysis of ARGs is equally important to ARBs in order to keep a check on the spreading resistance.

It is very crucial to state here that efforts are not only made in the diagnosis of antimicrobial resistant ESKAPE pathogens in the community or in the wastewater or application of WBE but also toward the treatment of these wastewaters to get rid of these pathogens. Hiller et al. 2019 have shed light on the removal of AMR by various conventional as well as advanced treatment strategies and have focused on the advanced strategies such as membrane filtration, ozonation, UV irradiation, or chlorination to be better at AMR removal (Hiller et al. 2019). Simultaneous analysis of resistome, plasmidome and bacterial communities in influent and effluent samples of wastewater treatment plants in Portugal revealed efficacious removal of antibiotic-resistant genes pertaining to ESKAPE pathogens (Lira et al. 2020). Also, principal component analysis (PCA) revealed that the resistome and the bacterial communities cluster in influent but fail to do so in treated effluent samples (Lira et al. 2020). A metagenomic analysis of effluents from wastewater treatments plants as well as upstream and downstream samples from rivers in Korea have clearly defined the role of resistomes and mobilomes in the dissemination of ARGs in environment (Raza et al. 2021).

## 29.7.2 India National Status

The high population density in India along with compromised medical and sanitation facilities may lead to spread of these infections further deteriorating the existing conditions. The presence of these pathogens in the Indian population particularly in the area under the study has not yet been analyzed. Additionally, it has been observed that these pathogens co-infect with several other microorganisms further worsening the infections. Besides that, multiple preliminary work on the community-acquired infections caused by ESKAPE pathogens suggests the prevalence of these pathogens (Li and Webster 2018). Persistent use of antibiotics has provoked the emergence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) bacteria, which have rendered even the most effective drugs ineffective. The development of novel therapeutics to treat drug-resistant infections, especially those caused by ESKAPE pathogens, is the need of the hour. Different therapies such as use of antibiotics in combination or other strategies are widely reported worldwide. The increasing prevalence of ESKAPE in Indian population, and the study and analyses of these bacteria have gained importance. The prevalence of ESKAPE reported in different populations-based studies from India is highly variable. Apart from failing of current antibiotics, the slow and inefficient manufacturing of new antibiotics is somewhere responsible for the high level of infections. An analysis of pathogenic infections in several hospitals in India by *S. Gandra et al.* focused on mortality rates due to MDR pathogens among them ESKAPE pathogens and have stated that MDR and XDR strains are the most common ones (Gandra et al. 2019). A recent study conducted at Umaid Hospital, Jodhpur reported out of 2176 samples 437 (86.87%) ESKAPE pathogens were isolated. Also, another study was conducted in Vellore, India by *N.K.D. Ragupathi et al.* focused on identifying the plasmids associated with AMR genetic determinants in ESKAPE pathogens (Ragupathi et al. 2019).

In line with the guidelines by AMR, WHO, multiple efforts have been made by Indian health authorities to combat the same, but the havoc by AMR is still undermining all the efforts. Most of the analysis done so far is concerned with the humans, while it is very clear now that environment particularly wastewaters plays a crucial role in the spread and dissemination of ARBs and ARGs. The few studies done in India have clearly suggested a dire need for the analysis of urban wastewater and sewage wastewater to combat dissemination of AMR, but not many efforts have been made in this direction so far (Yadav and Kapley 2021). Besides, inefficient and inadequate treatment systems in India further worsen the situation. In line with the international studies done, the presence of carbapenem-resistant bacteria and associated genes has also been observed in hospital wastewater samples of urban India, specifically New Delhi (Lamba et al. 2017). This study was further extended to the evaluation of coliforms (total and fecal), carbapenem-resistant enteric bacteria, and associated genes and their correlations in different hospitals (Lamba et al. 2018), suggesting an urgent need for coverage of appropriate wastewater treatment facilities across the city.

The treatment strategies for the removal of these resistant pathogens from the wastewaters, though few, have also been attempted in India. One of the very few successful attempts to get rid of resistant bacteria during wastewater treatment is the reduction in NDM-positive bacteria in the treated hospital wastewater in Mangalore (Divyashree et al. 2020). Also, previous studies have shown that analysis of secondary treated wastewater from WWTPs consists of chlorine-tolerant bacteria suggesting the inefficacy of the plant to remove pathogens (Shekhawat et al. 2020). Further analysis by our group suggested the efficacious removal of chlorine-tolerant *Klebsiella* via a short exposure of UV radiation (about 60 s) (Shekhawat et al. 2021). A preliminary study has also shown promising results in the removal of ARBs and ARGs from clinical wastewater with the help of vermifiltration technology, which is a sustainable and a natural treatment technology (Arora et al. 2020, 2021a, b).

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# Fundamentals of SARS-CoV-2 Detection in Wastewater for Early Epidemic Prediction and Key Learnings on Treatment Processes for Removal of Viral Fragments

# 30

Sudipti Arora, Devanshi Sutaria, Ekta Meena, and Aditi Nag

## Abstract

Wastewater Based Epidemiology (WBE) is an approach gaining attention exceedingly after the onset of pandemic COVID-19. This pandemic has highlighted the usefulness and efficient application of the WBE approach in early disease spread prediction, hotspot predictions based on actual biomarker detection, and is useful in identifying the asymptomatic patients in a comprehensive and yet cost-effective manner. This approach has been proposed to be used as a complementary diagnostic approach to clinical testing to monitor and check the spread of any epidemics. The most interesting feature of this approach is that it can be applied on mega scales such as city and community levels to even micro-level monitoring at the society level. However, despite being so used, this approach faces certain challenges and is yet to be accepted as a popular way to keep tabs on the spreading pandemic in the majority of the world. This chapter analyzes the fundamentals of SARS-COV2 detection in wastewater for early surveillance of COVID-19 and shed light on the treatment processes for the removal of viral fragments from wastewater. The fate of Coronaviruses (CoVs), and in particular SARS-CoV-2 in wastewater treatment plants (WWTPs), has not been completely understood yet, but an adequate knowledge on the removal performances in WWTPs could help to prevent waterborne transmission of the virus that is still under debate. The chapter also includes a case study for the successful application of WBE for 19 WWTPs in the context of Indian urban cities like Jaipur, Haridwar, and Roorkee.

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**Keywords**

Cost-effective approach · hotspot prediction · SARS-CoV-2 surveillance · Wastewater treatment · WBE

## 30.1 Introduction

SARS-CoV-2 is a Baltimore class IV positive-sense single-stranded RNA virus that causes severe acute respiratory syndrome in humans (SARS-CoV). It has zoonotic origins and is genetically linked to bat coronaviruses (Ye et al. 2020). The SARS-CoV-2 virion measures 50–200 nm in diameter. The four structural proteins of SARS-CoV-2 are the S (spike), E (envelope), M (membrane), and N (nucleocapsid); the N protein holds the RNA genome, while the S, E, and M proteins form the viral envelope. The spike protein enables the virus to stick to and fuse with the membrane of a host cell; its S1 subunit catalyzes attachment, while the S2 subunit catalyzes fusion. SARS-CoV-2 has a strong affinity for the human cell receptor angiotensin converting enzyme 2 (ACE2), which it uses as a cell entry mechanism. Despite the fact that SARS-CoV-2 is primarily a respiratory pathogen, the virus's survival and replication in the gastrointestinal (GI) tract, as well as shedding through feces, has been proven (Wang et al. 2020; Xiao et al. 2020a; Zhang et al. 2020; Young et al. 2020; Wölfel et al. 2020; Mohan et al. 2021). According to recent findings, 2–10% of COVID-19 patients had gastrointestinal problems such as diarrhea (Chan et al. 2020; Chen et al. 2020; Gao et al. 2020; Wang et al. 2020). Although the precise mechanism of COVID-19-induced gastrointestinal symptoms is yet unknown (Gu et al. 2020), a recent research found that SARS-CoV-2 infects gastrointestinal glandular epithelial cells (Xiao et al. 2020). Angiotensin-converting enzyme 2 (ACE2) has been identified as the cellular receptor for both SARS-CoV-2 and SARS-CoV (Yan et al. 2020), and the receptor ACE2 is abundantly expressed in the small intestine, as well as the lung and oral mucosa (Hamming et al. 2004; Xu et al. 2020). This evidence is pointing to SARS-CoV-2 replication in the gastrointestinal system.

Several independent studies have revealed the presence and replication of SARS-CoV-2 in the gastrointestinal tract, as well as the prolonged shedding of SARS-CoV-2 viral material through feces during and beyond the active infectious stage (Wölfel et al. 2020; Holshue et al. 2020; Kitajima et al. 2020; Cai et al. 2020; Ling et al. 2020; Wu et al. 2020; La Rosa et al. 2020; Xiao et al. 2020; Ahmed et al. 2020a; Wurtzer et al. 2020). In this case, wastewater-based epidemiology (WBE) investigations are appropriate for understanding and estimating viral distribution in a specific community in order to conduct effective disease monitoring. WBE was formerly used to identify and manage viral infections such as polio and rotavirus (Ahmed et al. 2020a; Usman et al. 2020; Murakami et al. 2020; Lodder and de Roda Husman 2020; Hata and Honda 2020; Mohan et al. 2021). Meanwhile, wastewater surveillance might provide an unbiased approach of analyzing the spread of illness in various places, even where clinical diagnosis resources are limited and reporting mechanisms are missing or impractical, as in poor nations. Furthermore, wastewater monitoring can aid in the detection of variations in circulating strains via phylogenetic analysis, allowing for comparisons between regions and the assessment of virus genome evolution over time, as demonstrated repeatedly for enteric viruses (Bisseux et al. 2018; Lodder et al. 2013), and more recently for SARS-CoV-2 (Nemudryi et al. 2020). Furthermore, because a substantial proportion of the population is asymptomatic and sheds the virus, WBE enables scientists and health officials to watch the progression of the epidemic even if a huge proportion of the population is asymptomatic. The relevance of wastewater monitoring is further underscored by its capacity to identify low levels of viruses; this can occur when the number of infected patients decreases as a result of public health initiatives, as has been the case with successful instances such as poliovirus eradication efforts (Asghar et al. 2014).

## 30.2 Shedding of Virus and Wastewater Surveillance

In the COVID-19 infected patients, but also in asymptomatic individuals, the SARS-CoV-2 virus can be excreted from the gastrointestinal tract in the stool as well as from other bodily secretions (saliva, sputum, urine) which are subsequently disposed of in wastewater (Medema et al. 2020; Kitajima et al. 2020). SARS-CoV-2 may be present in stool even after respiratory symptoms have ended. In this way, the virus is discharged from feces into the sewer network and can reach WWTPs within a few hours.

The shedding profile of infected individuals directly determines the wastewater viral load and is hence regarded as one of the most critical factors in WBE. With what we've seen about viral dynamics in humans and fecal shedding, we predict wastewater surveillance to outperform diagnostic testing by a week or more. Shedding can occur shortly after infection, but an infected individual discovered through the health system must show symptoms and seek treatment before being diagnosed. The discovery by Peccia et al. 2020 describes sludge RNA lacks predictive ability when compared to diagnostic testing (excluding delays in test processing) and calls the early warning theory into doubt. However, epidemiological statistics in the early days of the outbreak were hampered by factors such as testing capacity constraints and changes in testing instructions over time. Before we dismiss the possibility of using wastewater as an early warning system, additional study with more rigorous epidemiological data is required to adequately assess its potential. Furthermore, in places where testing delays continue, the significance of wastewater surveillance as an early warning system is clearly obvious.

Another advantage of wastewater surveillance is that it does not have the biases that traditional indicators have in determining where disease transmission is happening, rising, or decreasing. The cumulative frequency of diagnosed cases was an important signal in the early days of the epidemic. Later, additional emphasis was placed on hospitalizations, fatalities, and, most recently, test positive rates and serologic data (Larsen et al. 2020). While useful, these indicators contain biases.

For example, the number of cases is dependent on diagnostics availability, which has been restricted during the epidemic, and the possibility of isolation and quarantine might deter individuals from getting tested. Hospital admissions and fatalities are transmitted weeks later. Rates of test positive, like total instances, are affected by testing regimens, procedures, and availability. Daily testing of SARS-CoV-2 RNA in wastewater could offer information comparable to daily random testing of hundreds of people in a community, but it would be less costly and less intrusive. Wastewater data might potentially be used to assess the dependability of epidemiological trends derived from patients diagnosed. More study is needed to understand how SARS-Cov-2 found in wastewater correlates to COVID-19 transmission, and whether it may serve as an early warning sign. This study should take into account variables such as varied transmission patterns, different geographical locations, and different solids collecting techniques at wastewater treatment plants. It will also be necessary to compare influent and sewage from the same wastewater treatment facility in order to identify whether the method is more sensitive to lowercase numbers in a community. Wastewater systems provide a realistic method for identifying viruses emitted in an entire region's feces. Using this technique, it is feasible to monitor the epidemiology of virus infections even if clinical monitoring does not reveal them, especially because standard epidemiological approaches may be hampered by the asymptomatic character of many viral illnesses and clinical cases under diagnosis. The epidemiological modeling of the prevalence of SARS-CoV-2 in communities serve as well as a warning signal to communities attempting to mitigate the spread of the infection.

## 30.3 Epidemiological Modeling

Wastewater-based epidemiology is a concept based on the presence and detectability of biomarkers like the genome of a pathogen and its fragments, stress-related molecules, byproducts and illicit drug molecules, etc. due to their excretion by humans into the wastewater inadvertently. Thus one might say that the basis of WBE is if it is present in the body, it can be excreted out and, if it is excreted, it can be detected in wastewater. The approach has been widely used for the past several years to detect illicit drug use in a particular community. With the emergence of the SARS-CoV-2 pandemic in Wuhan, China people became aware of its presence in the human hosts. This led the scientists of the field to look for any traces of the viral genome which could be shed from the body. This led to a few investigations showing the presence of viral particles in patient feces. Thus, many groups who were involved in wastewater monitoring at the time tested for the viral presence in it. Among the very first reports to be shared with the scientific community was from (Medema et al. 2020) who showed the presence of this virus in the wastewater of the Netherlands in the samples collected from February 2020. After that, several studies from different types of water samples, countries, and sewage systems have been published (Wright et al. 2004). More interestingly, it was reported (Wurtzer et al. 2020; Ahmed et al. 2020a, b) that the detection of SARS-CoV-2 was not strictly

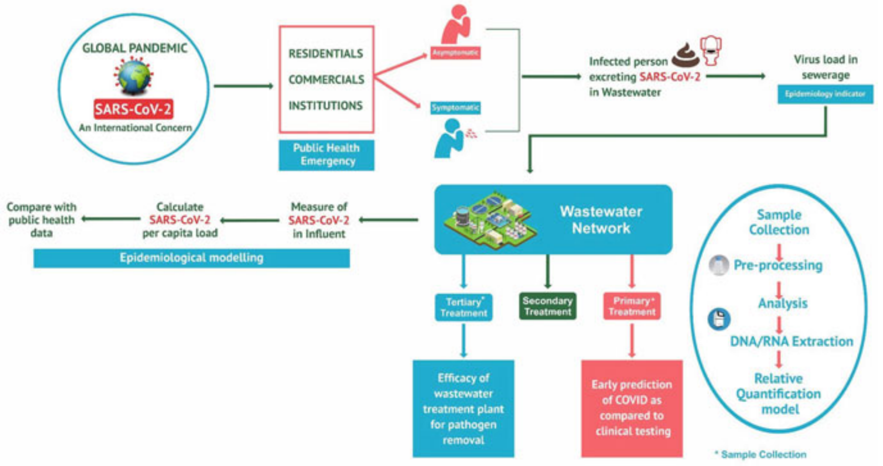

**Fig. 30.1** Sewage surveillance for SARS-CoV-2 detection

matching the number of cases in the community. It was observed that there was an advance window of time in viral detection in sewage samples as compared to the actual caseload of patients, which could only be explained by the fact that the virus shedding started even before the patients showed symptoms at all. The studies which tested fecal and rectal swab samples from patients also reported that the viral genome could be detected much after the nasopharyngeal swab started testing negative for the presence of water (Heneghan et al. 2021). All the above findings have highlighted the significance of WBE application in detection of the pandemic agent virus early on and in the communities where patients are manifesting no or very mild symptoms. This ability of WBE approach leads to the prediction of potential hotspots of the next pandemic outburst in the area. Further studies have consolidated the validity of viral detection and monitoring through WBE. The studies which came out later showed the presence of the binding receptor ACE2 in the alimentary canal. This receptor is known to assist viral entry into the cells by attaching the Spike protein of SARS-CoV-2. Along with these findings, it was also reported that feces samples of half of the patients positive for SARS-CoV-2 tested positive as well. Not only that, during the first wave of the pandemic, a Hong Kong-based study claimed that 17.6% of the patients showed gastrointestinal symptoms. It was later reported that gastrointestinal symptoms constituted one of the six major symptom clusters of the COVID-19 disease (Saawarn and Hait 2020). Depending on the stage of disease progress, it is also reported that the probability of viral RNA detection in the infected patients' fecal samples varies between 15% and 83%. The load of SARS-CoV-2 in feces was also investigated in order to understand the correlation between the number of patients in a community to the genome load and it was found to be in a range of 5 × 103–107.6 RNA copies/mL (Foladori et al. 2020). Figure 30.1 outlines the schematic flow of Sewage Surveillance for SARS-CoV-2 detection.

RNA quantification is the most used approach and the recently proposed standard for wastewater-based surveillance by the EU recommendation (Kassim and Tholoniat 2021). However, this approach does not provide data on the infectivity of the virus, because the presence of fragments of viral RNA in wastewater does not necessarily imply that the virus is structurally intact and viable. Conversely, cell culture-based approaches would be the gold standard for assessing the infectivity of isolated viruses (Hamza et al. 2011). However, not all viruses are easy to propagate as they may replicate too slowly, the cell lines for their propagation may not be widely available or, in other cases, the methods are very difficult to be used routinely. Moreover, cell culture may be time-consuming and may have limited detection sensitivity. In the case of CoVs and SARS-oV-2, the presence of the envelope may result in a difficult isolation and detection of infectious virions in feces and sewage (Sbaoui et al. 2021). Due to these drawbacks, PCR-based methods are commonly used in the analysis of CoVs and SARS-CoV-2 in wastewater, while the detection of infectious virions has rarely been investigated (Rimoldi et al. 2020; Bivins et al. 2020).

For this reason, data are not yet enough to prove the potential fecaloral transmission route; that is still under debate (Amirian 2020; Kitajima et al. 2020). For example, the infectivity of SARS-CoV-2 resulted null in both inlet and outlet from some WWTPs, despite the presence of viral RNA in the samples (Rimoldi et al. 2020). However, due to the limited information, a precautionary approach in risk assessment is advised and the potential presence of viable viral particles cannot be excluded (Rimoldi et al. 2020). Also, the role of wastewater in fecal–aerosol/droplet and fecal–fomite transmission has to be better understood (Olusola-Makinde and Reuben 2020).

Although the presence of SARS-CoV-2 in wastewater has been extensively reported and linked to the onset of clinical illness in the associated community, WBE approaches have yet to be used in a systematic fashion to analyze and forecast viral illness epidemics for worldwide communities. Wastewater-based epidemiology has the ability to anticipate the emergence of viral illness at “critical places” and at “critical moments.” The success of the suggested technique depends on designing geographical and temporal sampling that is appropriate for the region of concern, as well as simulating viral destiny. There has been no proven studies as of now, which could detect a live virus in the feces of human patients; however it might pose a danger of transmission and infection to others, and more future studies are required in this aspect. Despite the fact that fecal oral transmission has yet to be proven, precautions should be taken to reduce the risk. The routes via which human and animal excreta might possibly transfer the virus back to humans Sewage and subsequently Wastewater Treatment Plants (WWTPs) are at the heart of this possible transmission pathway, and WWTPs can help identify and limit SARS-CoV-2 transmission.

## 30.4 Wastewater Treatment for Virus Removal

The persistence and inactivation of CoVs and, in particular SARS-CoV-2, in wastewater and related treatments are largely unknown and undocumented. Some knowledge can be derived from studies using surrogates, such as feline infectious peritonitis virus and human CoV 229E used by Gundy et al. (2009), mouse hepatitis virus (MHV strain A59) and *Pseudomonas* phage *Φ6* (surrogates for human enveloped viruses) used by Ye et al. (2016), transmissible gastroenteritis virus (TGEV) and MHV used by Casanova et al. (2009), bacteriophage *Φ6* as a surrogate of enveloped viruses used by Casanova and Weaver (2015), and murine hepatitis virus indicated as a good surrogate also for SARS-CoV-2 (Ahmed et al. 2020c).

However, it is worth noting that the stability in water of different types of viruses may be variable, and surrogates may be insufficient to describe exactly the behavior of a specific virus of interest such as in the case of SARS-CoV-2 (Aquino de Carvalho et al. 2017; Kitajima et al. 2020).

The WHO emphasized the significance of safe wastewater management in its interim guideline issued on April 23, 2020. Limiting SARS-CoV-2 spread into the environment is critical, given that numerous species have already been identified as possible hosts and that recovered water may be utilized for agriculture. Gerba et al. (2002) reported the virus elimination reduction factor that wastewater treatment plants must offer based on the amount of reclaimed water used. They show that a 6 $\log_{10}$ reduction is sufficient for irrigation of edible crops, but a 12 $\log_{10}$ removal is required to make the water drinkable. The same objectives for removal have been reported by Ahmed et al. (2020a, b). Removal of CoVs in WWTPs cannot be derived from typical physico-chemical parameters of the influent/effluent such as COD (Chemical Oxygen Demand) or $BOD_5$ (Biochemical Oxygen Demand) because there is no correlation between these routine parameters and the viral load. However, COD and $BOD_5$ in raw wastewater are used for estimating the population size of the sewershed served by the WWTP, aimed at calculating the virus loads per capita per day required for surveillance of SARS-CoV-2 and WBE applications (Kassim and Tholoniat 2021).

### 30.4.1 Membranes' Use in Wastewater Treatment for Virus Removal

Water filtration membranes are categorized by their pore size and their distributions in four categories: Reverse Osmosis, Nanofiltration, Ultrafiltration, and Microfiltration.

#### 30.4.1.1 Reverse Osmosis

Wlodarczyk and Kwarciak-Kozlowska (2020) recently reviewed the treatment of waterborne pathogens by reverse osmosis. They provide complete description of the type of pathogens that are waterborne, which are divided into three different groups: protozoans (5–100 μm); bacteria (0.5–1.0 μm); and viruses (0.01–0.1 μm); as well as requirements for Reverse Osmosis membrane materials. They indicated that Reverse

Osmosis is seldom used to remove pathogens from water even if it is one of the techniques reported by the EPA with indicative log removals above 6. This is because reverse osmosis is typically coupled with a pre-treatment system, often ultrafiltration, to reduce foulants which may interfere with the reverse osmosis process. However, RO can be used in combination with such a suitable pretreatment, to remove particulate matter and, post treatment, to complete the removal of any remaining contaminants (Prado et al. 2019). The efficiency of the system led to a virus concentration below the detection level, which would mean, considering the initial virus concentration and the detection limit of the analysis, a $\log_{10}$ removal higher than 6 or 7.

#### 30.4.1.2 Ultrafiltration

As ultrafiltration is often used as a pre-treatment step prior to RO treatment, its efficacy in removal of viruses is more widely reported in the scientific literature than for other membrane-based technologies. Recently Al Aani et al. (2020) reviewed the use of Ultra filtration. Lee et al. (2003) used a combination of coagulation and ultrafiltration (UF) on a pilot scale for wastewater reclamation. They improved removal of MS2 bacteriophage by optimizing the pH of the secondary effluent to a range of 5–6 depending on the effluent. Once improved, the virus removal factor was in the 6.8–7.5 $\log_{10}$ range. Additionally, to obtain a more stable transmembrane pressure, an additional sedimentation step was required. The ideal combination was a hybrid coagulation-sedimentation-UF system.

#### 30.4.1.3 Membrane Bioreactor

O'Brien and Xagoraraki (2020) reported that membrane bioreactors, under the correct conditions are capable of a 7 $\log_{10}$ reduction in virus concentration. From a comparison of a number of studies, it was found that the presence of a biofilm on the membrane was important in virus removal efficiency, where a biofilm, while reducing water flux, also restricted viral penetration of the membrane. It was concluded that there must be a trade-off between maintaining a biofilm to keep viral removal efficiency high with backwashing of the membrane to remove biofilm to maintain acceptable water fluxes. They also concluded that model viruses do not necessarily show the same removal efficiency as viruses which are pathogenic to humans, with data obtained using harmless bacteriophages likely to lead to overestimation of viral removal efficiencies for pathogenic viruses. Miura et al. (2018) investigated the removal of three virus families in a full scale membrane bioreactor plant. The best results were obtained at pH 4 with a $\log_{10}$ reduction range of 2.3–4.5.

#### 30.4.1.4 Role of Biofilm in Treatment

Viral transmission from an infected person or an animal to a new host can occur by direct or indirect routes. During indirect transmission, contaminated surfaces can play an important role. This kind of transmission is dependent on several factors, which include the quantity of viral particles excreted by an infected organism, their stability in the environment, the potential to spread within a closed environment, as well as interaction of the virus and the host organism. Large numbers of viral particles can be shed via various body fluids, including blood, feces, vomit, saliva, urine, and respiratory secretions from infected individuals or carriers. The more viruses are shed, the greater their chance is to survive and reach a new host organism (Rzeżutka and Cook 2004). A critical factor of viral transmission is its ability to survive in the environment. Previous studies have shown that viral particles can persist for extended periods on surfaces such as medical devices, fomites, or human skin (Abad et al. 2001; Sattar et al. 1987; Todd et al. 2009). Even if some viruses survive relatively poorly in the environment, the low infective dose suggests that these viruses are able to persist in sufficient numbers to act as a source of infection for several days, weeks or, in some cases, months (Barker et al. 2001; Boone and Gerba 2007). Surfaces can be contaminated directly by their contact with body secretions and fluids or indirectly through virus contaminated aerosol or other contaminated fomites. Once a surface is contaminated, the transfer of infectious viral particles may easily occur between inanimate or animated objects, or vice versa, e.g., hands and work surfaces with the potential of subsequent transfer to food or direct hand-to-mouth transfer (Goldmann 2000; Rzeżutka and Cook 2004). Rapid spread of viral infections through contaminated surfaces is common particularly in crowded indoor establishments such as schools, day-care facilities, nursing homes, business offices, hospitals, or transport systems (Barker et al. 2001). The influences of environmental factors on the stability and spread of bacterial infections are well documented, especially in hospitals.

Respiratory viral infections are a major health burden (Winther et al. 2006). Although these diseases are often regarded as trivial, hospital admission and mortality rates among infants or elderly are considerable. For instance, respiratory viruses cause more than 400,000 hospitalizations per year in children less than 18 years of age in the United States (Henrickson et al. 2004). Viral agents that primarily infect the upper or lower respiratory tract include influenza and parainfluenza viruses, adenoviruses, respiratory syncytial virus (RSV), corona viruses, human metapneumovirus, rhinoviruses, and enteroviruses (Kesson 2007). It is generally accepted that respiratory viruses are spread from person to person by aerosol transmission due to sneezing or coughing (Goldmann 2000). Additionally, there is evidence to suggest that a significant proportion of flu viruses and other respiratory viruses are spread via contaminated hands and fomites (Tiwari et al. 2006; Winther et al. 2007). RSV was recovered from counters for up to 6 h, from rubber gloves for up to 1.5 h, from clothing gowns and paper tissue for 30–45 min, and from skin for up to 20 min. Additional experiments demonstrated that infectious RSV could be transferred to hands by touching these contaminated surfaces and could be further recovered from these hands for up to 25 min (Hall et al. 1980). A study done by Winther et al. (2007) was designed to assess rhinovirus contamination of surfaces and rhinovirus transfer from these surfaces to fingertips during normal daily activities of adults. Common virus-positive sites were door handles, pens, light switches, TV remote controls, faucets, and telephones. Rhinovirus was transferred from surfaces to fingertips in 60% trials 1 h after contamination and in 33% after 18 h after contamination. Influenza viruses, and parainfluenza viruses are also able to survive on hands long enough to permit self-inoculation. Their survival in the environment is affected by a combination of biological, physical, and chemical factors (Fig. 30.1). To date, complete information regarding the influence of the environment on all viruses and their stability in external conditions does not exist. Most studies have used only a few target viruses or their surrogates. Since experimental conditions and methods vary, it is difficult to draw conclusions from these studies (Carter 2005a, b). Once the surface is contaminated, it can be a source of contamination for other animate or inanimate objects, e.g., contaminated door handles and hands were found to be an efficient vector of viruses. At least 14 persons could be contaminated or infected by touching a polluted door handle. Successive transmission of virus from one person to another could be followed up to the sixth contact person (Rheinbaben et al. 2000). It was also found that contaminated fingers could subsequently transfer a virus from up to seven clean surfaces (Barker et al. 2004).

## 30.5 Viruses Within Biofilms

From a public health point of view, biofilms have already been regarded as a common cause of bacterial infections. It has also been hypothesized that produced exopolymeric substances (EPS) may protect biofilms from viruses, especially phage penetration (Sutherland et al. 2004). Recent studies have revealed that, even in the absence of specific enzymatic reactions, viral particles are able to penetrate inside the EPS structure of mucoid biofilms. After penetration inside the polymeric matrix, the viruses may take advantage from the specific ‘biofilm lifestyle,’ and benefit from protection against environmental stress, such as desiccation or other actions of antimicrobial agents. Moreover, during biofilms erosion or sloughing, protected immobilized viral particles may be released in the environment, and then contact their target host, commencing the viral infectious cycle (Briandet et al. 2008; Helmi et al. 2008; Lacroix-Gueu et al. 2005; Lehtola et al. 2007; Quignon et al. 1997). Viral attachment rates to biofilms vary greatly and may depend on many factors such as the biofilm or viral characteristics (size, shape, isoelectric point) and concentration of viral particles (Helmi et al. 2008). Infectious viral particles were detected in the drinking water biofilm for up to 6 days after inoculation, whilst the viral genome was still detectable at Day 34. Hock and Botzenhart (2002) injected phages MS2, uX174 and poliovirus 1 at comparable concentrations into a laboratory reactor containing approximately 100 mL of drinking water. After the contact time of 1 h, concentrations of 0.04%, 0.13%, and 0.27% were recovered from the biofilm. MS2 showed less adsorption to the biofilm than uX174 and poliovirus 1 (Hock and Botzenhart 2002; Skraber et al. 2005). Results published by Quignon et al. (1997) showed that water biofilm can protect viruses form inactivation. This hypothesis is supported by the study of Storey and Ashbolt (2001). Phages MS2 and B40-8 were recovered from biofilm in the presence of the average concentration of 0.2 mg free chlorine/L during a 30-day period. In comparison, Duran et al. reported that phages MS2 and B40-8 showed 3.2 and 1.7 log10-units reduction after only 10 min in groundwater containing 0.5 mg free chlorine/L. Storey and Ashbolt (2003) compared the inactivation of two phages (B40-8 and MS2) within biofilms. Results indicated that the subpopulation of approximately 0.01% of phages had the potential to persist over 100 days for B40-8 and almost 10 times longer for MS2 in the presence of free chlorine. The level of viral protection may depend on parameters such as the composition, thickness, or structure of biofilms. Various studies have suggested that biofilms may trap and accumulate virus-sized particles and produce a potential reservoir of human or bacterial pathogens. In natural environments, biofilms are mixed microbial cultures normally consisting of predominantly prokaryotes with some eukaryotes (Sutherland et al. 2004).

## 30.6 Effectiveness of Wastewater Treatment Plants for Degrading Viruses

It is commonly known that raw municipal wastewater contains many pathogenic and opportunistic microorganisms, including those resistant to antibiotics, mainly of fecal origin. The presence of pathogenic microorganisms in wastewater is dangerous, as they may lead to epidemic effects, allergic reactions, toxic or immunotoxic health interactions in humans, animals, and other environmental species. Despite very high, reaching 99% reduction of bacteria in wastewater during its treatment, wastewater treatment plant effluent may still contain from $10^5$ to $10^7$/mL of indicator fecal coliform (Campos et al. 2016; Oleńczuk-Neyman 2001). Among all waterborne pathogens, viruses have the smallest size and therefore are the hardest to be removed by sedimentation and filtration. Though disinfection has been adopted for pathogen removal, neither UV nor chlorine achieved satisfactory virus removal in wastewater treatment (Simmons and Xagoraraki 2011). This observed insufficient virus removal could pose a threat to public health (Vergara-Alert et al. 2017). On the basis of the literature data (Michałkiewicz et al. 2011), one may find that mechanical treatment processes enable the decrease of total number of bacteria by 20% and of Salmonella species and tuberculosis mycobacteria by 90%, whereas for biological wastewater treatment by means of activated sludge, removal efficiencies reach 90–98% for total number of bacteria, 55–98% for Salmonella species, and 45% for tuberculosis mycobacteria. Conventional wastewater disinfection, including decomposition of byproducts which are formed during the treatment, is known as specific disinfection. It can be run using physical and chemical methods. Among former techniques, one can distinguish: pasteurization, UV irradiation, thermal drying, ionization radiation, ultrasonication and membrane filtration (UF/MF). Chemical methods of disinfection rely mainly on the addition of oxidizing compounds, such as chlorocompounds (chlorine, sodium hypochlorite, bleaching powder, calcium hypochlorite, chlorine dioxide, calcium hydroxide or oxide), ozone, per acetic acid, or on the application of alternative techniques. The issue related with the presence of pathogenic microorganisms in biologically treated wastewater has become important due to their reuse or water reclamation in industry and agriculture (Zanetti et al.

2010). Similarly, as in the case of water treatment, MF and UF are mentioned to be suitable physical wastewater disinfection technique, as they guarantee high removal rate of viruses and microorganisms, colloids, suspensions, and high molecular weight organic compounds (Bodzek 2015; Bodzek et al. 2019; Michałkiewicz et al. 2011). Virus removal in water reuse should not solely rely on disinfection. In full-scale wastewater treatment plants, the contribution of secondary treatments on virus removal is much larger than that of disinfection, probably due to the high concentration of nutrients in wastewater increasing the consumption of disinfectants (Simmons and Xagoraraki 2011). Unlike disinfection, the improvement of virus removal in the secondary treatment does not rely on augmenting the disinfectant dosage. Hence, effective disinfection of wastewater is assured.

## 30.7 New Learnings and Experiences from Our Studies (Arora et al. 2020, 2021)

The application of WBE in India effectively is a challenge on its own due to the non-connected rural areas and highly fragmented sewage networks in the urban areas. However, a few groups, including ours, have been trying to apply this approach in monitoring the pandemic. Despite the above mentioned challenges, it turns out that with proper site selection and planning it is possible to use WBE with its effectiveness in detection and monitoring of the pandemic hotspots. Our group has studied samples collected from about 19 STPs collected across two states (Rajasthan and Uttarakhand) and correlated the viral presence and detection to the communities with potentially high numbers of COVID-19 patients (Arora et al. 2020, 2021) during the first wave. Furthermore, it was also observed that the wastewater treatment systems used in the hospitals and community wastewater at the time was sufficient in viral removal from the treated wastewater. However, this observation was changed during the second wave in the city of Jaipur where the patient case load went 3–5 times higher in the community. At that time it was observed that the treatment systems reached their capacity and were unable to remove the viral genome presence above a load of log4 copies of the genome indicating that current secondary and tertiary treatments might not be sufficient during the pandemic wave peak in the city (Arora et al. 2021). Given that no study has yet ruled out any possibility of transmission through wastewater, two things become very pertinent. First of all, viability studies in wastewater are needed to assess the public health risks, especially for those who come in direct contact with it. Secondly, until such definite proofs are here, there is an urgent need to re-examine the treatment strategies and the strictness measures to follow these guidelines through. These guidelines include the proper treatment of hospital wastewater before being dumped into the community wastewater amongst other things. To add further, there is a need to update the disinfection and treatment policies in the rural areas, especially of the sludge, as there is hardly any sewage treatment mechanism in rural India, and the untreated wastewater and improperly treated sludge end up in irrigation and farming purpose, which is a great risk to public health. Until such steps are taken, presence of viral genome remains a major concern currently, as natural degradation of virus may take 4–14 days in wastewater (La Rosa et al. 2020). We have also observed that, even at higher ambient temperatures, the virus remains detectable, thus adding to the concerns of public health risk. However, regardless of the current status of treatment strategies, WBE has proven to be an efficient approach in monitoring the viral load circulating in the community. The approach can prove to be more sensitized with incorporation of additional data like the connected population size, flow rate, etc. and applied to correlate the morbidity and mortality associated with the spread of COVID-19. In another of our study by Arora et al. (2021), which tracked the city-wide dynamics of severe acute respiratory syndrome-corona virus 2 (SARS-CoV-2) RNA in the wastewater from nine different wastewater treatment plants (WWTPs) in Jaipur during second wave of COVID-19 outbreak in India. A total of 164 samples were collected weekly between February 19 and June 8, 2021. SARS-CoV-2 was detected in 47.2% (52/110) influent samples and 37% (20/54) effluent samples. The increasing percentage of positive influent samples correlated with the city's increasing active clinical cases during the second wave of COVID-19 in Jaipur. Furthermore, WBE-based evidence clearly showed early detection of about 20 days (9/9 samples reported positive on April 20, 2021) prior to the maximum cases and maximum deaths reported in the city on May 8, 2021. The study has successfully proven the global implications of WBE for India, highlighting the role of WBE through application of scalable and cost-effective protocol reported in the study for societal benefit and third wave improved management.

A study further observed the presence of SARS-CoV-2 RNA in treated effluents at the time window of maximum active cases in the city even after tertiary disinfection treatments of UV and Chlorine. The average genome concentration in the effluents and removal efficacy of six commonly used treatments; Activated Sludge Process + Chlorine disinfection (ASP + $Cl_2$), Moving Bed Biofilm Reactor (MBBR) with Ultraviolet radiations disinfection (MBBR + UV), MBBR + Chlorine ($Cl_2$), Sequencing Batch Reactor (SBR) and SBR + $Cl_2$ were compared with removal efficacy of SBR + $Cl_2$ (81.2%) > MBBR + UV (68.8%) > SBR (57.1%) > ASP (50%) > MBBR + $Cl_2$ (36.4%). The study observed the trends and prevalence of four genes (E, RdRp, N, and ORF1ab gene) based on two different kits and found that prevalence of N > ORF1ab > RdRp > E gene suggested that the effective genome concentration should be calculated based on the presence/absence of multiple genes. Hence, it is imperative to say that using a combination of different detection genes (E, N, RdRp, and ORF1ab genes) increases the sensitivity in WBE.

## 30.8 Conclusions

Early diagnosis and containment of the new severe acute respiratory syndrome corona virus 2 (SARS-CoV-2) is critical for controlling Coronavirus Disease 2019 community outbreaks (COVID-19). Clinical testing for every person in a community is both impracticable and costly. Hundreds of towns across the world are already employing SARS-CoV-2 wastewater surveillance. Wastewater monitoring covers the void created by insufficient individual testing. Nonetheless, the use of WBE has been hampered by the difficulty of connecting the incidence of the virus in wastewater to the number of infected people. Environmental monitoring, on the other hand, may provide a continuous, cost-effective way of monitoring areas for early indicators and the progression of epidemics. WBE (wastewater-based epidemiology) is a potential method for assessing COVID-19. There are several WBE applications, including drug detection, pathogenic enteric virus monitoring, and assessment of possible industrial chemical exposures. SARS-CoV-2 is shed in the feces of both symptomatic and asymptomatic patients, and investigations have revealed the presence of SARS-CoV-2 genes in raw sewage and primary sewage disposal sludge. WBE based monitoring has many advantages, if applied smartly. It can prove to be highly informative while being a cost-effective measure to surveil the circulating pathogens. This approach should be used in complementation to the clinical diagnosis in such a way that individual level clinical testing can be minimized and streamlined to focus on a particular community where wastewater samples test positive for COVID-19. This saves time, effort, costs, provides an advance window to find the hotspots, and take appropriate actions to stop further transmission and, above all, circumvents the dependence of detection on the willingness and accessibility of individuals to get tested.

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# COVID-19 mRNA Vaccines

# 31

Yuan-Yeu Yau, Subhrajyoti Mishra, Mona Easterling,
and Ashwani Kumar

## Abstract

COVID-19 global pandemic, as of Mid-Oct. 2021, has reached 238,000,000 recorded cases and a death toll that climbed to more than 4,860,000. Several different strategies have been used to reduce the spread of SARS-CoV-2. These include lockdowns, social-distancing, gathering-size limits, room capacity adjustments, mask-wearing protocols, and proper handwashing techniques. However, the most effective way to reach herd immunity and eliminate this disease is vaccination. A few COVID-19 vaccines have been developed, trialed (with high efficacies), and granted emergency use authorizations (EUA), including mRNA vaccines. Vaccination rollout efforts began near the end of December 2020 in several countries, including Israel, the UK, and the US. Later, more countries applied vaccines to fight COVID-19 outbreaks. Since then, real-world data have shown that vaccines work effectively to reduce symptomatic infections, severe COVID-19 illness such as hospitalization and death. This chapter will cover the development of COVID-19 vaccines with a focus on mRNA-based COVID-19 vaccines. This will include vaccine efficacy from both clinic trials and real-world vaccination studies. The four major SARS-CoV-2 variants of concern (VOC) will

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also be explored. The impact of VOCs on the first-generation vaccine, developed based on the original Wuhan SARS-CoV-2 genome sequence, will also be discussed.

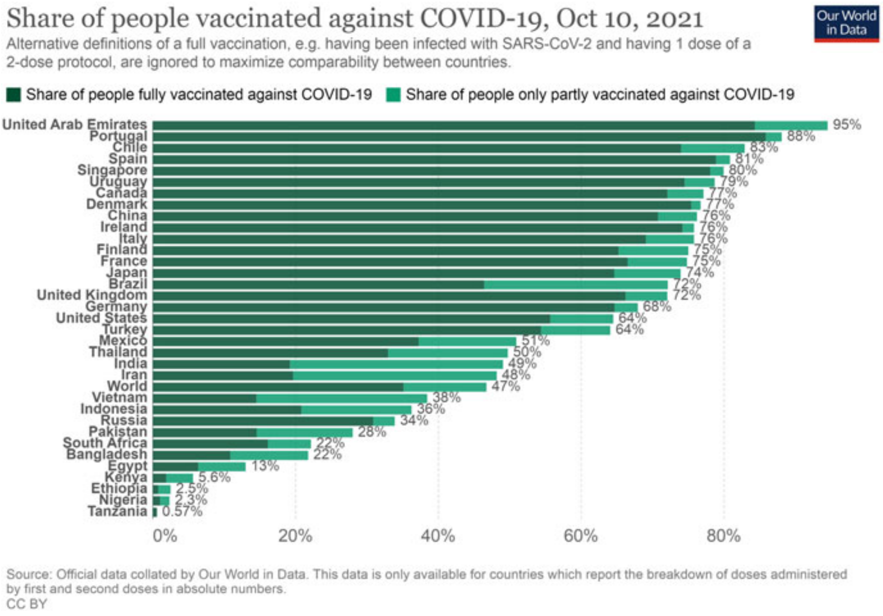
**Keywords**

Pfizer-BioNTech COVID-19 vaccine · Breakthrough infection · COVID-19 mRNA vaccine · Moderna COVID-19 vaccine · SARS-CoV-2 · Vaccine efficacy

## 31.1 Introduction

COVID-19 disease is caused by SARS-CoV-2 novel coronavirus. SARS-CoV-2 genome sequence is closely related to the virus causing SARS-CoV. This deadly COVID-19 disease was first discovered and documented in Wuhan, Hubei province of China. The deadly Coronavirus has spread globally and has mutated into several variants. As of Mid-October 2021, the number of confirmed global COVID-19 cases has reached 238,000,000 and the death toll has reached 4,860,000, according to COVID-19 tracker at Center for Systems Science and Engineering (CSSE) from Johns Hopkins University (https://coronavirus.jhu.edu/map.html). The US has suffered the greatest number of per capita COVID-19-related deaths worldwide. As of Oct. 15, 2021, more than 714,000 Americans have died of the disease. Estimated average American life expectancy fell 1 year in 2020 (January to June), compared to 2019 (Arias et al. 2021). Death rates due to COVID-19 infection are impacted by age, health status, and personal immunity level. Due to the rapid spread and heavy death toll of this novel coronavirus, the World Health Organization (WHO) declared a pandemic on March 12, 2020. Strategies for COVID-19 infection prevention include wearing an appropriate facial covering/mask, keeping social distance of 6 ft or more, frequent handwashing, and vaccination. Of these, vaccination is the most effective method for slowing the pandemic and establishing a new normal with herd immunity (Nag et al. 2021). Numerous drug-makers rushed to develop a COVID-19 vaccine with various platforms after the 'prototype' SARS-CoV-2 genome was sequenced in China. Several vaccines are currently available and have been used for the global COVID-19 vaccine campaign since the end of 2020. Figure 31.1 shows numbers of COVID-19 vaccine doses administrated in different countries (from *Our World in Data*). In the US, three COVID-19 vaccines have obtained FDA Emergency Use Authorization (EUA) as of October 2021. These vaccines are manufactured by (1) Pfizer-BioNTech, (2) Moderna, and (3) Johnson & Johnson.

There are several vaccine-producing platforms, and one of them is the mRNA-based vaccine. This technology uses virus mRNA information (typically virus-spike protein) to generate an engineered mRNA fragment as the vaccine core ingredient. These engineered mRNA fragments are delivered subcutaneously through a nanoparticle. Although researchers have studied mRNA-based vaccines and drugs for many years, no mRNA-based vaccine has previously been approved for use. Pfizer



Share of people vaccinated against COVID-19, Oct 10, 2021

Alternative definitions of a full vaccination, e.g. having been infected with SARS-CoV-2 and having 1 dose of a 2-dose protocol, are ignored to maximize comparability between countries.

Source: Official data collated by Our World in Data. This data is only available for countries which report the breakdown of doses administered by first and second doses in absolute numbers.
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**Fig. 31.1** Share of vaccination doses administered (as of October 10, 2021). (Chart is from *Our World in Data*, https://ourworldindata.org/covid-vaccinations; under CC-BY license for unrestricted reuse)

(US based)-BioNTech (Germany based) and Moderna (US based) have both successfully developed different versions of COVID-19 mRNA vaccines. Vaccines typically require years of research, testing, and trials before reaching clinical application. A vaccine regularly goes through (1) pre-clinical testing on lab animals such as mice and monkeys for immune response study; (2) Phase 1 safety trials on a small group of volunteers for vaccine safety; (3) Phase 2 expanded trials; and (4) Phase 3 efficacy trials involving tens of thousands of volunteers. Unlike previous vaccines, the development of Pfizer-BioNTech and Moderna mRNA vaccines has been completed in record time, taking less than 1 year to move from design to ready-for-use. Table 31.1 shows Pfizer-BioNTech and Moderna mRNA vaccine timelines.

Trial results show both mRNA vaccines with extremely high efficacies for COVID-19 disease. This chapter overviews the basic concept of generating an mRNA vaccine with a focus on Pfizer-BioNTech and Moderna mRNA vaccines and how mRNA vaccines can be used to end the pandemic. Real-world data will be presented in terms of the effectiveness of mRNA vaccines against COVID-19, and the likelihood of allergic reactions induced by the vaccines. Four major SARS-CoV-2 variants of concern (VOCs) and numerous variants of interest (VOIs) have arisen through genetic mutation during the course of the pandemic. In this chapter, we also discuss the disease COVID-19 and the virus plus its variants that causes the disease, and review the production/storage/administration of COVID-19 mRNA vaccines.

**Table 31.1** Timelines of Pfizer-BioNTech and Moderna COVID-19 vaccines

| | |
|---|---|
| December 31, 2019 | COVID-19 first reported |
| January 10, 2020 | SARS-CoV-2 genome sequenced (Wu et al. 2020) |
| January 17, 2020 | NIH (USA) designed mRNA vaccine in collaboration with Moderna |
| March 16, 2020 | Phase 1/2 trial began (Moderna) |
| April 16, 2020 | US Government support (Moderna) |
| May 2, 2020 | Phase 1/2 trial began (Pfizer-BioNTech) |
| July 14, 2020 | Phase 1/2 trial results published in NEJM (Moderna) |
| July 27, 2020 | Phase 3 trial began (Moderna) |
| July 28, 2020 | Phase 3 trial began (Pfizer-BioNTech) |
| August 12, 2020 | Phase 1/2 trial results published in *Nature* (Pfizer-BioNTech) |
| November 9, 2020 | Pfizer-BioNTech announced interim analysis efficacy >90% |
| November 16, 2020 | Moderna issued a preliminary data readout of its COVID-19 vaccine, suggesting an efficacy rate of 94.5% |
| November 18, 2020 | Pfizer-BioNTech announced COVID-19 vaccine final efficacy 95% |
| November 20, 2020 | First COVID-19 vaccine EUA submitted to US FDA by Pfizer-BioNTech |
| November 27, 2020 | United Airline flights prepared distribution of Pfizer-BioNTech throughout the US |
| December 10, 2020 | Pfizer-BioNTech reported safety and COVID-19 vaccine efficacy findings from the phase 2/3 trial (online) |
| December 10, 2020 | US FDA external review for Pfizer-BioNTech vaccine Emergency Use Authorization (EUA) (Committee voted 17:4 to back vaccine) |
| December 11, 2020 | US FDA issued EUA for emergency use of Pfizer-BioNTech COVID-19 vaccine |
| December 13, 2020 | First truck departed the Pfizer plant in Portage, Michigan (US) with the first batches of COVID-19 vaccine |
| December 17, 2020 | US FDA external review for Moderna COVID-19 vaccine EUA (Committee voted 20-0, with 1 abstention) |
| December 18, 2020 | US FDA issued EUA for emergency use of Moderna COVID-19 vaccine |
| December 31, 2020 | Moderna published Phase 3 Trial results in *The New England Journal of Medicine* (online) |
| January 25, 2021 | Moderna announced they are preparing to develop a new vaccine targeting South Africa (B.1.351) variant of concern |
| February 24, 2021 | Pfizer and BioNTech announced a new clinical trial to test a third booster. The extra booster is designed to fight off the new variants |
| February 24, 2021 | Moderna announced their booster shot is now ready for human trials |
| May 7, 2021 | Pfizer-BioNTech applied for full US FDA approval, a Biologics License Application (BLA), for its COVID-19 vaccine |

(continued)

**Table 31.1** (continued)

| | |
|---|---|
| June 1, 2021 | Moderna applied for full FDA approval, a Biologics License Application (BLA), for its COVID-19 vaccine |
| May 10, 2021 | US FDA granted emergency use authorization to Pfizer-BioNTech COVID-19 mRNA vaccine for use on 12–15 year-olds |
| August 23, 2021 | Pfizer-BioNTech COVID-19 vaccine received full approval from US FDA (https://www.fda.gov/news-events/press-announcements/fda-approves-first-covid-19-vaccine) |
| September 20, 2021 | Pfizer-BioNTech announced positive results of COVID-19 *vaccine* on children aged 5–11 *years* (https://www.pfizer.com/news/press-release/press-release-detail/pfizer-and-biontech-announce-positive-topline-results) |
| September 22, 2021 | FDA authorized booster dose of Pfizer-BioNTech COVID-19 vaccine for certain populations (https://www.fda.gov/news-events/press-announcements/fda-authorizes-booster-dose-pfizer-biontech-covid-19-vaccine-certain-populations) |
| October 7, 2021 | Pfizer-BioNTech submitted an emergency request to the U.S. FDA for Authorization of its COVID-19 vaccine for 5–11 years old children |

Variant impacts related to current mRNA vaccines will be discussed as well. Finally, vaccine equity and forecasts for the future of COVID-19 vaccines will be covered.

## 31.2 COVID-19 and SARS-CoV-2

COVID-19 was first reported in Wuhan, Hubei Province of China. The virus causing this disease was originally named 2019-nCoV. But this changed on February 11, 2020 when the International Committee for the classification of viruses designated *severe acute respiratory syndrome coronavirus 2* (SARS-CoV-2), when genome analysis provided a definite relation with SARS-CoV (Wang et al. 2020). The origin of the virus remains unclear. Bats seem to be the virus' potential reservoir, and pangolins the virus' possible intermediate host responsible for virus amplification. The original epicenter of COVID-19 outbreak in Wuhan is close to local Seafood Wholesale Market Huanan. Some suspect the disease was spread through wild animals sold from this wet market. However, no direct evidence of this has been found. In January 2021, a World Health Organization (WHO) team of experts traveled to China to investigate the origins of the COVID-19 pandemic. They provided some results, but key questions about the exact timing, location, and transmission origin of the virus remain unclear (Mallapaty 2021).

SARS-CoV-2 is an enveloped single-stranded RNA virus, and the virions are approximately spherical. Figure 31.2 shows SARS-CoV-2 virions, using a cryo-Electron-Microscopy (cryo-EM) (https://nanographics.at/projects/coronavirus-3d/). SARS-CoV-2 genome (RNA) is buried inside a lipid bilayer (Fig. 31.3). There are four major structural proteins for SARS-CoV-2: the nucleocapsid protein (N), the membrane protein (M), the envelope protein (E), and the spike protein (S) (a large surface glycoprotein) (Wang et al. 2020). Part of the spike proteins (or S trimers) protrude from the surface of the envelope, making the virus particle look like a

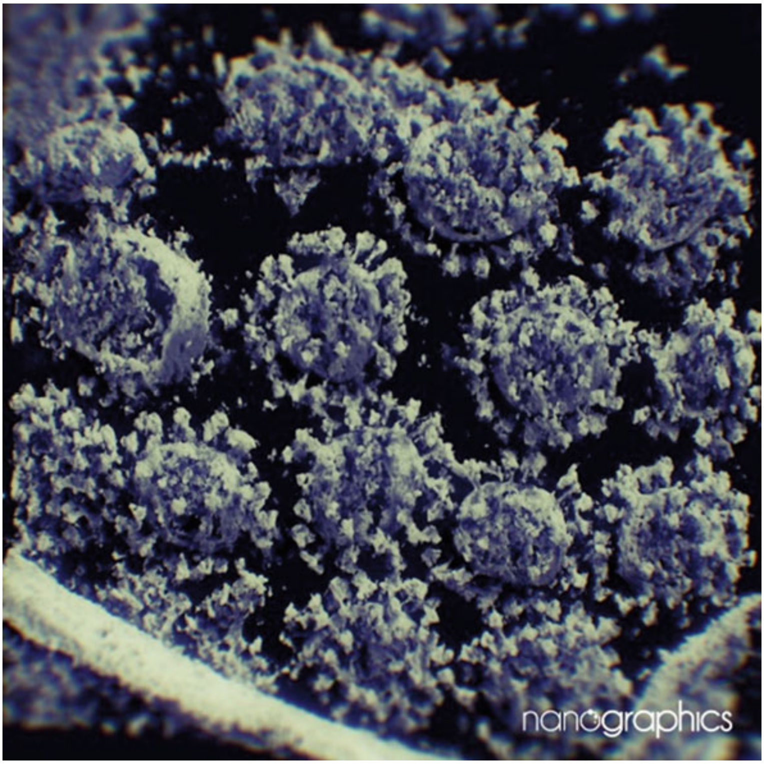

**Fig. 31.2** SARS-CoV-2. (Image from: https://nanographics.at/projects/coronavirus-3d/; under CC-BY license for unrestricted reuse)

"corona" and resulting in the name 'coronavirus' (Fig. 31.4) (Ke et al. 2020). SARS-CoV-2 uses its spike protein to gain entry into host cells by interacting with human cellular receptor *Angiotensin-converting enzyme 2* (ACE2). ACE2 is composed of the S1 subunit and S2 subunit. S2 subunit fuses host and viral membranes, allowing the virus to release its genome into host cells. The receptor-binding domain (RBD) located at subunit S1 specifically interacts with ACE2 (Awadasseid et al. 2021). ACE2 is the receptor of SARS-CoV, the virus responsible for SARS disease. The genome size of SARS-CoV-2 is approximately 29.8 kb, annotated to possess 14 Open Reading Frames (ORFs) encoding 27 proteins. The four structural protein genes (N, M, E, and S) are located at the 3′-N terminus of its genome. Figure 31.4 shows SARS-CoV-2 genome organization and its spike protein molecular structure.

SARS-CoV-2 is transmissible between humans. The virus can also spread from human to animals, and from animals to human. SARS-CoV-2 is transmitted through respiratory droplets when a COVID-19 patient sneezes, coughs, shouts, talks, or sings. The virus presents in salivary glands, where it can replicate, resulting in salivary transmission as well (Tan et al. 2021). Respiratory droplets are composed of both large droplets and micro-droplets. Droplets exist in aerosol form and can be

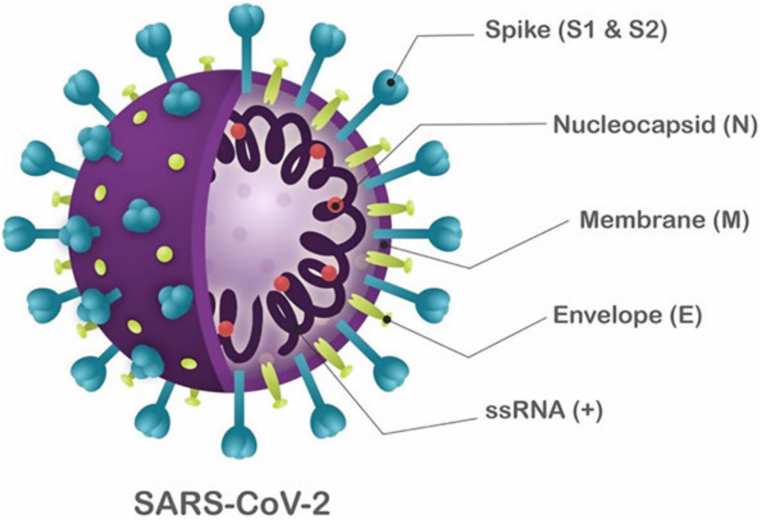

**Fig. 31.3** Schematic structure of SARS-CoV-2. The viral structure is primarily formed by the structural proteins such as spike (S), membrane (M), envelope (E), and nucleocapsid (N) proteins. The S, M, and E proteins are all embedded in the viral envelope, a lipid bilayer derived from the host cell membrane. The N protein interacts with the viral RNA in the core of the virion. (Figure from: Santos IA, Grosche VR, Bergamini FRG, Sabino-Silva R and Jardim ACG [2020] Antivirals Against Coronaviruses: Candidate Drugs for SARS-CoV-2 Treatment. *Front. Microbiol.* 11: 1818. doi: https://doi.org/10.3389/fmicb.2020.01818; under CC-BY copyright license for unrestricted reuse)

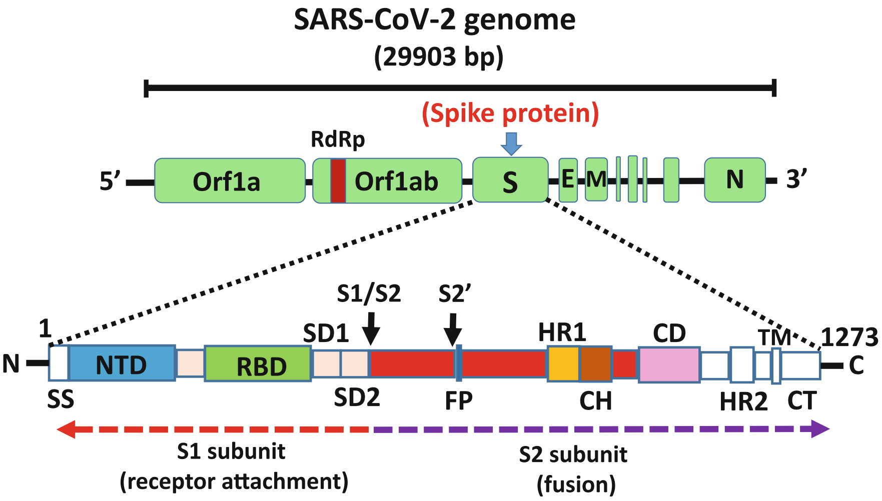

**Fig. 31.4** Primary genome structure and spike protein genes of SARS-CoV-2. Orf, Open reading frame; SS, signal sequence; RBD, receptor-binding domain; NTD, N terminal domain; S2′, S2′ protease cleavage site; FP, fusion peptide; HR1, heptad repeat 1; CH, central helix; CD, connector domain; HR2, heptad repeat 2; TM, transmembrane domain; CT, cytoplasmic tail. Arrows denote protease cleavage sites

inhaled by people nearby or far away. SARS-CoV-2 viral particles located in droplets larger than 100 μm typically fall to the ground in seconds. However, viruses in micro-droplets (smaller than 100 μm) can remain suspended in the air for many seconds to hours with the potential to be inhaled by passersby (Prather et al. 2020). Inhaled droplets enter via the mouth or nose and travel the airways to the lungs. Eyes also provide entry for SARS-CoV-2. Studies first suggested the potential for SARS-CoV-2 transmission through the ocular surface. Researchers found conjunctival congestion in lab-confirmed COVID-19 patients (Qing et al. 2020). Contaminated surfaces can be a transmission route for the virus, but the risk is very minimal, as this is not an important route (Tang et al. 2021). More and more scientific studies have shown the importance of aerosol transmission. CDC (US) recently also acknowledged that very fine *aerosol particle transmission is* one important route of infection, in addition to 'close-contact' transmission. CDC statement could be found here: https://www.cdc.gov/coronavirus/2019-ncov/science/science-briefs/sars-cov-2-transmission.html.

Animals can be infected with the virus causing COVID-19. Several animal species have been infected with SARS-CoV-2. Those include (1) tiger, lions, and gorilla at zoos, (2) domestic cats and dogs, (3) aquarium otters, and (4) farmed minks (Oreshkova et al. 2020). In November 2020, Demark culled 17 million farmed minks due to SARS-CoV-2 infection, in fear that this mink-related SARS-CoV-2 Y453F variant could jump species to human beings (Larsen and Paludan 2020). The World Organization for Animal Health, the Office International des Epizooties (OIE), has compiled a list of wild and domesticated animals' susceptibility rates for COVID-19 infection. High susceptibility species include mink, ferret, raccoon, dog, rabbits, cat, hamster, large cat, and gorilla. Dog species are low. Pig and cattle species are extremely low, and poultry has no susceptibility to COVID-19 (https://www.oie.int/scientific-expertise/specific-information-and-recommendations/questions-and-answers-on-2019novel-coronavirus/). To prevent COVID-19 virus infections among zoo animals, US zoo keepers began vaccinating zoo animals with Zoetis animal COVID-19 vaccine (Tizard 2020).

## 31.3 Vaccine Types for COVID-19

After the first genome sequence of novel coronavirus discovered in China was published (Wu et al. 2020), companies rushed to design and produce COVID-19 vaccines based on that sequence. Most vaccines are designed to target the virus spike protein (particularly, the RBD domain). Figure 31.4 shows spike protein, which is an important interaction region for human cell receptor ACE2 binding to allow viral entry into cells. There are several approaches to produce COVID-19 vaccines. So far, there are four major vaccine types: (1) "Whole virus" vaccine; (2) Viral vector vaccine; (3) "Protein subunit" vaccine; and (4) mRNA vaccine. "Whole virus" vaccine includes *live attenuated vaccine* and *inactive vaccine*. For live attenuated vaccine, virus is treated so that it is weak but it still can replicate. Replication of virus in live attenuated vaccine cannot cause human sickness. For inactive vaccine, the virus genetic material was modified that it cannot replicate but it still can induce an immune response. Viral vector vaccines contain a different virus (modified) as a vector to deliver SARS-CoV-2 genetic material into human cells. A segment of genetic material from SARS-CoV-2 genome (spike protein gene) is then inserted into the viral vector for delivery. The final product is the main ingredient of viral vector vaccines. Protein subunit vaccines contain a specially selected and harmless protein derived from SARS-CoV-2 in its ingredients. Once the protein is injected into the human body, it elicits an effective immune response. Hepatitis B vaccine is another example of protein vaccine. Protein subunit vaccines have less side effects, but it induces a weaker immune response, compared to other types of vaccines. Protein subunit vaccines are usually delivered alongside adjuvants (agents that stimulate the immune system). mRNA vaccines contain mRNA of SARS-CoV-2 spike protein. mRNA molecule is delivered into cellular cytosol, where it is translated into protein and elicits an immune response (see next paragraph). Detailed COVID-19 vaccine tracker from WHO can be viewed here: https://www.who.int/publications/m/item/draft-landscape-of-covid-19-candidate-vaccines.

## 31.4 mRNA Vaccines

As mentioned in the previous paragraph, there are several platforms for vaccine production. mRNA (messenger RNA)-based vaccine emerged before all others as a rapid and versatile platform for quick response to this challenge. mRNA is a fragile biological molecule to deliver. It is easily degraded by cellular nucleases. Safe delivery of the mRNA molecule into cells was critical technology to development of the mRNA vaccine. After years of study, scientists developed lipid nanoparticle (LNP) as a vehicle to deliver mRNA molecules to human cells. This is one of the most critical steps toward successful development of mRNA vaccines. We will discuss LPN later in this chapter.

Pfizer-BioNTech and Moderna become the two first pharmaceutical companies to produce mRNA COVID-19 vaccines. Pfizer-BioNTech mRNA vaccine is named BNT162b2 and Moderna mRNA vaccine is termed mRNA-1273. The human trial efficacy of these two mRNA vaccines are 95% and 92%, respectively (Polack et al. 2020; Baden et al. 2021). These results were obtained from trials against the early version of SARS-CoV-2. Both Pfizer-BioNTech and Moderna vaccines induce 'active' immunization to prevent COVID-19 infection. This is opposed to 'passive' immunization, where antibodies are used to vaccinate. One example of passive immunization includes the use of monoclonal antibodies. In the beginning, Pfizer-BioNTech was authorized for use in individuals aged 16 or older. Moderna vaccine was authorized for use in individuals aged 18 and older. However, recently, both vaccines were approved for emergency use for teens 12–15 years old in the US, the UK, Germany, and other countries. Both mRNA vaccines require two doses. For Pfizer-BioNTech vaccine, the two doses are administered 3 weeks apart. For Moderna vaccine, the two doses are given 4 weeks apart. The UK was the first country to authorize the Pfizer-BioNTech mRNA vaccine for emergency use. The

US was the second country to allow the Pfizer-BioNTech vaccine for emergency use (EUA). Later, more countries followed suit.

Israel is leading the world as the most vaccinated country in the early stage of global COVID-19 vaccine rollout. As of June 1, 2021, nearly 63% of its population has received at least one shot of COVID-19 vaccine (from *Our World in Data*). The Israel Heath ministry stated a recent data analysis found Israelis who have received **both doses** of the Pfizer-BioNTech vaccine saw a dramatic drop in both illness and hospitalization. On April 18, 2021, Israel lifted their requirement for wearing masks in public, due to the dramatic drop in new confirmed COVID-19 cases. The launch of the COVID-19 mRNA vaccine has been a huge success. In the following paragraphs, we will discuss mRNA COVID-19 vaccine production, function, and possible allergic reactions with a focus on Pfizer-BioNTech and Moderna mRNA vaccines. Pfizer-BioNTech mRNA vaccine will serve as the main production example.

## 31.4.1 Making of mRNA Vaccine

### 31.4.1.1 Engineering of SARS-CoV-2 Spike Protein mRNA Fragment

After the SARS-CoV-2 genome sequence became available, scientists used it to design and produce the core mRNA vaccine ingredient, mRNA molecules. Scientists synthesized a critical RNA fragment from the spike protein region, which was modified to form a final version of the ingredient mRNA. The mRNA molecule of Pfizer-BioNTech's mRNA vaccine is used for explanation here. The structure of this particular RNA fragment is CAP-5′UTR-Signal-(spike protein coding region)-3′UTR-(poly-A-tail). The 5′ untranslated region ('UTR') is composed of nucleotides as follows: GAAΨAAACΨAGΨAΨΨCΨΨCΨGGΨCCCCACAGACΨCAGAGA GAACCCGCCAC. The Ψ replaces U, because years of research have shown replacing the U in RNA with a slightly modified molecule, pseudouridine (Ψ), allows for immune system evasion and increased gene expression. The 5′UTR is ribosome landing pad for protein synthesis. The 'signal peptide' is "S (spike) glycoprotein signal peptide (a leader sequence)," which takes the synthesized protein to exit cells through "endoplasmic reticulum." The signal peptide sequence is 'codon optimized' through *codon usage*. The actual Spike protein gene (3777 nucleotides in length) is also codon-optimized. Reports indicate that mRNAs with an optimal combination of codon and modified nucleotides give the most robust gene expression in human vaccines (Lu et al. 2020). The third and fourth codons (K and V amino acids) of spike protein gene are both replaced with a 'P' (or proline) codon. These two consecutive proline substitutions occurred in the S2 subunit (Fig. 31.4). Double Proline substitution (also termed 2-P substitution) renders 'prefusion' configuration/structure of SARS-CoV-2 spike proteins, even without being integrated within the whole virus. 2-P substitution prevents collapse, allowing the protein to maintain the appropriate structure. This works because Proline is a very rigid amino acid (Pallesen et al. 2017). The detailed explanation of the Pfizer-BioNTech mRNA vaccine mRNA fragment can be found here: https://berthub.eu/articles/posts/reverse-engineering-source-code-of-the-biontech-pfizer-vaccine/.

#### 31.4.1.2 Ingredients of Pfizer-BioNTech and Moderna mRNA Vaccines

Some vaccine ingredients are potential allergens to allergic individuals, capable of triggering life-threatening reactions. Therefore, knowing the ingredients of a vaccine is important. People can do a preventive skin test for those ingredients before they take the vaccine. For example, scientists suspect PEG could be an allergen to some people who develop serious side effects after taking mRNA vaccines. IgE-mediated allergy to PEG is well described in the literature, with these cases testing positive in PEG-skin tests (Banerji et al. 2021). Below are ingredient lists for Pfizer-BioNTech and Moderna mRNA vaccines.

The Pfizer-BioNTech COVID-19 Vaccine includes the following ingredients: "spike protein mRNA, lipids ((4-hydroxybutyl)azanediyl)bis(hexane-6,1-diyl)bis(2-hexyldecanoate), 2 [(polyethylene glycol)-2000]-*N*,*N*-ditetradecylacetamide, 1,2-Distearoyl-sn-glycero-3-phosphocholine, and cholesterol), potassium chloride, monobasic potassium phosphate, sodium chloride, dibasic sodium phosphate dihydrate, and sucrose" (https://www.fda.gov/media/144414/download).

The Moderna COVID-19 Vaccine contains the following ingredients: "messenger ribonucleic acid (mRNA), lipids (SM-102, polyethylene glycol [PEG] 2000 dimyristoyl glycerol [DMG], cholesterol, and 1,2-distearoyl-sn-glycero-3-phosphocholine [DSPC]), tromethamine, tromethamine hydrochloride, acetic acid, sodium acetate, and sucrose" (https://www.fda.gov/media/144638/download).

#### 31.4.1.3 Delivery of mRNA with Lipid Nanoparticles

Vaccine mRNA molecules must be delivered into cells for protein production. There are barriers to delivery before mRNA molecules enter cells, including potential degradation by extracellular ribonucleases (ex. RNase) that are abundant in skin and blood. RNA molecules are more chemically labile than DNA. Therefore, mRNA can rapidly degrade before cell entry. Safe transport of mRNA molecules to cells requires a special protection device. The mRNA vaccines developed by Pfizer-BioNTech and Moderna use a lipid-based nanoparticle carrier system called Lipid Nanoparticle (or LNP) (Fig. 31.5). Each mRNA molecule is buried inside the lipid bilayer of LNP, preventing rapid cellular enzymatic degradation to facilitate in vivo delivery. An LNP typically includes (1) an ionizable or cationic lipid; (2) a zwitterionic lipid resembling lipids in the cell membrane, ex DSPC; (3) cholesterol; and (4) PEG (Kowalski et al. 2019). Cholesterol plays several roles, including filling gaps in the particle, stabilizing the LNP lipid bilayer, limiting LNP-protein interactions and possibly promoting membrane fusion (Buschmann et al. 2021). The lipid-based nanoparticle carrier system is further stabilized by a hydrophilic polyethylene glycol (PEG) 2000 providing a hydrophilic layer and prolonging half-life (Buschmann et al. 2021). DSPC helps form a stable bilayer underneath the PEG surface.

Although it took decades to develop LNPs for mRNA vaccine delivery, the exact mechanism of LNPs for cell entry is still not fully understood (Hou et al. 2021).

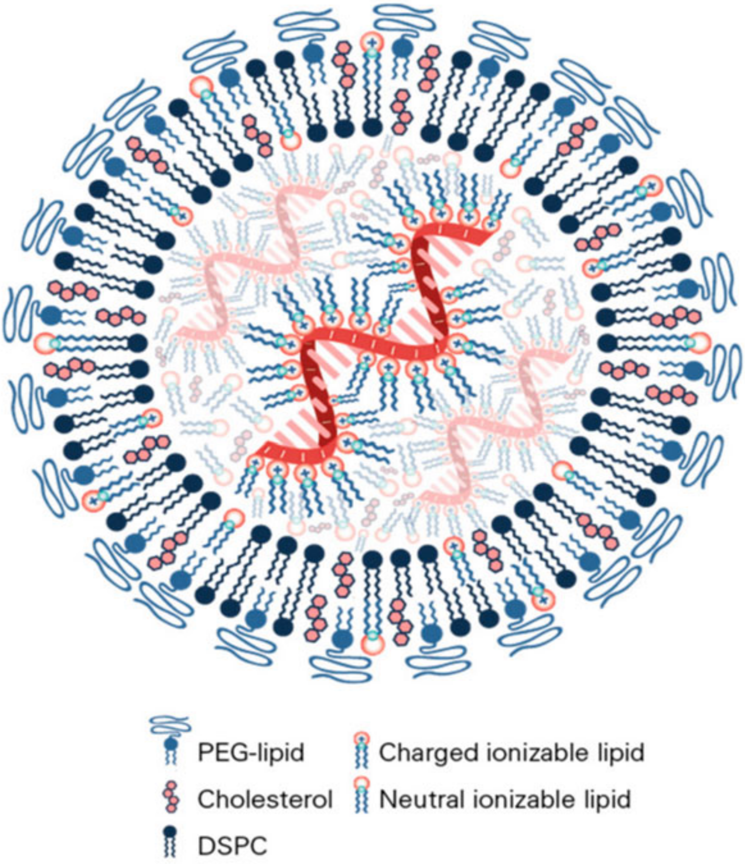

**Fig. 31.5** mRNA lipid nanoparticle (LNP) structure. Recent studies using cryo-Electron Microscopy, small-angle neutron scattering and small-angle X-ray scattering have shown that the mRNA lipid nanoparticle includes low copy numbers of mRNA (1–10) and that the mRNA is bound by the ionizable lipid that occupies the central core of the LNP. The polyethylene glycol (PEG) lipid forms the surface of the lipid nanoparticle, along with DSPC, which is bilayer forming. Cholesterol and the ionizable lipid in charged and uncharged forms can be distributed throughout the LNP. (Figure is reproduced from paper by Buschmann MD, Carrasco MJ, Alishetty S, Paige M, Alameh MG, Weissman D. (2021) Nanomaterial delivery systems for mRNA vaccines. *Vaccines*. 9(1):65. https://doi.org/10.3390/vaccines9010065, under copyright license CC-BY for unrestricted reuse)

However, most accept the general LNP uptake route to be the endocytosis pathway. LNPs are able to electrostatically attach and fuse with the cell membrane using inverted non-bilayer lipid phases to enter cells. Once inside the cell, LNPs are in the form of endosomes. mRNA nano-construct then undergoes endosomal escape and releases mRNA into the cytosol for protein synthesis (Kowalski et al. 2019; Wu and Li 2021). Figure 31.6 illustrates how an LNP is uptaken by human cells.

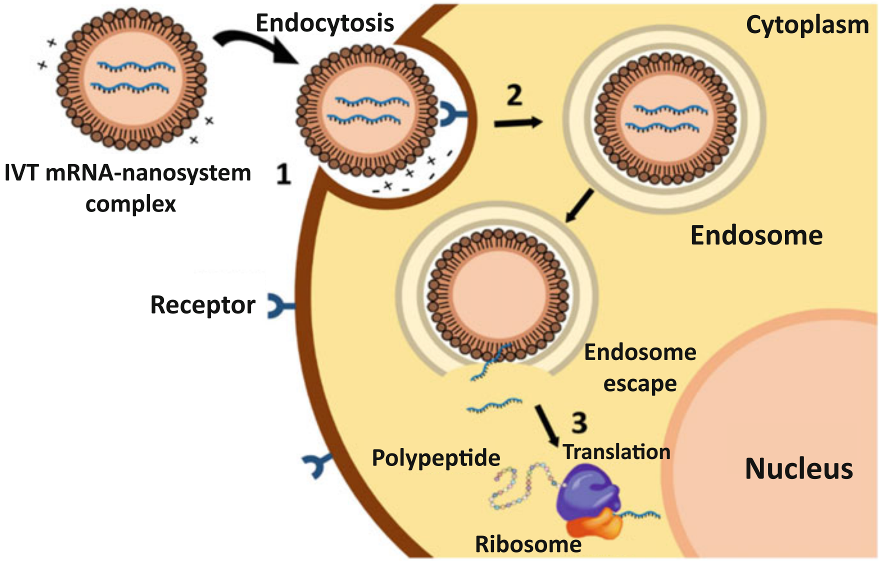

**Fig. 31.6** Intracellular barriers for in vitro transcribed (IVT) mRNA delivery: (1) Interaction between the delivery system and cell membrane, (2) endocytosis, and (3) endosomal escape and release of the mRNA to start the translation process. Figure from Gómez-Aguado I, Rodríguez-Castejón J, Vicente-Pascual M, Rodríguez-Gascón A, Solinís MÁ, Del Pozo-Rodríguez A (2020) Nanomedicines to deliver mRNA: state of the art and future perspectives. *Nanomaterials* (Basel). 10(2):364. DOI: https://doi.org/10.3390/nano10020364. Under copyright license CC-BY for unrestricted reuse

### 31.4.1.4 mRNA Vaccine Storage

Current COVID-19 vaccines, including the two mRNA vaccines, are not manufactured in pre-filled syringes like most other vaccines used in high-income countries. One reason for this measure is ‘COVID-19 vaccines are needed for emergency use’. Companies cannot produce pre-filled syringes fast enough for vaccine rollout. Initially, both COVID-19 mRNA vaccines were manufactured in vials, and was charged by vials. Pfizer-BioNTech vial contains five doses with doses past five considered extra doses (more than 5 doses/vial has been observed). However, Pfizer-BioNTech recently requested and received approval from the US FDA (and some other countries) to charge based on doses rather than vials. Moderna recently requested US FDA approval for an increase in the amount of vaccine per vial to facilitate vaccine production too.

Pfizer-BioNTech originally stated their mRNA vaccine required storage in an ultra-cold freezer between −80 °C and −60 °C (or −112 °F and −76 °F). Pfizer-BioNTech mRNA vaccines must be transported inside a box with dry ice. These boxes include GPS devices to track location and temperature. For Pfizer-BioNTech

COVID-19 vaccine US distribution Fact Sheet, see https://www.pfizer.com/news/hot-topics/covid_19_vaccine_u_s_distribution_fact_sheet.

Although ultra-cold freezers are recommended for long-term Pfizer-BioNTech vaccine storage, there are additional options for hospitals and clinics seeking short-term storage options (https://www.cdc.gov/vaccines/covid-19/info-by-product/pfizer/downloads/storage-summary.pdf). Recently, the company published new guidelines for vaccine storage between −25 and −15 °C for a total of 2 weeks with European Medicines Agency (EMA) and FDA (US) approval (Mar. 26, 2021; https://www.pfizer.com/news/press-release/press-release-detail/ema-approves-new-storage-option-pfizer-biontech-vaccine). Another recent major change for Pfizer-BioNTech vaccine storage was that unopened, undiluted, thawed vials can be stored in a refrigerator (2–8 °C) from original 5 days to 1 month (https://www.fda.gov/news-events/press-announcements/fda-brief-fda-authorizes-longer-time-refrigerator-storage-thawed-pfizer-biontech-covid-19-vaccine). This change will make it much easier for community doctors to receive, store, and administer the vaccine. Moderna's mRNA vaccine requires storage in a freezer at temperatures between −25 and −15 °C (−13 and 5 °F). The vaccine can be stored in the refrigerator between 2 and 8 °C (36 and 46 °F) for up to 30 days (https://www.cdc.gov/vaccines/covid-19/info-by-product/moderna/downloads/storage-summary.pdf).

## 31.5 Efficacy and Effect of Pfizer-BioNTech and Moderna Vaccines Against Ancestral SARS-CoV-2 Strains

From trial results, efficacies of Pfizer-BioNTech and Moderna vaccines for the ancestral virus (based on genome sequence used for vaccine development) are 95% and 92%, respectively, against the early versions of SARS-CoV-2. In real-world vaccination data analysis, the efficacy is also as high as the efficacy reported from earlier human trials. Even one dose of the mRNA vaccines can provide strong protection against the COVID-19. Here are some examples (Note: the following reports were based on pre-Delta-variant data):

### 31.5.1 In the UK (First Country to Use Pfizer-BioNTech Vaccine)

UK was one of the first countries to begin a massive vaccination campaign near the end of December 2020. By January 25, 2021, reports revealed deaths from COVID-19 had fallen by 62% among individuals 80 years of age and above (the first age group got vaccinated). By February 16, 2021, one-third of that age group had some level of immunity against coronavirus, by receipt of their first vaccine dose within the previous 2 weeks (https://www.theguardian.com/society/2021/feb/16/encouraging-signs-covid-vaccine-over-80s-deaths-fall-england). Another UK study found that "for healthcare workers under the age of 65, one dose of the vaccine reduced the risk of infection by 72% after 3 weeks, while two vaccine doses reduced the risk of infection by 85%". This high level of protection also extended to the virus variant, B.1.1.7, first identified in the UK in December 2020 (https://www.cnn.com/2021/02/22/health/uk-phe-study-pfizer-biontech-intl-gbr/index.html). As of the middle of May 2021, the UK had reached their goal of zero COVID-19 deaths per day.

### 31.5.2 In the USA (Second Country to Use Pfizer-BioNTech Vaccine)

A study involving 3950 health care workers was conducted in the US to check both Pfizer-BioNTech and Moderna mRNA vaccines' effectiveness against COVID-19 in the real world. Results showed the risk of becoming ill due to COVID-19 was reduced by 80% two or more weeks after administration of the first dose and by 90% two or more weeks after the second dose (Thompson et al. 2021). As of the beginning of June 2021, the confirmed new COVID-19 cases in the US have reached the lowest numbers since the beginning of pandemic. US CDC also advised that fully vaccinated people can go maskless, except at some facilities.

### 31.5.3 In Israel (the Most Vaccinated Country)

From a landmark mass vaccination study in Israel, Pfizer-BioNTech COVID-19 mRNA vaccine was found to be 94% effective in the data, including 1.2 million people (i.e., 0.6 million vaccinated people against the 0.6 million control group of unvaccinated people) (Dagan et al. 2021). In the study, vaccine effectiveness for first and second doses respectively are (1) for infection, 46% and 92%; (2) for symptomatic Covid-19, 57% and 94%; (3) for hospitalization, 74% and 87%; and (4) for severe disease, 62% and 92% (Dagan et al. 2021) (Fig. 31.7).

### 31.5.4 In Spain

Guijarro et al. (2021) reported that COVID-19 infections dramatically dropped among the health care workers even after the first dose of Pfizer-BioNTech COVID-19 mRNA vaccine. They found new SARS-CoV-2 infections in 1820 health care workers declined by 62% at 2–4 weeks after first dose administration and virtually no infections after the second dose of vaccine.

### 31.5.5 Other Results

Another study also showed even before a second dose, BNT162b2 developed by Pfizer-BioNTech to be highly efficient with a vaccine efficacy of **92.6%**. A finding similar to the first-dose efficacy of **92.1%** reported for Moderna's mRNA-1273 vaccine. The author of this research advocates postponing the second dose, in order to vaccinate more people with the first dose for the purpose of saving lives (Vergnes

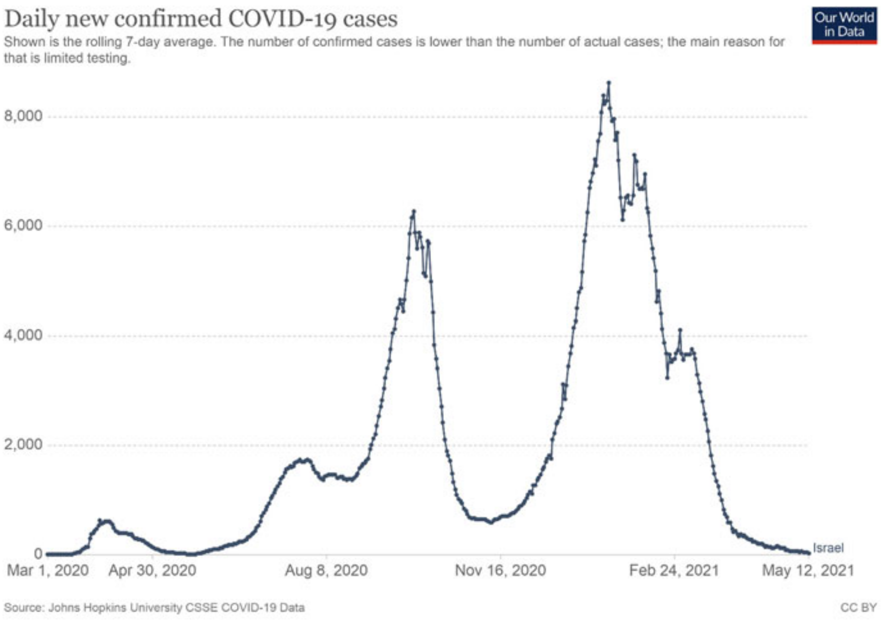

**Fig. 31.7** Israel: Coronavirus Pandemic Country Profile before Delta variant wave (Daily New Confirmed Cases). (Chart is from *Our World in Data*, under CC-BY license for unrestricted reuse. https://ourworldindata.org/coronavirus/country/israel)

2021). Study from Scotland also demonstrated that first dose of Pfizer-BioNTech mRNA vaccine is associated with a vaccine effectiveness of 91% for COVID19-related hospitalization at 28–34 days after vaccination (Vasileiou et al. 2021). Due to the high effectiveness of mRNA vaccines, researchers are advocating to "defer the second dose to accelerate and maximize coverage of the first dose in the population" (Leshem and Wilder-Smith 2021).

## 31.6 How Long Do Pfizer-BioNTech and Modern mRNA Vaccines Provide Protection?

### 31.6.1 Types of Neutralizing Antibodies (nAb)

According to one study, five distinctive patterns of neutralizing antibodies were observed in patients who recovered from COVID-19 infection. The authors did a 180-day observation following symptom onset. These five patterns are (1) negative (no nAb was produced); (2) rapid waning (nAb produced but disappear fast); (3) slow waning; (4) persistent; and (5) delayed response. Delayed response type includes people who developed neutralizing antibodies 90–180 days after symptom onset (Chia et al. 2021). It is not yet known if vaccine-induced neutralizing antibodies share these type patterns.

### 31.6.2 Longevity of Vaccine-Induced Neutralizing Antibodies (nAb)

Pfizer-BioNTech announced on April 1, 2021, "*The ongoing Phase 3 clinical trial of BioNTech/Pfizer's coronavirus vaccine confirms its protection remains high for at least six months after the second dose. Protection likely lasts even longer than that, but they say having data showing good protection* ***six months*** *after people were vaccinated is good news. The vaccine remains more than 91% effective against disease with any symptoms for six months* (https://www.pfizer.com/news/press-release/press-release-detail/pfizer-and-biontech-confirm-high-efficacy-and-no-serious)." On April 6, 2021, Moderna also reported that its vaccine provided at least **6 months** of protection in *The New England Journal of Medicine* (Doria-Rose et al. 2021). The protection can be even longer than 6 months or years. Indeed, from a recent *Nature* paper, it reveals that SARS-CoV-2 induced long-lived bone marrow plasma cells in humans (Turner et al. 2021). Long-lived bone marrow plasma cells (BMPCs) are a persistent and essential source of protective antibodies (Turner et al. 2021). However, antibody levels seem to wane with increasing age of participants. For example, the titer levels of antibodies averaged over 92,000 in vaccinated people aged 18–55 at 6 months after full immunization. The level dropped to about 62,000 in people aged 56–70, and to just over 49,000 in people >71 years old (Doria-Rose et al. 2021). All data shows COVID-19 mRNA vaccines provide strong and lasting protection against ancestral SARS-CoV-2 strains. However, many research studies have shown that first generation mRNA COVID-19 vaccines have waned significantly across different age groups during Delta variant wave.

### 31.6.3 Vaccine Breakthrough Infection

It is worth noting that in rare cases fully vaccinated persons can contract COVID-19, in what are termed '*breakthrough infections*'. In Washington State, US, 102 breakthrough cases were observed in one million fully vaccinated individuals. These breakthrough cases were seen since February 1, 2021 and represent 0.01% of vaccinated people in Washington (https://www.doh.wa.gov/Newsroom/Articles/ID/2720/Cases-of-COVID-19-vaccine-breakthrough-confirmed-in-Washington-state). In Texas, Houston health department reports 142 breakthrough infections were observed out of more than 577,000 fully vaccinated individuals. The Houston rate is approximately 0.02% (https://www.khou.com/article/news/health/coronavirus/vaccine/houstonians-test-positive-covid-19-after-vaccine/285-b50aba7f-5f23-4ae0-bec8-05f71af300a5). The CDC (US) summarized national breakthrough infections. From Jan. 1 to April 30, 2021, more than 87 million Americans were fully vaccinated against COVID-19. Nationally, 10,262 breakthrough infections had been reported. Sixty-three percent of cases were female. Some patients were hospitalized and some were deceased. However, after May 1, 2021, CDC (US) only monitors vaccine breakthrough cases which lead to hospitalizations or deaths (https://www.cdc.gov/vaccines/covid-19/health-departments/breakthrough-cases.html).

Breakthrough infection is considered rare for the earlier virus strains. Studies have shown evidence of increased breakthrough infection rates of SARS-CoV-2 *variants* in Pfizer-BioNTech vaccinated individuals (Kustin et al. 2021). Certain variants of concern are capable of immure escape. Actually, in a recent paper, the authors demonstrated that 20 vaccine breakthrough cases detected at their institution, all 20 (100%) infections were due to variants of concern (VOC) (McEwen et al. 2021). During the middle of 2021, the Delta VOC has become dominant in many countries and caused more breakthrough infection, due to its evasion of immunity. Estimated Pfizer-BioNTech mRNA vaccine efficacy for symptomatic infection has dropped to 50–60% at best at Delta variant wave.

### 31.6.4 Should COVID-19 Survivors Take COVID-19 Vaccine?

According to the CDC (US), people with prior COVID-19 infections should take the vaccine. More than a dozen studies have shown that individuals with prior SARS-CoV-2 infections can elicit a strong immune response even after just one dose of Pfizer-BioNTech or Moderna mRNA vaccine. Dozen researches have shown that the response is greater than that from two vaccine doses on infection-naives. Many experts have therefore advised that COVID-19 survivors should take only 1 dose of COVID-19 vaccine to save vaccine doses and avoid unnecessary side effects (Ebinger et al. 2021; Frieman et al. 2021).

### 31.6.5 Which mRNA Vaccine Can Elicit Stronger Immune Response?

Two recent papers have shown that Moderna mRNA vaccine elicits stronger immune response than Pfizer-BioNTech mRNA vaccine does. The possible causes attributed to that are (1) higher vaccine dosage is used in Moderna COVID-19 vaccine, 100 μg, while Pfizer-BioNTech COVID-19 vaccine uses 30 μg.; (2) The schedule for two vaccine doses are different. For Moderna vaccine, the two doses are 4 weeks apart, and for Pfizer-BioNTech vaccine, the two doses are 3 weeks apart. Moderna’s COVID-19 vaccine also wanes slower than Pfizer-BioNTech’s.

## 31.7 SARS-CoV-2 Variants and Surveillance

### 31.7.1 SARS-CoV-2 Variants

Typically, RNA viruses undergo constant mutation, and this is the case for the SARS-CoV-2 RNA genome. A typical SARS-CoV-2 virus accumulates one to two single nucleotide mutations in its genome per month. This is ½ the mutation rate of influenza and ¼ that of HIV (Duchene et al. 2020). A recent publication showed a total of 149 mutations have been found in 103 sequenced ‘strains’ (or ‘variants’) since the early stage of this pandemic (Awadasseid et al. 2021). These mutations can be single nucleotide polymorphisms (SNPs) or single RNA nucleotide changes, representing deletion and recombination (Li et al. 2020). Mutations can alter viral pathogenesis, virulence, and/or transmissibility. However, most mutations do not cause an impact.

The WHO has proposed working definitions for SARS-CoV-2 variants. They are *Variants of Interest* (VOI) and *Variants of Concern* (VOC). The WHO defines SARS-CoV-2 isolate as VOI "if it is phenotypically changed compared to a reference isolate or has a genome with mutations that lead to amino acid changes, and has been identified to cause community transmission (forming COVID-19 disease clusters)." On the other hand, any isolate is defined as VOC if (1) it has been associated with increase in transmissibility or virulence to detrimentally change COVID-19 epidemiology; (2) a decrease in effectiveness regarding public health/social measures, available diagnostics, or vaccines (https://apps.who.int/iris/bitstream/handle/10665/339859/nCoV-weekly-sitrep25Feb21-eng.pdf?sequence=1).

Since SARS-CoV-2 spike protein (S) plays an essential role in viral entry and antibody recognition, any mutations in this region will be worthy of attention. The first VOC described is the SARS-CoV-2 D614G (Asp$^{614}$-to-Gly) variant. This gain-of-function mutation made the virus easier to spread. This SARS-CoV-2 D614G variant eventually became the dominant strain early in the pandemic during June 2020 and replaced the 'prototype' from Wuhan City, China. In the US, the CDC uses the terms 'wild-type'/'background type' to describe the virus that emerged from China. SARS-CoV-2 D614G variant exhibits efficient replication ex vivo and transmission in vivo (Hou et al. 2020). The D614G substitution enhances SARS-CoV-2 infectivity, competitive fitness, and transmission in human cells. However, this mutation also made the new variant more susceptible to neutralizing antisera (Hou et al. 2020). Therefore, D614G did not gain much attention.

Later on, four major VOC appeared. They are (1) variant 20I/501Y.V1, VOC 202012/01, or B.1.1.7, first identified in the UK; (2) variant 20H/501Y.V2 or B.1.351, first identified in South Africa; (3) variant P.1, first identified in Brazil; and (4) variant B.1.617, first identified in India. P.1 and B.1.351 have three virtually identical mutations: E484K, K417N/T and N501Y. B1.1.7 has rapidly spread in many countries and regions worldwide. However, in the middle of 2021, B.1.617 (especially the B.617.2) has replaced B.1.1.7 and become the dominant variant in many countries. According to a Variant Tracker (Rambaut et al. 2020), as of August 2021, at least 154 countries reported cases (sequences) of B.1.1.7 (UK) variant. The B.1.351 (South Africa) variant has been reported in 104 countries, while 62 countries have reported P1 (Brazil) variant. Meanwhile, at least 105 countries reported B.1.617.2 sequence as of August, 2021 (https://cov-lineages.org/global_report.html). According to the US CDC, rates of the four major VOCs are as follow (as of the beginning of August 2021): (1) B.1.617.2 was >90% of cases sequenced; (2) B.1.1.7 variant represents 3% COVID-19 cases; (3) P.1 variant: <1%; and (4) B.1.351: <0.5% (https://covid.cdc.gov/covid-data-tracker/#variant-proportions). B.1.617.2 variant (dominant variant in August 2021) completely takes over B.1.1.7 variant in only a few months in the US. B.1.1.7 was a dominant VOC in May 2021 in the US.

B.1.1.7 variant was the dominant UK SARS-CoV-2 strain before B.1.617.2 became dominant later. B.1.1.7 is 30–50% more transmissible and more deadly than the earlier strains. According to The New and Emerging Respiratory Virus Threats Advisory Group (NERVTAG), an advisory committee to the UK government, B1.1.7 is associated with an increased risk of hospitalization and death (https://www.gov.uk/government/groups/new-and-emerging-respiratory-virus-threats-advisory-group). One study confirmed that B.1.1.7 increased in deaths from 2.5 to 4.1 per 1000 detected cases (Challen et al. 2021). Another study found that B.1.1.7 was associated with increasing case fatality by more than two-thirds compared to the previously circulating virus among an unvaccinated population (Grint et al. 2021). In the US, B.1.1.7 has spread more than any VOC and comprises ~72% cases (as of mid-May 2021).

The B.1.351 lineage spread rapidly as well. Genomic sequence data shows B.1.351 to be the dominant SARS-CoV-2 variant in South Africa since November 2020 (Tegally et al. 2021). P1 variant is devastating Brazil now. A recent study from Institut Pasteur, Paris, France showed that B1.351 and P.1 variants are able to efficiently infect and replicate in mice without human ACE2 to extend the SARS-CoV-2 host range to mice. This abrogation of the species barrier raises a concern that wild rodents can serve as a secondary reservoir for variants (Montagutelli et al. 2021).

The B.1.617 variant was initially referred to as “double mutant”, although it is misleading, since it has more than two mutations in the spike protein. B.1.617 variant lineage has evolved to become three sub-lineages: B.1.617.1, B.1.617.2, and B.1.617.3. B.1.617.2 has recently caused India’s second wave COVID-19 outbreak. Recently, it also caused the UK a new COVID-19 case number surge. This variant was also reported in the US and other countries. As of August 2021, B.1.617.2 has been identified from genome sequences submitted by 105 countries (https://www.sciencemediacentre.org/expert-reaction-to-cases-of-variant-b-1-617-the-indian-variant-being-investigated-in-the-uk/). A recent UK study revealed that B.1.617.2 is 64% more transmittable than B.1.1.7. It also caused 2.2 times hospitalization rate than B.1.1.7 did.

Recently, WHO has renamed major VOCs with Greek alphabets. B.1.1.7 is called Alpha. B.1.351 is called Beta. P1 is called Gamma. B.1.617.2 is called Delta (https://www.nature.com/articles/d41586-021-01483-0).

In addition to these four major global VOCs, regional variants have also been reported. For example, US reported its home-grown variants: B.1.427/B.1.429 from the State of California (Zhang et al. 2021) and B.1.526 from New York City, New York (West et al. 2021). There are two versions of B1.526. One is B1.526 (E484K) and the other B1.526 (S477N). In addition to the original mutation, both have a D614G mutation in the spike protein. The B1. 526 E484K spike-protein mutation is the same mutation present in variants B1.351 and P1. In November 2020, B1.526 began showing up in samples collected in New York City. By the end of March 2021, genomic sequencing found this variant has accounted for >50% confirmed new cases. Scientists are working to determine the impact of these variants.

These variants concern virologists and public health experts, because they possess characteristics that could complicate the global vaccine effort and prolong the pandemic. Not only do these variants spread fast, they can also evade the immune response ("immune evasion") leading to re-infections and vaccine resistance issues (Garcia-Beltran et al. 2021; Tillett et al. 2021). Different VOCs can also co-infect a person (Francisco et al. 2021). Co-existing VOCs within the human body could undergo genetic recombination resulting in a new generation of dangerous VOCs (Jackson et al. 2021).

### 31.7.2 Genomic Surveillance

The best way to track SARS-CoV-2 variants is by genomic surveillance, which involves sequencing specimens of confirmed cases. Global Initiative on Sharing All Influenza Data (GISAID) is a popular public platform for researchers worldwide to deposit their SARS-CoV-2 sequences. By May 13, 2021, more than 1.5 million SARS-CoV-2 sequences have been deposited in GISAID (Maxmen 2021).

In the US, SARS-CoV-2 Sequencing for Public Health Emergency Response, Epidemiology, and Surveillance (SPHERES) is a new national genomics consortium for COVID-19 response to strengthen COVID-19 surveillance. It coordinates and standardizes SARS-CoV-2 virus sequencing across the nation. More than 100 institutions are participating in the SPHERES platform to work together and share genomic data. These include federal agencies and laboratories, academic institutions, corporations, state/local health laboratories, and non-profit public health or research laboratories. SPHERES is led by CDC's Advanced Molecular Detection (AMD) program, which invests in pathogen genomics to track infectious disease. A detailed description of SPHERES can be found at this link: https://www.cdc.gov/coronavirus/2019-ncov/covid-data/spheres.html. Since January 2021, US sequencing has increased. As of August, 2021, ~700,000 sequences were deposited in GISAID, and 400,000 were deposited in NCBI (https://covid.cdc.gov/covid-data-tracker/#published-covid-sequences).

Several other useful websites have been set up to track development of various variants. These sites are:

1. **Global Initiative on Sharing All Influenza Data** (**GISAID** 2020) repository (www.epicov.org). A non-profit organization allows researchers worldwide to deposit SARS-CoV-2 genome sequences into this repository. Researchers are required to register to gain access to the database.
2. **Pango Lineages** (https://cov-lineages.org/) provides information about currently circulating global lineages. In addition, the site updates case number counts and data regarding countries reporting cases of the three major variants.
3. **Nextstrain** (https://nextstrain.org/sars-cov-2/) explores SARS-CoV-2 evolution. Researchers can compare their SARS-CoV-2 genomic sequences to the SARS-CoV-2 reference sequence and assign them to clades to see where they fall on a SARS-CoV-2 tree.

4. **Global.health** (https://global.health/) is a relatively new, massive Google-funded COVID database. According to its site description, this global data repository and visualization platform enables open access to real-time epidemiological line-list data protecting the identity of individuals. Launching on February 25, 2021, the open repository gives free access to more than 160 million data points with details about individual infections. The site will be used to track variants and immunity.
5. **Outbreak.info** (https://outbreak.info/) tracks SARS-CoV-2 mutations with a variant report and detailed mutation map of each major VOC. The site was created by the Center for Viral Systems Biology (CViSB) at Scrippe Research (US) (https://cvisb.org/launch-of-outbreak-info-variant-tracking/).
6. **CoVariants** (https://covariants.org/). This site overviews SARS-CoV-2 variants, and percentage of different variants in different countries.
7. **COVID Data Tracker (Variant Proportions)** (https://covid.cdc.gov/covid-data-tracker/#variant-proportions). This site is sponsored by US CDC. It tracks the variant proportions in the US.

Genomic surveillance should also include samples collected from community sewage. Sewage surveillance helps detect SARS-CoV-2 variants early. For example, Missouri (US) has reported only one confirmed B.1.1.7 variant case (as of March 4, 2021), but genetic sequencing found evidence of the variant in more than 13 community wastewater sites around the state during the same week One useful site is “COVIDPoops19”, a dashboard maintained by researchers at University of California-Merced. The site tracks and reports wastewater surveillance of COVID-19 variants globally. Their goal is to provide a world map of SARS-CoV-2 wastewater testing so the public can easily see where testing is happening in their community (https://ucmerced.maps.arcgis.com/apps/opsdashboard/index.html#/c778145ea5bb4daeb58d31afee389082).

## 31.8 Effectiveness of COVID-19 mRNA Vaccines vs. SARS-CoV-2 VOCs

Both Pfizer-BioNTech and Moderna have conducted performance research on their mRNA vaccines against the three major VOCs.

### 31.8.1 Against B.1.1.7 (First Identified in the UK)

1. This was the major SARS-CoV-2 VOC in the UK, the US, and some other countries before it was replaced by B.1.617.2 VOC. Several scientific studies have indicated that the B.1.1.7 variant had no significant effect on neutralization by serum obtained from participants who had received the Pfizer-BioNTech BNT162b2 or Moderna mRNA-1273 vaccine. This is good news regarding a highly transmissible, high-death rate variant. However, two non-RNA vaccine efficacies dropped approximately 10% (Novavax and AstraZeneca) trials.

2. Recent publication further confirms that B.1.1.7 is not a neutralization-escape VOC for COVID-19 vaccines, indicating B.1.1.7 is unlikely to increase the risk of SARS-CoV-2 re-infection (Shen et al. 2021a, b). Authors observed B.1.1.7 remains sensitive to neutralization at moderately reduced levels (~2-fold), by serum samples from convalescent individuals and recipients of Moderna mRNA-1273 vaccine, and a protein nanoparticle vaccine (NVX-CoV2373, Novavax) (Shen et al. 2021a, b).
3. Lui et al. studied Pfizer-BioNTech BNT162b2 vaccine's effect against the three major VOCs (2021). They observed neutralization of B.1.1.7-spike and P.1-spike viruses to be roughly equivalent when compared to neutralization of USA-WA1/2020 used as a control in the study (Liu et al. 2021).

### 31.8.2 Against B.1.351 (First Identified in South Africa)

mRNA vaccines from Pfizer-BioNTech and Moderna, and non-mRNA vaccines from other companies (ex. Novavax and Johnson & Johnson) have been studied for efficacy against the South African B.1.351 VOC. Unfortunately, efficacies of all vaccines dropped significantly against this variant. For example, neutralization of the B.1.351-spike virus was weaker by Pfizer-BioNTech BNT162b2 vaccine-induced antibodies, by approximately one third, compared to neutralization of USA-WA1/2020, the study's designated control (Liu et al. 2021). Authors also observed viruses containing mutant residues in receptor-binding sites (K417N, E484K, and N501Y) to be more poorly neutralized (Liu et al. 2021). Both B.1.351 and P.1 variants have this set of mutations in their genome. Moderna's mRNA-1273 vaccine-induced serum was also used against (1) lab-generated recombinant vesicular stomatitis virus (rVSV)-based SARS-CoV-2 (a pseudovirus-based model). The pseudoviruses bore spike protein either from (1) original Wuhan-Hu-1 isolate; (2) the D614G variant; (3) the B.1.1.7; or (4) B.1.351 variant for comparison. The results were compared. The neutralization efficiency of the neutralizing antibodies in serum for D614G and B.1.1.7 were similar. However, the efficiency dropped when it was used to against B.1.351 (Wu et al. 2021). For non-mRNA vaccines, the Novovax vaccine showed 90% efficacy in the United Kingdom, but only 49% in South Africa cases dominated by the South Africa variant. Similarly, the efficacy of Johnson & Johnson vaccine presents at 72% in the United States, but drops to 57% in South Africa.

However, in spite of reductions in neutralization ability, Pfizer-BioNTech's mRNA COVID-19 vaccine against the South Africa B.1.351 variant remains robust. A recent trial in South Africa, where the B.1.351 lineage is prevalent, reported 100% participant protection from COVID-19. On April 1, 2021, Pfizer reported a trial enrolling 800 participants with only nine cases of B.1.351 infection and they were exclusively in the placebo group, indicating vaccine efficacy of 100% (https://www.pfizer.com/news/press-release/press-release-detail/pfizer-and-biontech-confirm-high-efficacy-and-no-serious).

On February 24, 2021, to ensure vaccines with high efficacy against the three VOCs, both Pfizer and BioNTech announced a new clinical trial to test a third vaccine booster. The third booster is designed to better protect individuals from SARS-CoV-2 variants, such as B.1.1.7 and B.1.351. They recruited 144 early-phase trial volunteers to volunteer again. Since that time, Moderna also announced a new clinical trial for the novel booster designed against the B.1.351 variant. Additional booster shots of authorized vaccines are one approach COVID-19 vaccine developers are taking to generate a competent vaccine regimen against new variants. Some other approaches include development of variant-specific and multivalent candidates (https://www.biocentury.com/article/634561/actions-covid-vaccine-makers-are-taking-to-tackle-variants-data-byte).

### 31.8.3 Against P1 (First Identified in Brazil)

Dejnirattisai et al. (2021) found Pfizer-BioNTech vaccine antibodies reduced by 2.6-fold neutralization of variant B1.1.7, compared to the original strain. A 3.3-fold reduction in neutralization of P1 was found with a 7.6-fold reduction in neutralization of B.1.351. Although both B.1.351 and P1 have the same set of mutations (E484K, K417N/T, and N501Y), P.1 is significantly less resistant to naturally induced or vaccine-induced antibody responses than B.1.351. Data suggests mutations outside the RBD region impact antibody-mediated neutralization (Dejnirattisai et al. 2021).

### 31.8.4 Against B.1.617.2 (First Identified in India)

A research was conducted to study the effectiveness of the BNT162b2 (Pfizer-BioNTech) against B.1.617.2. In the study, it demonstrated that effectiveness (against symptomatic infection) was significantly lower after 1 dose of vaccine with B.1.617.2 cases (33.5%) compared to B.1.1.7 cases (51.1%). After two doses with Pfizer-BioNTech vaccine, the effectiveness reduced from 93.4% with B.1.1.7 to 87.9% with B.1.617.2 (Bernal et al. 2021). However, more recent studies from the UK and Israel showed that efficacy of Pfizer-BioNTech vaccine against B.1.617.2 has dropped significantly, to 50–60% at best. The lowered efficacy has caused more breakthrough infection. Data also shows that Delta-infected fully vaccinated people have high viral load, which is similar to those unvaccinated or not fully vaccinated, and readily spread to other people (Brown et al. 2021). Researches from the UK and Denmark show that Delta virus increases hospitalization risk to about 2.5×, compared to the Alpha variant.

### 31.8.5 Against B.1.526 (First Identified in New York)

A study was conducted to compare the effectiveness of convalescent sera and vaccine-elicited antibodies on ancestral strain G614G, and variants B1.1.7, B.1.35, B1.526 (E484K), and B1.526 (S477N). Convalescent sera and vaccine-elicited antibodies retain full neutralization against the B.1.526 (S477N) variant, but neutralization of the B.1.526 (E484K) version declined with a modest 3.5-fold decrease in titer compared to the original variant (D614G). This variant has out-completed by B.1.627.2.

### 31.8.6 Against B.1.429 (First Identified in California)

Serum from people vaccinated with Moderna's mRNA vaccine (mRNA-1273) was used in a neutralizing B.1.429 variant test (Shen et al. 2021a, b). Results showed a modestly lower value in neutralization titers against the B.1.429 variant, similar to previously observed neutralization of the B.1.1.7 variant tested with the same assay using serum samples obtained from recipients of mRNA-1273 vaccines. Results suggest mRNA vaccine-elicited neutralizing antibodies are likely to remain effective against the B.1.429 variant. This variant has also been replaced by B.1.627.2.

In summary, research reports indicate highest rates of immune evasiveness with B.1.351 to date. The degree of immune evasiveness: Ancestral strain (Wuhan strain) = D614G < B.1.1.7 ("UK") < B.1.429 ("California") < B.1.617 ("-India") < B.1.526-E484K ("New York") < P1 ("Brazil") < B.1.617.2 ("-India") < B.1.351 ("South Africa"). A recent noteworthy study found neutralizing antibodies elicited by B.1.351 variant to have strong responses against both the original variant (D614G) and P1 variant, indicating high levels of cross-reactivity (Moyo-Gwete et al. 2021). As of August 2021, New York and California local variants have been taken over by VOC B.1.6172.

## 31.9 Do COVID-19 Vaccines Block Virus Spread?

Preventing COVID-19 illness is different than preventing COVID-19 transmission. Currently, we know COVID-19 mRNA vaccines prevent COVID-19 illness. It is not yet clear whether or not the vaccines can prevent transmission of COVID-19 virus. This question is not yet answered, and scientists are racing to discover these impacts. A US research project is underway, "Prevent COVID U". It was started by COVID-19 Prevention Network based at Fred Hutchinson Cancer Research Center in Seattle, Washington (https://preventcovidu.org/the-study/#needtodo). Twelve thousand students from 21 US universities are involved in the project. Students will be divided into two groups. One group will be vaccinated with Moderna's mRNA vaccine immediately. The other group will not be vaccinated until July 2021. Simultaneously, the students will invite their 'close contacts' (25,000 people in total) to the study. The results from this study will help determine if those vaccinated against COVID-19 prevent spread of the virus. However, two recent studies shows that household members of vaccinated health care workers had lower risk of COVID-19, compared to household members of unvaccinated health care workers (Shah et al. 2021; Salo et al. 2021). A current study also showed evidence that vaccination can protect non-vaccinated people in a community, especially for children (Milman et al. 2021).

## 31.10 The Safety of COVID-19 mRNA Vaccines

### 31.10.1 Common and Rare Side Effects of mRNA COVID-19 Vaccines

Common vaccine side effects are mild and include pain, swelling, and arm tenderness at the injection site. Some individuals also experience chills, tiredness, and headache according to US CDC (https://www.cdc.gov/coronavirus/2019-ncov/vaccines/different-vaccines/Pfizer-BioNTech.html). In general, these side effects last only a few days. However, there are some reports of delayed injection-site reactions (or "Delayed Large Local Reactions") with the Moderna COVID-19 mRNA-1273 vaccine used against SARS-CoV-2 (Blumenthal et al. 2021a, b). They are also called Moderna COVID-19 vaccine induced "COVID ARM" (Wei et al. 2021). People that take Pfizer-BioNTech vaccine don't have such side effects. The cause of this side effect is unknown. "COVID TOE" was also reported, but the cause is also unclear (Baeck and Herman 2021).

In a study published, researchers from the US CDC analyzed safety data from Vaccine Adverse Event Reporting System (VAERS; duration between December 14, 2020 and January 13, 2021), from 13.7 million Americans after receipt of either Pfizer-BioNTech or Moderna COVID-19 vaccines. Among 6994 total side effects reported to the agency, 78.7% were reported by women, despite a 61.2% administration of vaccine rate for women (Gee et al. 2021). However, 90.9% VAERS reports were for non-serious events and involved exclusively local and systemic symptoms.

Serious allergic reaction, anaphylaxis, have been reported for people receiving vaccines. Anaphylaxis symptoms involve more than one body system. Presenting involvement can include the respiratory system (ex. inducing shortness of breath), cardiovascular system (ex. inducing dizziness, fainting, and hypotension), skin (ex. showing hives, itching, swelling of lips and faces) or neurological system (ex. experiencing impending doom) (https://www.cdc.gov/vaccines/covid-19/downloads/recognizing-responding-to-anaphylaxis-508.pdf). Without timely treatment, anaphylaxis is a life-threating disorder. According to a study of Mass General Brigham (MGB) employees who had received their first dose of an mRNA COVID-19 vaccine, anaphylaxis resulting from vaccination was estimated to occur in 2.5–11.1 cases per million vaccine doses (Blumenthal et al. 2021a, b). Most of those individuals had a personal history of allergic reaction. Women account for almost all of rare anaphylactic reactions to COVID-19 vaccines. CDC reports each of the 19 individuals experiencing such a reaction to the Moderna COVID-19 vaccine has been female, and 44 of the 47 anaphylactic reactions to the Pfizer-BioNTech vaccine were also female (https://jamanetwork.com/journals/jama/fullarticle/2776557).

Doctors report using 'graded dosing' can effectively administer the second dose when individuals have serious COVID-19 vaccine allergic reactions (such as anaphylaxis) with their first dose (Mustafa et al. 2021). In this method, patients are not provided a full dose at once, instead small doses are administered sequentially every 15 min. The paper provided this protocol:

1. 0.05 mL of 1:10 vaccine dilution (diluted with sterilized water)
2. 0.05 mL of full-strength vaccine
3. 0.1 mL of full-strength vaccine
4. 0.15 mL of full-strength vaccine
5. 0.2 mL of full-strength vaccine.

Rare cases of acute Myopericarditis (***inflammation of heart muscle***) were also reported following COVID-19 mRNA vaccine injection. The most common symptom of Myopericarditis is chest pain 1–5 days after receiving the second dose of this vaccine (Das et al. 2021). It occurs especially in male adolescents and young adults. Myopericarditis can be treated medically, and most patients response well to the treatment (https://www.cdc.gov/coronavirus/2019-ncov/vaccines/safety/myocarditis.html). However, after a study, several countries (such as Finland and Sweden) are restricting the use of Moderna mRNA COVID-19 vaccine to vaccinate young adults over the concern of Myopericarditis.

## 31.11 Conclusion and Final Remarks

Several high-efficacy COVID-19 vaccines, including mRNA vaccines, have been developed. Real-world data have shown vaccines effectively preventing COVID-19 illness and reducing both hospitalization and death. However, there are still many things to address in the face of the COVID-19 outbreak. These include: (1) **Vaccine Hesitancy** is a key barrier to ending the COVID-19 pandemic. Misinformation and disinformation from social media and antivaxxers reduces vaccination willingness and slows the opportunity to reach herd immunity as soon as possible. According to a recent poll from Monmouth University, about 20% of Americans are not willing to take a COVID-19 vaccine. https://www.monmouth.edu/polling-institute/reports/monmouthpoll_us_041421/. It is vital to gain public trust to reduce vaccine hesitancy, based on the vaccine's safety records. Many vaccine-hesitant individuals express concern about possible vaccine side effects. Governments can educate the public about each vaccine to reduce fear and promote public safety. Hopefully, the recent full approval of Pfizer-BioNTech and Moderna COVID-19 vaccines by the US-FDA can increase the vaccination rate. Vaccine mandates from public sectors and private sectors can also boost the vaccination rate. (2) **Vaccine Equity** is a cause for global concern. The COVID-19 pandemic has had a brutal impact on the global economy by destroying livelihoods and causing the death of more than two million people worldwide. Vaccine introduction could prevent the loss of $375 billion to the global economy each month. Global equitable vaccine access is the only way to migitate the public health and economic impact of the pandemic. It is crucial to protect health care workers and those most at risk (https://www.who.int/initiatives/

act-accelerator/covax). However, most available vaccines to date have been purchased by high-income countries. Only a handful of poorer countries have access to COVID-19 vaccines. According to Launch & Scale Speedometer, the confirmed COVID-19 vaccine doses purchased by high-income countries is 4,601,070,000 (as of March 8, 2021), while for low-income countries doses are around 670,000,000 (https://launchandscalefaster.org/COVID-19#Timeline%20of%20COVID%20Vaccine%20Procurement%20Deals). In May 2020, WHO and other groups (Gavi, the Vaccine Alliance, and the Coalition for Epidemic Preparedness Innovations (CEPI)) created the COVID-19 Vaccines Global Access (COVAX) Facility to address expected vaccine inequities. The goal of this group is to ensure 92 poor countries can access COVID-19 vaccine as early as possible. A number of high-income countries have already pledged donations to support the program. The COVAX platform will additionally support research, development, and manufacture of a wide range of COVID-19 vaccine candidates. It also has a goal of fairly distributing two billion COVID-19 vaccine doses by the end of 2021. In addition to COVAX effort, WHO also has initiatives to make COVID-19 tests and vaccines more affordable to poorer countries, such as Act-Accelerator (https://www.who.int/initiatives/act-accelerator/covax). (3) **Universal vaccine development** will be required. As we already know, SARS-CoV-2 has evolved to evade current vaccine immunity. Some vaccines already exhibit reduced effectiveness against new variants, such as the B.1.351 or the Delta variant. Different vaccines need to be developed to effectively fight mutated novel SARS-CoV-2 variants. It is important to develop universal Coronavirus vaccines capable of simultaneously neutralizing several coronaviruses, inculding SARS-CoV-2 and its variants. As a matter of fact, scinetists have recently developed a vaccine which induced cross-neutralizing antibody (cross-nAb) responses against batCoVs, SARS-CoV-1, SARS-CoV-2, and SARS-CoV-2 variants B.1.1.7, P.1, and B.1.351 in macaque (Saunders et al. 2021).

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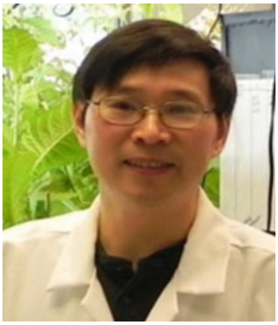

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# CRISPR-Cas Technology: A Genome-Editing Powerhouse for Molecular Plant Breeding

# 32

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## Abstract

Global human population is growing rapidly. The latest world population projections indicate human population will reach ten billion in the year 2055 (The World Bank). Simultaneously limited cultivable land, climate change, and plant diseases greatly impede the crop yield improvement necessary to feed the growing population. Breeders and farmers face a grand challenge in sustaining production to accommodate the population numbers in a race against time. Precision and rapid breeding are effective ways to tackle this great challenge (Hickey et al., Nat Biotechnol 37:744–754, 2019). Conventional genetic-engineering (GE) transgenic technology is an important approach in modern plant breeding, and several transgenic plant varieties with improved yields, disease-resistance,

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and drought-tolerance have been developed. However, the process of producing them is extremely laborious and time-consuming. These GE products also face a lengthy government regulation process before their release and commercialization. They also have a history of being poorly received by consumers. Novel genome-editing technology, especially the CRISPR-Cas-based system, has revolutionized bioscience fields from medicine to agriculture. CRISPR-Cas system displays several advantages over earlier gene-targeting technologies, such as TALEN and ZFN. Its simple design provides precise, targeted editing of living cell genomes with unparalleled editing efficiency and lower costs than other technologies. With these considerations, this review describes the implications of genome-editing tools in plant breeding and crop improvement.

**Keywords**

CRISPR-Cas · Designer nucleases · Genome editing · Knock-in · Knock-out · Precision targeting · Targeted mutagenesis

## 32.1 Introduction

Targeted genome editing provides a prominent degree of control and accuracy when changing target DNA sequences (Nasti and Voytas 2021). For many years, initiation of a gene disruption capable of modifying the function of a coded protein sequence at a preferred site remained a formidable task in plant genome editing (Chen et al. 2017a, b). This changed with the discovery of a few sets of genome-editing tools, zinc finger nucleases (ZFN) and transcription activator-like effector nucleases (TALEN) (Gaj et al. 2013; Kamburova et al. 2017). For these systems, targeted genome editing relies on the use of site-specific nucleases to create precise modification at specific locations that are subsequently repaired by the cellular repair mechanisms in the genome. Early versions of the genome-editing tools, ZFN and TALEN, required encoding of proteins through large DNA segments for each new target sequence and development of large vector sets for each target sites. These motivated researchers to find novel genome-editing tools (Gupta and Musunuru 2014; Kamburova et al. 2017). In 2013, the introduction of a simple yet powerful tool, *clustered regularly interspaced short palindromic repeats* with its associated *Cas9* protein (CRISPR-Cas9) allowed apparent genome-editing in prokaryotes and eukaryotes (Jinek et al. 2012; Barakate and Stephens 2016). CRISPR-Cas9 system has superseded other genome-editing tools (i.e., ZFN and TALEN) with its ease of design, precision, efficiency, versatility, and low cost. CRISPR-Cas9 technology utilizes a combination of Cas9 proteins and short RNAs (sgRNA) to aim for specific DNA cleavage sites (Gupta and Musunuru 2014). The cleavage induces site-specific double-stranded breaks (DSBs). The DSB activates cellular repair systems that repair the break in the DNA strands. Scientists began by taking advantage of the cell's error-prone *non-homologous end joining* (NHEJ) repair system for targeted gene mutagenesis. The *homologous-directed recombination* (HDR) repair system was used for gene insertion or replacement (Fauser et al. 2014). Over time, other CRISPR-Cas systems were discovered and applied to genome manipulation. Since the first mention of the bacterial CRISPR-Cas9 system's potential for precise eukaryotic genome-editing in 2012 (Jinek et al. 2012), a flurry of studies demonstrated site-specific DNA editing in eukaryotic cells through the heterologous expression of Cas9 with a guide RNA (Cong et al. 2013). Since that time, the technology has gone viral and revolutionized every bioscience field from modern medicine to agriculture. According to a report by B2B research company *MarketsandMarkets* (www.marketsandmarkets.com), the global plant breeding and CRISPR plants market is projected to for a significant increase from 7.57 billion USD in 2018 to 14.55 billion USD by 2023. CRISPR technology is opening doors for an increasing number of applications each day, so more and more plant-based researches adopt the technique in their studies. To save resources and avoid redundant work, a database has been set up to host all the information of gene-edited plant mutants. It is called Plant Gene Editing Database (PGED; http://plantcrispr.org/cgi-bin/crispr/index.cgi) (Zheng et al. 2019) (Table 32.1).

CRISPR-Cas technology is often used to knock out a specific allele of a gene for gene function and trait study, through NHEJ. CRISPR-Cas9 system can also be used to target allele insertion through HDR pathway (Schiml et al. 2014; Nasti and Voytas 2021). It virtually works on any gene of interest as long as the genome sequence is available. Although a genetic modification in plants through a CRISPR-Cas system could feasibly be followed by HDR pathway repair, it is ineffective compared to gene knock-out through NHEJ (Svitashev et al. 2015; Zong et al. 2017; Rozov et al. 2019). CRISPR-Cas technology is also able to rapidly create artificial genetic variation in breeding stocks for plant breeding. Crop improvement breeding is greatly enhanced when genetic mutations or variations are introduced into new crop varieties with certainty.

In this review, we summarize CRISPR-Cas-based technologies *in planta* genome editing, some of the remarkable advancements in CRISPR-based plant basic researches, and various applications for plant breeding. The plant applications discussed include the use of CRISPR-Cas technology in (1) creating mutant lines for gene function studies; (2) deleting chromosomes, regulatory elements, transposon elements, and selectable maker genes (SMG) from transgenic plants; (3) gene stacking at the same locus *in planta*; (4) metabolic pathway engineering; (5) developing quality crops with increased yield; (6) food safety (ex. heavy-metal-free or allergen-free foods); (7) breeding double-haploid (DH) lines, apomixis crops, parthenocarpic crops, or gynoecious crops; (8) generating male-sterility lines (ex. CMS) for hybrid seed production; (9) producing 'clonal' hybrid seeds from hybrid plants; (10) creating 'genetic variation' from elite gene pools or exotic germplasms as breeding stocks; (11) generating new crop cultivars adapted to new regions; (12) developing new crop varieties adapted to new regions (ex. high latitude region); (13) de novo domestication (or re-domestication) of wild species related to currently cultivated/elite crops; (14) eliminating crop pests using *gene drive*, and increasing favorable alleles in a population with *allelic drive*. This review also discusses the advantages and limitations of CRISPR-Cas tools for generating stress-tolerant crops

**Table 32.1** Applications of CRISPR-Cas systems in plant genome editing

| Plant | RNA promoter | Transformation method | Target gene | PAM sequence | Mutation efficiency (%) | References |
|---|---|---|---|---|---|---|
| *Bambusa oldhamii* | OsU3, OsU6 | *Agrobacterium* | *PDS* | AGG | 6.6 | Lin et al. (2017) |
| *Setaria italica* | | | | | 10.2 | |
| *Oryza sativa* | | | | | 7.3 | |
| *Zea mays* | | | | | 1.1 | |
| *Arabidopsis thaliana* | | | | | 6.5 | |
| *Brassica oleracea* | | | *BolGA4.a* | | 75.2 | |
| *B. napus* | | | *BolGA4.a* | | 56.8 | |
| *Nicotiana tabacum* | | | *PDS* | | 15 | |
| *Solanum lycopersicum* | | | *PDS* | | 3.7 | |
| *A. thaliana* | U6-26 | Agro transformation | *ISU1*, *CNI1* | TGG | 4–69 | Durr et al. (2018) |
| *Medicago sativa* | AtU6 | Agro transformation | *SPL9* | NGG | 2.2 | Gao et al. (2018) |
| *M. truncatula* | OsU6 | | | | 10.4 | |
| *Theobroma cacao* | T7 | *Agrobacterium* | *TcNPR3* | NGG | 27 | Fister et al. (2018) |
| *Triticum aestivum* | TaU6 | Neon electroporation | *TaLox2*, *TaUbiL1* | TGG | 92 | Bhowmik et al. (2018) |
| *N. tabacum* | U6 | Agro transformation | *pds* | TGG | 33.3–67.1 | Chen et al. (2018a, b) |
| *O. sativa* | OsU3 | Agro transformation of immature embryo | *EPFL9* | NGG | 69 | Yin et al. (2017) |
| | ZmUbi, OsU6 | Agro transformation of callus | *OsPDS*, *OsDEP1* and *OsROC5* | NGG | 25 | Tang et al. (2017a, b) |

| | | | | | | |
|---|---|---|---|---|---|---|
| *S. lycopersicum* | U6-26 | Agro transformation of leaf disk | *SIIAA9* | AGG | 100 | Ueta et al. (2017) |
| | CaMV 35S, AtU6 | *Agrobacterium* | *ALC* | CGG | 7.69–72.73 | Yu et al. (2017) |
| *O. sativa* and *S. lycopersicum* | U6 | *Agrobacterium* | *ALS* | AGG | 4.3–18.3 | Shimatani et al. (2017) |
| | AtU6 | | *DELLA*, *ETR1* | TGG | 26.2–53.8 | |
| *M. truncatula* | pMtU6 | Agro transformation | *PDS3*, *PDS4*, *PDS10*, *PDS18*, *PDS21* | AGG | 10.35 | Meng et al. (2017) |
| *Gossypium hirsutum* | AtU6 | Agro transformation | *GhCLA1*, *GhVP* | AGG | 47.6–81.8 | Chen et al. (2017a, b) |
| | | | | TGG | | |
| *O. sativa* (Indica rice line IR58025B) | U3/U6 | Agro transformation of Callus | *DEP1-S1* | TGG | 96 | Wang et al. (2017d) |
| | | | *S2*, *S3*, *S4* | CGG | 98 | |
| | | | | TGG | 98 | |
| | | | | GGG | 97 | |
| *B. napus* | AtU6-26, U6-29 | Agro transformation | *RGA*, *DA1*, *DA2*, *FUL* | TGG | ~65.3 | Yang et al. (2017) |
| *B. carinata* | CaMV 35S, U4-2, U6-26 | *Agrobacterium rhizogenes* | *BcFLA1* | CTG | 20 | Kirchner et al. (2017) |
| *Pharbitis nil* | AtU6 | Agro transformation | *InDFR-B* | TGG | 84 | Watanabe et al. (2017) |
| *O. sativa* | OsU6 | Agro transformation | *OsALS* | TGG | >1 | Endo et al. (2016a, b) |
| | OsSPL14 and OsmiR156 | | *Gn1a* | NGG | 42.5 | Li et al. (2016) |
| | | | *GS3* | | 57.5 | |
| | | | *IPA1* | | 27.5 | |
| *S. tuberosum* | AtU6 | PEG-protoplast transformation | *IAA2*, *ALS* | TGG | 83.60 | Andersson et al. (2016) |

(continued)

**Table 32.1** (continued)

| Plant | RNA promoter | Transformation method | Target gene | PAM sequence | Mutation efficiency (%) | References |
|---|---|---|---|---|---|---|
| *A. thaliana* | AtU6 | Agro transformation | *OST2*, *ABI4*, *GL1* | GGT | 32.84–97 | Osakabe et al. (2016) |
| | | | | GGG | | |
| | | | | CGG | | |
| | | | | TGG | | |
| | AtU6-26, AtU3b, At7SL-2 | | *6-PYR/PYL* (*pyl112458*) | NGG | 13–93 | Zhang et al. (2016b) |
| | AtU6-26 | Agro transformation | *API*, *TT4* | CGG | 3–56 | Mao et al. (2016) |
| *S. lycopersicum* | AtUBQ, AtU6-26 | Agro transformation of cotyledon | *SlPDIS* | NGG | 72–100 | Changtian et al. (2016) |
| *Glycine max* | GmU6-16g-1, AtU6-26 | Agro transformation of cotyledonary nods | *GmPDS* | NGG | 43.4–48.1 | Du et al. (2016) |
| *Vitis vinifera* | AtU6 | Agro transformation | *IdnDH* | NGG | 100 | Ren et al. (2016) |
| *Z. mays* | ZmU3 | Agro transformation | *Zmzb7* | NGG | 19–31 | Feng et al. (2016) |
| | ZmU6 | | *PSY1* | NGG | 0.8–78.83 | Zhu et al. (2016) |
| | ZmU6 | | *ZmMADS*, *ZmMYBR*, *ZmRPL*, *ZmPPR* | GGG, CGG, AGG, TGG, CCT, CCG | 57.1–71.4 | Qi et al. (2016) |
| *S. lycopersicum* | AtU6-26 | Agro transformation | *SlPDS*, *SlPIF4* | NGG | 76.7–100 | Pan et al. (2016a, b) |

| | | | | | | |
|---|---|---|---|---|---|---|
| *Petunia hybrid* | | Leaf disc agro transformation | *PDS* | AGG | 55.6–87.5 | Zhang et al. (2016a) |
| *N. benthamiana* | U6-26 | Agro infiltration | *XT* | CGG | 11 | Vazquez et al. (2016) |
| *N. tabacum* | U6 | Agro transformation | *mCherry* | TGG | – | Mercx et al. (2016) |
| *Cucumis sativus* | AtU6 | Agro transformation | *elF4e* | CGG | – | Chandrasekaran et al. (2016) |
| *S. lycopersicum* | CaMVE35S, AtU6 | *Agrobacterium* | *ANT1* | – | 29 | Cermak et al. (2015) |
| *Z. mays* | ZmUBI, ZmMDH | PEG-protoplast | *Ms26*, *Ms45*, *ALS1*, *ALS2*, *LIG1* | NGG | 1–3.9 | Svitashev et al. (2015) |
| *O. sativa* and *A. thaliana* | OsU3 OsU | *Agrobacterium* | Gramineae genes | NGG | 81.4–90 | Ma et al. (2015) |
| *A. thaliana* | ICU2, U6 | Agro transformation by floral dip | *FT* | CCG | 90 | Hyun et al. (2015) |
| | U6-26 | Agro transformation | *AtCRU3* | NGG | 2.5–92 | Johnson et al. (2015) |
| | AtU6-26 | | *ADH1* | NNAGAA | 6.1–98.5 | Steinert et al. (2015) |
| | AtU6-26 | | *BRi1* | TGG | 4.3–90.5 | Yan et al. (2015) |
| *Populus tomentosa* | AtU3b, AtU3d, AtU6-1, AtU6-29 | Leaf disc agro transformation | *PtoPDS* | – | 51.7 | Fan et al. (2015) |
| *Populus tomentosa* | CaMVE35S, MU6.6 | Leaf disc agro transformation | *4Cl1*, *4CL2* | NGG | 100 | Zhou et al. (2015) |
| *S. tuberosum* | CaMVE35S, StU6P | Agro transformation | *StIAA2* | NGG | – | Wang et al. (2015a, b) |

(continued)

**Table 32.1** (continued)

| Plant | RNA promoter | Transformation method | Target gene | PAM sequence | Mutation efficiency (%) | References |
|---|---|---|---|---|---|---|
| *B. oleracea* | U6-26 | Agro transformation | *BolC.Ga4.a* | CGG | 10 | Lawrenson et al. (2015) |
| *G. max* | AtU6 | Electroporation | *Bar*, *GmFE11*, *GmFE12* | NGG | 10–93.3 | Cai et al. (2015) |
| | AtU6, GmU6 | Agro transformation | *GmPDS11*, *GmPDS18* | CCG | 1.7–48.1 | Jacobs et al. (2015) |
| | AtU6-26, GmU6-10 | | *Glyma* | NGG | 3.2–20.2 | Sun et al. (2015) |
| | MtU6.6 | Particle bombardment | *ALS1* | CCG | 59 | Li et al. (2015) |
| | | Agro transformation | *GFP*, *DDM1* | NGG | >70 | |
| *G. max* and B *M. truncatula* | CaMVE35S, GmUbi, U10, U6 | Agro transformation | *GS1*, *CH120* | CGG | – | Michno et al. (2015) |
| | | | *GUS* | TGG | | |
| *N. benthamiana*, *A. thaliana* and *O. sativa* | AtU3, AtU6, OsU3, OsU6 | Agro transformation | *NbFLS2*, *NbBAK1*, *OsYSA*, *OsROC5*, *miR319*, *AtPAP1* | CCT | 95 | Lowder et al. (2015) |
| *N. benthamiana* | CaMVE35S, AtU6-26 | Agro transformation | *NbIspH* | AGG | – | Yin et al. (2015) |
| *N. tabacum* | CaMVE35S, AtU6-26 | Agro transformation | *NtPDS*, *NtPDR6* | NGG | 81.8–87.5 | Gao et al. (2015) |
| *O. sativa* | OsU3 | Agro transformation | *CDKA1*, *CDKA2*, *CDKB1* | NGG | 76.9 | Endo et al. (2015) |
| | | | *OsAOX1a*, *OsAOX1b*, *OsAOX1c* | NGG | 41 | Xu et al. (2015a, b) |
| | | | *YSA*, *CDKB2* | AGG | 7.6–68.7 | |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | | | | Mikami et al. (2015) |
| *S. lycopersicum* | AtU6 | Agro transformation | *RIN* | AGG | – | Ito et al. (2015) |
| *S. tuberosum* | AtU6 | Agro transformation | *StALS1* | TGG | 3–60 | Butler et al. (2015) |
| *A. thaliana* | PcUbi4-2, AtU6-26 | Agro transformation | *ADH1*, *TT4*, *RTEL1* | CCT | 2.5–70 | Fauser et al. (2014) |
| | | | *ADH 1* | CCT | 42.8 | Schiml et al. (2014) |
| | AtU6 | | *sgR-MRS*, *CHLI1* and *CHLI2* | AGG | 1.4 | Mao et al. (2013) |
| *T. aestivum* | TaU6 | PEG-protoplast transformation | *MLO-A1* | CCG | 36 | Wang et al. (2014) |
| *Z. mays* | ZmU3 | PEG-protoplast transformation | *IPK* | NGG | 16.4–19.1 | Liang et al. (2014) |
| *Citrus sinensis* | CaMV35S | Leaf Agro infection | *CsPDS* | AGG | 3.2–3.9 | Jia and Wang (2014) |
| *O. sativa* | OsU6 | Agro infection of callus | *SWEET1a-1b-11-13* | TGG | 2–100 | Zhou et al. (2014) |
| | | PEG-protoplast transformation | *KO1*, *KOL5*, *CPS4*, *CYP99A2*, *CYP76M5*, *CYP76M6* | | | |
| | ZmU3 | Agro infection of callus | *PDS*, *PMS3*, *EPSPS*, *DERF1*, *MSH1*, *MYB5*, *MYB1*, *ROC5*, *SPP*, *YSA* | – | 21.1–66.7 | Zhang et al. (2014) |
| | AtU6 | | *BAL* | CGG | 2 | Xu et al. (2014) |
| *S. lycopersicum* | AtU6 | *Agrobacterium rhizogenes* | *GFP*, *SHR* | GGG | – | Ron et al. (2014) |

(continued)

**Table 32.1** (continued)

| Plant | RNA promoter | Transformation method | Target gene | PAM sequence | Mutation efficiency (%) | References |
|---|---|---|---|---|---|---|
| | | Agro transformation of cotyledon | *SlAGO7*, *Solyc08gN 041770*, *Solyc07g 021170*, *Solyc12g 044760* | | 75–100 | Brooks et al. (2014) |
| *Marchantia polymorpha* | MpU6 | Agro transformation of sporelings | *ARF1* | TGG | 11 | Sugano et al. (2014) |
| *T. aestivum* | TaU6 | Particle bombardment | *TaLOX2* | AGG | 45 | Shan et al. (2014) |
| *A. thaliana* and *N. benthamiana* | AtU6 | PEG-protoplast transformation | *AtPDS3*, *FLS2* | AGG, TGG | 1.1–5.6 | Li et al. (2013) |
| | | | *RACK1b*, *RACK1c* | | 2.5–2.7 | |
| | | | *NbPDS3-1* | AGG | 37.7–38.5 | |
| | | Leaf agro infection | *NbPDS3-2* | AGG | 4.8 | |
| *A. thaliana*, *N. benthamiana*, *O. sativa* and *Sorghum bicolor* | AtU6-26, CaMV35S, OsU6 | Agrobacterium infiltration | *OsSWEET14* and *OsSWEET11* | AGG | – | Jiang et al. (2013) |
| *N. benthamiana* | AtU6 | Leaf agro infection | *AtPDS3* | AGG | 1.8–2.4 | Nekrasov et al. (2013) |
| *O. sativa* | OsU3 | Agro infection and Particle bombardment | *CAO1*, *LAZY1* | AGG, GGT, GGG | 91 | Miao et al. (2013) |
| | OsU6, CaMV35S | PEG—protoplast transformation | *MPK5* | | 3–8 | Xie and Yang (2013) |
| *T. aestivum* | CaMV35S | Agro infection in immature embryo | *PDS*, *INOX* | CGG | 18–22 | Upadhyay et al. (2013) |

| | | | | | | |
|---|---|---|---|---|---|---|
| *O. sativa* and *T. aestivum* | OsU3 | PEG—protoplast transformation | *OsBADH2, OsMPK2* and *Os02g23823* | AGG | 14.5–38 | Shan et al. (2013) |
| | TaU6 | | *TaMLO* | | 28.5 | |
| *A. thaliana* and *O. sativa* | CaMV35S, AtU6-26, OsU6-2 | *Agrobacterium* | *BRI1*, *GAI*, *JAZ1* | CGG, TGG | 26–84 | Feng et al. (2013) |
| | | | *ROC5*, *SPP*, *YSA* | | | |
| | | | *OsMYB1, YFFP* | GGG, GGA | 50 | Mao et al. (2013) |
| | | | *TT4*, *CHLI1*, *CHL12* | NGG | 38–89 | |

from recent studies. In addition, various advances in precision breeding and newly popular genome-editing systems are also mentioned.

Factors affecting CRISPR editing efficacy are also discussed. These factors include (1) location of the gene to be edited in the genome; (2) target site selection of the gene; (3) sgRNA design; (4) Cas9 gene expression strength (promoters used to drive Cas nucleases); (5) the use of Cas variants (ex. Cas-NLS, codon-optimized Cas); (6) methods used for delivery; (7) various formats of CRISPR components (plasmid, mRNA, or protein) used for delivery; and (8) the sensitivity of Cas protein to temperature, etc. We also discuss how to increase the efficiency of HDR-mediated, site-directed gene insertion. In addition, applications of CRISPR-Cas technology beyond genome editing are highlighted. This includes CRISPR-based gene activation and repression systems (i.e., CRISPRa and CRISPRi), and a live cell imaging tool. Governmental regulation policies can directly affect the process of any biotech company investment in CRISPR crop production. Some examples of regulation policies for CRISPR-edited products from a few countries are also discussed.

## 32.2 Principle Mechanism of CRISPR-Cas System, Cas9 Variants and Cas9 Orthologs

The *clustered regularly interspaced short palindromic repeats* (CRISPRs) and CRISPR-associated (Cas) protein are an important type of programmable endonuclease characterized as an adaptive immune mechanism against invasive plasmid transfer and phage infection in both archaea and prokaryotes (Wiedenheft et al. 2012). The first CRISPR locus was discovered by Ishino et al. (1987) in *Escherichia coli* genome while sequencing the *iap* gene. CRISPR systems have since been found in 40% bacteria and 90% archaeal species. In 2007, Barrangou and colleagues discovered CRISPR spacers conferring potent resistance to bacterial viruses (bacteriophage) bearing matching DNA sequences when working with *Streptococcus thermophilus* (Barrangou et al. 2007). In 2013, after 6 years, the CRISPR system was developed for use as a genome-editing tool for eukaryotic cells.

### 32.2.1 Classification of CRISPR-Cas System

CRISPR systems are classified into two classes. Class 1 systems rely on the multi-subunit Cascade complex to identify and destroy nuclear acid targets. While, Class 2 systems function with single protein effectors (Makarova et al. 2015). For example, Class 1 CRISPR systems need different nucleases to complete different processes (pre-crRNA processing, spacer sequence loading, and targeted cleavage processing). In contrast, Class 2 systems perform all of the listed functions with a single protein. Each Class contains different Types, based on the *cas* operon composition. The Class 1 systems contain Type-I, Type-III, and Type-IV. The Class 2 systems contain Type-II and Type-V, which cleave double-stranded DNA *via* Cas9 or Cpf1 (now also known as Cas12a), respectively. While Type-VI, cleaves single-stranded RNA *via* Cas13 (previously known as C2c2).

#### 32.2.1.1 Mechanism of Class 2 Type-II CRISPR-Cas System

Due to its simplicity and wide use in research, this review will focus mainly on CRISPR-Cas9 systems, which belong to Class 2 Type-II CRISPR-Cas system. Class 2 Type-II CRISPR-Cas systems recognize and target pathogen genomes in three steps: adaptation (spacer acquisition), expression (crRNA processing and target binding), and interference (target cleavage) (for review, see Barrangou and Marraffini 2014; Makarova et al. 2015).

##### Acquisition (or Adaptation)

The adaptation process involves selection/recognition and integration of invading nucleic acid protospacer into the CRISPR array (locus). There are two types of spacer acquisitions, naive and primed. Acquisitions are naive when there is no record of invasion in the CRISPR locus. Primed acquisitions indicate the invasion was previously recorded in the CRISPR locus (Fineran and Charpentier 2012). The adaptation phase stores the pre-recorded genetic memory and utilizes this information in the interference phase (Rath et al. 2015). The adaptation phase is a two-step process, involving selection of the protospacer and the integration of the spacer into the CRISPR array. Spacer selection depends on certain sequence elements within the target. Target sequences are predicted with the presence of a short motif next to the target sequence, named *protospacer adjacent motif* (PAM) (Mojica et al. 2009). Different types of CRISPR-Cas systems follow different PAM-recognition mechanisms during spacer acquisition. In the Type-II-A system, Cas9 is responsible for identifying PAM. This is vital, as mutations disabling Cas9's PAM recognition result in acquisition from protospacers without PAM.

##### Expression and Interference

Transcription of CRISPR repeats spacer elements initiates in the leader region. This leader region contains promoter elements, the regulatory protein binding site and an element essential for integration of the spacer. All are required for the production of precursor CRISPR RNA (pre-crRNA). Cas gene transcription is directed by the second promoter, which is located upstream from the Cascade complex (Pul et al. 2010). Once Cas9 binds to crRNA, the complex searches for complementary target sites in penetrating the DNA. Target search and recognition requires complementary-base-paring activity between short crRNA (20-nt spacer sequence) and protospacer sequence of the invading DNA (Jinek et al. 2012). CRISPR-Cas9 complex search for appropriate target PAM sequence results in an RNA-DNA heteroduplex (Nishimasu et al. 2015). The Cas9 protein is activated, and the target dsDNA cleaved by the Cas9 domains. The double-stranded target DNA cleavage occurs upstream of the protospacer region by three to four base pairs.

CRISPR systems Type-I and Type-III both follow a similar mechanism for processing. In the Type-II system the pre-crRNA repeat sequence forms a complementary pair to trans-activating crRNA (tracrRNA) and degrades invading DNA using double-standard (ds) RNA specific ribonuclease RNase III in the presence of the Cas9 protein (Deltcheva et al. 2011; Jinek et al. 2012). Another distinct feature of the Type-II system is crRNA trimming by an unknown nuclease, while the crRNA-tracrRNA remains bound to Cas9 (Jinek et al. 2012). Type-II CRISPR systems require PAM and conserved complementary pairing between target protospacer and the crRNA-Cas9 complex and so called 'seed region' (Semenova et al. 2011). After the successful degradation of foreign DNA, the system stores the genetic memory of invaders for the purpose of recognizing and disabling future infections.

### 32.2.2 Cas9 Variants

Researchers create various Cas9 variants for different application purposes. Among them: (1) creation of novel PAM specificities; (2) increase in Cas9 enzyme fidelity to reduce off-target editing; and (3) fusing of DNA-binding domain or other genetic elements to Cas9 (Cebrian-Serrano and Davies 2017; Ali et al. 2020). Today, there are many Cas9 variants available for genome-editing in animal and plant species. Below are some published Cas9 variants and their new functions:

PAM sequence is required for Cas9 target-site recognition to induce DSB at appropriate site. However, a PAM is not always available near the locus selected for editing. The widely used *Streptococcus pyogenes* Cas9 (SpCas9) requires motif NGG as a PAM for genome editing. Although SpCas9 is a powerful genome-editing tool, it is limited in targeting genomic locus lacking of NGG PAM. Engineering a Cas9 variant capable of broad PAM compatibility will be very useful. Cas9 variants have been engineered with novel PAM specificities earlier, such as NGCG and NGAG (Kleinstiver et al. 2015). Two recently engineered SpCas9 variants, **xCas9** (Hu et al. 2018a) and **Cas9-NG** (Nishimasu et al. 2018), show promising potential in both broadening the targeting range and improving targeting specificity. xCas9 is generated through direct evolution to recognize a broad range of PAM sequences such as NG, GAT, and GAA with high DNA specificity. Another variant, SpCas9-NG, can recognize relaxed NG PAMs. In plants, xCas9 was first tested for genome editing in rice genome (Wang et al. 2019d). The studies demonstrate it works in rice with reduced efficiency (Wang et al. 2019d). The authors found that xCas9 displays ability to edit at non-canonical GAA PAM sites. However, the efficiency (4.17–10.42%) at canonical GGG PAM site was significantly reduced compared to *Sp*Cas9 efficiency (75–77%) (Wang et al. 2019d). Later, Hua et al. (2019) found that xCas9 can efficiently induce mutations at target sites with NG and GAT PAM sequences in rice. They also found SpCas9-NG exhibited a robust editing activity at sites with various NG PAMs. In another study, the efficiencies of xCas9 and Cas9-NG for PAM usage in rice genome were evaluated (Zhong et al. 2019). The authors found that xCas9 is a high-fidelity nuclease for targeting canonical NGG PAMs and Cas9-NG as preferred variants for targeting relaxed PAMs. Ge et al. (2019) also report that engineered xCas9 and *Sp*Cas9-NG variants could, to a certain degree, expand the use of CRISPR *in Arabidopsis*, although with a reduced efficiency. These studies suggest xCas9 and Cas9-NG have the potential to expand the scope of genome editing with some non-canonical PAMs *in planta*.

With wild-type *Sp*Cas9 structure optimized, high-fidelity Cas9 variants have been identified or created to reduce off-target editing. They are ***Sp*Cas9-HF1**, **e*Sp*Cas9 (1.1)**, **HypaCas9**, **evoCas9**, **Sniper-Cas9**, **xCas9(3.7)**, and ***Sp*Cas9-NG** (He et al. 2019). Raitskin et al. (2019) recently compared the engineered variants of Cas nucleases e*Sp*Cas9 (1.0), e*Sp*Cas9 (1.1), e*Sa*Cas9, and xCas9 (3.7) in *N. benthamiana* protoplasts. Improved specificity was observed at some targets with these high-fidelity Cas9 variants. A recent study on two high-fidelity Cas9 variants, *Sp*Cas9-HF1 and HypaCas9, found that these variants improve discrimination of DNA cleavage by slowing the DNA cleavage rate by 100-fold (Liu et al. 2020a).

**Cas9 nickase (or Cas9n)** was created to improve the specificity of Cas9-mediated genome editing and lower off-target editing (Cong et al. 2013; Ran et al. 2013). Cas9 nickase was developed through a point mutation of a key catalytic residue in the domains of native Cas9 protein (either D10A for HNH or H840A for RuvC). Cas9 nickases are capable of introducing a *single-strand* cut with the same specificity as a regular CRISPR-Cas9 nuclease (Gasiunas et al. 2012). Studies indicate only '*paired*' Cas9 nickase activity leads to site-specific DSBs and NHEJ, as single-stranded nicks generated from a single Cas9 nickase are predominately repaired by other repairing systems (Mali et al. 2013). Paired Cas9 nickase induces breaks containing two long overhangs (sticky ends) on each of the cleaved ends instead of blunt ends, which significantly reduces off-target activities and provides greater control over precise gene editing (Ran et al. 2013). In plants, paired Cas9 nickase has been successfully applied to edit *Arabidopsis* endogenous genes. Results show Cas9 nickase cannot efficiently induce NHEJ-based mutagenesis, but exclusively HR (Fauser et al. 2014). Recently, *Sp*Cas9 nickase (D10A), fused to an evolved tRNA adenosine deaminase, was used as adenine base editors (ABE) in plants. This method has utilized A•T to G•C conversions to generate targeted point mutation in rice and wheat from Dr. Caixia Gao's lab. Base editing will be discussed in a later section.

The dead Cas9 (**dCas9**) is inactive catalytically. Nuclease-deficient dCas9 was derived from mutating the nuclease domains of Cas9 from *S. pyogenes* (through a H840A mutation in the HNH domain and a D10A mutation in the RuvC domain) (Qi et al. 2013). The applications of CRISPR/dCas9 also include gene activation/repression, base editing, chromatin imaging, epigenome editing, and chromatin topology. CRISPR/dCas9 platform has also been applied to plant fundamental studies and improvement in recent years (for review, Moradpour and Abdulah 2020). dCas9-derived tools have successfully executed epigenetic modification, such as histone acetylation and methylation or DNA methylation (Vora et al. 2016). CRISPR-dCas9-(Activator or repressor domain) fusion provides a new way of engineering *artificial transcription factors* (ATFs), which can be used for genes' transcription activation and repression studies (Lowder et al. 2018a, b). In plants, scientists have used dCas9 proteins fused to known transcriptional activator domains (VP64) or repressor domains (SRDX) to activate *AtPAP1* and repress *AtCSTF64* gene expressions in *Arabidopsis thaliana* (Lowder et al. 2017). In another report, dCas9-VP64 has been used for transcription activation of protein-coding genes and an LTR/COPIA transposable element (Papikian et al. 2019). Papikian et al. (2019) used dCas9-SunTag system (which contains the catalytic domain of the *Nicotiana tabacum* DRM methyltransferase) to efficiently target DNA methylation to specific loci, resulting in phenotypic changes in plant development. In the study, the system effectively induced an ectopic methylation on a flowering gene promoter, *FWA* promoter, in *Arabidopsis thaliana* (Papikian et al. 2019). Gentzel et al. (2020) demonstrated that CRISPR/dCas9 can be a useful tool for maize functional gene analysis of maize, using protoplasts.

RNA-guided FokI-nucleases (RFNs) are a Cas9 variant created for genome editing with high specificity, containing a Fok1 nuclease domain fused to deactivated Cas9 (dCas9), **FokI-dCas9** (or **fCas9**) (Guilinger et al. 2014; Aouida et al. 2015; Pan et al. 2016b; Saifaldeen et al. 2020). This approach requires simultaneous binding of two FokI-dCas9 monomers at adjacent target sites. These two monomers are guided by two separate and independent sgRNAs to respective target sites. An increase of 140 times has been reported with fCas9s specificity over wild-type Cas9 in human cells (Guilinger et al. 2014).

Another Cas9 variant is **Cas9-pDBD**. In Cas9-pDBD, Cas9 protein is fused to a *programmable DNA-binding domain* (pDBD) such as TALE domains or Zinc Finger DNA-binding domains. This design allows the complex to cleave at alternative PAM sequences to improve precision over the sequence-specific 3′ NGG PAM (Bolukbasi et al. 2015). Due to the off-target activity, the re-designing of Cas9-pDBDs with specificity reduces the interaction with non-specific DNA (Slaymaker et al. 2016).

Scientists have recently published a paper in *Cell* regarding a novel programmable/controlled Cas9 variant called **ProCas9** (Oakes et al. 2019). The authors circularly permuted native Cas9 into circular permutants (Cas9-CPs). These Cas9-CPs are not in active state. However, the presence of a specific protease from a pathogen, such as Zika, West Nile, or plant viruses, will unlock the Cas9-CPs and activate induction of DSB. ProCas9 is enzymatically activated by sequence-specific proteases. This new variant obtains its name from **pro**tease-activated “ProCas9.” Oakes et al. (2019) demonstrated that ProCas9 was activated by *Flavivirus* proteases to induce target editing.

### 32.2.3 Cas9 Orthologs

Cas9 protein exhibits orthogonal feature with conserved domains with less sequence similarity and length variability (~900 to 1600 amino acid residues), which activates when combined with crRNA and performs dsDNA cleavage (Fonfara et al. 2014). Cas9 protein orthologs have been identified within a wide variety of species. Currently, the most studied and extensively used Cas9 protein in genome editing is from *Streptococcus pyogenes* (***Sp*****Cas9**). *Sp*Cas9 contains 1368 amino acid residues. Cas9 orthologs from *Staphylococcus aureus* (***Sa*****Cas9**) and *Neisseria meningitides* (***Nm*****Cas9**) show similar efficiency compared to SpCas9 (Ran et al.

2015). Cas9 protein from *Streptococcus thermophiles* (***St*Cas9**) was first identified in bacterium. This variant (***St*Cas9**) shows better cleavage and homologous recombination compared to NmCas9, *Treponema denticola* (***Td*Cas9**) and SpCas9 (Esvelt et al. 2013). The Cas9 protein from *Francisella novicida* (***Fn*Cas9**) is significantly larger than all other Cas9 orthologs, and it uniquely recognizes dual-RNA and PAM sequences and alternative 5′-YG-3′ PAM for their function (Sampson and Weiss 2013; Hirano et al. 2016). A new Cas9 protein identified from *Campylobacter jejuni* (***Cj*Cas9**) (Kim et al. 2017a), is one of the smallest Cas9 orthologs with 984 amino acid residues. The *Cj*Cas9 shows same level of editing efficiency but better specificity, compared to SpCas9 and SaCas9 (Kim et al. 2017a). Both *St*Cas9 and *Sa*Cas9 have been used to induce error-prone NHEJ-mediated targeted mutagenesis in the model plant *Arabidopsis thaliana* (Steinert et al. 2015). Recently, another Cas9 variant was isolated from bacteria *Streptococcus canis* (***Sc*Cas9**). The sequence of *Sc*Cas9 nuclease bears striking similarity (89.2% sequence homology) to that of *Sp*Cas9, but differs in one important aspect. It requires a less stringent PAM sequence 5′-NNG-3′ (instead of 5′-NGG-3′ for *Sp*Cas9) for its activity, largely expanding the genomic target for CRISPR editing (Chatterjee et al. 2018). Recently, researchers analyzed the protospacer adjacent motif (PAM) and guide RNA (gRNA) requirements of 79 Cas9 proteins, and identified at least seven distinct gRNA classes and 50 different PAM sequence requirements (Gasiunas et al. 2020).

The Cas9 protein recently obtained from thermophilic bacterium *Geobacillus stearothermophilus* (***Geo*Cas9**) catalyzes RNA-guided DNA cleavage at high temperatures of up to 75 °C (Harrington et al. 2017; Doudna 2017). *Geo*Cas9 has been found to have an extended stability in human plasma and sustains cleavage activity at mesophilic temperatures, exhibiting potential for mammalian genome editing (Harrington et al. 2017). Recently, a ***thermo*Cas9** from the thermophilic bacterium *Geobacillus thermodenitrificans* T12 was identified and characterized (Mougiakos et al. 2017). Fundamental and translational research will be necessary to increase the efficiency of CRISPR-Cas systems, which emerge as dominant and cutting-edge tools for genome editing. Researchers recently identified a novel ***CaldoCas9*** which showed stable nuclease activity in vitro at temperatures up to 65 °C. The authors found that the preference for the protospacer adjacent motif (PAM) specificity of *CaldoCas9* is 5′-NNNNGNMA (Adalsteinsson et al. 2021).

### 32.2.4 Other Class 2 CRISPR-Cas Systems

Class 2 CRISPR systems designated Type-V are classified as V-A, V-B, V-C, or V-U. The distinctive feature of Type-II and Type-V CRISPR-Cas sequences is the presence of a RuvC-like nuclease domain in their multi-domain effector proteins. RuvC domain similarity is the only Type-V subtype similarity. V-A was identified from *Francisella* cf. *novicida* and named as **Cas12a**, which exhibits unique mechanisms for target recognition that result in increased specificity (Gao et al. 2016). The V-B (**Cas12b**) and the V-C (**Cas12c**) were identified from *Alicyclobacillus acidoterrestris* and *Oleiphilus* sp., respectively. Also, the various

V-U (Type-V-uncharacterized) were classified as V-U1 to V-U5 from *Anabaena variabilis*, *Cyanothece* sp., *Rothia dentocariosa*, *Bacillus thuringiensis*, and *Gordonia otitidis*, respectively. These V-U types are much smaller proteins and highly similar to TnpB proteins (Shmakov et al. 2017).

CRISPR system from *Prevotella* and *Francisella* 1 (Cas12a; also known as Cpf1) is a newly identified Class 2 Type-V CRISPR-Cas endonuclease (Zetsche et al. 2015). Cpf1-mediated cleavage is guided by a crRNA (42–44 nt) and utilizes a thymidine (T)-rich PAM sequence, TTTN, as opposed to the G-rich sequences for Cas9 (Gao et al. 2016; Zhang et al. 2018b; Zhong et al. 2018). Cpf1 is capable of producing more structured pre-crRNA molecules, which fully develop into crRNAs. This would enable its use for both pre- and mature crRNAs in genome-editing process (Fonfara et al. 2016; Moreno-Mateos et al. 2017; Fernandez et al. 2018). Interestingly, Cpf1 enzyme is a dual nuclease which cleaves target DNA as well as its own crRNA (Fonfara et al. 2016; Wang et al. 2017b; Zetsche et al. 2017). Cpf1 creates double-strand breaks in a staggered manner at the PAM-distal position. Cpf1 is guided by a single crRNA, no *trans*-acting crRNA is needed. The known Cpf1 family members which have been used for plant genome editing include ***Fn*Cpf1** derived from *Francisella novicida,* ***Lb*Cpf1** derived from *Lachnospiraceae* bacterium ND2006, and ***As*Cpf1** derived from *Acidaminococcus* spp. BV3L6. Two variants (FnCas12a-EP15 and FnCas12a-EP16) increased the targeting range of FnCpf1 approximately fourfold.

Recently, the Class 2 system diversity increased with Type-VI RNA-editing CRISPR-Cas13 system (Cox et al. 2017). Type-VI is classified into three subtypes: VI-A (**Cas13a; previously known as C2c2**), VI-B (**Cas13b1 and Cas13b2**), VI-C (**Cas13c**). These were identified from *Leptotrichia shahii* (Cas13a), *Prevotella buccae* (Cas13b) and *Bergeyella zoohelcum* (Cas13b), and *Fusobacterium perfoetens* (Cas13c), respectively. VI type variants contain an effector protein with two higher eukaryotes and prokaryotes nucleotide (HEPN)-like binding domains (Shmakov et al. 2017). The Cas13a has a similar mechanism to Cas12a, but its activation yields non-specific RNase activity. RNase destroys collateral RNA, which results in programmed cell death (East-Seletsky et al. 2016). Investigation is required to determine if the other types of VI effectors such as Cas13b and Cas13c share similar properties with Cas13a.

CRISPR-Cas13-based RNA editing can be applied to plant research as a diagnostic tool for identifying specific pathogens in plants. Also, variant dCas13 could be applied to block virus replication and improve plant stress tolerance (Ali et al. 2018). **Cas13** specifically targets and cleaves single-stranded RNA in eukaryotic cells, causing downregulation of a specific transcript. Abudayyeh et al. (2017) used a most effective Cas13a ortholog from *Leptotrichia wadei* (*Lwa*Cas13a) to cleave mammalian and plant endogenous gene transcripts. *Lwa*Cas13a was expressed in rice protoplasts and efficiently knocked down (efficiency greater than 50%; maximum efficiency can reach up to 78%) three intended endogenous gene transcript (Abudayyeh et al. 2017). In a pioneering work, Aman et al. (2018) used CRISPR-Cas13a for RNA virus interference study in plants. Codon-optimized *Leptotrichia shahii* Cas13a (*Lsh*Cas13a) was used in this study with a target of *Turnip mosaic* *virus* (TuMV), a member of *Potyviruses*. *Potyviruses* are plant-infecting viruses which cause serious damage for a wide range of crop plants. Results demonstrate tobacco (*Nicotiana benthamiana* L.) plants overexpressing components of the CRISPR-Cas13a RNA interference system showed interference activity against TuMV (Aman et al. 2018). The simultaneous application of multiple editing systems (ex. DNA- and RNA-editing systems) in the same cell offers promising new options for plant research (review see Wolter and Puchta 2018). A catalytically inactive Cas13 variant (namely, dCas13) can also be used as a tool to direct adenosine-to-inosine (A to I) conversion for RNA editing (Cox et al. 2017). RNA editing has multiple advantages over traditional DNA-editing. It avoids permanent changes in DNA structure within the genome and off-target editing. Recently, CRISPR-Cas13-based technology has been used for pathogens and viruses' gene detection (Abudayyeh et al. 2019; Aquino-Jarquin 2021).

### 32.2.5 CRISPR-Cas Systems with the Smallest Cas Enzymes

Two new CRISPR-Cas systems, CRISPR-CasX and CRISPR-CasY, were discovered from little-studied, uncultivated nano-archaea and expressed in *E. coli* (Burstein et al. 2017). They stand out as two of the most compact, naturally occurring CRISPR systems by far. These new proteins, **CasX** and **CasY**, are composed of about 980 and 1200 amino acids, respectively. This is promising, as delivery of small genes into cells is much easier than delivery of large genes (Cross 2017). In the CRISPR-CasX system, the CRISPR array encodes *cas1, cas2, cas4*, and *casx* genes (Yang and Patel 2017). CasY protein shows unique characteristics that are distinct from any other reported CRISPR-Cas proteins (Yang and Patel 2017). Recently, CasX was classified as Cas12e. The CasY effector was classified as Cas12d (Makarova et al. 2018). In vivo data show that CasX is active for *Escherichia coli* and human genome modification (Liu et al. 2019c). In 2020, a start-up company used CasX for the main Cas enzyme for gene targeting (https://cen.acs.org/pharmaceuticals/gene-therapy/CasX-startScribe-Therapeutics-raises-100/99/i12).

## 32.3 Application of Bacterial CRISPR-Cas System for Eukaryotic Genome Manipulation

CRISPR-Cas9 systems promote genome editing in eukaryotic cells by inducing a double-stranded breaks (DSBs) at a target genomic site. DSBs are repaired through two cellular pathways: non-homologous end joining (NHEJ) or homology directed repair (HDR). NHEJ mediates religation of broken DNA molecules with no repair template (Weterings and Chen 2008). NHEJ steps include (1) DNA end recognition (DSB) and stabilization of the NHEJ complex at DSB; (2) Bridging the DNA ends; (3) Processing of DNA end; (4) ligation of the broken ends and promotion of end stability; (5) DNA end processing; and (6) ligation of the broken ends and dissolution of the NHEJ complex (Davis and Chen 2013). NHEJ is error-prone and results in insertions and deletions (indels) within the repaired target sequences (Cong et al. 2013). The indels result in frame-shift mutations that silence gene expression. Therefore, this repair pathway is most effective in creating gene knockouts and loss-of function alleles. It seems that there are patterns for the sizes of indels, which are the outcomes of repair. Vu et al. (2017) found that 1-bp to <1000-bp deletion and/or very short insertions, deletions >1 kb (all due to NHEJ) and deletions combined with insertions between 5-bp and >100 bp were the most frequent repair outcomes at three target loci of their focus in *Arabidopsis*. They also observed higher frequency and size of insertions when highly similar *cis* sequences were available (Vu et al. 2017).

HDR is a faithful, cellular-repair pathway. HDR pathway is cell-cycle-dependent and acts mainly in *S*- or *G2*-phase. HDR pathway requires homologous DNA sequences; and in nature, sister chromatids can serve as template for repair (Zaboikin et al. 2017). HDR can be used to repair a damaged DNA in the presence of a 'repair template', which can be inserted into the target by homologous recombination (Mali et al. 2013). Because HDR is restricted to *S* and *G2* phases of the cell cycle, differences in cell cycle phases can have a significant potential impact on the outcomes of genome editing experiments. Gutschner et al. (2016) engineered a Cas9 variant, Cas9-hGem (1/110), with a cell-cycle-tailored expression, low levels in *G1* but high expression in *S/G2/M*, for HDR-mediated genome-editing in animal cells, and observed that Cas9-hGem (1/110) increased the rate of HDR by up to 87% compared to wild-type Cas9.

Scientists take advantage of these mechanisms (CRISPR to generate DSB at specific locus and cellular repair pathways-NHEJ and HDR) to generate various gene knockouts, gene knock-ins and allele-replaced plant lines for basic research and application (Steinert et al. 2016). For a review on CRISPR-mediated HDR gene-targeting in plants, see Hahn et al. (2018).

## 32.4 Favorite CRISPR Systems Used for Genome Editing in Plants

Due to its simplicity, Type-II CRISPR systems are the most studied and broadly used in eukaryotic genome manipulation. Type-II CRISPR-Cas9 from *Streptococcus pyogenes* (SpCas9) became the first system developed for this purpose (Zhang et al. 2017). To date, most genome-editing work done in plants with CRISPR system uses SpCas9.

The second most used system for plant genome-editing is CRISPR-Cpf1 (Zaidi et al. 2017). CRISPR-Cpf1 system has established potent genome-editing activity in a few plant species such as *A. thaliana* (Tang et al. 2017b; Malzahn et al. 2019; Raitskin et al. 2019; Bernabé-Orts et al. 2019), tobacco (*N. tabacum* L.) (Endo et al. 2016a, b; Kim et al. 2017b), tobacco (*N. benthamiana* L.) (Raitskin et al. 2019; Bernabé-Orts et al. 2019), rice (*O. sativa* L.) (Endo et al. 2016a, b; Begemann et al. 2017; Hu et al. 2017b; Tang et al. 2017b; Wang et al. 2017b; Xu et al. 2017; Zhong et al. 2018; Malzahn et al. 2019; Li et al. 2019b), maize (Malzahn et al. 2019), soybean (*G. max* L.) (Kim et al. 2017b), and tomato (*S. lycopersicum*) (Bernabé-Orts et al. 2019).

Worth noting: Recently, Raitskin et al. (2019) directly compared efficiency and specificity of multiple wild-type *Sp*Cas9, *Sa*Cas9, *Fn*Cpf1, and *Lb*Cpf1 in tobacco and *Arabidopsis* protoplasts, and found *Sa*Cas9 most efficient for inducing mutations. However, this experiment incubated protoplasts at 24 °C, and differing Cas protein complexes differ in temperature sensitivity. In a previous study, Cas9 editing efficiency improved at higher temperatures in both *Arabidopsis* and citrus (Ma et al. 2015). In a most recent paper, Malzahn et al. (2019) compared *As*Cpf1, *Fn*Cpf1, and *Lb*Cpf1 activities at different temperatures in rice, maize, and *Arabidopsis* and observed *As*Cpf1 to be the most sensitive to temperature. It requires a temperature over 28 °C for high activity and can, at this temperature, generate rice mutants with frequencies up to 93% among $T_0$ lines (Malzahn et al. 2019). For ortholog *Lb*Cpf1, editing activity at 22 °C was barely detectible in *Arabidopsis*, but activity improved in transgenic plants growing at 29 °C (Malzahn et al. 2019). Therefore, study results from Raitskin et al. (2019) could have differed if a favorite temperature was applied for best performance of Cas nuclease. Similar results were observed in another study, where no edited plants were found using *As*Cpf1 construct to edit (Bernabé-Orts et al. 2019). Again, tobacco plants used for CRISPR reagents' infiltration were grown at 24 °C (light)/20 °C (darkness), not an optimum temperature for *As*Cpf1 enzyme activity (Malzahn et al. 2019) (Fig. 32.1).

### 32.4.1 CRISPR Vectors and Promoters of Cas9 and sgRNA for Plant Cell Expression

CRISPR vectors can be built in the lab to suit the specific purpose of the research project (Wu et al. 2018; Tang et al. 2018; Wang et al. 2018a, b, c, d; Gasparis et al. 2018; Castel et al. 2019). For a cloning lab, this is an easy task. For other labs, there are alternatives to obtain CRISPR vectors. For example, CRISPR vectors can also be acquired from Addgene (https://www.addgene.org/), a non-profit plasmid depository organization, by paying a fee. A lot of original plasmids mentioned in published papers are deposited in Addgene. People can purchase and replace the original target sequence with the desired target sequence of the work. Other important components (such as promoter::*cas9* and sgRNA-scaffold) are already built in the vector. Purchase of the vector includes instructions concerning restriction enzyme(s) needed and appropriate reaction conditions (LaManna and Barrangou 2018). The advantage of those vectors are that they had been proven as working in plants. Several commercial vectors are also available for expressing Cas9 (or Cas9 variants) and gRNAs in plants. They usually contain screening markers (ex. GUS) or selection markers (ex. kanamycin-resistant, hygromycin-resistant, or *bar* gene). This includes a series of all-in-one ready-to-use plant CRISPR vectors from Sigma-Aldrich, either for *Agrobacterium*-mediate or particle bombardment method.

Feng et al. (2018) pointed out from several research results that the mutation efficiency of CRISPR-Cas9 system largely depends on the expression efficiency of

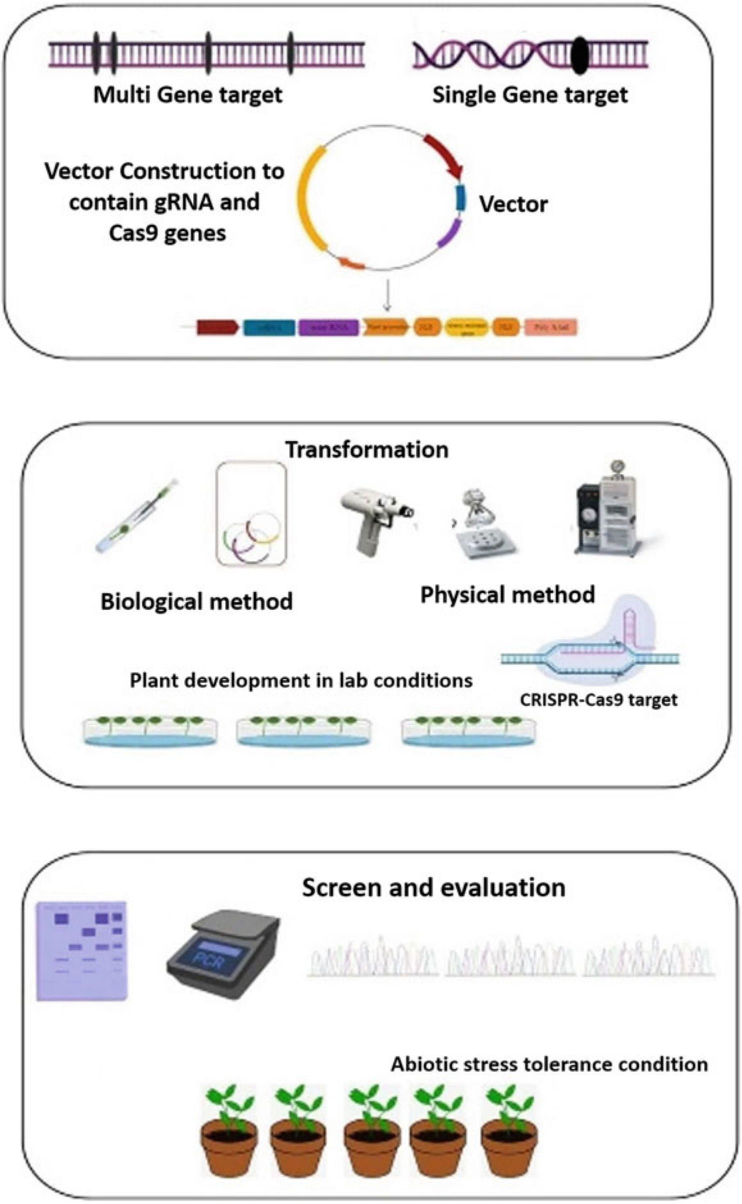

**Fig. 32.1** Schematic representation of plant genome editing

both Cas9 and sgRNAs. Optimization of the promoters used for the CRISPR/Cas9 system is important for enhancing the efficiency of targeted genome editing in plants. An array of promoters has been employed to drive CRISPR components (Cas9 and sgRNA) in plant genome editing. In most cases, Cas9 gene is driven by Cauliflower mosaic virus 35S promoter (CaMV35S) (for dicots) or ubiquitin promoters (for monocots), while the sgRNA is usually driven by U6 promoters (ex. AtU6 [*Arabidopsis]*; TaU6 [wheat]; OsU6 or OsU3 [rice]) (Belhaj et al. 2013). Other promoters are being tested and used to drive Cas9 gene or sgRNA to enhance expression. For example, after observing CaMV35S-driven Cas9 production of mosaic mutant lines in the first generation ($T_1$) of *Arabidopsis* (Feng et al. 2014), researchers began seeking other promoters to improve Cas9 expression. Both egg cell-specific promoter EC1.2 Wang et al. (2015b) and cell division-specific promoters, YAO and CDC45 (Feng et al. 2018) were tried. In another case, maize DMC1 promoter has been used to drive Cas9 expression in maize. In $T_0$ maize plants, highly efficient editing has been reported at target sites with homozygous or bi-allelic mutants accounting for about 66% (Feng et al. 2018). Researchers have also used the *native* U6 promoter derived from plant species currently used in the focus of their studies (instead of using U6 promoters from other species) for their research. For example, recently a CRISPR-mediated genome-editing soybean study increased editing efficiency ~1.8–6.3-fold when native GmU6-10 promoter was used to drive the sgRNA gene, compared to AtU6-26 promoter (Sun et al. 2015).

Cas9 gene is usually codon-optimized in plant species of interest to enhance expression (Bortesi and Fischer 2015). Cas9 codon-optimization to a specific plant species might not function efficiently in a different plant species. For example, Klimek-Chodacka et al. (2018) compared three different codon-optimized Cas9 in carrot (dicot) genome editing. They compared AteCas9 (codon-optimized to *Arabidopsis*), mCas9 (codon-optimized to maize), and Cas9P (codon-optimized to work for plants in general, for both dicots and monocots; 'P' = plant). The authors observed that *Arabidopsis* codon-optimized AteCas9 gave the highest editing efficiency, up to 90%. Perhaps due to dicot status of both *Arabidopsis* and carrot.

Nucleus localization signal (NLS) is also frequently fused to the Cas9 gene for more efficient targeting of Cas9 protein to the nucleus (Belhaj et al. 2013). Studies have shown numbers of NLS and position of NLS on Cas protein (N- or C-terminus) can influence editing efficiency. Li et al. (2013) demonstrated adding NLS to both N- and C-termini delivers increased nuclear targeting efficiency over one NLS. In another study, Osakabe et al. (2016) compared mutagenesis efficiency using Cas9 with one NLS and Cas9 with two NLS signals. They found Cas9 with two NLS signals demonstrated higher CRISPR-mediated mutagenesis rate, and considered it to be an important factor in increasing CRISPR editing efficiency in experiments. Cas9 proteins with higher number of NLS signals are also used. For instance, three NLS signals are commercially available at idtDNA (www.idtDNA.com). However, Hu et al. (2018b) provide evidence that NLS may not be crucial in increasing editing efficiency in Zebrafish. They compared various Cas9-NLS variants (NLS-fused Cas9 proteins [N-NLS-Cas9, Cas9-NLS-C, N-NLS-Cas9-NLS-C, Cas9 protein without NLS]) for genome-ending efficiency in Zebrafish. Cas9 protein without an

NLS was shown to generate mutation rates comparable to the Cas9 variants with an NLS. This study concluded high levels of mutagenesis can be achieved regardless of fused NLS position in Zebrafish (Hu et al. 2018b). Due to conflicting reports, empirical studies on other species regarding NLS impacts on genome-editing efficiency are needed.

## 32.4.2 Delivery Systems

### 32.4.2.1 Delivery Tools

The effective delivery of CRISPR-Cas cassette into plant genomes is crucial for efficient modification. This remains an enormous challenge due to the plant cell wall. The most common methods for vector delivery include *Agrobacterium*-mediated delivery, agroinfiltration, biolistic delivery, electroporation, virus-based delivery, and PEG-mediated or direct delivery into protoplasts (Ran et al. 2017). These methods vary according to plant species and desired target. Each method has unique advantages and disadvantages. Effective delivery systems are needed to optimize applications of CRISPR-Cas9 in plant species, particularly polyploidy plants. Improvement of delivery systems helps increase on-target efficiency by minimizing off-target cleavage. Most current applications of CRISPR-Cas9 in plants use *Agrobacterium*-mediated transformation, which introduce T-DNA carrying both the Cas9 and sgRNA expression cassettes directly into the plant genome by a type IV secretion mechanism. However, other methods have recently demonstrated enhanced control with CRISPR-Cas9 delivery into plants.

***Agrobacterium***-mediated T-DNA transformation is a common way to deliver CRISPR-Cas9 expression cassettes to plant cells (Lowder et al. 2016). Genomes of different plant species such as *A. thaliana* (Zhang et al. 2015), soybean (*Glycine max* L.) (Sun et al. 2015), barley (*Hordeum vulgare* L.) (Lawrenson et al. 2015), rice (*O. sativa* L.) (Zheng et al. 2016), and wheat (*Triticum aestivum L.*) (Howells et al. 2018) have been successfully edited with CRIPSR-Cas9 using the *Agrobacterium* method. Stable, edited, transgene-free wheat lines can be obtained within 36 weeks using only two generations (Howells et al. 2018). Castel et al. (2019) reported T-DNA architecture (promoters, terminators, sgRNA backbones, Cas9 allele and their orientation in a T-DNA) can be important for editing efficiency. Optimized T-DNA architecture usually results in stable (i.e., homozygous) mutations in the first generation. The major drawback of ***Agrobacterium***-mediated delivery using CRISPR-Cas9 technology is transformation-recalcitrant plants. This limitation has necessitated alternative systems for CRISPR-Cas9 delivery in plant cells.

**Particle bombardment** is also used to generate genome-edited plants (Shan et al. 2014; Wang et al. 2014). However, one weakness of this method is high-copy-number complex insertions at multiple loci. As a consequence, segregation of Cas9-sgRNA cassettes in subsequent generations can be challenging. A recent study showed biolistic-delivery-based ‘transient’ CRISPR-Cas9 successfully generated genome-edited wheat (Hamada et al. 2018). Transient expression avoids permanent insertion of foreign genes within the genome. Bombarding CRISPR-Cas9 components designed to directly target specific genes into *shoot apical meristems* (SAM)-exposed embryos of imbibed seeds yielded results. 5.2% (11 out of 210) of plants derived from bombarded seeds carried the mutant allele, and the mutant allele from three of the 11 plants were stably inherited in the subsequent generation. Liu et al. (2019a) also reports successful genome-editing for *cinnamyl alcohol dehydrogenase* (CAD) and *phytoene desaturase* (PDS) genes in sorghum through biolistic bombardment.

Recently, mRNA of CRISPR reagents were successfully delivered in vivo to animal cells using lipid nanoparticles (Liu et al. 2019b). The nanoparticles simultaneously delivered Cas9 mRNA and sgRNA into kidney cells with up to 90% efficiency (Liu et al. 2019b). Nanocarriers were also used to deliver DNA, mRNA, and proteins into plant cells (Cunningham et al. 2018). Since plant cells have rigid cell walls, bombardment is a preferred method for plant cell delivery. Once delivered, cargos can be released slowly. For example, a mesoporous silica nanoparticle was used to deliver Cre protein into maize cells to excise a DNA fragment flanked by *loxP* sites (Susana Martin-Ortigosa et al. 2014). The advantage of delivering mRNA and proteins into cells with nanoparticles is protection of the cargo from cellular RNase and proteinase degradation. No foreign genes are permanently inserted into the genome. To authors' best knowledge, there is no report of using nanoparticles to carry CRISPR components into plant cells.

**PEG**-mediated CRISPR-Cas9 component delivery in plant cells is another way (Lin et al. 2017). Protoplasts are used for PEG-mediated genetic transformation. This method has been successful in a few plant species, including rice (Jiang et al. 2013; Shan et al. 2014), maize (Liang et al. 2014), wheat (Shan et al. 2014), soybean (Cai et al. 2015), tomato (Cermak et al. 2015), and potato (*Solanum tuberosum* L.) (Andersson et al. 2018), and tobacco (*Nicotianna tabacum L.) (*Lin et al. 2017*).* Lin et al. (2017) were able to generate amphidiploid tobacco plants with all four *phytoene desaturase* (*NtPDS)* alleles mutated, without *Cas9* DNA being present in most regenerated plants. This same research group also developed protoplast isolation protocols for several crops, ornamental species, and model plant *Arabidopsis.* These are very useful for researchers using protoplasts for CRISPR-mediated gene editing (Lin et al. 2017). However, protoplast experiments are primarily used to evaluate efficiency of various sgRNA designs. Not every crop species has an efficiency protocol for regeneration from protoplasts. For example, maize has no protocol for regeneration from protoplasts (Doll et al. 2019).

Compared to the three laborious and highly technical *Agrobacterium*-mediated, biolistic, or protoplast transformations, viral systems offer a simple yet effective means for delivering CRISPR-Cas9 components. Virus-based sgRNA delivery in plants has substantial advantages compared to conventional constitutive-promoter-driven expression due to its rapid and robust amplification of sgRNAs during virus replication and movement (Hu et al. 2019). *Vi*rus-based sgRNA delivery system for CRISPR-Cas9 mediated plant *g*enome *e*diting (or **VIGE**) is widely applicable to diverse crop taxa (Hu et al. 2019). This includes plant DNA viruses, such as geminivirus (Yin et al. 2015). Yin et al. (2015) used a *Cabbage Leaf Curl virus* (CaLCuV) vector to express sgRNAs in stable transgenic plants expressing

Cas9. Several plant RNA viruses were also used in model plants (tobacco and *Arabidopsis)* for targeted genome editing: *tobacco rattle virus* (TRV) (Ali et al. 2015a, b), *pea early-browning virus* (PEBV) (Ali et al. 2018), *tobacco mosaic virus* (TMV) (Cody et al. 2017), and *beet necrotic yellow vein virus* (BNYVV) (Jiang et al. 2019). More recently, Hu et al. (2019) applied VIGE on two economically important crops, wheat and maize, using *barley stripe mosaic virus* (BSMV) as the vector. Targeted mutagenesis of wheat *TaGASR7* and maize *ZmTMS5* genes was achieved with up to 78% and 48% efficiency, respectively (Hu et al. 2019).

Syngenta researchers devised a cleaver scheme for delivering CRISPR to transformation-recalcitrant crop varieties. In their approach, pollen of a transformation-friendly corn variety (variety A) carrying a frame-shift-mutated *MATRILINEAL* gene was used to harbor CRISPR-Cas9 components. *MATRILINEAL* gene encodes a pollen-specific phospholipase. Maize plants carrying mutated *MATRILINEAL* gene generates pollen capable of triggering ‘haploid induction (HI)’ (Kelliher et al. 2017). Variety A pollen was used to pollinate variety B, which was recalcitrant to CRISPR application. Once fertilized, pollen chromosomes spread sgRNA and Cas9 editing machinery to the haploid genome of variety B’s egg, and underwent haploid induction to eliminate male chromosomes from pollen. Newly created plants will be haploid, instead of traditional diploid. Edited haploid plants lack both haploid-inducer parental DNA and editing machinery. This HI-Edit approach HI-Edit has confirmed function in both monocots (corn and wheat) and dicots (*Arabidopsis*) (Kelliher et al. 2017).

#### 32.4.2.2 Format of CRISPR Components for Delivery into Plant Cells

Sequence-specific nucleases (SSNs), such as TALEN nuclease or Cas9, may be delivered by DNA, mRNA, or protein format into plant cells for genome-editing (review see Ran et al. 2017). Typically, DNA constructs are delivered to either constitutively or transiently express the nucleases. Although rarely used in a plant application, mRNA has demonstrated effectiveness in genome-editing. Use of Cas9-gRNA ribonucleoprotein complexes (RNPs) is an alternative way to deliver CRISPR-Cas components into plants for gene editing (review see Zhang et al. 2021b). Transient delivery results in stable transgenic events, and greatly reduces off-targeting effects (Stoddard et al. 2016). In *Z. mays* and *T. aestivum,* pre-assembled RNPs delivery showed no off-target mutations (Svitashev et al. 2016; Liang et al. 2017), conferring immense applicability to the RNP approach (Wolter and Puchta 2017). Direct transfer of purified Cas9-gRNA ribonucleoprotein complexes (RNPs) to protoplast was recently successful in *A. thaliana*, *Lactuca sativa*, tobacco (*N. tabacum* L.), rice (*O. sativa* L.) (Woo et al. 2015), maize (*Z. mays*) (Svitashev et al. 2016), soybean (Kim et al. 2017b), wheat (*T. aestivum* L.) (Liang et al. 2017), and potato (*Solanum tuberosum* L.) (Andersson et al. 2018). This strategy is limited to certain plant species due to lack of established protocols for protoplast transformation for other plant species. Transformation of pre-assembled Cas9-gRNA RNPs is an alternative for use in plasmid delivery. This alternative eliminates the possibility of introducing foreign DNA to the host genome (Cho et al. 2013; Woo et al. 2015). However, the organization of transported

RNPs and their activity regulation are poorly understood (McClintock et al. 2018). More recently, a Japanese group directly delivered Cas9-gRNA RNPs into rice zygote cells produced by in vitro fertilization of isolated gametes (Toda et al. 2019). The use of zygote cells as genome-editing material can effectively reduce generation of chimera in later generations.

Lipofection reagents have been successfully developed for protein delivery of the Cas9-gRNA RNP complex into mammalian cells, but not in plants (Yu et al. 2016). Until recently, a novel lipofection-mediated transfection approach was reported to deliver pre-assembled Cas9-gRNA RNPs into plant cells (Liu et al. 2020b). Two lipofection reagents (Lipofectamine 3000 and RNAiMAX) were used to deliver Cas9-gRNA RNPs into tobacco BY-2 protoplasts for genome editing. They reported the optimal efficiencies for Lipofectamine 3000- and RNAiMAX-mediated protein delivery were 66% and 48%, respectively (Liu et al. 2020b). The method appears to confer higher targeted mutagenesis frequency than the PEG-mediated DNA delivery method (Liu et al. 2020b).

## 32.5 Utility of CRISPR-Cas Systems for Precise Molecular Plant Breeding

### 32.5.1 Rapid Production of Desired Homozygous Mutation Lines for Gene Function Study

CRISPR-Cas9-created homozygous mutations are readily identified in $T_0$ plants. Numerous CRISPR papers report these results in a variety of plant species with high frequency. Traditional knock-out method (ex. T-DNA insertional mutagenesis) usually obtains hemizygous status of the mutated allele in $T_0$ generation (or first generation [$T_1$] of *Arabidopsis*). This requires extra generations of genotyping (i.e., selfing and progeny test) to identify homozygous mutation lines. A CRISPR approach saves time and effort for breeders to generate the desired mutant lines for gene function study. For example, disruption of an amino-acid-transporter *LHT1* gene leads to growth inhibition and low yields in rice (Wang et al. 2019c). In another study, authors examine Isoamylase 1 (*ISA1*) gene's role in rice starch formation (Shufen et al. 2019). The gene encodes an isoamylase-type debranching enzyme. Two homozygous mutants, *cr-isa1-1* and *cr-isa1-2*, were generated using CRISPR-Cas9. Authors observed seed sizes of both *cr-isa1-1* and *cr-isa1-2* were affected, and these two mutant lines also displayed a shrunken endosperm with significantly lower grain weight (Shufen et al. 2019). Zhang et al. (2020a) efficiently generated homozygous *Medicago truncatula* mutants using a hairy root system with CRISPR-Cas9 system.

#### 32.5.1.1 Produce Homozygous Mutant Lines with Less Chimera

Obtaining high-frequency, homozygous mutation plants at $T_0$ (or first generation [$T_1$] of *Arabidopsis*) generation is possible with CRISPR editing. However, CRISPR-Cas9 edits can result in chimera plants. Use of proper promoters to drive

Cas9 improves the situation and reduces chimera frequency. Wang et al. (2015a, b) used an egg cell-specific promoter *EC1.2* to drive Cas9 in *Arabidopsis* for CRISPR-Cas9 editing. The expression of CRISPR machinery components in egg cells and one-cell stage embryos efficiently generated homozygous or biallelic mutants with multiple target genes in *Arabidopsis* by the first generation ($T_1$) Wang et al. (2015b). In a similar study, Mao et al. (2016) evaluated 3 germline promoters (the 5′ regulatory sequences of the *Arabidopsis SPOROCYTELESS* (*SPL*) promoter, *DD45* promoter, and the tomato *LAT52* promoter) to drive Cas9 expression in germline cells. *SPL* specifically expresses in microsporocytes. *DD45* is an early embryo-specific promoter. *LAT52* gene is expressed exclusively in pollen cells. The vast majority (70%) of $T_2$ *Arabidopsis* mutant population generated with constitutively expressed CRISPR-Cas9 systems were chimeras, while germ-line-specific Cas9 systems produced 29% chimeras (Mao et al. 2016). These results suggest directing Cas9 expression in germline cells significantly increases rates of heritable mutations and reduces the proportion of chimeras. Similarly, Feng et al. (2018) reported using cell division-specific promoters, *YAO* and *CDC45*, to improve mutation rates to 80–100% levels in the first generation ($T_1$) of *Arabidopsis*. Both *YAO* and *CDC45* promoters are shoot apical meristem-active promoters. pYAO-Cas9 and pCDC45-Cas9 systems produced more homozygous mutations (25–49%). Gametogenesis expression of *YAO* and *CDC45* may produce mutations in both egg and sperm genomes during meiotic cell division (Feng et al. 2018).

#### 32.5.1.2 Produce Transgene-Free Homozygous Mutant Lines

Edited plants containing transgenes are subject to the same regulations as conventional GM products. Removal of transgenes (*cas9-sgRNA*) is a prerequisite for regulatory approval of CRISPR-edited crops with commercial applications. Transgene remains are a metabolic burden for plants and can induce unwanted off-target editing. Removal of CRISPR components from the edited plant genome is necessary, but producing transgene-free, homozygous-mutation plants with an edited genome can be time-consuming. Transgene-free, CRISPR-edited plants can be obtained through genetic segregation (i.e., selfing or backcross) and PCR genotyping. This is a labor-intensive and time-consuming process, particularly for practical-breeding programs producing many plants that must be screened. Recently, Lu and Zhu (2017) devised a novel approach to RNAi cassette integration to a CRISPR-Cas9 construct. This enables a PCR-free, phenotype-based identification of genome-edited $T_0$ plants. Subsequently, *transgene-free genome-edited* $T_1$ plants are selected using the herbicide bentazon. The RNAi cassette supresses *CYP81A6* expression, which encodes a P450 cytochrome protein conferring resistance to bentazon in rice. Another group, He et al. (2018), devised a quick, simple method for screening transgene-free CRISPR-mediated lines. By integrating *REG2-BARNASE* and *35S-CMS2* into the CRISPR cassette, CRISPR constructs are eliminated—by destruction of any embryos and male gametophytes containing a CRISPR cassette. *REG2* is an embryo promoter, and *CMS2* causes male sterility. Transgene-free seeds can be yielded from $T_0$ plant. Fluorescence markers (ex. GFP) can be used to indicate the presence of cas9-sgRNA, and this phenotype accelerates isolation of heritable and Cas9-free plant mutants (He et al. 2018). Aliaga-Franco et al. (2019) also rapidly identified transgene-free CRISPR-edited plants of rice, tomato, and *Arabidopsis* by observing DsRED fluorescence in dry seeds.

### 32.5.2 Multiplex Editing to Modify Multiple Alleles at Multiple Genomic Loci

Simultaneously mutating multiple alleles in an organism's genome is vital to gene function study. Multiplex editing is especially useful for crops with numerous functionally redundant genes/paralogues, numerous members of a gene family, or parallel pathways, as all of these factors hamper gene-function analysis. Triple-mutant *Arabidopsis* has been historically generated through inter-cross of three single-mutant T-DNA insertion lines. Understandably, it is a labor-intensive and time-consuming approach to obtain all mutated alleles with homozygous status (Ulker et al. 2008; O'Malley and Ecker 2010). CRISPR-Cas9 can edit several genes simultaneously with unprecedented ease and speed. Multiplex editing with CRISPR-Cas9 simply requires the monomeric Cas9 protein and any number of different sequence-specific sgRNAs. Here are some examples:

1. Two research groups independently edited three *homoeo-alleles* in hexaploid bread wheat to enhance powdery mildew resistance (Wang et al. 2014; Zhang et al. 2017). Zhang et al. (2016b) used a CRISPR-Cas9 multiplex system to target 6 of 14 PYL families of the ABA-receptor genes in a single transformation experiment. One edited line with mutations in all six targeted PYLs (sextuple PYL mutant) was identified from 15 $T_1$ plants. Sun et al. (2018) generated single mutants and double mutants of homologous genes, *BaPDS1* and *BaPDS2*, to determine their biological function in Chinese kale (*Brassica oleracea* var. *alboglabra*). The leaves of plants with edited *PDS1* and *PDS2* were pure albino, and those with *PDS1* or *PDS2* single mutations have a mosaic-albino phenotype.
2. CRISPR technology provides a technically simple, yet high-throughput approach for generating a collection of lines with targeted mutations. Jacobs et al. (2017) transformed tomato plants with a CRISPR library targeting the immunity-associated, leucine-rich-repeat subfamily of XII genes. Heritable mutations were recovered in 15 of the 54 genes targeted.
3. Yang et al. (2017) tested efficiency of simultaneous gene mutations for three gene families (*BnaRGA*, *BnaDA2*, and *BnaFUL*) in allotetraploid *B. napus*. Two multiple-gene-targeted sgRNAs were used to successfully edit conserved sequences of all nine gene family members in the three families simultaneously.
4. Many important traits are often regulated by multiple genes. Therefore, to improve such traits requires editing of multiple genes. An efficient CRISPR multiplex system is very useful for this purpose. For example, Zhou et al. (2017) conducted multiplex QTL editing of grain-related genes and improved yield in elite rice varieties. They mutated three yield-related QTL genes, *OsGS3*, *OsGW2*, and *OsGn1a*, which negatively regulate grain size (Zhou et al. 2017).

This resulted in 68% and 30% yield per panicle increases in triple mutants for two of the three elite, edited rice lines.

5. Multiplex editing is also used to study the relationship between '*gene dosage*' and 'phenotype variation'. Morineau et al. (2017) studied dosage effect on lipid profile, using three CRISPR-mutated, delta-12-desaturase (*FAD2*) genes in the hexaploid oilseed crop, *Camelina sativa*. Silencing of *FDA2* led to reduced levels of polyunsaturated, fatty acids and increased accumulation of oleic acid. They observed various lipid profiles, ranging from 10% to 62% oleic acid accumulation, in the single, double, and triple CRISPR-Cas9-edited mutants (Morineau et al. 2017).

   The allohexaploid genome of common wheat (*Triticum aestivum*, $2n = 6x$, genomes *AABBDD*) has many three-copy genes on each homoeologous (AA or BB or DD) chromosome. For example, the *TaGW2* gene has one copy on the AA chromosome, a copy on the BB chromosome, and another copy on the DD chromosome. Wang et al. (2018b) used CRISPR-Cas9 to generate a series of knockouts of this gene. When functionally active copies of *TaGW2* were mutated from all three (A, B, and D) chromosomes (yielding genotype: *aabbdd*) or from B and D genomes (yielding genotype: *AAbbdd*), the grain weight increased significantly (Wang et al. 2018b). For *aabbdd* mutant, the total grain weight (27.7%), grain width (10.9%) and grain length (6.1%) were significantly increased, compared to the non-edited plants (Wang et al. 2018b). CRISPR-Cas9 can be successfully applied to engineer for higher plant yields, per these results.
6. Sánchez-León et al. (2018) used CRISPR-Cas9 to target α-gliadin genes for production of low-gluten wheat. Two sgRNAs were designed to target conserved regions adjacent to immunodominant epitope coding sequence in wheat gluten. One of the edited lines knocked out 35 of 45 different genes identified in wild type. α-gliadins are encoded by approximately 100 genes and pseudogenes in bread wheat. These knockouts reduced immunoreactivity by 85% (Sánchez-León et al. 2018).
7. Doll et al. (2019) described CRISPR-Cas9-based mutagenesis for 20 genes associated with kernel development in maize. A total of 28 different single guide RNAs (sgRNAs) were generated on 12 plasmids to target these 20 genes. Depending on the transformation strategies, at least one mutant allele was obtained for 18 of 20 genes. Double and triple mutants were also created in a single step (Doll et al. 2019).

Several published platforms are useful tools to efficiently assemble several sgRNAs on a vector. The popular tRNA-processing sgRNA expression system is a prime example (Qi et al. 2016; Port and Bullock 2016; Wang et al. 2016a, b, c, d, 2017a, b, c, d; Ma et al. 2019).

### 32.5.3 For Removal of Entire Chromosome, Gene, or Short DNA Fragment

The CRISPR-Cas9 system presents an adaptable tool for creating variable deletion mutations ranging from 1 bp to a chromosome arm or complete chromosome (Zhao et al. 2016; Zhang et al. 2017; Cai et al. 2018). Researchers can study the function of a gene or regulatory element by deliberately deleting these fragments. Endogenous alleles can also be deleted and replaced through HDR-mediated gene targeting. As Zhao et al. (2016) have successfully used it to delete miRNA gene regions (*MIR169a* and *MIR827a*) preparing to knock-in new genes later. In another report, Yan et al. (2016) used CRISPR-Cas9 and two sgRNA to generate heritable DNA fragment deletions for study of a regulatory element function. They deleted a portion of the second intron (450 bp) for flower-development gene *AGAMOUS* in *Arabidopsis*. Analysis revealed deletion of this intron fragment yields a 40% decrease in *AGAMOUS* gene expression, with no change to the gene's splicing profile. This intron serves as a regulatory element per the results, functioning as an activator of *AGAMOUS* gene expression (Yan et al. 2016). This same research group removed the entire *SEPALLATA3* (*SEP3*) gene (5.1 kb) with two sgRNAs (Yan et al. 2016). Zuo et al. (2017) was first to report complete elimination of an entire Y chromosome *via* multiple DNA cleavages on the targeted chromosome using 14 sgRNA in mammalian cells. This could be a potential therapeutic strategy for treating human aneuploidy diseases involving additional chromosomes. DNA deletion can also be used to remove unwanted DNA fragments, or to study the biological function of a specific gene or a non-coding regulatory sequence, examples follow. CRISPR-Cas9 system-mediated DNA deletion is described *in planta*. In soybean, Cai et al. (2018) successfully used CRISPR-Cas9 to delete DNA fragments from *GmFT2a* and *GmFT5* genes.

Durr et al. (2018) successfully used CRISPR-Cas9 to create heritable chromosomal deletions to study gene function in plants. A genomic region flanking the *ISU1* locus (~12 kb), containing a large cluster of defensin-like genes and non-coding regulatory sequences, was selected for deletion. ISU1 encodes a protein targeted to mitochondria for iron-sulfur cluster biogenesis. From the edited 152 independent transgenic lines, 77 (51%) plants displayed targeted deletions. In such mutants, early globular embryo development was arrested, and seeds display a significant reduction in size (Durr et al. 2018). In 2020, Osakabe et al. used a novel Class 1 type I subtype CRISPR-Cas system, type I-D (TiD), for long-range deletions in genomic regions of interest in plant with low off-target effects. This could be an effective genome editing tool for removing complex genome structures.

Transposable elements (TEs) can affect adjacent gene expression, leading to phenotypic changes. Modification of TEs *via* genome editing can lead to the production of novel plant cultivars. For this, Saika et al. (2019) used CRISPR-Cas9 to excise *Tos17* retrotransposon from the rice genome. *Tos17* retrotransposon is flanked by long terminal repeat (LTR) sequences at both ends. The group designed specific sgRNA to target the two LTRs. Breaks in flanking LTRs release remaining

*Tos17*. In the next generation, plants homozygous negative for *Tos17* element were recovered.

CRISPR-Cas9 was also used in plant transgenesis to remove selection-marker gene (SMG). SMG in transgenic products raises concerns for consumers and regulatory agencies. Removal of SMGs from transgenic crops increases public acceptance of GM crops. The removed SMG can be reused in a gene-stacking project for improvement of complex traits (Yau and Stewart 2013). SMG flanked with two sgRNA-targeting site can be recognized by two specific sgRNAs. DSBs induced at two sgRNA-targeting sites will release the SMG (Srivastava et al. 2017). Srivastava et al. (2017) designed two sgRNAs to target each end of a 1.6 kb *GUS* gene and deleted *gus* gene in rice. Homozygous excision lines can be readily recovered in the $T_0$ generation. However, it is important that both sgRNAs should have similar high-targeting efficiency. Dissimilar sgRNA efficacies could result in low rates of deletion (Pathak et al. 2019).

Recently, a *T. fusca* derived novel Type-I CRISPR system, CRISPR-**Cas3**, has been successfully used to edit the human genome (Dolan et al. 2019). This system has an unprecedented ability to target and erase long stretches of DNA, not just by cutting but by shredding the target. The authors have successfully removed up to 100 kb of targeted DNA sequences in human embryonic stem cells. This system has the potential to erase specific genetic elements in a genome. An analysis of lost function can help determine the role of these genetic elements (Dolan et al. 2019). This approach has the potential to erase ectopic viruses, such as herpes and hepatitis B, which are detrimental to human health. Similarly, this new approach holds promise for plant breeders for removal and prevention of pathogenic viral diseases.

### 32.5.4 Utility of CRISPR System to Reverse Gene Silencing

Gene silencing widely occurs in genetically transformed plants (Day et al. 2000). Methylation of a transgene promoter or methylation of the transgene can cause gene silencing of any particular transgene (Yamasaki et al. 2011). For example, progressive 35S promoter methylation was observed increasing rapidly during vegetative development in transgenic *Nicotiana attenuata* plants (Weinhold et al. 2013). Commercial varieties display differing degrees of promoter and transgene methylation. Vilperte et al. (2016) have reported different degrees of gene methylation in four single-transgene corn varieties from Monsanto Company. Katayama et al. (2016) conducted a study replacing a methylated promoter with an unmethylated one using CRISPR-Cas9 in mammalian cells. The approach was sufficient to activate expression in cells used for the experiment (Katayama et al. 2016). Although not yet applied in plants, breeders have a potential tool for saving valuable breeding lines from gene silencing due to promoter methylation.

### 32.5.5 For Allele Repair or Replacement Through HDR-Mediated Gene Targeting

After a DSB is induced by a CRISPR system, in the presence of a 'repair template,' eukaryotic cells may repair a DSB using HDR instead of NHEJ. Repair templates are designed to contain homology arms with sequences homologous to DNA flanking the DSB. Repair templates can be knocked-in to the DSB location through double homologous recombination (Huang and Puchta 2019). For instance, dual-sgRNA CRISPR-Cas9 system was successfully used to delete miRNA gene regions (*MIR169a* and *MIR827a*) in *Arabidopsis* (Zhao et al. 2016). The deleted region of the locus was replaced with a *TERMINAL FLOWER 1* (*TFL1*) gene. HDR approach was performed for this knock-in. Hahn et al. (2018) also used HDR to successfully repair a defect *gl1* gene, which has a 10 bp deletion in the coding region. *GLABROUS1* (*GL1*) gene is the R2R3-MYB transcriptional master regulator for trichrome formation. In this study, the 10 bp (-CTGCCGTTTA-) were flanked with homology arms (~830 bp each) and used as repair template.

Shi et al. (2017) devised replacement of *ARGOS8* native promoter with a native maize GOS2 promoter, which is a moderate-constitutive promoter, using CRISPR-Cas9 and HDR-mediated insertion. The generated variant transforms *ARGOS8* gene expression from non-constitutive to constitutive expression. This will be a very attractive approach to make a specific gene overexpressing *in planta*. For example, a 2x35S promoter can be knocked-in to replace a native promoter of a specific gene for overexpression. Researchers conventionally clone out the gene first and place it downstream of a 2x35S promoter in T-DNA on a binary vector (in case of *Agrobacterium* transformation). The vector is then transformed back into the plant-species-of-interest for overexpression study. However, T-DNA usually contains other cassettes (ex. *nptII* gene cassette) with other promoters which could interfere with 2x35S promoter (phenomenon of 'transcription interference') (Bhullar et al. 2009). T-DNA has a predisposition for random insertion, easily knocking-out essential endogenous genes or showing 'position effect'. Scientists can also replace a native gene *in planta* with a reporter gene, such as *gus*, for investigation of a gene expression profile or determination of promoter strength.

Since HDR occurs with much lower efficiency than NHEJ in plants, it is inefficient to use HDR for gene knock-in. HDR often restricts to certain cell-cycle stages or cell types. Although approaches to increase gene knock-in efficiency through HDR have been suggested (Liu et al. 2019d), scientists continue to seek improvements to gene targeting through HDR. Potential methods include the use of different Cas orthologs (Wolter and Puchta 2018), the use of different promoters to drive Cas protein (Wang et al. 2015b; Wolter and Puchta 2018) or the use of a different transformation approach (ex. 'sequential transformation method') (Miki et al. 2018).

Wolter and Puchta (2018) compared (1) *Streptococcus pyogenes* Cas9 (*Sp*Cas9) and *Staphylococcus aureus* Cas9 (*Sa*Cas9) nuclease and (2) different promoters driving Cas9 protein for HDR-mediated gene targeting efficiency. Promoters selected to use were from: *Arabidopsis* stem-cell-identity-regulating CLV3 gene, the YAO gene, and egg-cell specific EC1.1 gene (described in Wolter and Puchta 2018). Results demonstrate (1) *Sa*Cas9 performs better than *Sp*Cas9 nuclease, and (2) egg-cell specific EC1.1 promoter improves *in planta* gene targeting, over CLV3 and YAO promoters (Wolter and Puchta 2018).

Miki et al. (2018) reported inefficient HDR-mediated 'heritable' editing events using the conventional 'all-in-one' (i.e., 35S::*Cas9* + AtU6::*sgRNA*) construct design for genetic transformation in *Arabidopsis*. This was consistent for even using germline-specific promoters, such as 'egg cell- and early embryo-specific' DD45 promoter, pollen-specific promoter *Lat52*, shoot apical meristem-active promoters YAO and CDC45 (Miki et al. 2018). However, by using 'sequential transformation' (transforming *Arabidopsis* with Cas9-expressing cassette [Pro::*Cas9* + 35S::*hpt*] first, then selected strong *Cas9*-expressing lines for second construct [AtU6::*sgRNA*-(repair template)-35S::*bar*] transformation), the heritable knock-in mutation efficiency through HDR was greatly improved (Miki et al. 2018). They were able to edit several endogenous genes in *Arabidopsis* genome. The authors also used 'sequential transformation' method to successfully knock-in a *gfp* gene through HDR gene targeting, next to an endogenous gene *DME* for a 3′ in-frame fusion (*DME-GFP*) and 5′ in-frame fusion (*GFP-DME*). *DME* is an endogenous DNA glycosylase gene (Miki et al. 2018). Among these, the egg-cell promoter DD45 yielded the best results. This supports findings of Wolter and Puchta (2018), in which egg cell EC1.1 gave the best performance.

### 32.5.6 Utility of CRISPR System for Gene Stacking at the Same Locus

To stack genes at the same locus ensures those genes can co-segregate together in subsequent generations. Researchers successfully executed several strategies to stack genes at the same locus in plants. These include using recombinase-mediated DNA cassette exchange (RMCE) system (Li et al. 2010), ZFN genome-editing system (Ainley et al. 2013), site-specific recombination (SSR) systems (Hou et al. 2014), and an *Agrobacterium*-based gene stacking system (Collier et al. 2018). However, CRISPR-mediated gene stacking has not yet been widely used, despite the system being considered much easier than ZFN for genome manipulation. Trait genes (with promoters) can theatrically be incorporated into the same locus by HDR sequentially, although knock-in efficacy is low in plants. Rational design of repair templates can strongly enhance HDR efficiency, such as lengths of homology arms used. Promoters used to drive Cas protein are also important in improving editing efficiency, as mentioned above. Researchers should refer to many studied strategies for increasing HDR-mediated gene knock-in efficiency and then chose optimal HDR conditions for your own species of interest. Researchers recently have combined used CRISPR-Cas9 and site-specific recombination to stack genes at a maize genome locus (Gao et al. 2020).

The HDR method for gene insertion or stacking at a single locus is not exclusive. Transgenes should also be able to be stacked into a DSB site through targeted *Agrobacterium*-mediated T-DNA integration, since T-DNA preferentially integrates into double-strand DNA breaks. Researchers have successfully targeted a T-DNA fragment into a CRISPR-induced DSB site. The T-DNA fragment used contains RB-[*pro-hptII-ter*]-[*pro-sgRNA*]-[*pro-cas9-ter*]-[*pro-mCHERRY-ter*]-LB, where *pro* is promoter, *ter* is terminator, RB and LB are borders of the T-DNA and mCHERRY is a red-florescent-protein gene. The Cas9/sgRNA generates DSB at the target locus, then linear T-DNA fragment insertion at the DSB occurs. Single-copy transgene integrants were generated with an overall integration efficiency near 5%. Among these integrants, both orientations of T-DNA integrations were detected. In another report, researchers observed 6.7–83.3% of *Arabidopsis* $T_1$ lines carrying T-DNAs inserted at Cas9 cut sites. Therefore, a CRISPR-Cas9 system combined with an *Agrobacterium*-mediated T-DNA delivery method will theoretically be a simple and efficient approach for gene insertion and stacking *in planta*. However, in those studies, authors permanently integrated both selectable-marker gene and CRISPR machinery in the genome, which is not a desirable outcome. Other systems can be added to remove these components following a T-DNA integration. Site-specific recombination systems (SSR) can be used for this purpose (Yau and Stewart 2013). One approach for removal would be SSR-mediated autoexcision, where recombinase is driven by a tissue-specific promoter (ex. pollen promoter) (Somleva et al. 2014).

A new method for gene (PCR product) insertion has been used with DSB that did not require homology arms on the PCR product. This approach has been called "*Knock-in blunt ligation*" (Geisinger et al. 2016). Relying on the machinery of canonical NHEJ to insert a linearized, blunt-ended, double-stranded DNA fragment into a genomic DSB with a high degree of precision and minimal loss of nucleotides results in a less complex vector design compared to conventional homology-arm-based repair templates. The concise protocol can be found at https://bio-protocol.org/e2163 (Geisinger and Calos 2017). This group successfully inserted fluorescent protein cassettes into various loci, with efficiencies up to 36% in mammalian cells. This method has not yet tested on plants. Plant researchers could utilize a similar approach using plant protoplasts for gene integration and stacking. The addition of a selectable marker has the potential to speed up selection of integrants. Protoplasts can then be directly used for evaluation after 2 or 3 days, or be embedded in agar-MS medium for the purpose of callus selection. Selected calli can be generated into plantlets and used for next-run gene stacking (Yau et al. 2011).

Very recently, another novel method using *CRISPR-associated transposase* (CAST)-mediated DNA insertion was reported in *Science* (Hou and Zhang 2019). Insertion action is guided by CRISPR-Cas12k. The transposase renders a transposon [on a plasmid with (**left end**)-(cargo DNA)-(**right end**)] for insertion at a specific locus. The inserted transposon can include 'cargo DNA' for genetic engineering (Hou and Zhang 2019). Unlike Cas9- or Cas12-based editing tools, CAST systems do not require DSB repair by host HDR pathways and may enable high efficiency gene knock-ins in a wide variety of cell types and tissues (Strecker et al. 2019). It will be interesting to discover if this system functions in plant cells to increase integration efficiency outside of the HDR pathway. The only drawback of this method is the ‘left end’ and ‘right end’ footprint remaining behind in the genome, rendering conventional GMO end products.

### 32.5.7 For Single-Base Editing

Precise *base editing* is highly desirable in plant functional genomic research and crop molecular breeding. Novel tools have been developed to precisely perform programmable base editing (or base substitution) of C•G to T•A or A•T to G•C. Base-editing was first described for mammalian cells (Komor et al. 2016) and quickly adopted to plant genomes (Review see Marzec and Hensel 2018). Two types of base-editing are available: cytosine and adenine base editors (CBEs and ABEs). Adenine base editor (ABE)-nCas9 (nickase-type Cas9 variant) converses A•T to G•C. Precise A•T to G•C base editing was first described in rice by Lu and Zhu (2017). In their study, six out the 23 lines showed expected T to C substitutions at a target region, achieving an editing efficiency of 26% (6/23). It is so precise that no other mutations resulted in the locus. Later, four other independent groups published similar A•T to G•C base editing in rice using ABE-based system (Hua et al. 2018; Yan et al. 2018; Hao et al. 2019). In rice, mutated allele *Wx-mq* contains a T to C substitution at position 595. This mutation results in replacement of a tyrosine by a histidine at residue 191, and low amylose in the plants. Hao et al. (2019) has used ABE to fix the mutant amylose gene *Wx-mq* through A•T to G•C base editing. By base-editing the acetolactate synthase (ALS) and acetyl-coenzyme A carboxylase gene, Zhang et al. (2019) created herbicide-tolerant wheat varieties, conferring tolerance to sulfonylurea-, imidazolinone- and aryloxyphenoxy propionate-type herbicides for weed management. In 2019, Tang et al. demonstrated that STU-nCas9-PmCDA1 is a very efficient base-editing system in generating plants with targeted C to T base changes (Tang et al. 2019). Base editing technique has also been applied to other important monocots, notably wheat and maize (Zong et al. 2017). For current advances, limitations and applications of base-editor in crops, refer to recent review by Mishra et al. (2020). Recently, Nakazato et al. (2021) used base-editing technology to target *Arabidopsis thaliana* plastid genome. The Cs to Ts substitution were observed homoplasmically in some T1 plantlets.

Like in other applications using CRISPR-Cas9, off-target editing is problematic for base-editing. Two early studies, one in mouse embryos (Zuo et al. 2019) and one in rice (Jin et al. 2019), showed that CRISPR-guided cytosine base editor (CBE) can cause hundreds of unwanted mutations in other parts of the genome. In the rice study, authors declared little to no off-target events were detected in ABE (Jin et al. 2019). More recently, base editors causing wide-ranging off-target mutations in transcriptomes (RNA) were reported, indicating base editors can edit DNA and

RNA at the same time (Grunewald et al. 2019). One way to reduce RNA off-target editing is to engineer novel variants.

### 32.5.8 CRISPR-Based Diagnostic Tool for Rapid Detection of Viral Infections, GMO and DNA Quantification

Researchers at Broad Institute and Harvard have turned the powerful CRISPR-Cas system into a diagnostic tool for DNA and RNA detection or pathogen identification. Their diagnostic platform was named *Specific high-sensitivity enzymatic reporter unlocking* (or SHERLOCK). SHERLOCK technology was originally designed to diagnose human infectious diseases such as Dengue or Zika single-stranded RNA virus (Gootenberg et al. 2018). It might also help in diagnosis of other viruses, such as Lassa fever, an epidemic, in Nigeria that killed more than 69 people in 2019 (Maxman 2019). The diagnostic system does not rely on heavy instrumentation. It is also fast, low cost, portable, and easy to use. Now the system is used to detect SARS-CoV-2, which causes COVID-19, during pandemic. It generates a fluorescent or colorimetric readout, when Cas13 recognizes the target nucleic acid sequence (Abudayyeh et al. 2019). Scientists have further expanded SHERLOCK technology to detect and quantify plant genes for agricultural applications, including trait detection during breeding, pest surveillance, and pathogen identification (Abudayyeh et al. 2019). SHERLOCK platform offers a much easier platform for quantifying gene copies as Southern assay; qPCR and other methods currently used for gene quantification are still considered laborious and time consuming (Collier et al. 2017). As the procedure is fast (approximately ~15 min for minimum plant material preparation) and quantitative, rapid screening of putative single copy transgenic lines is possible. CRISPR-Cas13-based platform is also a powerful tool for rapid SARS-CoV-2 detection during COVID-19 pandemic (Aquino-Jarquin 2021). The other CRISPR-based diagnostic tool is Cas12a system, which targets DNA, instead of RNA (Chen et al. 2018a, b). Cas12a kit is currently used to diagnose HPV, which is linked to both cervical and anal cancer.

### 32.5.9 For Rapid Creation of Novel Genetic Diversity and Variation for Plant Breeding Stock

Genetic variation is vital to plant breeding. Limited genetic variation is a significant obstacle in plant breeding. Humans have a long history of exploiting natural genetic variation *via* sexual hybridization in plants by domestication of crops from wild species. Scientists introduce man-made genetic variation in crops by artificial introduction of mutations, using radiation and chemical mutagens (ex. EMS). CRISPR-Cas technology now enables promising new opportunities to generate genetic diversity for breeding in an unprecedented way (Wolter et al. 2019). For example, Shen et al. (2017) used multiplex CRISPR-Cas9 technology to create heterozygous and homozygous mutation lines (including homozygous sextuple, septuple, and octuple mutants) with various phenotypes. In the past, the majority of *cis*-regulatory elements remain poorly characterized. *Cis*-regulatory variation represents an untapped resource for expanding allelic diversity for plant breeding (Rodriguez-Leal et al. 2017). Recently, Rodriguez-Leal et al. (2017) used CRISPR systems to create mutations at *cis*-regulatory elements of quantitative trait genes in tomato. They rapidly and efficiently generated dozens of novel *cis*-regulatory alleles for three quantitative trait genes, which might be possible to regulate fruit size. This project demonstrates CRISPR-Cas9 can be used to generate novel variations in plants where naturally existing variations are not available. CRISPR technology can also be used for directed evolution in plants to identify novel traits, expand the range of traits, and accelerate trait development and improvement. Butt et al. (2019) demonstrated using CRISPR for directed evolution of the spliceosome to resist splicing inhibitors.

### 32.5.10 Combined Use of CRISPR and Microspore Technology for Double Haploid (DH) Breeding

Microspores are an important source for CRISPR-mediated genome-editing. Single celled microspores are haploid cells, and haploid cells can develop into double haploid (DH) plants in the lab by tissue-culture. DH plant lines have been utilized extensively as an advanced breeding tool to produce homozygous plants or pure lines within one generation (Brew-Appiah et al. 2013; Yan et al. 2017; Bhowmik et al. 2018). Combined use of microspore breeding technology and CRISPR-Cas9 technology, for trait discovery and improvement in crops, was recently described and reported in common wheat *(Triticum aestivum* L.) (Bhowmik et al. 2018; Lv et al. 2020). Multiple Cas9-sgRNA constructs were used to modify one exogenous *DsRed* gene and two endogenous genes, *TaLox2* and *TaUbiL1* in cultivars Bobwhite and AC Nanda (Bhowmik et al. 2018). Electroporation using a minimum of 75,000 cells and 10–20 μg DNA at a pulsing voltage of 500 V was found to be optimal for wheat microspore transfection (Bhowmik et al. 2018). In this study, authors did not induce edited pollen into DH plants, instead edited pollens were co-cultured with living mature ovaries for embryo development. Engineering the essential *CENTROMERIC HISTONE* (*CENH3*) gene induces paternal **haploid induction** (HI), such as in *Arabidopsis* and wheat (Lv et al. 2020). Researchers have employed *CENH3* HI technology in wheat, with HI rate of ~7% (Lv et al. 2020). HI can be useful for hybrid breeding in crops.

### 32.5.11 Utility of CRISPR System to Generate Male Sterility Lines to Facilitate Hybrid Breeding

Hybrid plants possess the value of '*hybrid vigor*' (or *heterosis*). Hybrid varieties exhibit superior performance compared to parent plants. For instance, hybrid rice varieties yield 15–20% more than inbred varieties. Today, most modern crops and vegetable species are grown from $F_1$ hybrid seeds. There are different practices for preparing pollen-free female plants for hybrid production. For example, male parts in corn are de-tasselled by machine at 70% with laborers cleaning up the remaining 30%. In tomato, flower stamens are hand-emasculated, and male sterility lines are used to produce hybrids. This method is also used for rice and carrots. Use of male sterility lines brings down cost in hybrid seed production.

Zhou et al. (2016) used CRISPR-Cas9 to induce specific mutations in *TMS5* gene and generated 'transgene-free' thermo-sensitive genetic male sterility (TGMS) for a 2-line hybrid rice breeding system. In another study, Cao's group also used CRISPR-Cas9 to knock out maize *thermosensitive genic male-sterile 5* (*TMS5*) gene, *ZmTNS5*, and generated male-sterile line for maize hybrid seed production (Li et al. 2017). At the low temperature of 24 °C, *tms5* mutants were male-fertile. At the high temperature of 32 °C, *tms5* mutants were male-sterile (Li et al. 2017). These mutants are valuable breeding stock for maize 2-line hybrid breeding programs. A group of researchers had deployed CRISPR-Cas9 system to simultaneously disrupt three TaNP1 homoeoalleles in wheat. The resulting *Tanp1* triple mutant lines showed complete male sterility (Li et al. 2020). These lines are valuable breeding stocks to accelerate hybrid wheat production.

Cytoplasmic male sterility (CMS) is a type of male sterility. CMS is often linked to aborted pollen development and an absence of seed setting. Frequently, it is used to produce $F_1$ hybrid seeds in agriculture. Mitochondrial genes are the suspected cause of CMS. Several years ago, genes linked to CMS were compared to mitochondrial genome sequences of sterile and fertile lines. However, the cause remains unclear. Modern day genome-editing tools can help find genes responsible for CMS by knocking out candidate genes. *Nature Plants* published Kazama et al. (2019) in the first reported use of TALEN system to knock out CMS candidate gene *orf79* in CMS rice plants to restore fertility. More researches will be moving forward with the CRISPR system, due to ease of use, simplicity in design, and rapid operation.

### 32.5.12 Utility of CRISPR System to Produce 'Clonal Seeds' from Hybrid Plants

Performance of hybrid $F_1$ is superior to both parent plants due to 'hybrid vigor' (or *Heterosis*). However, hybrids do not breed true. Seed companies develop inbred lines to produce hybrid seeds for farmers to purchase. Since hybrids do not breed true, farmers have to purchase hybrid seeds each year. Seeds cannot be saved from plants derived from the hybrid seeds for future use, ending hybrid vigor. Scientists can now produce 'clonal seeds' from plants derived from hybrid seeds without sexual pollination. This is a great breakthrough. Wang et al. (2019e) manipulated two sets of genes, allowing *japonica* rice hybrid Kitaake to clone its own seeds. The first gene is *BABA BOOM1*. They placed a promoter in the female copy *BABY BOOM1* to trigger development of a seed embryo. Normally, pollen male copy *BABY BOOM1* triggers seed embryo development. In addition, they modified by disabling a second set genes crucial for meiosis in rice. This CRISPR-Cas9-based modification, switched mother plans to asexual reproduction. Those cloned seeds grew into plants capable of cloning themselves. Right now, only 30% of parents are able to produce viable clone seeds (Wang et al. 2019e). This research team is looking for new approaches to boost efficiency. The cost for hybrid seed production would be dramatically reduced for seed companies with access to this method.

### 32.5.13 Utility of CRISPR System to Generate Apomixis Plants

Apomixis is an asexual reproduction or cloning via seed. The seed-derived progenies of apomixis are genetically identical to their maternal parent. There are three forms of apomixis: (1) adventitious embryony, (2) diplospory, and (3) apospory (Hand and Koltunow 2014). Since apomicts produce clonal offspring, any edited parents can produce identical edited offspring. Even F1 hybrids are able to transfer identical genome makeup from one generation to next (Sailer et al. 2016). Gaafer et al. (2018) induced apomixis from an Egyptian rice hybrid line, Hybrid-1. They successfully demonstrated fixation of heterosis in the progeny of Hybrid-1. Several genes controlling apomixis have been found in natural apomictic species, but there has been little success to date integrating them into sexual plants. Since several mutants from sexual plants have mimicked aspects of apomixis, scientists could generate apomixis from a sexual plant by engineering the specific endogenous genes involved. Xie et al. (2019) attempted to generate apomictic rice (*Oryza sativa*) by mutating 3 *MiMe* (*Mitosis instead of Meiosis*) genes, *OsSPO11-1, OsREC8, OsOSD1*, and *OsMATL* gene in one generation using CRISPR-Cas9 technology. *OsMATL* is associates with haploid induction ability. One quadruple mutant was generated, and meiocytes of this quadruple mutant underwent mitotic-like division instead of meiosis, producing unreduced clonal diploid gametes (Xie et al. 2019). This quadruple mutant is expected to produce apomictic offspring. However, more studies are needed in order to establish an efficient CRISPR-Cas-medicated apomixis induction system (Scheben and Hojsgaard 2020).

### 32.5.14 For Rapid Breeding of Parthenocarpic Crops

Development of fruits without pollination and fertilization is called parthenocarpy. Parthenocarpic fruits are seedless. Climate change can negatively impact pollen production, fertilization, and fruit production. Therefore, parthenocarpy is an efficient way to produce fruits under adverse environmental conditions without pollination or fertilization required. This process has the potential to stabilize or increase both production and productivity, because no pollinator plants are needed. Consumers could drive increased demand for seedless fruits, such as parthenocarpic cucumber. Parthenocarpy is controlled by interactions between phytohormones. Regulators are involved in auxin signalling, Aux/IAA9 (IAA9) is one of many involved in tomato fruit development. It functions to repress fruit initiation without fertilization (Ueta et al. 2017). Ueta et al. (2017) used CRISPR-Cas9 technology to induce mutations in tomato (*Solanum lycopersicum* L.) *IAA9* gene (*SlIAA9)*—a key gene controlling parthenocarpy—for parthenocarpic crops. The mutations were heritable. Fruit development was initiated before fertilization, resulting in parthenocarpy (Ueta et al. 2017). Klap et al. (2017) carried out knockout of *Slagamous*-like 6 (*SlAGL6*) gene in tomato (*S. lycopersicum*) to produce parthenocarpic fruits under heat stress conditions that restrict fertilization-dependent fruit set severely. The mutant gene was stable, and seedless tomato fruits were regular in shape and weight, contained viable pollen and were capable of sexual reproduction.

### 32.5.15 For Rapid Breeding of Gynoecious Crops from Monoecy

Many well-known vegetable and fruit crops such as cucumbers, squash, zucchini, bitter gourd, melons, and other vine crops are monoecious, having separate male and female flowers on the same plant. Gynoecious plants can produce predominantly female flowers on one plant. Gynoecious varieties often outproduce standard varieties when a pollinizer (monoecious variety) is present, because most flowers on the same plant are female flowers (Iowa State University Extension and Outreach). Therefore, in the viewpoint of breeding hybrid, the gynoecious line is an excellent inbred stock for use as a female line. There are several methods for producing gynoecious plants. Some select from a population of a monoecious line to cross with another monoecious line. However, the process is time-consuming and laborious. Other methods are worth exploring for rapid generation of gynoecy. Martin et al. (2009) found that mutation of *CmWIP1* conferred a gynoecious phenotype in melon. Hu et al. (2017a) made use of this, and mutated *CmWIP1* ortholog in cucumber, *CsWIP1*, using CRISPR-Cas9 to develop cucumber gynoecious inbred lines. *Cswip1* $T_0$ mutants displayed a gynoecious phenotype, with upper nodes bearing only female flowers Hu et al. (2017a). In addition, *Cswip1* T0 produced seven times more female flowers than wild-type. Zhang et al. (2020b) also used CRISPR-Cas technology to produce gynoecious watermelon by editing C2H2 zinc finger transcription factor gene, *ClWIP1*.

### 32.5.16 To Enhance Crop Production

Miao et al. (2018) simultaneously mutated the genes encoding the ABA receptors *pyrabactin resistance 1-like* (*PYL1*), *PYL4*, and *PYL6* genes. This resulted in improved growth and increased grain yield in rice. *pyl1/4/6* exhibited the best growth and improved grain productivity in natural paddy field conditions. Results revealed a successful genetic strategy to enhance crop productivity.

In *Japonica* rice, dominant *dep1* (*dense and erect panicle 1*) allele is a gain-of-function mutation, caused by deletion. Rice plants with *dep1* mutant allele carry phenotypes of dense and erect panicles, reduced inflorescence internode length, increased grain number per panicle, and increased grain yields Wang et al. (2017b). To create *Indica* rice with improved grain yields, Wang et al. (2017b) used a similar strategy by generating *dep1* mutants with CRISPR-Cas9 technology. They successfully deleted fragments of *dense and erect panicle1 (DEP1)* gene from public elite *Indica* rice line IR58025B. The phenotypes of *dep1* mutants generated by CRISPR-Cas9 included reduced plant height and dense-and-erect panicles, matched previously described phenotypes. As mentioned earlier, CRISPR was used to knock out three genes *OsGS3*, *OsGW2*, and *OsGn1a* in yield-related trait QTLs, and showed 68% and 30% yield increase per panicle in triple mutants (Zhou et al. 2017). In common wheat ($2n = 6x$), triple knockouts of gene *TaGW2* resulted in increases to total grain weight (27.7%), grain width (10.9%), and grain length (6.1%). These are significant compared to non-edited plants (Wang et al. 2018b).

### 32.5.17 For Studying and Improving Symbiotic Nitrogen Fixation

*Lotus japonicus* (diploid) is a model plant for genome studies involving legume symbiotic nitrogen fixation. *Symbiotic nitrogen fixation* (SNF)-related genes, symbiosis receptor-like kinase (SYMRK), and leghemoglobin loci (*LjLb1, LjLb2, and LjLb3*), were knocked out in *L. japonicus* (Wang et al. 2016b). SYMRK is an essential enzyme for symbioses in legumes. Overexpression of SYMRK initiates nodule formation in the absence of rhizobia. *LjLbs* genes are essential for establishing low-free-oxygen concentration and high energy status (ATP/ADP) within nodules for effective SNF (Wang et al. 2016b). Significantly increased ‘white’ nodules (instead of pink ones) could be observed in roots transformed with CRISPR-Cas9 reagents which target *LjLbs* genes, after inoculation with *M. loti* (Wang et al. 2016b). Rapid creation of homozygous mutants in this model will advance the study of molecular mechanisms of nodule development and SNF in legumes. In 2019, Wang et al. employed CRISPR-Cas9 technology to generate knockout lines of leghemoglobin (Lbs)-genes in *Lotus japonicus*, to study leghemoglobin biofunction. They observed that the lack of Lbs results in early nodule senescence (Wang et al. 2019a).

### 32.5.18 For Breeding New Crop Varieties to Adapt to New Regions

Scientists have used CRISPR-Cas technology to create soybean mutants adapted to high latitude areas, paving the way for new soybean varieties. Soybean is known as a short-day (SD) (or long-night) plant that accelerates transition from vegetative phase to reproductive phase in SD conditions. Soybean flowers under long-day (LD) conditions, but much later than in SD conditions (Cai et al. 2020). Soybean, as an SD crop (a crucial trait for current elite soybean varieties) adapting to locations with higher latitudes (LD conditions) is to reduce or to remove inhibition of flowering by LD conditions. Therefore, studying responses of flowering to photoperiod has great significance in soybean regional introduction and domestication (Wang et al. 2016d). Cai et al. (2020) used CRISPR-Cas9 to create mutations of *GmFT2a* and *GmFT5a* to study regulation of flowering time in soybean (*Glycine max* L.). They observed *GmFT5a*, not *GmFT2a*, to be essential for soybean adaptation at higher latitude regions, and *ft2aft5a* double mutants showed late flowering (by about 31.3 days under SD conditions), resulting in increased vegetative size.

Significant increases in pods and seeds per plant were observed compared to wild type (Cai et al. 2020).

### 32.5.19 Using CRISPR-based 'Gene-Drives' or 'Allelic-Drives' for Agricultural Pest Control or Augmentation of Favorable Alleles in a Breeding Population

CRISPR-based 'gene-drives' can spread a specific gene through a wild population more rapidly than by normal Mendelian inheritance patterns. Gene drives have been used in significant disease vectors to decrease the probability of disease transmission. Notably in mosquito for malaria or dengue fever. Scientists used this method for complete population suppression in caged mosquitoes (Kyrou et al. 2018). In agriculture applications, Buchman et al. (2018) implemented a gene-drive system to control a worldwide crop pest, *Drosophila suzukii*. They observed biasing of Mendelian inheritance rates with up to 100% efficiency (Buchman et al. 2018).

More recently, Guichard et al. (2019) used an 'allelic drive' to remove unwanted allele and increase favorable allele frequency in a *Drosophila* population. This system directs the CRISPR system to cut undesired variants of a gene and replace it with a preferred version of the gene (Guichard et al. 2019). This approach could also be applied to increase favorable alleles in a crop population for yield or other important argotic traits. Recurrent selection is one commonly employed breeding method in crop improvement programs. Recurrent selection aims to simultaneously increase the frequencies of favorable alleles at multiple loci in a breeding population through inter-cross of selected individual parents (Moose and Mumm 2008). Recurrent selection involves random insertion. Use of CRISPR-based allelic drives increase precision and speed for 'fixation' of these alleles in a population. Recently, gene drive was also applied in plants to generate homozygous F1 plants through zygotic conversion using a CRISPR/Cas9-based gene drive (Zhang et al. 2021a).

### 32.5.20 Utility of CRISPR System for Changing Fruit Ripening Time, Metabolic Pathway Engineering and Plant Architecture Study (Tomato Research as an Example)

CRISPR-Cas9 system has also been used for metabolic pathway engineering to change phenotypic characteristics or boost production of specific nutrients in fruits. Tomato is an example for discussion of the implementation of CRISPR technology in plant breeding (Pan et al. (2016a). Tomato is the fruit from plant *Solanum lycopersicum*, which belongs to the family *Solanaceae*. It is one of the most important solanaceous vegetable crops cultivated worldwide under outdoor and indoor conditions. Scientists continue to breed tomato with innovated techniques to improve yield and quality. Recently, tomato breeders altered tomato (*Lycopersicon esculentum* Mill.) fruit ripening time (Li et al. 2018c), increased tomato fruit quality (Li et al. 2018b), engineered nutritional components (ex. lycopene and GABA), and produced various disease-resistant tomato varieties (Nonaka et al. 2017) with CRISPR technology. Below are some case studies.

Li et al. (2018c) used CRISPR-Cas9 to knock out a key ripening-related long non-coding RNAs (lncRNAs), *lncRNA1459*. The tomato ripening process was significantly repressed in *lncRNA1459* mutants. Both ethylene production and lycopene accumulation were repressed in resulting mutants. Further analysis revealed lycopene biosynthesis genes (*PSY1*, *PDS*, and *ZDS*) and ethylene biosynthesis genes were both downregulated in the loss-of-function *lncRNA1459* mutants (Li et al. 2018c). Results show *lncRNA1459* is an 'activator' for tomato ripening.

Tomatoes are a major dietary source of the antioxidant lycopene, which is a bioactive compound linked to many health benefits. A major goal of tomato breeding is an increase in lycopene content in tomato fruit. Li et al. (2018e) used CRISPR-Cas9 to boost tomato lycopene content through suppression of conversion of lycopene to β- and α-carotene. Five genes (i.e., *SGR1*, *LCY-E*, *Blc*, *LCY-B1*, and *LCY-B2*) from a carotenoid metabolic pathway were knocked down by CRISPR-Cas9 in the tomato genome to promote the biosynthesis of lycopene (Li et al. 2018e). Lycopene content in edited fruit increased fivefold (Li et al. 2018e). Similarly, Klimek-Chodacka et al. (2018) tried to study carrot *flavanone-3-hydroxylase (F3H)* gene function by interrupting it, and tested for blockage of the anthocyanin biosynthesis in a purple-colored carrot callus. Callus color changed from purple to yellowish in edited calli.

Tomato (*Solanum lycopersicum* L.) contains higher levels of γ-aminobutyric acid (GABA) than other major crops (Takayama and Ezura 2015). GABA has received much attention as a health-promoting functional compound. Glutamate decarboxylase (GAD) is the key enzyme in GABA biosynthesis. High concentrations of GABA are usually observed at green-fruit stage, but levels decrease at red-fruit stage. Plant GADs generally have an '*autoinhibitory domain*' at C-terminus to regulate its own enzymatic activity. Deletion of this domain increases GAD activity. Nonaka et al. (2017) used CRISPR-Cas9 system to knock out two of five GAD genes, *SlGAD2* and *SlGAD3*, where *SlGAD2* and *SlGAD3* are expressed during tomato fruit development. Edited lines have promoted GABA accumulation seven- to 15-fold, at red-fruit stage. In another report, Li et al. (2018d) tried to manipulate five key genes in the 'γ-aminobutyric acid (GABA) shunt' (a metabolic pathway for GABA) to increase tomato GABA content with multiplex CRISPR-Cas9 system, which generated single to quadruple mutants. The γ-aminobutyric acid accumulation in both leaves and fruits of edited plants was significantly increased. Especially in quadruple mutants, which increased 19-fold over wild *S. lycopersicum* plants (Li et al. 2018d).

CRISPR-Cas9-mediated targeted disruption of *SlMYB12* gene in four elite *red*-fruited inbred lines lead to production of pink tomato fruits (Deng et al. 2018). Pink tomato can be more appealing to a certain region customer, particularly in Asia (Deng et al. 2018). CRISPR-mediated method can dramatically reduce the time for pink-tomato line development, compared to using conventional breeding method (ex. advanced backcross breeding strategy), which may take up to 5 years (Deng et al. 2018). Besides, backcross breeding usually associates with the undesirable 'linkage drag' phenomenon.

CRISPR-Cas9 system was also used to produce j2-CR and ej2-CR null mutations in *S. lycopersicum* and validated that the *Solyc12g038510* is j2 where the resulting j2-CR plants showed jointless un-branched inflorescences (Soyk et al. 2017). Uncovered dosage relationships between the allelic series of natural and induced MADS-box mutations by dissecting interactions of meristem expressed *SEP4* gene through breeding. Further, to validate this phenomenon, CRISPR-Cas9 system was used to aim *LIN* in the elite cherry *S. lycopersicum* Sweet 100 cultivar and produced mutant lines with quite a few branched inflorescences and elevated the flower production (Soyk et al. 2017).

It is noteworthy that researchers have transformed the cultivated white rice into red rice (Zhu et al. 2019). Red rice contains high levels of anthocyanins, which have been recognized as health-promoting nutrients. Red coloration of rice grains is controlled by two genes, *Rc* and *Rd*. Rice with *RcRd* genotype produces red pericarp in wild species *Oryza rufipogon*, whereas most cultivated rice varieties produce white grains. Loss-of-function associated with red pericarp production is due to a 14-bp frame-shift deletion mutation in an exon of the *Rc* allele (Zhu et al. 2019). Zhu et al. (2019) used CRISPR-Cas9 system to functionally restore the recessive *rc* allele through reverting the 14-bp frame-shift deletion to in-frame mutations. These approaches have successfully converted three elite white pericarp rice varieties into red ones. High accumulation level of anthocyanidins were detected in rice seeds from $T_1$ in-frame *Rc* edited lines, with no significant difference between wild-type and edited lines in major agronomic traits (Zhu et al. 2019).

### 32.5.21 Utility of CRISPR Systems in Tolerating Plant Biotic Stress

One great challenge for food security is improvement of yield stability through development of disease-resistant crops (Piquerez et al. 2014). Plants are continuously being damaged by various pathogens, including bacteria, fungi viruses, and parasitic weeds, resulting in 20–40% losses in crop yield (Borrelli et al. 2018). Plant breeders are compelled to search for and make the best use of the diverse disease resistance alleles from current available germplasms or exotic germplasms for elite crop cultivar improvement (Wang et al. 2017a). CRISPR gene-editing technology is a powerful tool for exploring the mechanisms of resistance regulated by these resistance alleles.

#### 32.5.21.1 For Fungus Resistance

Disease-related genes have been edited in plants *via* genome-editing technologies to fight disease. Rice blast disease caused by the **fungus** *Magnaporthe oryzae* is a costly damaging disease, costing $66 billion annual loss (Zhao et al. 2018). Li et al. (2012) used TALENs to disrupt a specific susceptibility gene *OsSWEET14* in rice to confer resistance to bacterial blight disease. In another study, CRISPR-Cas9 was used to knock-out *ERF Transcription Factor* gene *OsERF922* in rice, and generated mutant lines displayed enhanced resistant to rice blast disease. The number of blast lesions formed following pathogen change was significantly reduced in all tested mutant lines compared with wild-type plants at both the seedling and tillering stages (Wang et al. 2016a). Recently, CRISPR-Cas9 targeted mutation of *Ptr* gene in a resistant rice cultivar led to blast susceptibility, validating gene function in disease resistance (Zhao et al. 2018). CRISPR mutagenesis provides a strategy for developing blast-resistant rice cultivars. In another study, both TALEN and CRISPR systems were used to successfully target three homoeoalleles encoding *mildew-resistance locus O* (*MLO*) in hexaploid bread wheat. Resultant mutant lines exhibited broad spectrum resistance to **fungal** disease powdery mildew (Wang et al. 2014). *MIO*-like genes present in both monocots and dicots. The gene confers susceptibility to fungus causing the powdery mildew disease. A similar study was reported by Nekrasov et al. (2017), where *MLO* (*SlMlo1*) conferring susceptibility to fungi *Oidium neolycopersici* was knocked out in tomato variety Tomelo. *SlMlo1* is primary *MLO* of the 16 *MLO* genes found in tomato. In the study, CRISPR-Cas9 with double sgRNA targeted two positions within the gene resulting in a 48-bp deletion. Using this method, powdery mildew-resistant tomato line can be generated within 10 months. In another study, Huibers et al. (2013) reported silencing of both susceptibility (*S*) genes, *SlDMR1 and SlPMR4* using RNAi, resulted in resistance to the tomato powdery mildew fungus *Oidium neolycopersici*. Koseoglou (2017) targeted its ortholog in tomato *SlPMR4* using CRISPR-Cas9 technology. Edited plants exhibited partial resistance against *O. neolycopersici*. *Botrytis cinerea* is a necrotrophic fungus. Its most notable hosts are wine grapes. Wang et al. (2018c) used CRISPR-Cas technology to knock out *VvWRKY52* transcription factor gene in grape (*Vitis vinifera L.*) to increase the resistance to *Botrytis cinerea*.

### For Bacterial Resistance

Citrus canker, caused by *Xanthomonas citri* subsp. *citri* (*Xcc*), is an import **bacterial** disease to the citrus industry. Citrus canker is highly contagious and can be spread rapidly, which causes premature leaf and fruit drop (Florida Department of Agriculture). *CsLOB1* was previously revealed to confer susceptibility for citrus canker in citrus (Hu et al. 2014). Activation of *CsLOB1* expression promotes citrus canker development. Recently, two independent research groups applied CRISPR technology to knock-out *CsLOB1* gene function, generating canker resistant citrus varieties (Peng et al. 2017; Jia et al. 2017). Peng et al. (2017) employed CRISPR-Cas9-targeted to edit the susceptibility gene *CsLOB1* promoter region to improve citrus canker resistance. With pinprick inoculation evaluation, eruption of pustules slowed on leaf surfaces of edited mutant lines compared to wild type (Peng et al. 2017). In a similar study, Jia et al. (2017) used CRISPR-Cas9 technology to modify the canker susceptibility gene *CsLOB1* in grapefruit. In another study, Fister et al. (2018) transiently introduced CRISPR-Cas9 machinery components into cacao (*Theobroma cacao*) leaves and cotyledons, targeting *Non-Expressor of Pathogenesis-Related 3* (*TcNPR3*) gene (a defense response suppressor), to confer resistance to *Phytophthora* infection.

#### 32.5.21.2 For Virus Resistance

Apart from fungal and bacterial pathogens, crop yield is also affected by various **viral** diseases. The first reports of exploitation of CRISPR-Cas9 system to produce virus resistance in plants came from Baltes et al. (2015) and Ji et al. (2015), working on geminivirus. Mahfouz group delivered sgRNAs (specific to *tomato yellow leaf curl virus*) (TYLCV) in tobacco plants. When plants were challenged with TYLCV, delayed or decreased viral DNA was observed. This eliminated or attenuated TYLCV symptoms of infection (Ali et al. 2015a, b), indicating *in planta* viral interference. Similarly, Baltes et al. (2015) successfully engineered *bean yellow dwarf virus* (BeYDV) resistant tobacco by introducing mutations within the viral genome and reduced virus copy number through CRISPR-Cas9 technique. Transgenic plants expressing CRISPR-Cas9 components showed reduced symptoms when challenged with BeYDV (Baltes et al. 2015). Iqbal et al. (2016) proposed a scheme to use CRISPR-Cas9 to develop resistance in cotton against *cotton leaf curl disease* (CLCuD).

Eukaryotic translation initiation factor *eIF4E* plays a crucial role in plant for potyvirus infection (Ruffel et al. 2006). In earlier studies, silencing *eIF4E* gene can provide broad spectrum RNA virus resistance for the plants (Mazier et al. 2011). With a similar strategy, CRISPR-Cas9 was used to knock-out *eIF4E* gene in cucumber. $T_3$ progeny exhibited resistance to various viruses such as *cucumber vein yellowing virus, zucchini yellow mosaic virus*, and *papaya ring spot mosaic virus* (Chandrasekaran et al. 2016). Similarly, Pyott et al. (2016) introduced point mutations in *eIF4E* of *Arabidopsis thaliana* to obtain resistance to *turnip mosaic virus* (TuMV). Aman et al. (2018) also employed another CRISPR system, CRISPR-Cas13a (known previously as C2c2), to engineer interference with an RNA virus, Turnip Mosaic Virus (TuMV) in transient assays and stable overexpression lines of *Nicotiana benthamiana*. Macovei et al. (2018) employed CRISPR-Cas9 system to knock-out *eIF4G* gene in a rice variety (IR64) susceptible to *rice tungro spherical virus* (RTSV), and the resultant mutant lines exhibited resistance to RTSV infection. For review on targeting various susceptible (*S*) genes, including *eIF4E*, with genome-editing technology to confer disease resistance in plants, see Zaidi et al. (2018). In 2021, Pramanik et al. used CRISPR-Cas9-technology to generate pathogen-resistant tomato plant against *Tomato Yellow Leaf Curl Virus* and Powdery Mildew (Pramanik et al. 2021).

Except fungi, bacteria, and virus, parasitic weeds are another source to cause plant biotic stress. For example, Witchweed (*Striga hermonthica* [Delile] Benth) is a parasitic weed on Sorghum (*Sorghum bicolor* [L.] Moench). The grain yield of sorghum can be adversely affected by Striga plants (Nzioki et al. 2016).

#### 32.5.21.3 Some Concerns of Using Genome-editing Tools for Generating Disease Resistance in Plants

##### Could Generate Unwanted Virus Resistance

Positive results establish the potential for genome-editing tools to develop virus-resistant crops. However, one should bear in mind that genome-editing technology can confer resistance to virus strains. Researchers tried to use CRISPR-Cas9 interference to generate a ‘designer plant’ conferring resistance to *African cassava mosaic virus* (ACMV) (*Begomovirus*; *Geminiviridae*) and transformed Cassava with CRISPR-Cas9 machinery, to protect Cassava from the virus infection. However, the CRISPR system failed to provide effective resistance to this specific virus (Mehta et al. 2019). The pressure CRISPR-Cas9 applied to the virus applied selective pressure and increased viral resistance to CRISPR. 33–48% of edited viruses in the study developed novel mutation of a conserved single-nucleotide, conferring resistance to CRISPR-Cas9 cleavage.

#### The Pleiotropic Effect and the Trade-offs of Resistance

Use of CRISPR-Cas9 to knock out susceptibility genes, such as *eIF4E*, for disease resistance can be problematic. As susceptibility genes are also essential for plants. Editing *of eIF4E* in lettuce results in resistance to *Lettuce mosaic potyvirus*, but it negatively affects development of the edited plant (Abdul-Razzak et al. 2009). Huibers et al. (2013) also found that although resistance to *O. neolycopersici* was established in *SlDMR1*-silenced tomato (using RNAi), plant growth was severely reduced. In another study, disruption of *OsSEC3A* by CRISPR-Cas9 in rice resulted in enhanced resistance to fungal pathogen *Magnaporthe oryzae*, but edited plants developed a dwarf phenotype. Main roots of *Ossec3a* plants were also reduced (Ma et al. 2018). Thus, an understanding of pleiotropic effects of resistance loci is critical for crop improvement (Nelson et al. 2018).

### 32.5.22 Utility of CRISPR Systems in Tolerating Plant Abiotic Stress

Differing abiotic stressors, including salinity, drought, heat, flooding, radiation, and metal toxicity, hinder agricultural production. Understanding molecular mechanisms underlying plant response to environmental stressors has been a primary emphasis of investigation in past decades (Jain 2015). Although genome editing by CRISPR-Cas9 tool has been successful (Puchta and Fauser 2014; Bortesi and Fischer 2015; Zhang et al. 2016a), advances in applicability and selectivity are still needed for the system to be applicable widely in various plant species (Osakabe et al. 2016). For instance, use of CRISPR-Cas9 to generate stress tolerant crops garnered little focus at the launch of this technology (Jain 2015). Only a handful of reports have been published to determine if CRISPR-Cas technology can address robustness and adaptability for abiotic stress tolerance in crop plants (Mushtaq et al. 2018).

#### 32.5.22.1 Drought Tolerance

Osakabe et al. (2016) worked on abiotic stress-responsive factor in *Arabidopsis* with CRISPR system and truncated sgRNAs (tru-sgRNAs) for study. *OPEN STOMATA 2 (OST2) (AHA1)* gene codes for a major plasma membrane *Arabidopsis* $H^+$-ATPase involved with stomata response. The rate of stomata closing in mutant plants was higher than in the wild type. A lower rate of transpirational water loss in mutant plants was the result, indicating enhancement of stomatal response in edited plants

(Osakabe et al. 2016). *OsSAPK2* may be the primary mediator of ABA signalling in rice, making it a target for developing drought-stress resistance. Expression levels of *OsSAPK2* gene are strongly upregulated under conditions of high salinity, drought, and polyethylene glycol treatments. Functional properties of *osmotic stress abscisic acid activated protein kinase 2* (*OsSAPK2*) gene was elucidated through the loss-of-function mutants produced with CRISPR-Cas9 system in rice (Lou et al. 2017). The *sapk2* mutants exhibited an ABA-insensitive phenotype and were more sensitive to drought stress and reactive oxygen species (ROS) than wild-type plants. These results indicate *SAPK2* gene has a crucial role in drought-stress response in rice (Lou et al. 2017). The negative regulatory gene, *ARGOS*, enhances ethylene response. Constitutive overexpression of **maize** *ARGOS8* gene has improved grain yield under drought stress conditions. Breeding stock of ~400 maize inbreds have been screened for high-level ARGOS8 expression. None of these lines present with *ARGOS8* mRNA expression comparable to the transgenic line. Shi et al. (2017) devised an approach to replace *ARGOS8* native promoter with a native maize GOS2 promoter through CRISPR-Cas9 technology. GOS2 is a moderate constitutive promoter. *ARGOS8* variants enhanced the maize grain yield under severe, drought-stress conditions in the field. Variants also showed maximum seed production of five bushels per hectare under flowering-stress conditions and revealed no yield loss under well-watered conditions (Shi et al. 2017). Drought treatment stimulated upregulation of both genes, *dehydration responsive element binding protein 2* (*TaDREB2*) and *ethylene responsive factor 3* (*TaERF3*). CRISPR-Cas9 system successfully targeted *TaDREB2* and *TaERF3* in **wheat** protoplasts with transient expression of sgRNA and Cas9 protein (Kim et al. 2018), providing an efficient system for wheat gene study. However, authors did not culture edited protoplasts into plants for further analysis of their involvement in drought stress. *Mitogen activated protein kinase 3* (*MAPK3*) gene was induced by drought stress in **tomato** (*Solanum lycopersicum*). Wang et al. (2017c) used CRISPR-Cas9 system to generate *SlMAPK3* mutants, *Slmapk3*, for examination of gene function and heritability of mutations. With three mutant lines, the resulting $T_1$ plants and wild type were analyzed for drought tolerance. The mutant lines showed more severe leaf wilting and more bent stems under drought condition (Wang et al. 2017c). Higher proline, malondialdehyde and hydrogen peroxide content were also observed in mutants under severe drought stress compared to wild type plants, which revealed fewer symptoms under identical conditions (Wang et al. 2017c). Results indicate SlMAPK3 mutagenesis led to reduced drought tolerance in tomato. A similar study was also conducted on **tomato** *NPR1* gene. Plant gene *NPR1*, *non-expressor of pathogenesis-related gene 1*, is clearly a defensive response to pathogen. It is considered as a vital part of systemic acquired resistance (SAR). However, its role in abiotic stress is still not clear, although a variety of related *cis*-elements are located at tomato *NPR1* promoter. Li et al. (2019a) generated tomato *NPR1* (*SlNPR1*) mutants using CRISPR-Cas9 technology to study the role of the gene in drought-stress response. Their results showed *slnpr1* mutants exhibited reduced drought tolerance with increased stomatal aperture, higher electrolytic leakage, malondialdehyde (MDA), and hydrogen peroxide ($H_2O_2$) levels, and lower activity levels of antioxidant enzymes. Compared to wild-type plants, *SlNPR1* indicates involvement in the regulation of tomato plant drought response (Li et al. 2019a).

#### 32.5.22.2 Salinity Tolerance

*Zhang et al.* (2019) *used CRISPR-Cas9 to mutate one salt-related gene, OsRR22. OsRR22* encodes a 696-amino acid B-type response regulator transcription factor involved in both cytokinin signal transduction and metabolism. Loss of gene function significantly increases plant salt tolerance (Zhang et al. 2019). Results from significant difference analysis of shoot fresh weight, shoot dry weight, and plant height indicate T2 homozygous mutant lines were significantly enhanced in salinity tolerance compared to wild-type plants (Zhang et al. 2019).

The key enzyme in ABA biosynthetic pathway is *9-cis-epoxycarotenoid dioxygenase* (*NCED*). Rice *NCED* gene, *OsNCED3,* is highly induced under NaCl, PEG, and $H_2O_2$ stress. Overexpression of rice *OsNCED3* enhanced drought-stress tolerance. Huang et al. (2018) used CRISPR-Cas9 to mutate *OsNCED3* gene and studied its function. *nced3* mutant plants had increased sensitivity to drought stress and $H_2O_2$ stress and increased stomata aperture under drought, indicating *OsNCED3* is a gene related to abiotic stress tolerance (Huang et al. 2018).

#### 32.5.22.3 Cold Tolerance

Nawaz et al. (2019) used CRISPR to produce knockouts of *OsPRP1*. This gene encodes a proline-rich protein that confers enhanced cold sensitivity to rice in seedling stage. *OsPRP1* mutant plants displayed more sensitivity to cold stress, showed low survival rates, and decreased root biomass versus wild-type (Nawaz et al. 2019). Mutant lines demonstrated less antioxidant enzyme (superoxide dismutase [SOD], peroxidase [POD], and catalase [CAT]) activity and lower levels of proline under cold stress. Researchers hypothesized *OsPRP1* enhances cold tolerance by modulating antioxidants and maintaining cross talk through signalling pathways (Nawaz et al. 2019).

### 32.5.23 Utility of CRISPR Systems for Food Safety

Cadmium (Cd) is a highly toxic heavy metal for humans. Once absorbed, it is retained throughout life (Bernard 2008). Rice is an important staple food for many countries, particularly in Asia. Unfortunately, rice contaminated with cadmium is frequently reported in this area. Production of elite rice varieties containing less cadmium is a challenging task using conventional breeding methods, and new approaches are urgently needed (Tang et al. 2017a). Tang et al. (2017a) devised *Indica* rice lines with low cadmium accumulation (<0.05 mg/kg) by CRISPR knockout of metal transporter gene, *natural resistance associated macrophage protein* (*Nramp5*). This was accomplished without introducing transgenes (Tang et al. 2017a). Non-edited wild-type *Indica* line can accumulate cadmium from 0.33 to 2.90 mg/kg. The good news is editing did not affect rice yield. Yang et al. (2019)

used CRISPR-Cas9 to edit *OsNRAMP5* gene and reduce cadmium (Cd) in *japonica* rice grains.

Liu et al. (2019e) did similar experiments, knocking out *OsLCT1*, a rice homolog of wheat low affinity cation transporter 1 and *OsNramp5* (natural resistance-associated macrophage protein 5). *OsLCT1* gene might be involved in translocation of Cd. *OsNramp5* is a major transporter of Cd into the root stele. Authors observed one *OsLCT1* knockout line contained approximately 40% (0.17 mg/kg) Cd concentration compared to wild-type parental line. This result is less than the current China National Food Safety Standard (0.20 mg/kg). *OsNramp5* knockout lines showed low Cd accumulation (<0.06 mg/kg) in grain when paddy grown at approximately 0.9 mg/kg Cd (Liu et al. 2019e). Of the two *OsNramp5* mutants evaluated, only one had normal growth and yield. The growth of the other line was severely impaired. Another group knocked out *OsNramp5* in rice Wang et al. (2019b). They found edited lines not only significantly reduced Cd accumulation in grains of mutants but also negatively affected rice yield and quality.

Food allergies can cause mild to severe reactions. Severe food allergies can be life-threatening, such as anaphylaxis (U.S. Food and Drug Administration). Scientists have been using biotechnology to decrease or remove plant-derived allergens in food crops through downregulation of allergen-producing genes. For example, RNAi technology was used to reduce allergen '**Ara h 2**' in peanuts (Dodo et al. 2008), allergen '**Lyc e 3**' in tomato (Le et al. 2006), and allergen '**Mal d 1**' in apples (Krath et al. 2009). However, RNAi 'knock-down' technology allows small amount of allergen to be produced. With CRISPR-Cas technology, allergen-producing genes can be completely knocked out, resulting in zero allergen foods. Gluten proteins from wheat, barley, and rye are an allergen for gluten-intolerant individuals with Coeliac disease, an autoimmune disorder. Recently, Sánchez-León et al. (2018) used CRISPR-Cas9 system to target α-gliadin genes. One edited line has knocked out 35 of 45 different genes native to wild type. The α-gliadins encompass approximately 100 genes and pseudogenes in bread wheat. Knock outs reduced immune-reactivity by 85% (Sánchez-León et al. 2018).

### 32.5.24 Utility of CRISPR System for Crop De Novo Domestication

Not only are CRISPR systems editing existing elite-crop cultivars for better quality, yield, and other purposes, scientists are also testing the idea of introducing commercially desirable traits. Starting with wild or ancestral relatives of modern crops broadens the base of genetic diversity. Wild relatives possess genes which do not exist in modern varieties, due to millennia of selective breeding. Long-term selective breeding has led to reduced genetic diversity in modern elite lines. Some beneficial traits of wild species, such as disease resistance or stress tolerance, can be lost during the progress of intense selection. A novel breeding approach dubbed as "*de novo* domestication," "Re-domestication," or "Domestication 2.0" is using to re-domesticate valuable crops from wild species.

Zsogon et al. (2018) edited six loci important for yield and productivity with CRISPR-Cas9 system in wild *Solanum pimpinellifolium* for de novo domestication. The morphology, size, number, and nutritional value of engineered fruits of *S. pimpinellifolium* were enhanced. CRISPR lines had a threefold increase in fruit size, a tenfold increase in fruit number, and lycopene content increased by 500% compared with frequently cultivated *S. lycopersicum* (Zsogon et al. 2018). In another report, Li et al. (2018b) attempted re-domestication of wild tomato by introduction of commercially valuable traits. The goal was to retain drought resistance in wild tomato plants, to better withstand effects of climate change.

## 32.6 CRISPR-Based Novel Tools (Beyond Genome-editing)

### 32.6.1 CRISPR-Based Imaging Tools

Imaging techniques of multiple genomic loci in living cells can enhance our ability to investigate the dynamics of chromosomes. Imaging studies provide spatial and temporal information of a defined genomic region, both during development and environment change. The ability to visualize chromosomal dynamics is essential for understanding many fundamental intra-nuclear processes (Chen et al. 2013; Shao et al. 2016; Ma et al. 2016). Chen et al. (2013) first reported adapting CRISPR-Cas9 system for imaging in living human cells, providing a robust method for tracking dynamics of both repetitive and non-repetitive genomic sites.

Shao et al. (2016) later devised a dual-color imaging system for tracking genomic elements based on CRISPR-Cas9. Labelling chromosomal loci with multiple colors allows spatial resolution. One example would be for observation of translocations induced by DSBs (Shao et al. 2016). The system enables imaging of repetitive elements of both telomeres and centromeres simultaneously. Due to system photobleaching tolerance, a genomic element can be dynamically traced for a longer term than previously possible (Shao et al. 2016).

*CRISPRainbow* is an advanced tool for labelling DNA in living cells based on Cas9 variant, dCas9, and a number of different protein-binding sgRNAs (Ma et al. 2016). Authors visualized up to six chromosomal loci simultaneously in individual live cells and documented large differences in the dynamic properties of different chromosomal loci (Ma et al. 2016). Similarly, a new altered sgRNA method has been developed to recognize dual-color, chromosomal loci imaging (Shao et al. 2016).

In plants, CRISPR systems were also used to image plant cell components. Dreissig et al. (2017) used fluorescence proteins (eGFP or mRuby2) fused to dCas9 (derived from *S. pyogenes* (SpCas9) and *S. aureus* (SaCas9)) to visualize telomere repeats in live leaf cells of tobacco (*Nicotiana benthamiana* L.). This method traced telomere movement of 2 μm over 30 min during interphase (Dreissig et al. 2017).

### 32.6.2 Regulatory Switch for Gene Transcription

CRISPR activation (CRISPRa) and CRISPR interference (CRISPRi) together can regulate transcript levels of endogenous genes over various orders of magnitude (Gilbert et al. 2014; Kampmann 2018). CRISPRa uses dCas9 fusion proteins for targeted gene activation through multiple transcription activators with one sgRNA (Dominguez et al. 2016). Previous applications of CRISPRa used simple fusions of dCas9 to an activator domain, most frequently VP64, but only moderate activation of the targeted gene was obtained with one sgRNA (Kampmann 2018).

CRISPRi is a genetic disruption method providing a sequence-specific repression of gene expression in prokaryotic and eukaryotic cells (Qi et al. 2013; Larson et al. 2013). Binding of dCas9 and sgRNA complex sterically to the promoter sequence hinders interconnection among key transcription factors *cis*- and *trans*-acting DNA motifs, leading to repression of transcription initiation (Larson et al. 2013). Unlike stable genetic modifications induced with nuclease Cas9, gene repression by CRISPRi is reversible. Further research is necessary to develop a more effective method to curb gene expression on a genome-wide scale (Qi et al. 2013; Dominguez et al. 2016). Although CRISPRa and CRISPRi are highly gene-specific, more studies are required to better understand the causes and effects of off-target binding. It will need to be strategically minimized in future applications (Dominguez et al. 2016).

CRISPRi is very potent in suppressing bacterial genes using dCas9. It is highly specific with few off-target editing and is multiplexable (Dominguez et al. 2016). CRISPRi has also demonstrated simultaneous and efficient repression of gene expression in multiple *E. coli* genes with no distinguishable off-target effects (Qi et al. 2013). CRISPRi technology has been applied to microalgae *Chlamydomonas reinhardtii* to enhance lipid production for potential biofuel production (Kao and Ng 2017). For mammalian research, CRISPRi-based technology has been implemented for genome-scale identification of functional long noncoding RNA loci in human cells (Liu et al. 2017). CRISPRi facilitates mechanistic studies of long-noncoding RNA functions and assists global exploration of the long-noncoding-RNA-expressing genome (Liu et al. 2017). Although CRISPRa and CRISPRi have been widely used for mammalian studies, and epigenetic reprogramming in plants is the ability to accomplish histone and DNA modifications and has the potential for novel effects on plant breeding (López-Calleja et al. 2019), CRISPRa/CRISPRi research on higher plants is still lacking. For a review on CRISPRa and CRISPRi and other CRISPR/dCas9 platform-based technologies, see recent paper by Moradpour and Abdulah (2020).

## 32.7 Concerns of Using CRISPR Technology

Target specificity is a foremost concern for all genome-editing tools, including CRISPR-Cas9 technology. Off-targeting can be highly problematic in plant reverse genetic (Hahn and Nekrasov 2019). It causes unintended changes in the genome, leading to potential undesired phenotypic changes in genome-edited crops. Rare, low, and high-frequency off-target mutagenesis induced by CRISPR-Cas9 has been reported in plants (Wolt et al. 2016; Zhang et al. 2018a; Li et al. 2018a; Li et al. 2019b). Shan et al. (2013) evaluated potential off-target effects of two Cas9-sgRNA constructs intended for targeting only *OsMPK2* or *OsPDS* in rice (*O. sativa*) and wheat (*T. aestivum*). They have found these sgRNA constructs were off-targeting to a nearly identical sequence, MPK2_NI-1 (Shan et al. 2013). Their result suggests off-target cleavage can occur in homologous sequences. Comprehensive studies exploiting genome-wide approaches, such as whole genome sequencing (WGS), are necessary to thoroughly address off-target issues concerning Cas9-sgRNA system (Shan et al. 2013).

Recently, a number of studies have thoroughly examined the specificity of CRISPR-Cas system in plants (mentioned in Sect. 32.1). Studies have demonstrated the 3′ end of the guide sequence within the sgRNA confers target specificity (Jiang et al. 2013; Belhaj et al. 2013). The off-target effect of an engineered sgRNA was established on an improperly paired genomic site with lesser genome-editing efficiency than the perfectly matched site. Xie and Yang (2013) proposed specific sgRNAs were expected to target more than 90% genes in rice (*O. sativa*). Young et al. (2019) also demonstrated high frequency (up to 90%) on-target editing with no evidence of off-target cleavage activity when sgRNAs were bioinformatically predicted to be specific. Later on, a basic method has been reported to manually design a shared sgRNA target site-specific for multiple homologous target genes by aligning coding sequences and carrying out a BLAST search to assess off-target potentials in model plants *A. thaliana* and *N. benthamiana* (Li et al. 2013).

To minimize off-target editing potential, a variety of approaches have been developed (Hahn and Nekrasov 2019). These include (1) using different Cas; (2) engineering of Cas9 protein. Vakulskas et al. (2018) engineered high-fidelity (HiFi) Cas9, which retained the high on-target activity of Cas9 while reducing off-target editing by introducing a single point mutation in the original Cas9. (3) Genome editing using ribonucleoprotein (RNP) instead of plasmids; (4) computational identification and selection of Cas9 target sites with low potential for off-target editing; and (5) empirical assessment of target specificity using biochemical and cellular assays. Young et al. (2019) also found off-target events can be minimized by designing sgRNAs from a target differing from other genomic locations by at least three mismatches (bases) combined with at least one mismatch base in the PAM proximal region. More recently, researchers have increased the specificity of CRISPR-Cas9 system by engineering a hairpin secondary structure of sgRNAs (hp-sgRNAs) (Kocak et al. 2019). This approach increases specificity by several orders of magnitude.

## 32.8 Regulation of CRISPR-Edited Crops

Although genome-editing crops possess various benefits over conventional transgenic crops and conventional breeding (Lassoued et al. 2019), crop regulated worldwide remains unclear. In general, two approaches of regulation exists:

(1) process-based regulation (Ex. European Union and Brazil) that consider techniques used to develop new crops; and (2) product-based regulation (Ex. United States and Canada) that focus mainly on final product risk. Since genetic changes introduced by GE tools are precise, researchers advocate 'product-based' regulation for genome-edited crops. Huang et al. (2016) have recommended five preliminary principles for regulation of genome-edited crops. Steps include: minimize risk of escape for genome-edited crops from labs or fields, demonstrate absence of foreign sequences in edited crops, document DNA sequence changes at target sites, and ensure primary target site integrity—absence of unintended secondary editing events and no off-target events (Huang et al. 2016). Stringent regulation will in turn affect development costs and delay commercialization of GE crops. With rigid regulatory proceedings, a minimum of 6 years and $30 to $50 million will be needed to bring a crop to market (Waltz 2018). This imposes a high barrier to development of genome-edited food crops. Public acceptance of genome-engineered crop plants also plays a critical role for regulatory agencies. It is necessary for the scientific community to accurately convey the benefits and significance of genome-editing technologies, so the general public can understand novel advancements in crop improvement.

Considerable progress is being made by regulatory authorities worldwide to devise sensible and pragmatic regulations for genome-edited crops. In March 2018, the United States Department of Agriculture (USDA) stated no further regulations will be imposed on certain categories of crops developed through genome-editing techniques (USDA-APHIS Press Releases 2018/3/28). It is important to note that USDA-APHIS had already approved the non-browning mushroom developed through CRISPR-Cas9-based genome-editing technology (Waltz 2016). This is a great relief for many GM crop companies, especially small companies (*Nature Plants* Editorial). However, this does not apply to genome-edited food animals. The U.S. Food and Drug Administration (FDA) has proposed new mandatory premarket animal drug regulatory evaluation for all food animals, whose genomes have been intentionally altered using modern molecular technologies—this includes gene editing technologies (Van Eenennaam et al. 2019).

In contrast, Court of Justice of the European Union has recently ruled (Case C-528/16) crops created with gene-editing techniques will be regulated with existing laws governing conventional GMO (Callaway 2018). The decision disappointed many researchers and biotechnology companies from the area. This might trigger relocation of R & D departments of biotech companies to other regions. In the USA, CRISPR-Cas crops are not covered by tough GMO regulation. Plant breeders can develop and market new varieties much faster in areas without these regulations (https://resource.wur.nl/nl.htm). Australian gene-editing rules adopt a 'middle ground', where technology is allowed to edit plant, animal, and human cells without government approval/regulation as long as 'breaks' are naturally repaired without the introduction of new DNA (Mallapaty 2019).

## 32.9 Concluding Remarks

Environmental changes and plant co-evolution with a wide range of pathogens have resulted in a diverse and advanced plant immune response, in which certain microbes have improved their anti-defense strategies. Since staple crops are sessile, they are often exposed to abiotic stress, resulting in significant yield reductions in conventional crops, placing global crop supply at high risk. Several gene editing techniques and plant breeding methods have traditionally been used to establish stress-tolerant crop varieties. The evolution of genome editing has accelerated this race, hastening the production of the next generation of crops that will support an increase in population.

New breeding strategies, such as genome editing, allow researchers to accurately and rapidly modify a single gene for desirable qualities, as opposed to the complicated, inconsistent, and time-consuming traditional breeding methods. CRISPR-Cas-based genome editing is a revolutionary breakthrough technology for them. The ease of use, low cost, precise positioning modification of genomes, DNA, and RNA, and high throughput of various integrated CRISPR systems have led to its rapid adoption as an effective tool for genetic manipulation in a wide range of plants. CRISPR-Cas technology is often used by scientists to establish genetic diversity, improve crop production, nutritional benefits, disease tolerance, and fight biotic and abiotic stresses. The discovery of various Cas9 orthologs and engineered novel Cas9 versions increases the versatility and efficiency of the CRISPR editor system to accommodate multiple breeding programs.

CRISPR is not only a framework for gene delivery and genetic modification but it is also a modern molecular technique for targeting identified transcription factors (for example, protein, RNA, or DNA) to particular genomic and transcriptomic loci, enabling CRISPR to be repurposed for a wide range of applications. This involves visualizing various regions of DNA and RNA by acquiring fluorescent proteins, activating or repressing gene expression, and creating diversity in order to advance the growth of new germplasm and sometimes even new crops. In the face of rapid urbanization, widespread hunger, and environmental issues, the technology would be crucial in resolving agricultural, financial, and ecological challenges, while fostering sustainable agriculture. Though CRISPR is a relatively quick and efficient method, it is not without drawbacks that may result in incorrect consequences. Despite the fact that many techniques have been applied to maximize on-target performance, the majority of work done is still experimental and needs further progress. Furthermore, the rules governing CRISPR products for health safety are also unclear in the majority of the countries. The government should establish a consistent policy on NBT-derived commodities. Consumer adoption and media participation will both play significant roles in deciding when and how NBTs will be produced and used in farmland.

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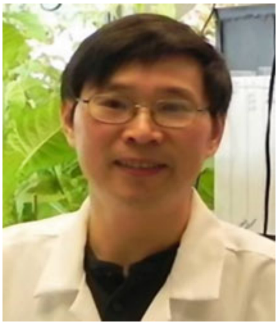

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# Recent Reductive Transformation from Lignin Derivatives to Aliphatic Hydrocarbons

# 33

Yumi Katayama and Yoshiharu Mitoma

**Abstract**

Cellulose and its derivatives, as main components of woody biomass, which are expected to be alternative materials to petroleum, are applied widely to the polymer chemical industry. Although lignin and its derivatives are also anticipated as new hydrocarbon alternatives, why would it not be used as an actual alternative? The answer is that polymer industrial applications of lignin and its derivatives are confronting several important challenges. First, lignin is a complex mixture. For that reason, extracting large amounts of simple and uniform chemical raw materials from wood is difficult. Moreover, extracts from lignin are often colored, which impedes industrial applications. Against this background, the reduction reaction of lignin is a key technology that is highly anticipated for the development of highly functional structures from the intrinsic aromatics. This section introduces some recent and reality-based reduction reactions of lignin under mild conditions.

**Keywords**

Lignin · Lignophenol · Hydrogenation · Reduction · Calcium

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## 33.1 Introduction

Interpreting the reduction of lignin derivates in a broad sense, lignin reduction reactions are of a “hydrogenolysis process,” by which a carbon-carbon or carbon-heteroatom single bond reacts with hydrogen to break itself, or of a “hydrogenation process,” by which an unsaturated bond transforms to the corresponding aliphatic hydrocarbon. For example, when hydrogenolysis of lignin derivatives is the main reaction, one can produce high yields of monomers, dimers, and/or oligomers without secondary condensation or polymerization reactions. For that reason, hydrogenolysis has become a powerful tool for elucidating the properties and binding modes of the constituent units of lignin derivatives. Furthermore, the analysis and separation of reduced products from lignin has dramatically improved in recent years. The chemical structures of most of the products have been clarified. Hydrogenolysis, as a mode of reduction, has played an immeasurably important role in the research of lignin at various stages. Moreover, the role of reduction is also important as a technique for converting a lignin derivative into commercially useful substances. When hydrogenolysis is the main reaction for reduction of lignin, phenol derivatives are obtained. When hydrogenation is also conducted in parallel, a ring-reduced product in which an aromatic ring is hydrogenated can also be obtained. However, to date, no case has been set to practical use on an industrial scale because the lignin derivatives are amorphous polyphenols. In addition, because the compositions are not uniform, conventional catalyst utilization is done without the development of a catalyst that is sufficiently adapted to the lignin reduction system and without the development of studies of a new utilization method.

Along with such circumstances, the sense of crisis related to exhaustion of petroleum resources has heightened further. Expectations for the use of lignin derivatives as materials are increasing because it represents a useful alternative resource that can be used even when oil is exhausted or when no effective alternative exists to petroleum as a raw material. In recent years, the development of bioethanol was hastily put forth as a solution to resource and energy problems, but one must recall that the development of bioethanol linked to food and feed resources caused food prices to soar. Naturally, it is necessary to establish a highly efficient bioethanol production method from sources other than food and feed resources too, but expectations for the development of lignin derivatives are increasing apace. Therefore, an increasing need exists to develop an effective hydrogenation method for future “resource recycling”: if it becomes possible to synthesize petroleum-like paraffins through hydrogenation, it will become possible to divert many excellent petroleum-reforming methods to synthesize useful materials. As examples, C6, C7, and C8 paraffins in the gasoline fraction might be reformed into their corresponding aromatic hydrocarbons (BTX: benzene (B), toluene (T), xylene (X)) of the same carbon number. In such processes, petroleum-derived sulfur is a catalyst poison. If petroleum-like paraffin is obtainable from a lignin derivative, then not only can it be used as a material, but also the problem of catalyst poisons can be avoided, leading to resource conservation by the use of rare metal catalysts.

Based on the background presented above, we introduce the latest technology for reducing the aromatic ring portion of lignin derivatives, aiming to induce it into the BTX line, which is synonymous with petrochemistry.

## 33.2 Conventional and Recent Reduction of Lignin Derivates

As described above, "hydrogenolysis" and "hydrogenation" are defined by particularly addressing the bond cleavage and hydrogen-addition mode. Severe reaction conditions such as high pressure and/or temperature were proposed in this cleavage reaction. Such conditions are barriers to practical use because of the formation of many complex mixtures (Yamaguchi et al. 2019a, b). Generally, hydrogenation occurs simultaneously with hydrogenolysis or is included in the hydrogenolysis process, hydrodeoxygenation (HDO), and other upgrading methods. Under these circumstances, hydrogenation has milder reaction conditions than those of hydrogenolysis. Hydrogenation of aromatic rings of lignin derivatives to investigate a realistic treatment method is examined specifically in this chapter. The notable reaction mechanism is hydrogenation of the aromatic ring portion of the lignin derivative. In these categories of hydrogen use, the reducing agents are classifiable roughly into the following three types: hydrogenation using hydrogen gas (hydrogenation), hydride reduction using hydrogen anions (hydride) as a hydrogen source, and electron transfer reduction, by which electrons generated from an electrode or a metal serve as a driving force. However, when the reduction reaction proceeds because of electron transfer, addition of a hydrogen source is required. Therefore hydrogenation follows. Furthermore, in lignin reduction, because the reaction solvent or the reducing agent and the lignin derivative must come into sufficient mutual contact, a hydrophilic aqueous solvent is often used. Therefore, it is unsuitable for hydride reduction, which cannot work in water. In fact, no reports describe hydride reduction of lignin derivatives. The hydrogenation and electron transfer reduction modes that are used frequently for lignin reduction are introduced hereinafter.

### 33.2.1 Hydrogenation of Lignin

Because hydrogenation usually requires a catalyst, it is called catalytic hydrogenation. A homogeneous reaction in which the catalyst dissolves in the system and a heterogeneous reaction in which the catalyst does not dissolve are known. In catalytic hydrogenation, hydrogen gas adsorbed onto the surface of the noble metal catalyst becomes atomic hydrogen (or hydrogen at the nascent stage) to generate a reducing power. Therefore, for catalytic hydrogenation, it is necessary to generate atomic hydrogen effectively and perform the process of transferring the atomic hydrogen to the reduced site of the lignin derivative efficiently. Various catalysts are used in hopes of promoting these actions.

#### 33.2.1.1 Hydrogenation Using Heterogeneous Catalyst

When aiming to construct a process on an industrial scale in the future, such as catalytic hydrogenation of lignin derivatives, reusing an expensive catalyst, will engender an important cost reduction. Typical heterogeneous catalysts such as zirconia-supported mono- and bimetallic noble metal (Rh, Pd, Pt) (Gutierrez et al. 2009), Pd/C + $H_3PO_4$ (Zhao et al. 2009; Zhao et al. 2011), Pt-Rh/$ZrO_2$ (Lin et al. 2011), Pt/$Al_2O_3$ (Runnebaum et al. 2012), Pt/ZSM-5 (Wang et al. 2012), Ru/HZSM-5 (Zhang et al. 2014a), Raney Ni + Nafion/$SiO_2$ (Zhao et al. 2010), or Ni/$SiO_2$-$ZrO_2$ (Zhang et al. 2014b) in acid and their nanoparticles (NPs) (Yan et al. 2010) were reported.

A method using a copper-chromium oxide catalyst was known in an early stage of development. In the presence of a catalyst, a lignin derivative derived from aspen is added to dioxane. Then *ca.* 20–35 MPa hydrogen gas is charged and subsequently heated from 250 °C to 260 °C for about 1 h. After that, the internal pressure is adjusted to 5 MPa. The total reaction time was set to 18–22 h to obtain a ring-reduced compound (Harris et al. 1938). The obtained ring-reduced products were 4-n-propylcyclohexanol, 3-(4-hydroxycyclohexyl)-propanol, and 4-n-propylcyclohexane-1,2-diol. The combined yield of these products reached 44%. After that, a method using copper-chromium oxide catalyst was improved as a method for obtaining a dimer (condition: treatment at 220 °C to 240 °C for 1 h under 7.85 MPa of hydrogen gas) (Matsukura and Sakakibara 1973). It was never used for catalytic hydrogenation of the moiety.

Raney nickel alloys are used for catalytic hydrogenation of aromatic rings of lignin derivatives. Raney nickel alloy is an alloy of nickel and aluminum in equal amounts. Immediately before use as a catalyst, aluminum in the alloy is dissolved using an acid or alkali aqueous solution. The porous nickel with increased surface area is used as a catalyst (Raney nickel catalyst). A typical reaction condition (Pepper and Lee 1970) is the following. They are to add a lignin derivative derived from a spruce material to an alkaline aqueous solution in the presence of a Raney nickel catalyst, heat it from 160 °C to 220 °C, fill it with 3.45 MPa of hydrogen gas, and react for 1.3–24 h. The results are presented in Table 33.1. Chromatographic analysis showed that a maximum of 56.68% of ring-reduced products such as cyclohexanol derivatives was achieved. A reaction mechanism (Parker et al. 1966) that occurs in the presence of Raney nickel catalyst has also been proposed. Presumably, the main reaction pathway is triggered by the hydrogen abstraction

**Table 33.1** GC/LC analysis of ethanol-soluble spruce after catalytic hydrogenation

| | [a]Abundance of chromatographable products/% | | | | | | |
|---|---|---|---|---|---|---|---|
| Catalyst | **1** | **2** | **3** | **4** | **5** | Others | Recovered lignin |
| Raney Ni | 0.660 | 0.830 | 1.69 | 40.9 | 12.6 | 9.19 | 16.5 |
| Ru/C | 1.56 | 3.49 | 0.320 | 6.95 | 4.52 | 16.0 | 11.8 |
| Ru/Alumina | 0.670 | 1.72 | 1.05 | 19.8 | 12.2 | 20.7 | 14.6 |

[a] **1**: 4-ethylcyclohexanol, **2**: 4-*n*-propylcyclohexanol, **3**: 3-cyclohexyl-1-propanol; **4**: 3-(4-hydroxycyclohexyl)-*n*-propanol, **5**: 3-(4-hydroxy-3-methoxycyclohexyl)-1-propanol

reaction from the phenol part by the alkali to induce the quinoid type, followed by catalytic hydrogenation to obtain the ring-reduced compound.

In recent years, important results have been obtained as many researches which focus on highly efficient hydrogenation under energy-saving conditions have been continuously carried out. In below, we will introduce the important reaction examples. Saffron, Jackson, and co-workers recently shown that Ru/C (Li et al. 2012) and Raney nickel (Lam et al. 2015) were effective cathodic catalysts for electrocatalytic hydrogenation and partial hydrodeoxygenation of phenolic compounds; better results were obtained under mild conditions ($\leq$80 °C and ambient pressure) compared to other reduction methods.

Catalytic reductive fractionation of lignocellulose was accomplished using a heterogeneous cobalt catalyst and formic acid or formate as a hydrogen donor. The catalytic reductive fractionation of untreated birch wood yields mono-phenolic compounds in up to 34 wt% yields of the total lignin, which corresponds to 76% of the theoretical maximum yield. Research of the model compound revealed that the main role of the cobalt catalyst is to stabilize the reactive intermediates formed during organosolv pulping by transfer-hydrogenation and transfer-hydrogenolysis reactions. Additionally, the cobalt catalyst is responsible for depolymerization reactions of lignin fragments through transfer-hydrogenolysis reactions that target the beta-O-4′ bond. The catalyst can be recycled three times with only a negligible decrease in efficiency, demonstrating the system robustness (Rautiainen et al. 2019).

Reforming of lignin to produce high-value chemicals selectively represents an enormous challenge for a biorefinery. 4-alkylphenol formed by breaking the robust Aryl-$OCH_3$ bonds in lignin-derived methoxyl alkylphenols was produced selectively over a $Co_1$-$Fe_{0.1}$ catalyst from real lignin oil as feedstock, which was obtained using a “lignin-first” strategy from either birch or cornstalk. A yield of 64.7 or 88.3 mol% of 4-propylphenol was obtained if birch lignin oil or eugenol was used as the substrate, respectively. Results of catalyst characterization and comparison indicated $CoN_x$ as the main active phase for demethoxylation and hydrogenation. The incorporation of Fe weakens the adsorption of 4-propylphenol to the catalyst, which inhibits the excessive hydrogenation of 4-propylphenol. Results obtained from this work demonstrate the potential to produce high-value-added 4-alkylphenol from renewable raw biomass (Liu et al. 2019).

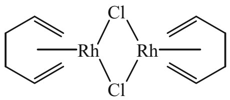
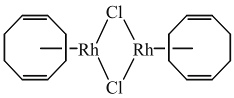
Recently, complete hydrogenation of eugenol, an important biomass-derived molecule, was achieved using ruthenium-containing nickel hydrotalcite (NiRu-HT)-type materials and isopropyl alcohol as a solvent. Some group VIII metals containing bimetallic Ni-HT-type materials were synthesized, characterized, and demonstrated to participate in eugenol conversion. Treatment by Ni-Ru-HT was assigned the highest selectivity toward alkyl cyclohexanol (Sreenavya et al. 2020).

To realize high-value-added utilization of alkali lignin, catalytic hydrogenolysis of alkali lignin was performed with assistance of formic acid (FA) acting as an internal hydrogen donor over the catalyst Ni-$ZrO_2$/γ-$Al_2O_3$ prepared using a chemical reduction. The increased temperature from 180 °C to 240 °C and the added catalyst (0.5 g catalyst/g-lignin) were beneficial for bio-oil yield. The presence of FA was advantageous for lignin conversion. The catalytic abilities were weakened along with the recycling of bio-oil. Furthermore, GC/MS analysis indicated the relative content of vanillin was high (65.65%) at 180 °C, whereas that of alkyl G-type phenols was dominant (around 70%) at 240 °C. Results demonstrated that the activity indicated excellent catalytic selectivity for specific phenolic products with cleavage of C–O–C/C–C bonds in alkali lignin, especially (Lu et al. 2020).

### 33.2.1.2 Homogeneous Hydrogenation

Many interesting examples exist of research using rhodium catalysts. When catalytically hydrogenating a low-molecular-weight lignin derivative model compound such as benzylacetone, 4-propylphenol, or 1,2-dimethoxy-4-propylbenzene, it is possible to obtain a ring-reduced compound under unprecedented milder conditions (Hu et al. 1997). For instance, in the presence of 0.063 mmol of rhodium catalyst (i), 0.40 mmol of tetrabutylammonium hydrogen sulfate and 6.3 mmol of benzylacetone are added to a two-phase reaction solvent of citrate buffer at pH = 7.5 and hexane. Hydrogen gas is bubbled constantly at atmospheric pressure and is subjected to a reduction reaction at 25 °C for a predetermined time to obtain the corresponding 4-cyclohexyl-2-butanone. The relation between the rhodium catalyst and the reaction time in this reaction is presented in Table 33.2. As shown there, the introduction of an aromatic ring into a corresponding aliphatic hydrocarbon under extremely mild conditions has succeeded. The reaction rate decreases as the number of methoxy substitutions on the aromatic ring increases. For example, with 2,6-dimethoxy-4-propylphenol, only 37% of the ring-reduced products are obtainable in 70 h. However, when the hydrogen gas was set to 1.36 MPa, 74% of the ring-reduced compound was obtained in 6 h. Then, the reduction reaction of the milled wood lignin derivative (derived from spruce wood) was conducted for 5 days under the same conditions using a $Rh_6(\eta^3\text{-}O)_4(OH)_{12}(DMF)_n$ catalyst (Hu et al. 1999). Comparison of the $^1H$ NMR spectra obtained before and after the treatment shows that partial reduction occurred because signals at $\delta = 2.08–1.42$, 1.24, and 0.86 were the hydroxyl and methoxy groups of the hydrogenated guaiacyl derivative. It was readily concluded, however, that the proton signal of the aromatic ring portion remained strongly observed.

**Table 33.2** Reaction time of lignin in various rhodium catalysts

| |  |  | $RhCl_3{\cdot}3H_2O$ |
|---|---|---|---|
| [a]Catalyst | **I** | **II** | **III** |
| Time | <24 h | 40 h | 64 h |

**I**: di-μ-chloro-bis($\eta^4$-1,5-hexadiene)-di-rhodium, **II**: di-μ-chloro-bis($\eta^4$-1,5-cyclooctadiene)-di-rhodium

### 33.2.2 Electron Transfer Reduction

In the solution, the metal element emits electrons by selection of an appropriate solvent. This solvent serves as a driving force for the reduction reaction. Catalytic hydrogenation occurs sequentially on the metal and the electrode. Herein, we introduce examples of partial reduction of aromatic rings by Birch reduction using metallic sodium and examples of new reactions by electrode.

#### 33.2.2.1 Reduction by Metals

Birch reduction is a useful method for the partial reduction of aromatic rings in organic synthetic reactions. It is effective not only for lignin derivative model compounds but also for catalytic hydrogenation of aromatic rings of original lignin (Pernemalm and Dence 1974) to use Birch reduction under typical reduction conditions. For example, anisole, a veratol derivative, or a diphenyl ether derivative was selected as a model compound; 1 mol of metallic sodium was added to 0.2 mol of a substrate in ammonia. Then the mixture was heated under reflux for 4 h. As a result, a phenol derivative was obtained in addition to the product obtained by adding hydrogen to a part of the aromatic ring. Therefore, it was revealed that about 1% of 2,5-dihydro-4-propyl anisole was produced by catalytic hydrogenation of lignin derivative derived from spruce wood under the same conditions. Consequently, although evidence of hydrogenolysis of the alkyl aryl ether bond in the Birch reduction was obtained, the results were insufficient for the catalytic hydrogenation of the aromatic ring moiety.

The other metals also are powerful for the reduction of lignin derivatives. Zerovalent metals (Al, Fe, Mg, and Zn) were reported to be active for hydrogenation of the C=C or C=O groups of lignin model compounds at room temperature under ambient pressure to obtain the reduced compounds (Liu et al. 2012). These methods are very stable and powerful as reduction for a variety of chemical structure. The obtained compounds which contained as corresponding hydrocarbons and alcohols etc. were identified by GC/MS. However, the conditions require stricter compared with reduction by catalyst like higher temperature, pressure, and longer reaction time.

Nano-sized materials are also powerful for the reduction of aromatic rings of lignin derivatives. A method of simple preparation was developed for magnetically separable nano-size Ru-based catalyst. Structural investigation revealed nano-size catalyst as a hierarchical and multiphased nano-size composite consisting of highly magnetic $Fe_3C$ nanoparticles. The $Fe_3C$ were distributed within a high-surface-area graphitic carbonaceous matrix that was decorated uniformly with highly active catalytic ruthenium as nanoparticles and/or its clusters. The nano-sized catalyst indicated an improved catalytic activity and selectivity for the hydro-deoxygenation of bio-resource-related compound such as eugenol compared to Ru/C. In combination with rapidly magnetic separation, it implied an enhanced potential for renewable resources and recycling of noble metals (Gyergyek et al. 2018).

### 33.2.2.2 Reduction by Electrochemical Method

Reduction of a lignin derivative using a Raney nickel electrode as a cathode for electrolysis was reported recently (Cyr et al. 2000). Finely powdered 45% Raney alloy and 45% nickel powder were mixed with 10% lanthanum phosphate. Then 40 g of this mixed powder was molded at 1000 kg/cm$^2$. The molded body was burned at 800 °C for 4 h under an argon atmosphere. It was then immersed in a 30% NaOH aqueous solution at 75 °C for 24 h to remove the aluminum. Then, the Raney nickel plate was used as a Raney nickel electrode by immersing the Raney nickel plate in a treatment solution prepared by adding trans-cinnamic acid to a 1 M NaOH solution for at least 18 h. As the electrolyte, 30 mL of 1 M NaOH aqueous solution is used. The decomposition temperature was changed from 25 °C to 75 °C. Then 0.75 mmol 1-(4-hydroxy-3-methoxyphenyl)-2-(methoxyphenoxy)-1-ethanol at 5 mA or 20 mA was electrolyzed. Graphite was used as the anode. The product was analyzed using HPLC. The results are presented in Table 33.3.

At a reaction temperature of 25 °C, 6% (Entry A, 5 mA) and 60% (Entry B, 20 mA) of unreacted raw materials were recovered, but the ratio for recovery of raw materials decreased gradually as the reaction temperature was raised from 50 °C and then to 75 °C in a step-by-step manner (Entries C–F). Cyclohexanol, which is the ring-reduced form of the lignin derivative, was produced at 0.2%–4.0% yield, but it could not be the main reaction. As the main products, 14–43% of guaiacol was obtained; α-methyl vanillyl alcohol was as high as 4–41%. Although one reported

**Table 33.3** Electroreduction of 1-(4-hydroxy-3-methoxyphenyl)-2-(methoxyphenoxy-)-1-ethanol in 1 M NaOH aqueous solution with Raney nickel electrode

| | Yield/% ($Q^b$ = 18 F/mol) | | | | | |
|---|---|---|---|---|---|---|
| | $T$ = 25 °C | | $T$ = 50 °C | | $T$ = 75 °C | |
| | $I$= | | $I$= | | $I$= | |
| | 5 mA | 20 mA | 5 mA | 20 mA | 5 mA | 20 mA |
| [a]Products | A | B | C | D | E | F |
| **1** | 6.0 | 60 | [c]nd | 10 | [c]nd | 1.0 |
| **2** | 0.3 | 0 | 2.5 | 0.2 | 4.0 | 2.5 |
| **3** | 2.0 | Traces | 7.0 | 2.0 | 13 | 7.0 |
| **4** | 43 | 14 | 37 | 40 | 24 | 37 |
| **5** | 0.7 | Traces | 8.0 | 2.0 | 24 | 7.0 |
| **6** | Traces | 0 | Traces | Traces | 2.0 | 1.5 |
| **7** | Traces | Traces | 1.2 | 0.6 | 3.0 | 2.0 |
| **8** | 1.3 | 1.6 | 7.0 | 7.0 | 5.0 | 12 |
| **9** | 1.0 | Traces | 6.0 | 3.0 | 2.5 | 3.0 |
| **10** | 41 | 21 | 21 | 31 | 4.0 | 18 |

[a] **1**: Recovery, **2**: cyclohexanol, **3**: phenol, **4**: guaiacol, **5**: 4-ethylphenol, **6**: 4-ethylguaiacol, **7**: 4′-hydroxyacetophenone, **8**: acetovanillone, **9**: 4-(1-hydroxyethyl)phenol, **10**: α-methyl vanillyl alcohol

[b] $Q$ = F/mol

[c] n.d = not detected

example (Zhang et al. 2002) is that of sponge-like lead used as a cathode, catalytic hydrogenation of an aromatic ring portion has progressed only slightly.

An effective method of producing renewable chemicals from corn stover lignin with electrochemical redox between the Cu/Ni-Mo-Co cathode and Pb/$PbO_2$ anode in sodium hydroxide solution was developed. Cyclic voltammetry tests and polarization curves were conducted to assess the electrochemical oxidation performance of Pb/$PbO_2$ anode and electrocatalytic hydrogenation of Cu/Ni-Mo-Co in a lignin alkali solution. Fourier transform IR spectroscopy (FTIR) and high-performance liquid chromatography (HPLC) were used to characterize the functional groups and molecular weight distribution of the lignin residue. Results of GC-MS analysis confirmed that there were compounds of 24 kinds in degradation products of alkali corn stover lignin. Moreover, some valuable chemicals including trans-ferulic acid, vanillin, 3-hydroxy-4-methoxyphenylethanone, syringaldehyde, acetosyringone, and 4-methoxy-3-methyl-phenol were identified (Cai et al. 2018).

## 33.3 Hybrid Reduction by Simultaneous Electron Transfer Reduction and Hydrogenation

As introduced up to this point, no report describes that the catalytic hydrogenation of the aromatic ring portion of the lignin derivative was achieved with sufficient efficiency. However, recent reports describe highly efficient catalytic hydrogenation of aromatic ring moieties of lignin derivatives under unprecedented mild conditions using a new reduction method that combines electron transfer reduction and catalytic hydrogenation.

### 33.3.1 Hybrid Reduction

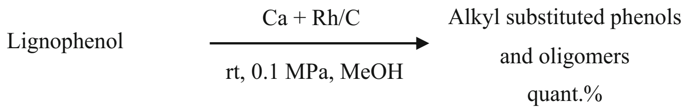
The new hybrid reduction method (Mitoma et al. 2006, 2009a, b) was developed for detoxifying chlorinated aromatic compounds such as dioxin-like compounds under mild conditions. Hybrid refers to a “fusion” technique that combines electron transfer reduction with catalytic hydrogenation. Dioxins are known as very stable compounds and highly toxic compounds. Their detoxification can be achieved by converting chlorine into inorganic chlorine by a dechlorination reaction or by catalytic hydrogenation of the aromatic ring portion to destroy the molecule planarity. Under the sealed condition, in the presence of Rh/C catalyst, reaction with metallic calcium as an electron source and lower alcohol such as methanol as a hydrogen source has been promoted efficiently for a dechlorination reaction and catalytic hydrogenation of the aromatic ring simultaneously. Consequently, highly efficient detoxification of dioxins was achieved perfectly. Because atomic hydrogen generated on the catalyst when metallic calcium with catalyst dissolves in methanol at room temperature, catalytic hydrogenation of the aromatic ring portion proceeds even though the internal pressure is as low as 0.15 MPa. This method is designated as a metal calcium catalyst method.

### 33.3.2 Reduction of Lignophenol by Calcium-Catalytic Reduction

A typical reaction example can be introduced as explained hereinafter (Scheme 33.1). A mixture of lignophenol extracted from 0.10 g of wood powder (Funaoka and Abe 1989), 10 mmol of metallic calcium, 0.10 g of Rh/C catalyst (Rh: 5 wt% supported on activated carbon, no pretreatment), and 5 mL of methanol were added and sealed in a glass pressure-resistant reactor (35 mL total volume). Next, the container itself was stirred for 24 h at room temperature using a swing rotor (60 rpm). Subsequently, a Kiriyama-type funnel was filled with diatomaceous earth and sodium carbonate filter material; the entire contents were put into the funnel. Solid matter and a solution were separated by suction filtration while washing with acetone. Acetone was removed by rotary evaporation under reduced pressure. Then the solvent was removed by vacuum drying (1–2 mmHg) for 24 h. The crude yield was 0.1476 g. In addition, when screening the reaction condition, the reagent was similarly placed in a SUS container equipped with a pressure sensor and a temperature sensor. The entire amount of this sample was transferred to an NMR measuring tube. The reduced state of the signal of the aromatic ring portion was measured by its $^{1}$H NMR spectrum (measuring solvent: $^{2}$H-labelled acetone).

The integrated intensity ratios of the signals obtained from the $^{1}$H NMR spectrum before and after the reaction are shown in Table 33.4. In untreated lignophenol, as presented in Table 33.4, protons adjacent to acetyl appeared near $\delta = 1.90$–$2.36$ ppm; benzylic protons were represented near $\delta = 2.68$–$3.05$ ppm. A proton at the methoxy position was found around 3.26–3.98 ppm. A methylene proton of cinnamic alcohol was found at $\delta = 4.70$–$5.19$ ppm. Also, an aromatic proton was indicated as about $\delta = 6.40$–$7.58$ ppm. Because a proton appearing around $\delta = 6.40$–$7.58$ ppm is bonded to the aromatic ring, the lignophenol clearly has an aromatic ring. All peaks are broadening, which is characteristic of polymers.

In the sample after the reduction treatment, as presented in Table 33.4, the aromatic protons at 6.40–7.58 ppm, which are high in abundance found in the raw material, had disappeared completely after the reaction. That finding indicates that, by this treatment, hydrogen was added to the aromatic ring of lignophenol. Then all the carbon-carbon double bonds in the aromatic ring were transformed single bonds. They were subsequently reduced to aliphatic hydrocarbons. All the aromatic rings have been hydrogenated catalytically, as indicated by the fact that the methylene protons of cinnamon alcohol of around $\delta = 4.70$–$5.19$ ppm in the raw materials disappeared because of the catalytic hydrogenation of aromatic rings.



**Scheme 33.1** Reduction of lignophenol by a mixture of metallic calcium-Rh/C in methanol

**Table 33.4** $^1$H NMR of spectra of lignin before and after calcium-catalytic reduction

| Before the reduction | | | After the reduction | | |
|---|---|---|---|---|---|
| $\delta$/ppm | Type of proton | Integral intensity ratio | $\delta$/ppm | Type of proton | Integral intensity ratio |
| 1.90 ~ 2.36 | Proton of a vicinal acetyl group | 5.1 | 0.85 ~ 0.90 | Aliphatic proton | –[a] |
| | | | 1.18 ~ 1.28 | Aliphatic proton and impurities | 8.30 |
| 2.68 ~ 3.05 | Benzyl proton | 2 | 1.74 ~ 1.91 | Proton of a vicinal olefin | –[a] |
| | | | 2.08 ~ 2.14 | Proton of a vicinal acetyl group | –[a] |
| 3.26 ~ 3.98 | Methoxy proton | 5.6 | 2.60 ~ 2.95 | Proton of a vicinal carbonyl group | 2.33 |
| | | | 3.80 ~ 4.00 | Proton of a vicinal hydroxyl group | 1.00 |
| 4.70 ~ 5.19 | Methylene proton of cinnamyl alcohol | 1 | 4.59 | Proton of a vicinal ester group | –[a] |
| | | | 5.23 | Olefin proton | –[a] |
| 6.40 ~ 7.58 | Aromatic proton | 6.8 | 6.19 | Proton of conjugated olefin | –[a] |

[a] Trace

Furthermore, the signals that were broadening in the $^1$H NMR spectrum of the raw material were shifted into the high magnetic field region around $\delta = 1.18$ ppm to 1.28 ppm and $\delta = 2.60$–2.95 ppm by the catalytic hydrogenation reaction. Moreover, the peaks became drastically sharp. When a low-molecular-weight anisole derivative is treated using this method, the ether bond can be cleaved so that the catalytic hydrogenation of the aromatic ring moiety and the bond between the constituent molecules of lignophenol specifically have hydrogen added to the ether bond. The bond is broken to obtain a hydrogenolysis product of low molecular weight. The strong signals near $\delta = 1.18$ ppm to 1.28 ppm and $\delta = 3.80$ ppm to 4.00 ppm are highly likely to represent methylene and methine protons of the cyclohexanol derivative, which is expected to be a ring-reduced compound.

This mechanism for powerful reduction was also investigated. Hydrogenation is a useful method that employs hydrogen atoms to reduce organic compounds. The double or triple carbon-carbon bonds, as well as double bonds of carbon-oxygen, are saturated during hydrogenation in the final products. The zeta potential of suspension particles in a mixed solution was applied as the principle. The interfacial condition (stern electric double layer) is estimated by the zeta potential. Because different atomic hydrogen-adsorbed and hydrogen-desorbed surfaces on a heterogeneous catalyst influence the zeta potential, the obtained potentials show the atomic hydrogen concentration on the catalyst surface. Consequently, results demonstrated that the dehydrogenation efficiency is linked to the atomic hydrogen concentration on the catalyst (Mitoma et al. 2016).

## 33.4 Conclusion

To achieve highly efficient catalytic hydrogenation of the aromatic ring portion of lignin derivatives, some common factors can be described specifically.

1. Choice of “water or alcohol solution”.
2. Selection of “alkaline solution”.
3. Increasing “atomic hydrogen concentration”.

First, common point 1 should be a water or an alcohol solution because good mixing of the lignin derivative and catalyst in the solvent must occur for all materials to have sufficient co-affinity (Katayama et al. 2019). Next, regarding common point 2, a mechanism by which catalytic hydrogenation of an aromatic ring having a phenol skeleton proceeds after inducing the phenol moiety into a quinoid type has been proposed, as introduced with the nickel catalyst. Considering this point, alkaline conditions are preferred. For common point 3, it is sufficient to increase the amount of atomic hydrogen. Moreover, increasing the molecular hydrogen (apparent hydrogen pressure) is not necessary. In a high-temperature reaction, the dissolved hydrogen concentration is lower according to Henry’s law: high concentrations of atomic hydrogen exist only with difficulty. For this reason, conventional research indicates that catalytic hydrogenation using a Rh homogeneous catalyst gave a small amount of ring-reduced compound at about 0.1 MPa.

Future studies will be conducted to examine lignin reduction in greater detail. A catalyst that is effective for catalytic hydrogenation of aromatic rings will be developed.

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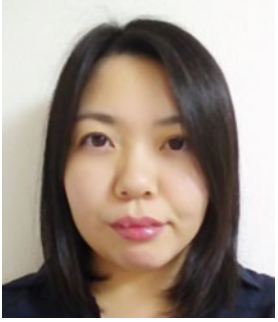

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# Understanding the Environment and Sustainability with Molecular Approaches

# 34

Aditi Nag

## Abstract

In the environment one of the fastest growing areas of biotechnology application is bioremediation. Due to economic cost and its public acceptability, bioremediation for environmental cleanup has become highly popular. Over the past few years, bioremediation has greatly and rapidly advanced because of the advent of molecular techniques in this field since the decade of 1990s. Application of molecular techniques has confirmed that the natural microbial flora and environment is much more diverse than previously observed using traditional culture methods. Nowadays, molecular ecological methods, such as directly isolating DNA from environmental samples, denaturing gradient gel electrophoresis (DGGE), methods based on polymerase chain reaction (PCR), nucleic acid hybridization, etc., can be used so that it can be studied and understood that which microbial consortia could be relevant to contexts of various types of pollutant degradation in the environment. Using these techniques have provided scientists with a means to better understand as well as control the environment along with the related processes, like bioremediation. This chapter covers an outline and applications of few such classic and advanced techniques which are simple yet easily applicable with high effectivity to understand the mechanisms going behind bioremediation processes.

## Keywords

Bioactive molecules · Bioremediation · Molecular markers · NGS · Omics · PCR-based techniques

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## Abbreviations

| | |
|---|---|
| AFLP | Amplified fragment length polymorphism |
| CFCs | Chlorofluorocarbons |
| DGGE | Denaturing gradient gel electrophoresis |
| DNA | Deoxyribonucleic acid |
| DTT | Dithiothreitol |
| ERMs | Environmentally relevant microorganisms |
| FISH | Fluorescence in situ hybridization |
| GMMs | Genetically modified microorganisms |
| GMOs | Genetically modified organisms |
| NGS | Next-generation sequencing |
| PCE | Perchloroethylene |
| PCR | Polymerase chain reaction |
| RAPD | Randomly amplified polymorphic DNA |
| RDT | Recombinant DNA technology |
| RFLP | Restriction fragment length polymorphism |
| RNA | Ribonucleic acid |
| SDS-PGE | Sodium dodecyl sulphate-polyacrylamide gel electrophoresis |
| TCE | Trichloroethylene |
| TNT | 2,4,6-trinitrotoluene |

## 34.1 Introduction

From historical times, mankind has been interested in using the biological processes for production of technology which increases the quality of life. These adaptations include the production of curd to production of vaccines, etc. When such products are commercially developed, it comes under the newly emerged branch of life sciences known as biotechnology.

Biotechnology has revolutionized the methods of studying life sciences not only by amalgamating applications of chemistry and physics but also taking the studies from classical approach to molecular level. The field of biotechnology was greatly boosted since 1972, which marked the birth of recombinant DNA technology (RDT). Since the advent of RDT, progress in biotechnology has been truly remarkable. Soon after the development of recombinant DNA technology, genetically modified organisms or the GMOs were already being used for making human enzymes, interferon, and hormones. Recombinant DNA technology and GMOs are reported to be widely used in the context of environment (Glick and Pasternak 1988; Cowan 2000).

Industrial revolution can be counted as the most significant turning point in the course of Earth's environment. The rise in industrialization has led to exploitation of many natural resources to the extent of extinction and depletion of habitats. The quality of air, water, and soil is increasingly degraded with the increase in economic growth and inventions over the past century. Major concerns are caused by the man-made products, their production, and the waste generated. These comprise a wide range from the air pollution caused by automobiles, the ozone hole caused by CFCs, oil spills in sea by the carriers or drills, and nondegradable poly bags increasing soil poisoning or increasing garbage created by human waste. Human intervention has now disturbed the fine ecological balance at multiple stages by deforestation of large land masses, mining, drilling, urban planning, etc. All the mentioned activities have contributed in disturbed monsoon cycles, abnormal weather patterns, change in wind patterns, global warming, etc. to mention a few effects. As a result of careless disposal of waste, chemical spills, and industrialization, a dangerous increase in the accumulation of recalcitrant compounds, e.g., TNT, PCE, TCE, etc., in the surface/ground waters, sediments, and soil has been recorded. Places like refineries, fuel storage areas, gasoline service stations, airports, etc. are reported to have accumulation of petroleum products due to spills, etc. which have sped up the strategies to clean the contaminants (Jansson et al. 2000).

Previously, various physical and chemical methods like adsorption, vaporization, extraction, etc. have been applied for remediating and cleanup of the contaminants from the environment. These methods however have been shown by studies to be less effective as they are often known to merely transfer the contaminants instead of degrading them, or have shown to disturb natural processes of ecosystems and destroy soil structure (Semple et al. 2001).

The focus now is thus being shifted to bioremediation. **Bioremediation** means the use of biological systems like microbes to accomplish the cleanup process of the contaminants. This approach has proved to be advantageous as in addition to solving the aforementioned drawbacks, it provides a cheaper and environmentally friendly way to degrade organic contaminants to simple inorganic molecules like $H_2O$, $Cl^-$, $CO_2$, etc.

There is a lot of interest in understanding the various ecosystems in the environment and conserving them by various approaches including bioremediation. Bioremediation is classified in two types: intrinsic and engineered.

**Intrinsic bioremediation** aims to utilize the self-sustaining feature of any ecosystem, wherein the native microflora is always engaged in the cleanup. In other words, it is the property of native microbes in a given habitat or ecosystem to survive and grow while utilizing the organic contaminants by breaking down of these complex wastes produced by humans, animals, and plants. Without this native activity of the microflora, many mineral cycles would be disrupted, and the Earth would have been covered in waste completely. Majority of the bioremediation approaches or even the natural bioremediation has been dependent on the innate capacity of waste turnover. Since the majority of this breakdown is carried out by microorganisms in the process of their growth and energy production, bioremediation is often linked directly to the microbes in the environment.

**Engineered bioremediation** means using molecular engineering for bioremediation by microbes. It is further categorized into two types: biostimulation and bioaugmentation (Plaza et al. 2015; Vogel 1996). Biostimulation is done by stimulating the existing abilities of the microbes to a higher level of expression with the help of certain stimulants. These stimulants can be anything from nutrients, electron donors, or acceptors like oxygen, induction factors, etc. which increases the microbial gene activity. In bioaugmentation the microbial consortia itself is augmented by certain specialized microbes which have the ability to degrade or to produce the molecules which degrade contaminants engineered into them. These engineered microbes may belong to the original microflora of an ecosystem wherein a gene responsible for contaminant degradation is engineered or can be introduced from the external environment. In many contexts an augmented microflora is introduced to the bioremediation process in order to improve performance at a particular step or aspect.

As mentioned above, RDT is a powerful tool in molecular and genetic engineering. Since innate microflora might not be as effective in the systems with high pollution load, there are many groups which focus on engineered bioremediation. In the case of bioremediation, the group of organisms which seem most relevant are usually microorganisms. Thus many studies are now focused on developing biostimulation and bioaugmentation approaches based on the use of genetically modified microorganisms (GMMs). Although testing of GMM use for such purposes is still in a preliminary stage and the studies so far have been in the systems which are at least partially contained, however, this strategy has already produced GMMs which have enhanced ability to degrade halogenated compounds, hydrocarbons, and aromatic compounds.

These GMMs have been successful on a laboratory or field trial stage, but using them freely and exposing the natural environment with these GMMs is a matter of big concern and risk which needs to be assessed carefully in each case. Releasing the GMOs or GMMs in the midst of existing microflora leads to changes in interaction, survival, etc. of native microflora (Tsai and Olson 1991; Saano and Lindstorm 1995). In order to have an accurate risk assessment, it is vital to know the microbial composition of a given ecology in the environment. This presents another challenge for researchers as the conventional methods of microbiology are not able to decipher the complex microflora composition. The available culture and microbiological media are capable of supporting cultures of only up to 10% of total microorganisms present in the environment. As is understandable, it is difficult to study or even identify the microbe without culturing. It is also difficult to directly isolate enough numbers of viable cells which help in studies; therefore, it poses a great challenge in the context of bioremediation strategy as well.

In addition to being useful for designing strategies for bioremediation, molecular tools are also proving to be critical in understanding the natural microflora and microbial identification (Fig. 34.1). Metagenomics is such a branch which helps in studying the whole genomes of the microbes isolated or present within the samples of biofilms, soil, water, air, etc., wherein the DNA from all cells is isolated directly from the sample and analyzed as a whole. Various algorithms are then employed to identify and classify the unknown microorganisms.

Just as important it is to understand the native microflora, it is also important to dissect out the microbes relevant to bioremediation in a context. Within the

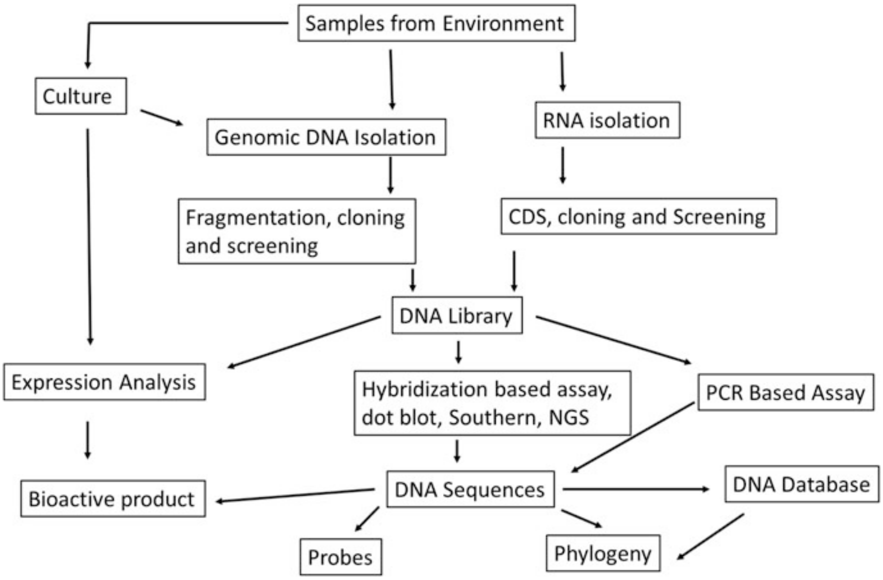


**Fig. 34.1** Flowchart depicting the study of native microflora and bioactive molecules significant in the bioremediation. The studies are based on both classical (through microbial culture) and newly developed (through noncultured microbe) molecular methods

microbial consortia present in an ecological system, there are only a few key microbes which are the primary decomposers of the toxic compounds/pollutant load. Other species are the secondary as they utilize the by products produced by primary decomposers for their survival. Such primary decomposers have been referred to as the environmentally relevant microorganisms (ERMs) (Watanabe and Baker 2000).

Next few sections discuss various molecular techniques followed by the omics approaches, used in the context of studying the native environment and ecology as well as their applications in the context of bioremediation.

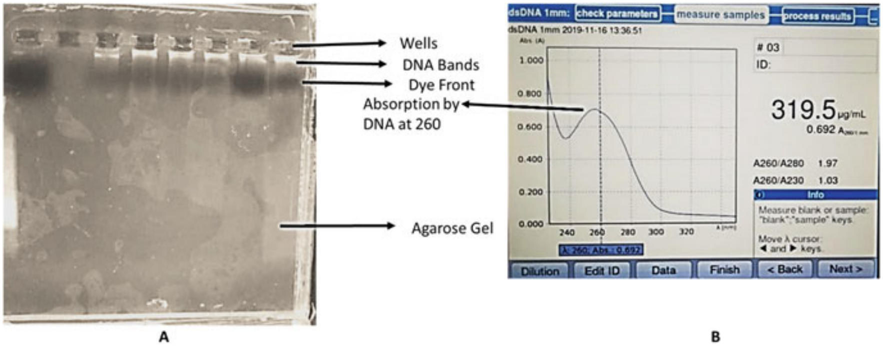
The most popular techniques which are used in environmental studies are DNA extraction, handling and study of genomes, polymerase chain reaction-based methods (e.g., RAPD, AFLP, etc.), classifying the microbes by phylogenetic analysis, the use of biomarkers (e.g., RFLP) to identify the consortia composition, reverse transcription-PCR to study RNA, denaturing gradient gel electrophoresis (DGGE), nucleic acid hybridization, fluorescence in situ hybridization (FISH), genotyping, quantitative real-time PCR (Q-PCR), SDS-PAGE, 2D gel electrophoresis, and many more. Here are a few of these discussed in detail:

## 34.2 Genomic DNA Isolation

The isolation and purification of DNA from cells is one of the most common procedures in molecular biology. It embodies a transition of studies from cell biology to molecular biology (from in vivo to in vitro). The isolation of DNA from bacteria is a relatively simple process. The organism to be used should be grown in a favorable medium at an optimal temperature and should be harvested in late log to early stationary phase for maximum yield.

The genomic DNA isolation needs to separate total DNA from RNA, protein, lipid, etc. Initially the cell membranes must be disrupted in order to release the DNA in the extraction buffer. SDS (sodium dodecyl sulphate) is an anionic detergent which is used to disrupt the cell membrane. Once a cell is disrupted, the endogenous nucleases tend to cause extensive hydrolysis. Nucleases apparently present on human fingertips are notorious for causing spurious degradation of nucleic acids during purification. DNA can be protected from endogenous nucleases by chelating Mg2++ ions using EDTA. Mg2++ ion is considered as a necessary cofactor for action of most of the nucleases. Nucleoprotein interactions are disrupted with SDS (sodium dodecyl sulphate), an anionic detergent, phenol, or proteinase K.

Protease enzyme is used to degrade the proteins in the disrupted cell soup. To obtain pure DNA, phenol and chloroform are used to denature and separate proteins from the DNA. Chloroform is also a protein denaturant, which stabilizes the rather unstable boundary between an aqueous phase and pure phenol layer. The denatured proteins form a layer at the interface between the aqueous and the organic phases which are removed by centrifugation. DNA released from disrupted cells is precipitated by cold absolute ethanol or isopropanol. DNA is then stored in TE buffer or nuclease-free water depending on the need for further experimentation. The DNA thus isolated can be assessed for quality and quantity by either gel electrophoresis (see Sect. 34.6 for details) or spectrophotometry (Fig. 34.2a, b).



dsDNA 1mm 2019-11-16 13:36:51

# 03

ID:

319.5 µg/mL

0.692 A

A260/A280 1.97

A260/A230 1.03

Measure blank or sample: "blank";"sample" keys

Move λ cursor: ◀ and ▶ keys

λ: 260; Abs.: 0.692

**Fig. 34.2** Assessment of the quality and quantity of the isolated DNA by (**a**) agarose gel electrophoresis; the brightness of bands informs of the amount of the DNA present. (**b**) Nanodrop device is used to measure DNA concentration; it also measures the purity of the sample by providing ratios of absorbance

The protocol of genomic DNA isolation has been modified and improved to be applied in environment studies. The technique is useful in studying the genomes and genes of the nonculturable microbes relevant in the context of bioremediation and environment sustainability. There have been studies establishing techniques which can be useful in isolating the genomic DNA directly from the samples like soil, sewage water, biofilms, activated sludge, etc. Thus, it is now possible to understand even those environmentally relevant microbes which are not culturable. Such modifications in the technique have made it possible to study the native microflora and their interactions comprehensively.

## 34.3 Polymerase Chain Reaction

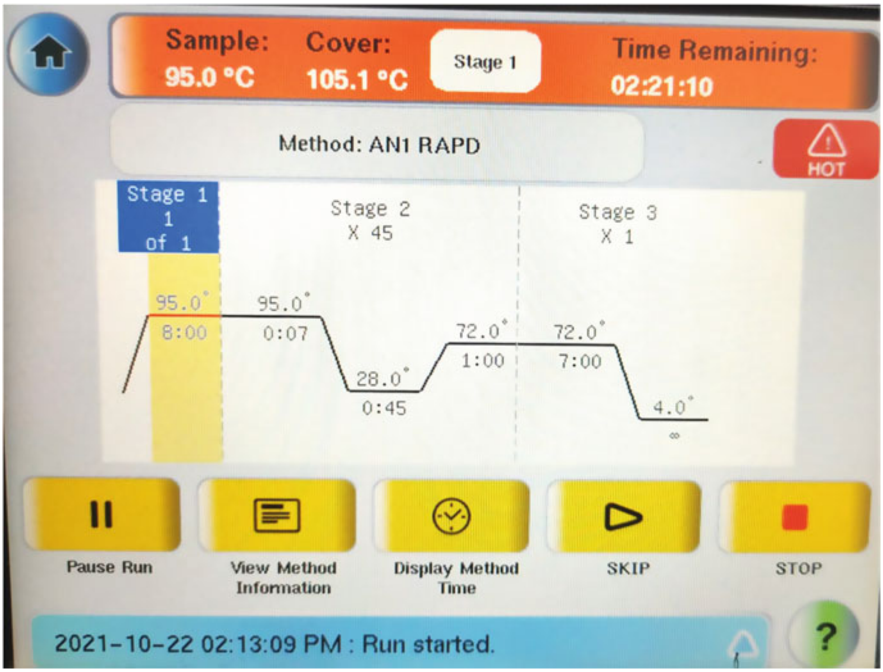
PCR is a technique used in molecular biology to amplify a single copy or a few copies of a segment of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. The technique was first developed in 1985 by Karry Mullis. PCR is an iterative process, consisting of three steps:

(a) Denaturation of the template by heat.
(b) Annealing of the oligonucleotide primers to the single-stranded target sequence (s).
(c) Extension of the annealed primers by a thermostable DNA polymerase (Fig. 34.3).

This technique has been modified to be used in resolving many environmental questions like identification of unknown microbes present within native microflora, molecular characterization, classifications, genotyping a gene related to bioactive compounds, etc. Following are a few of the known and widely used application of PCR:

### 34.3.1 Random Amplified Polymorphic DNA (RAPD)

RAPD analysis is a PCR-based molecular marker technique. Single short oligonucleotide primer is arbitrarily selected to amplify a set of DNA segments which might be present and distributed randomly throughout the genome. RAPD requires only one primer for amplification. The primers used in the PCR reaction are short arbitrary primers that are about 8–10 bases long. No knowledge of the DNA sequence of the targeted genome is required; thus, it is quite useful for the analysis of microbial genomes isolated from unknown microorganisms. The pattern of amplified sequences could be species or strain specific and be considered as an identity profile of an organism. This signature pattern can be compared with the signatures or markers from known microbes to identify or classify a particular microbe molecularly (Figs. 34.3 and 34.4).



Sample: 95.0 °C
Cover: 105.1 °C
Stage 1
Time Remaining: 02:21:10

Method: AN1 RAPD

2021-10-22 02:13:09 PM : Run started.

**Fig. 34.3** Protocol of a polymerase chain reaction. The whole setup is typically divided into three stages: stage one is set for the common denaturation process, stage two has the three key steps of the reactions coded in the program to run cyclically for the given number of times, and the final stage has a common final extension step. In the picture given, a protocol to run RAPD PCR is programmed to run 45 cycles at stage two

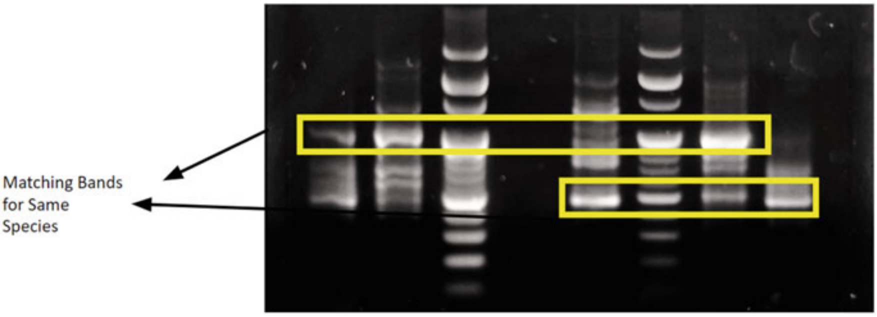


**Fig. 34.4** Bands as observed in agarose gel after a typical RAPD PCR reaction run. The pattern of polynucleotide sequences amplified by using common primers can be compared by comparing these bands, and similar strains can be identified based on similar band patterns

### 34.3.2 Amplified Fragment Length Polymorphism (AFLP)

AFLP is another PCR-based tool used in genetics research, DNA fingerprinting, and in the practice of genetic engineering. It is a method which combines the concept of restriction markers along with PCR. AFLP uses restriction enzymes to digest genomic DNA. This step is followed by ligation of known oligonucleotide sequences called adaptors to the sticky ends from restriction fragments. A set of selective primers are then used to amplify certain fragments of DNA. The primers are designed such that they have complementary sequences against the adaptor sequence, the restriction site sequence, and a few nucleotides present inside the restriction site fragments. Thus amplified fragments are then separated and visualized on denaturing gel electrophoresis. The resulting bands are then scored as presence or absence of polymorphisms.

It is a highly sensitive method for detecting polymorphisms in the DNA. The procedure of this technique is divided into following key steps:

1. Digestion of total cellular DNA with one or more restriction enzymes.
2. Ligation of sticky end adaptors to all restriction fragments.
3. Selective amplification of some of these fragments with PCR.
4. Electrophoretic separation and visualization of amplicons on a gel matrix.

## 34.4 Genotyping

Genotyping is the process of determining differences in the genetic makeup (genotype) of a microbe by examining its DNA sequence using biological assays and comparing it to that of another microbe's sequence or to a reference sequence. It reveals if a certain gene or its particular alleles are present in a given microbe. This technique is generally used in environmental studies where a particular microbe which has been isolated from a microflora is tested for presence or absence for a bioactive allele. For example, nirK and nirS genes are genotyped in the genome of microbes suspected to be involved in the denitrification process (Wojnowska-Baryła et al. 2010).

## 34.5 RFLP and Restriction Mapping

This molecular tool is developed by using a defense mechanism of bacteria. Restriction enzymes are present in bacterial cells that provide protection against invasion of the cell by foreign DNA like bacteriophage DNA. But the cells' own DNA is not cleaved by these restriction enzymes. This self-protection is achieved by the help of the specific DNA methyltransferase enzyme which will methylate the specific DNA sequence for its respective restriction enzymes by transferring methyl groups to adenine or cytosine residues to produce N6-methyladenine or 5-methylcytosine. An interesting feature of restriction endonuclease is that they commonly recognize sequences that are mostly palindromes—they show the same sequence when read 5′ to 3′ on the top strand and the bottom strand. In other words, they are nucleotide sequences or complementary strands that read the same in opposite direction.

Restriction endonucleases are the enzymes that are used to cleave DNA at specific target sites called restriction sites. Every restriction enzyme has a specific restriction site at which it cuts a DNA molecule. The most abundantly used restriction enzymes as molecular tools are the type II restriction enzymes which cleave at specific restriction sites only. These endonucleases usually have optimum pH of 7.4. The specific requirement of different enzymes may vary for ionic strength (sodium chloride and magnesium chloride) and is provided by the buffers designed specifically for such enzymes. These buffers also have a reducing agent such as dithiothreitol (DTT) which stabilizes the enzymes and prevents their inactivation. Any variation in the concentration of Na or Mg can lead to changes in the specificity of enzyme so that it can cleave at additional or nonstandard restriction sequences. The restriction endonucleases produce either sticky or blunt ends upon cleavage. Also based on the number of sequences identified for cleavage, they are called tetra cutter (length 4), hexa cutter (length 6), or octa cutter (length 8).

### 34.5.1 Restriction Fragment Length Polymorphism (RFLP)

The principle of restriction digestion is used to study the genetic markers present within a given DNA sample. RFLP detects differences in homologous DNA sequences by scoring for the presence of digested fragment fragments which are different in lengths with specific restriction endonucleases (Fig. 34.5). RFLP is very useful as a molecular marker as it is specific to a particular combination of nucleotides called as its target sequence.

Since in most cases both alleles in a heterozygous gene will be detected as RFLP markers, they are codominant and highly locus-specific. Sometimes, hybridization techniques are also used in combination with RFLP to detect a particular digested fragment/marker within the whole set of restricted fragments. For this a labelled probe (either with radioisotope label or with fluorescent label) is used to detect the complimentary restricted fragment which is fixed onto a matrix or membrane. For example, if a marker linked with a particular bioactive compound is known, that can be detected after doing southern hybridization for the molecular marker to know if the isolate has that particular molecular marker or not. The RFLP probes are also frequently used in genome mapping and in genotyping, etc.

### 34.5.2 Identification by Nucleic Acid Hybridization and Fluorescent In Situ Hybridization (FISH)

Nucleic acid hybridization methods are frequently used in analyzing native microflora, for example, a soil community. These methods help in the analysis to assess the following key features of a microflora like its composition, physical distribution

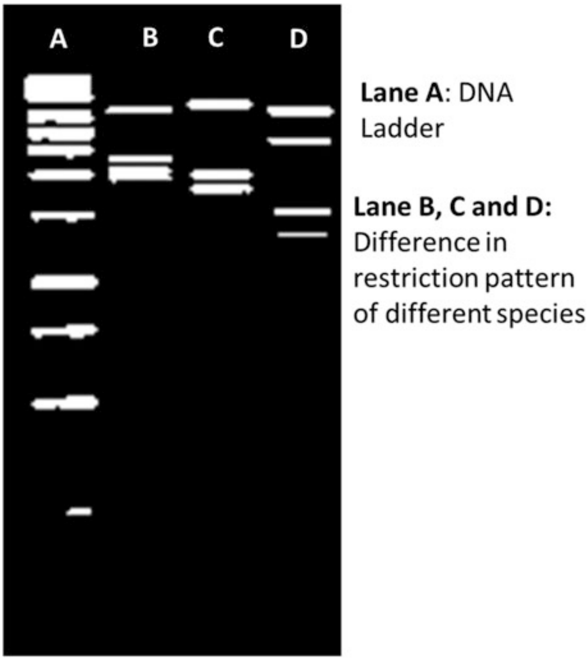


**Fig. 34.5** Restriction fragment length polymorphism. Bands here depict that same restriction enzyme digestion which can lead to the formation of completely unique band patterns for different microorganisms

in the ecosystem, abundance of a particular species or stain, relatedness of nucleotide sequences to the DNA, or RNA extracted from soil samples or present in the intact cells.

Nucleic acid hybridization methods are based on the tendency of complementary single-stranded nucleotide sequences to stabilize by forming nucleic acid duplexes. In this method, hybridization (or annealing) of a previously characterized/synthesized nucleic acid sequence (called as a probe) to its complementary sequence (called as target) within a mixture of DNA or RNA extracted from a given sample, helps to identify the sequence of interest. Probes used in hybridization are labelled with either a radioisotope (e.g., Southern blot) or fluorescent marker (e.g., FISH) for detection. The target is immobilized onto a solid support, such as a nylon membrane or a glass slide, to facilitate hybrid detection. Detection of rRNA is preferred for the quantification of the active community members because the rRNA numbers increase with cell activity. Microarray technology is now being used for high-throughput analysis (LaPara et al. 2000).

### 34.5.3 Expression Analysis by Reverse Transcriptase PCR and the Quantitative PCR

In addition to using various adaptations of PCR technique to analyze the genomic and ribosomal DNA of a given microflora, sometimes the researchers also need to identify which of the microbes within a community are more active and/or relevant in the context of the bioremediation activity. In such cases, instead of analyzing DNA, the expression pattern is analyzed by analyzing the RNA molecules produced, since it can be argued that the microbe active in any bioremediation will also have an enhanced expression of bioactive molecules. To analyze RNA content and quantity, the modifications of PCR such as RT-PCR and Q-PCR are used. These techniques help the researcher to understand the dynamics and interaction of active microbes within a native microflora. For example, analyzing RNA will easily differentiate between the dormant and active microbes of a given microbial community during wastewater treatment (Wojnowska-Baryła et al. 2010).

## 34.6 Gel Electrophoresis: Agarose Gel and SDS-PAGE

While using molecular techniques, it is often required in the experiment to separate various molecules like DNA or proteins to study them out of a mixture and individually. This separation is done using the principle of molecular separation on the basis of their size under the influence of an electric field or the gel electrophoresis. The next few techniques will discuss the various methods used by environmental biotechnologists to identify the bioactive molecule or the genes relevant in the bioremediation or resistance:

### 34.6.1 Separating DNA Fragments

The most effective way to separate varying sizes of DNA molecules is agarose gel electrophoresis. The sensitivity to separation in this technique is ranging from 100 bp to 25 kb. DNA is separated in a gel made from agarose which is obtained from the seaweed genera *Gelidium* and *Gracilaria*. It is composed of repeated arabinose (L- and D-galactose) subunits. During casting of gel, agarose polymers get linked non-covalently and form a network of bundles with pores whose varying sizes determine the molecular sieving properties of that gel. In order to separate DNA, wells are made while casting agarose gel. The DNA is then loaded into precast wells in the gel and a current applied. The backbone of the DNA (or RNA) molecules are negatively charged; hence, when an electric field is applied, DNA fragments start to migrate toward the positively charged anode. Since DNA molecules have uniform mass/charge ratio, they get separated by difference in their lengths/sizes, within a gel such that the log of the molecular weight of the DNA is inversely proportional to the distance travelled by it in the gel (Fig. 34.6a) (Lee et al. 2012). An additional and popular use of agarose gel electrophoresis is for visualization, checking the presence or absence of DNA as well as its qualitative analysis (Fig. 34.2a).

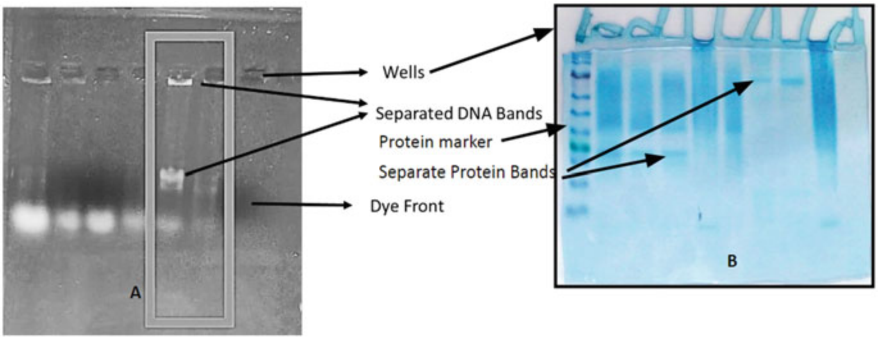


**Fig. 34.6** Separation of molecules on gel like (**a**) DNA and RNA molecules in agarose gel on the basis of their properties like molecular weight, etc. In this gel the genomic DNA which is much larger in size than the plasmid or RNA molecules travels the least amount of distance from the wells where it was loaded, while the smaller-sized molecules can even cross ahead of the reference loading dye front. (**b**) Separation of proteins on SDS-PAGE on the basis of molecular weight in kDa

### 34.6.2 Separating RNA and Proteins

Polyacrylamide gel electrophoresis (PAGE) has a higher resolution than agarose gel and has proved to be a powerful tool for RNA studies. Denaturing PAGE can be used to obtain information regarding a sample's composition as well as structural integrity of each individual RNA molecule. Non-denaturing gel electrophoresis on the other hand allows separation on the basis of various conformers or the alternatively folded RNA molecules (Petrov et al. 2013).

The technique is also very useful for separating molecules like proteins on the basis of their size and charge. One of the variants of PAGE is SDS-PAGE, which is often used to separate protein molecules by size. Sodium dodecyl sulfate (SDS) is a detergent, and it denatures secondary and tertiary (nondisulfide-linked) structures. SDS coats the protein molecules with a negative charge uniformly along their length, which allows their molecular weights to be estimated (Fig. 34.6b). In SDS-PAGE a discontinuous gel is cast with an upper gel used for stacking and a lower gel for resolving the molecules. These gels have different pH values and polyacrylamide concentrations. Here as well the smaller proteins move through the resolving gel more quickly as compared to the larger proteins (Brunelle and Green 2014).

## 34.7 Denaturing Gradient Gel Electrophoresis (DGGE)

DGGE is used to separate DNA fragments of short to medium length on the basis of their denaturation characteristics. The principle involves separation of DNA strands, based on the amount of CG and AT base pairs. During the polyacrylamide gel run, the DNA gets exposed to an increasing range of denaturant concentration. As the DNA sample progresses through the gel, it begins to get denatured at different points. The strands with higher GC content are harder to melt as compared with those of lower GC content which will melt more rapidly.

It has been frequently used in the studies related to complex ecosystem communities. This technique has been used for molecular fingerprinting and identifying single-nucleotide polymorphisms without DNA sequencing. This technique is also successful particularly in conjunction with amplification of microbial 16S rRNA genes. Here, the principles of DGGE, based on partial DNA strand separation at a given position in a gradient of chemical denaturant, are described, and an example protocol, optimized for fingerprinting of 200–300 bp fragments of bacterial 16S rRNA genes, is given (Strathdee and Free 2013).

## 34.8 2D Gel Electrophoresis

This technique is employed where better resolution than SDS-PAGE is required. In some cases, there are molecules which can be of similar sizes and thus cannot be separated properly by SDS-PAGE. In order to get these resolved, another level of separation is done on the basis of the difference in pKa properties of the protein.

Two-dimensional gel electrophoresis is therefore designed as a combination of the two techniques, isoelectric focusing (IEF), and SDS-PAGE. The first step is IEF, in which protein is separated based on its charge properties, whereas in the second step, the protein is separated on the basis of its mass. As explained above when the IEF-separated proteins are run on the SDS-PAGE gel, they get negatively charged due to SDS, and the electrophoresis leads to their separation on gel. Thus, the proteins that are separated on their isoelectric point are then separated by their molecular weights giving separate spots in detection (Büyükköroğlu et al. 2018).

## 34.9 The Omics Approach

Environmental studies have started using the recent developments in molecular biology and genetics like next-generation genome sequencing (NGS) and transcriptomic and proteomic methods. The NGS has made metagenomics studies and phylogenetic analysis possible for the researchers.

There have been many studies, which have collected samples from contaminated water sources, etc. to find out the natural microflora by using metagenomics approaches and NGS. This has helped the scientists to identify the microbial strains which are tolerant or resistant to the toxic compounds and the strains which might be capable of using the organic or toxic compounds for their metabolism and convert the contaminants in this process to less harmful products for the environment (Plewniak et al. 2018; Yadav et al. 2019).

In the genomic or metagenomics approach, samples of the inherent microflora are collected, and deep sequencing methods are employed. In this approach the genomic DNA is collected from the samples directly without culturing. As it is known that any environment changes have a direct effect on the composition of microflora, one

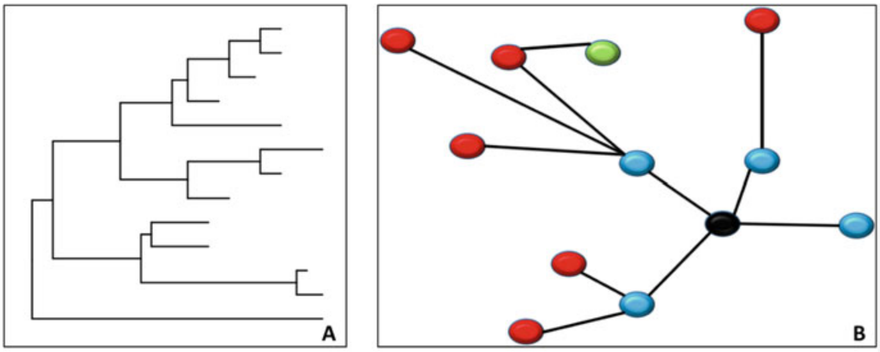


**Fig. 34.7** Omics approach can provide a plethora of information which can be used to understand the evolution and functional relationships between genes or molecules. Bioinformatics tools are being used to analyze such data and are used to prepare evolutionary relationships in form of (**a**) phylogenetic tree and (**b**) interactome

can assume that unique DNA combinations are expected to be isolated from each microenvironment. Each individual DNA fragment (Fig. 34.1) from isolated genomic DNA from a sample is sequenced, and the genomes are structured and reconstructed from those sequences. This information gives researchers an idea about the type of phyla and genera which thrive in a particular context, and preparing phylogenetic trees (Fig. 34.7a) can highlight the kind of chemical and environmental relationships going on in that particular microenvironment. It has also been known that microbes show changes in the physiology and metabolites according to the changes in their surrounding environment. Thus, studying the transcriptomes and metabolomes along with proteomes have provided insight to us regarding the knowledge of metabolic potential these microbes show and their ability to function in a certain way in the contaminated versus non-contaminated environments.

As mentioned above, microflora is composed of both actively bioremediation capable dominant species and inactive or rare species. Approach using deep genome sequencing of the whole community allows the researchers to reconstruct individual genomes as well as to functionally partition the analysis of both dominant and rare species present within these microflorae.

Two typical examples where omics approach has been particularly reported as a success are (a) acid mine drainage and (b) arsenic bioremediation. Multiple “omics” approaches have enabled the following in these cases: (I) determination of the microbial diversity distribution patterns in acid mine drainage based on the environmental changes. (II) Analyses have been done to uncover the response Vs adaptation mechanisms manifested in acidophilic microbes against different environmental conditions or stresses. (III) During the study of simple system communities, lately, the use of high-throughput sequencing of 16S rRNA/rDNA present within the given community has gained a lot of focus. Genomic and postgenomic methodologies have significantly advanced the current understanding of community function, microbial diversity, and evolution in such simple system environments (Huang et al. 2016). (IV). The use of functional genomic tools contributed to the establishment of the fact that certain species of microbes show a pleiotropic character in response to the presence of arsenic. (V) These approaches have proved to be a reliable means of deciphering the role of different microbes within a community without having the need to culture them. (VI) Another important contribution of these approaches is the prediction of genetic transfers as well as the possible occurrence of metabolic interactions within and out of microbes which could have brought about the evolution of traits like arsenic resistance. Studies with the help of bioinformatics have now been able to predict many such interactions called interactomes (Fig. 34.7b) (Huang et al. 2016; Andres and Bertin 2016).

## 34.10 Some Examples of Successful Applications of the Molecular Approaches

In the following section, a few examples are cited to highlight the diverse applications of the various molecular approaches described above. The interesting point which must be observed in these following examples is that the same basic molecular techniques can be easily applied to various types of bioremediation contexts, regardless of the type of environment, samples, biological agent, toxicant etc. in order to understand its mechanics.

### 34.10.1 Monitoring Bioremediation in Soil Microcosms Using Molecular Tools

Sagarkar et al. (2013) studied the bioremediation mechanism and the involved active microflora against the herbicide, atrazine, and analyzed that in a microcosm by process of bioaugmentation, biostimulation, and natural attenuation. Using quantitative real-time PCR, they were able to determine that the genes involved in the atrazine degrading pathway were atzA/B/C/D/E/F, trzN, and trzD. This amplified trzN gene was cloned and sequenced by NGS technologies for 16 s rDNA sequences. They could thus discover that the genes present in microcosm showed homology to genes reported in *Arthrobacter* and *Nocardioides*.

### 34.10.2 Bioremediation Effect of Plants and Earthworms on Contaminated Marine Sediments

Moreno et al. (2015) have tried to look for the effect of adding the healing effects of earthworms and phytoremediation to the bioremediations of PAH-contaminated marine sediments. They extracted genomic DNA from a year-long experimental sediment and tried to look for the diversity and the enrichment of the microflora present in the sediments through sequencing of 16s rDNA variable region 3. Moreno et al. have also tried to understand the effect of the prevailing contamination on the bacterial community structure and the metabolically active population by using PCR-DGGE, which lets them monitor the natural abundance, diversity, and dynamics of bacteria in the sediments for each of the bioremediation processes.

### 34.10.3 Asbestos Bioremediation by Microcosm Approach

Very recently Bhattacharya et al. (2021) have also used these basic molecular approaches to study the bioremediation of asbestos in siderophores. The group isolated various bacterial and fungal genomes from the iron-rich siderophores and performed 16sr DNA and 18sr DNA sequencing for the identification and understanding of the bioremediation microflora. The sequences obtained were subjected to a phylogenetic assessment by using a software. Based upon their observations, it was determined by the group that the bacteria *Bacillus subtilis* and *Bacillus atrophaeus* with the fungal species *Aspergillus tubingensis* and *Coemansia reversa* harbored effectivity toward reducing asbestos toxicity within their setup.

### 34.10.4 Toxicity of Ag+ on *Trifolium pratense* L. Seedlings with Special Reference to Phytoremediation

Mo et al. (2020) demonstrated that various dose-dependent changes occurred in RAPD profiles of DNA samples from Ag+ toxicity-treated seeds following the loss of normal bands in the gel, as well as the appearance of new bands. Based on their observations, the group suggested modulating the genotype of *Trifolium pratense L.* seedlings so that they may bear higher proportion of pollutants and are conducive to contamination site treatment.

### 34.10.5 Heavy Metal Accumulation Among Metallicolous and Non-Metallicolous Facultative Metallophyte *Biscutella laevigata* Subsp. *laevigata*

In another example, the study by Pošćić et al. (2015) used AFLP-based cluster analysis to demonstrate that the root-to-shoot accumulation rates in metals were unchanged or decreased as opposed to being enhanced in the metallicolous populations of *Biscutella laevigata* subsp. *levigata*, in contrast to the non-metallicolous population. They could also show that this population was genetically very distinct from the others, using AFLP-based cluster analysis.

### 34.10.6 Study of Subcellular Proteome-Wide Alterations of the Degradative System of *Penicillium oxalicum*

In 2018, Camacho-Morales et al. used 2D gel electrophoresis to demonstrate that ten and eight differential proteins were overexpressed in the cytosolic and microsomal sub-proteomes of *P. oxalicum*.

### 34.10.7 Microbial Diversity and Functional Profiling of Solid Tannery Waste

Verma and Sharma (2020) recently used next-generation sequencing Illumina HiSeq platform to understand the microbial diversity of solid tannery waste. They found that there was a presence of pollutant-degrading microbes such as *Bacillus*, *Clostridium*, *Halanaerobium*, and *Pseudomonas* in the waste samples they collected which strongly indicated the bioremediation potential on the sites. They also determined that serine- and metalloproteases belonging to *Bacillaceae*, *Clostridiaceae*, *Xanthomonadaceae*, *Flavobacteriaceae*, and *Chitinophagaceae* families were present which are known for proteinaceous waste degrading ability. Thus highlighting the depth of information obtained from the metagenomes.

### 34.10.8 Bacterial and Fungal Diversity and Their Bioremediation Potential from Sediments of River Ganga and Yamuna in India

Behera et al. (2020) have studied the presence of microbial communities with bioremediation potential in terms of relative abundance and taxonomic biodiversity in sediment samples of rivers Ganga and Yamuna present in India. Behera et al. used the NGS platform of “NextSeq 500 by Illumina” technology and were able to find several bioremediation bacteria. They showed that the bioremediation microbes belong to 45 bacterial genera with 92 species and 13 fungal genera with 24 species. Their study has revealed that in these samples, *Proteobacteria* was the most dominant bacterial flora, followed by *Actinobacteria*, *Firmicutes*, and *Deinococcus-Thermus*.

## 34.11 Conclusions

The field of bioremediation studies is slowly progressing in various contexts and has a high potential as a sustainable solution. The molecular technologies and approaches have opened a gateway to better understand the mechanics of these bioremediation processes. These techniques especially the ones related to culture-independent methods have become an important milestone and a step closer to the realization of bioremediation in real-world contexts.

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# Bxb1-*att* Site-Specific Recombination System-Mediated Autoexcision to Prevent Environmental Transgene Escape

# 35

Mona Easterling and Yuan-Yeu Yau

## Abstract

Transgenic plants are obtained experimentally with low frequency. Scientists use selectable marker genes (SMGs) and selection agents to select transformed cells from mostly untransformed cells in the production of transgenic plants. The SMG is usually an antibiotic or herbicide-resistance gene. However, the presence of SMGs in GM plants and subsequently in food, feed, and the environment has raised concerns from regulatory agencies and the public. In recent years, several strategies have been deployed to remove SMGs from GM plants. This chapter describes a case study highlighting the Bxb1-*att* site-specific recombination system for SMG removal in tobacco plants. Case study includes development-induced autoexcision cassette designed to delete both the SMG and the *bxb1* gene with a seed promoter derived from common bean *Phaseolus vulgaris* phaseolin (*phas*) gene to drive *bxb1* expression. GUS-positive $T_0$ lines transferred to soil for setting $T_1$ seeds. $T_1$ progeny and their $T_2$ generation are also obtained for study. Bxb1-mediated autoexcision events are identified in $T_1$ seeds and $T_1$ and $T_2$ plants through junction PCR analysis. Sequencing confirmed successful excision events. Chimeric plants containing both excised and intact T-DNA were observed in both $T_1$ and $T_2$ independent lines. However, two homogenous SMG- and *bxb1*-free $T_2$ lines were also obtained.

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**Keywords**

Autoexcision · Bxb1-*att* · CRISPR-Cas · Genome editing · Gene stacking · Marker free · Root promoter · Site-specific recombination · Unidirectional SSR · ZFN

## 35.1 Introduction

Genetic engineering can be used in plants to improve crop traits and yields by gene targeting to modify or control gene expression (Dong and Ronald 2021). Several available essential molecular tools can serve this purpose. Site-specific recombination (SSR) technology is one of them. SSR systems are well suited for use in both heterologous systems and synthetic genetic circuits. This is because that SSR recombination sites are small, easy to use, and do not require host-specific cofactors. They work well on both supercoiled DNA and linear DNA substrates. The most popular plant application for SSR systems is the removal of selectable marker genes from transgenic plants (GMOs) to produce marker-free GM crops. In addition, SSR systems have been used for gene integration and gene stacking.

*In planta*, the SSR system has been used in five specific tasks. (1) Removal of unwanted DNA fragments, particularly the selectable marker gene (SMG) for identifying successful genetic transformations, is a primary use (reviewed in Gidoni et al. 2008; Yau and Steward Jr 2013; Kleidon et al. 2020). Selectable marker genes serve no useful purpose after the initial selection phase. The constant presence and expression of SMG can be a metabolic burden to the transformed plant. Due to the small number of effective plant SMGs currently available, there is an advantage to marker removal. Transgenic plants with selectable markers removed have the potential for a future round of genetic transformation with the same SMG (reviewed, see Sang et al. 2013). The presence of SMGs in food and feedstock causes concern among consumers and regulatory agencies. SSR systems can be employed for genetic use restriction technologies (GURTs; also known as “terminator technologies”) to protect the intellectual property (IP) of genetically modified (GM) crops using SSR system-mediated site-specific deletion (Kaiser 2000; Sang et al. 2013; Lombardo 2014). (2) Integrating genes of interest (GOI) to a characterized locus for predictable gene expression is a secondary use (reviewed in Srivastava and Gidoni 2010). (3) Simultaneous integration of transgenes and excision of SMG in transgenic plants can be accomplished with two SSR systems (Nandy and Srivastava 2012). (4) SSRs are used to stack transgenes at the same locus *in planta* (Li et al. 2010; Ow 2011; Hou et al. 2014; Srivastava 2019; Pathak and Srivastava 2020). (5) SSRs can serve as genetic switches or tunable genetic circuits to regulate gene expression in eukaryotes. Unidirectional SSR systems, like ϕC31-*att* and Bxb1-*att*, are especially suitable for this application. Gomide et al. (2020) employed both ϕC31 and Bxb1 integrases and their *attB*/*attP* sites in *Arabidopsis* plants to serve as genetic switch controllers to turn a gene on or off. Bernabé-Orts et al. (2020) built a memory switch in tobacco plant (*N. benthamiana*) to control transcriptional states (on or off) of two genes using components of ϕC3-*att* SSR system.

Among these five, removal of transgenic plant SMGs is the most widely used. Application of SMG excision has moved beyond laboratories into commercial farming. Cre-*lox*-mediated SMG-free Monsanto corn LY038 was one of the first successfully produced and approved for marketing (Ow 2007). However, emergence of novel SSR systems has expanded the repertoire of site-specific genomic engineering *in planta*. Researchers recently generated recombinase-expression lines in maize, using Cre, R, FLPe, phiC31 integrase, or phiC31 excisionase gene for a variety of genetic engineering applications (Cody et al. 2020).

In this chapter, SSR systems are described generally, and plant applications are explored. Recombination outcomes for both bidirectional and unidirectional SSR systems are discussed. A case study using the unidirectional SSR system Bxb1-*att* for autoexcision removal of SMG in tobacco is presented. Finally, the combined use of SSR systems and the novel genetic editing tool CRISPR-Cas system is addressed for next-generation genome manipulation.

## 35.2 The Site-Specific Recombination (SSR) Systems

### 35.2.1 The Basics of SSR Systems

The majority of SSR systems are found in bacteria and their virus, bacteriophage. Only a few cases of SSR systems are found in eukaryotes, like yeast. These systems carry out various biological functions, including integration of bacteriophage DNA into host genomes. SSR systems are characterized by these three features: (1) recombination exclusively at specific sequences, known as *attachment sites* (or “recognition sites” and “recombination sites” by some authors); (2) no addition or deletion of DNA bases at recombination sites, known as “conservative” recombination; and (3) strand exchange occurring at small regions (or *core regions*) within the recognition sites.

SSR systems typically fall into two major families according to their mechanisms of catalysis—the *tyrosine* and the *serine* recombinase families. The tyrosine family utilizes a C-terminal catalytic tyrosine to mediate recombination, while the serine family utilizes an N-terminal catalytic serine to mediate recombination (Grindley 1997). Members of the tyrosine recombinase family include the well-studied λ integrase, Cre resolvase, Flp invertase, and many others (Grindley et al. 2006). Examples from the serine recombinase family include the mycobacteriophage Bxb1 integrase and the *Streptomyces* phage ϕC31 integrase.

Recombinase from each SSR system can catalyze site-specific recombination between the two corresponding recognition sites. Sequences of both recognition sites for a specific SSR system can be identical or dissimilar. Recombination between the two recognition sites results in “excision,” “integration,” “inversion,” or “translocation” of DNA depending on orientation and location for the two recognition sites (Fig. 35.1).

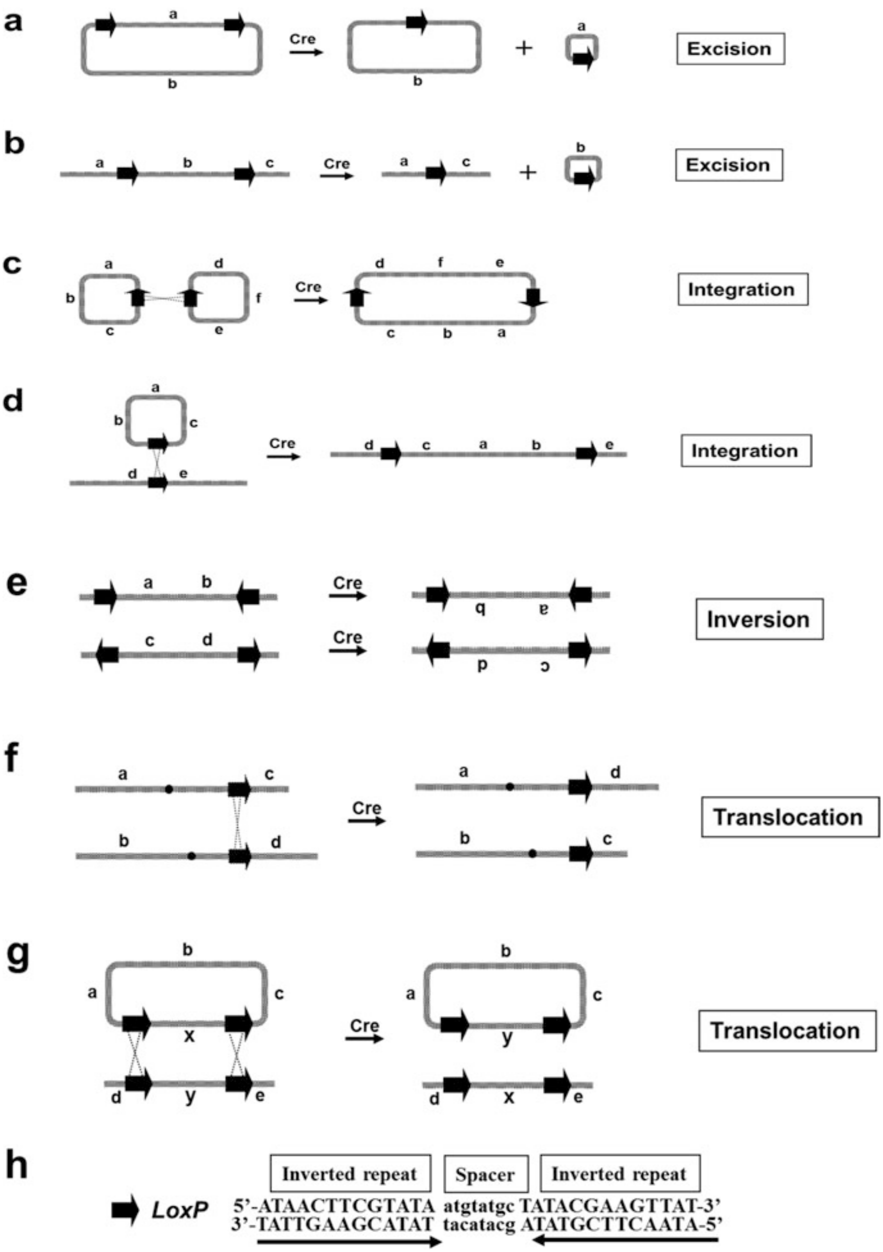

**Fig. 35.1** Cre-mediated DNA manipulation. (**a**) DNA fragment *a* flanking two *loxP* sites excised from plasmid. (**b**) DNA fragment *b* flanking two *loxP* sites excised from genomic site. (**c**) Co-integration of two plasmids through *loxP* × *loxP* site-specific recombination. (**d**) Plasmid integrated into a genomic site through *loxP* × *loxP* site-specific recombination. (**e**) Inversion of DNA fragment between two opposite-oriented *loxP* sites. (**f**) Cre-mediated translocation between two chromosomes. (**g**) Exchange of DNA fragments *x* and *y* through recombinase-mediated cassette exchange (RMCE). (**h**) Structure of *loxP* site from Cre-*lox* SSR system

Traditionally, SSR technology manipulated eukaryotic genomes by embedding SSR recognition sites into host genomes before recombination. The common use of *Agrobacterium*-mediating transformation resulted in random insertion of recognition sites. The number and orientation of recognition sites are arranged on the vector to either excise or add a DNA fragment later through site-specific deletion or integration. After sites are transformed into the host genome, successful recombination results in transient or stable expression of a recombinase DNA cassette. Since the recognition cassette is randomly embedded in the host genome, position effect plays a vital role in expression. Experimental results demonstrate that the genomic position of recognition sites determines the efficacy of site-specific recombination (Thomson et al. 2009).

There are numerous SSR systems in nature (Grindley et al. 2006). Recently, approximately 35 new phage integrases active in bacteria have been described (Yang et al. 2014). However, only a few SSR systems have been researched, and even fewer are currently being used for genome manipulation in mammals and plants.

### 35.2.2 Uni- Vs. Bidirectional SSR Systems

The resulting sequence from two identical recognition sites (e.g., *loxP* × *loxP*) yields the same sequence as *loxP*. This newly formed *loxP* site can again be used as a substrate for Cre-mediated site-specific recombination (Fig. 35.2a). Because of a tendency to catalyze both *integration* and *excision* in the presence of Cre recombinase (Fig. 35.2a), these systems are called *bidirectional* SSR systems. Examples of such SSR systems are Cre-*lox*, FLP-*FRT*, ParA-*MAR* (Gerlitz et al. 1990), and CinH-*R2* (Kholodii 2001).

On the other hand, SSR systems such as serine recombinases ϕC31 or Bxb1 promote unidirectional or irreversible reactions between *attP* and *attB* sites. These differ in sequence and produce two "hybrid sites," *attL* and *attR* (Fig. 35.2b, c). Such SSR systems are termed *unidirectional*. DNA sequences of *attL* and *attR* differ from *attP* and *attB* sequences and do not provide a substrate for their integrases. Once *attR* and *attL* sites are generated, free reversal will not occur in the presence of corresponding recombinase, until a phage-encoded accessory protein or recombination directionality factor (RDF) is provided. For example, the Bxb1-*att* SSR system RDF is gp47 protein (Ghosh et al. 2006, 2008), and the ϕC31-*att* RDF is 27.5 kDa gp3 protein (Khaleel et al. 2011). In the absence of RDF, integrases promote *attP* × *attB* recombination, while in the presence of RDF, the *attP* × *attB* recombination is inhibited, and *attL* × *attR* is stimulated (Ghosh et al. 2006; Khaleel et al. 2011). To date, only members of the tyrosine-recombinase family have shown bidirectional activity. Both the tyrosine and serine recombinase families demonstrate unidirectional function. Some members of the serine recombinase family are dedicated to a deletion reaction not capable of catalyzing inversion or integration reactions (Thomson and Ow 2006).

Bidirectional SSR systems catalyze bidirectional recombination between two identical sites. Figure 35.2a depicts catalysis of Cre protein, shown in the two

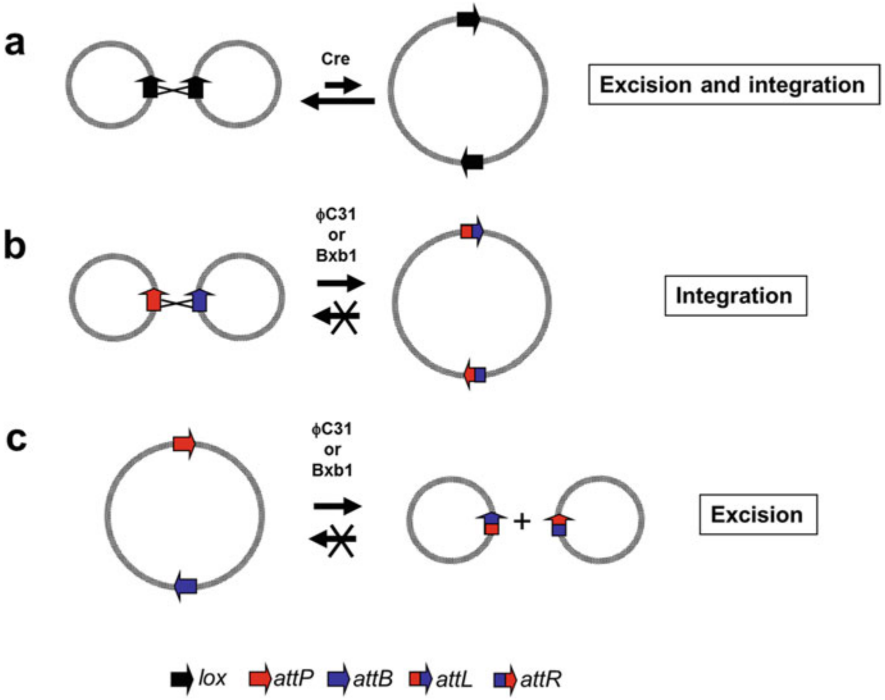

**Fig. 35.2** SSR system-mediated DNA integration and excision. (**a**) Bidirectional SSR system (Cre-*lox*)-mediated DNA integration and excision. (**b**) Unidirectional SSR system (ϕC31-*att* or Bxb1-*att*)-mediated two circular DNA co-integration. (**c**) Unidirectional SSR system (ϕC31-*att* or Bxb1-*att*)-mediated DNA excision. Black arrow: *loxP* site. Black arrow: *loxP* site; red arrow: *attP* site; blue arrow: *attB* site; *attL* and *attR*: hybrid sites

small circular DNA molecules each carrying a *loxP* site that co-integrate to form a larger, circular DNA molecule with two *loxP* sites similarly oriented. Continuous presence of Cre protein can perform intramolecular excision to convert the circular molecule back into two smaller DNA molecules through site-specific recombination of the two *loxP* sites. This occurs because the deletion reaction is kinetically favorable to the integration reaction. This system is most useful for carrying out site-specific deletions efficiently (Nagy 2000). In contrast, unidirectional recombinase reactions are irreversible following integration. The transgene is trapped, and the reaction cannot be reversed, as hybrid sites are no longer recognized by the recombinase. This renders these recombinase options especially useful for catalyzing integration reactions. Bidirectional SSR systems are also available for conducting transgene integration but require extra measures to stabilize integrated genes. These measures increase integration efficiency of bidirectional SSR systems in comparison to unidirectional systems. Some measures include the following:

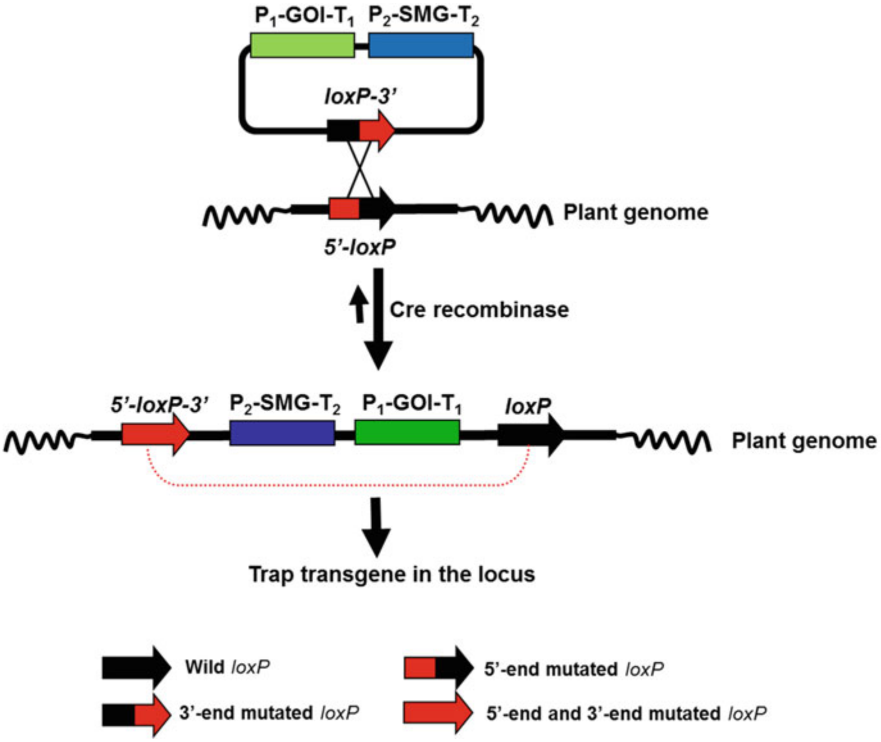

**Fig. 35.3** Cre-mediated site-specific integration with mutant *lox* sites. Integrated gene is stably trapped through recombination of two mutated *lox* sites (***loxP*-3′** and **5′-*loxP***), resulting in doubly mutated 5′-*loxP*-3′ and wild-type *loxP* sites, which reduces the reversible recombination reaction significantly. *GOI* gene of interest, *SMG* selectable marker gene

1. The use of partial mutation on the two identical recognition sites to prevent reversible recombination (Fig. 35.3). The brief history of using this strategy *in planta* is described in other reviews (Srivastava and Gidoni 2010; Nandy et al. 2015; Srivastava and Thomson 2016).
2. The use of limited or transient recombinase expression for temporary usefulness. The recombinases are provided temporarily. This is an option when the recombinase gene does not integrate the plant genome or when the *cre*-expression construct present in the target genome is displaced to abolish post-integration Cre activity (Albert et al. 1995).

Although the strategies mentioned above can increase efficacy of bidirectional SSR systems, no known strategy can reach the efficiency of unidirectional SSR systems for trapping an integrated gene. For example, the strategy (1) described above has been studied using reversible recombination (Fig. 35.3). The product derived through Cre-mediated DNA fragment excision between a wild-type *loxP* and a doubly mutated *lox* site (*lox*75/76) has been observed in rice (Srivastava et al.

2004). However, a small number of reversible recombination reactions between wild-type *loxP* and the doubly mutated *lox* site were observed. Reversibility of the inserted transgene reduces integration frequency.

Bidirectional SSR systems (e.g., Cre-*lox*) are also not suitable for iterative integration of multiple genes, due to recombination reversibility (Nandy et al. 2015). Specific strategies are needed to ensure integrated gene trapping when bidirectional SSR systems are used for iterative gene integration, known as transgene stacking. For example, Srivastava's group demonstrated the combined use of three systems (Cre-*lox*, ZFN, and I-SceI) for Cre-mediated transgene stacking at the same locus in rice (Nandy et al. 2015).

### 35.2.3 Unidirectional Bxb1-*att* SSR Systems

Mycobacteriophage Bxb1 is a temperate phage of *Mycobacterium smegmatis* isolated at Albert Einstein College of Medicine in 1990. The Mycobacteriophage Database contains additional details concerning this phage (Fig. 35.4a) (http://phagesdb.org/phages/Bxb1/). The genome size of Bxb1 mycobacteriophage is about 5 kb in length (GenBank AF271693). Bxb1 integrase catalyzes integration of Bxb1 phage genome into the *Mycobacterium smegmatis* chromosome through recombination of phage *attP* site and bacterial chromosomal *attB* site. Both *attP* and *attB* sites share an 8-bp common core (5′-GCGGTCTC-3′) (Fig. 35.4b), allowing DNA strand exchange during recombination. Recombination between *attP* and *attB* sites generates two hybrid sites, *attL* and *attR*. Hybrid sites flank inserted prophage genomic DNA. Bxb1 integrase belongs to the "large" serine recombinase group, named for their large size (300–500 amino acids). Previously discovered serine recombinases contain only 200 amino acids (Stark 2017).

Mycobacteriophage Bxb1 is a unidirectional SSR system and a powerful eukaryotic genome manipulation tool in several species, including some plants (Xu et al. 2013). Study results show Bxb1-*att* system is efficient *in planta* for DNA excision and integration. The system works well in plants with small genomes, including model plant *Arabidopsis* and more complex genomes like wheat. Bxb1 was first tested for functionality *in planta* using plant protoplasts (Yau et al. 2011). In this study, Bxb1 integrase facilitates site-specific integration. A plasmid (or *integration* plasmid) carrying Bxb1 *attB* sites integrates a transformed tobacco genome pre-embedded with the Bxb1 *attP* site. Integrants were obtained lacking Bxb1 recombinase gene detection, indicating transient Bxb1 recombinase activity. One of these integrants was used in a gene stacking study for further exploration. Later, Bxb1 integrase was used to successfully stack three GOIs (*gus*, *gfp*, *luc*) back-to-back at a tobacco genomic locus by the same research group (Hou et al. 2014). Functionality of Bxb1-*att*-mediated site-specific excision also confirmed in *Arabidopsis* and wheat (Thomson et al. 2012; Blechl et al. 2012). Final excised cassette was transmitted to the next generation of *Arabidopsis* plants. In wheat, Bxb1 gene demonstrated heritability to progeny. In 2014, Shao et al. demonstrated Bxb1-*att* capable of site-specific excision in the tobacco plastid genome (Shao et al. 2014).

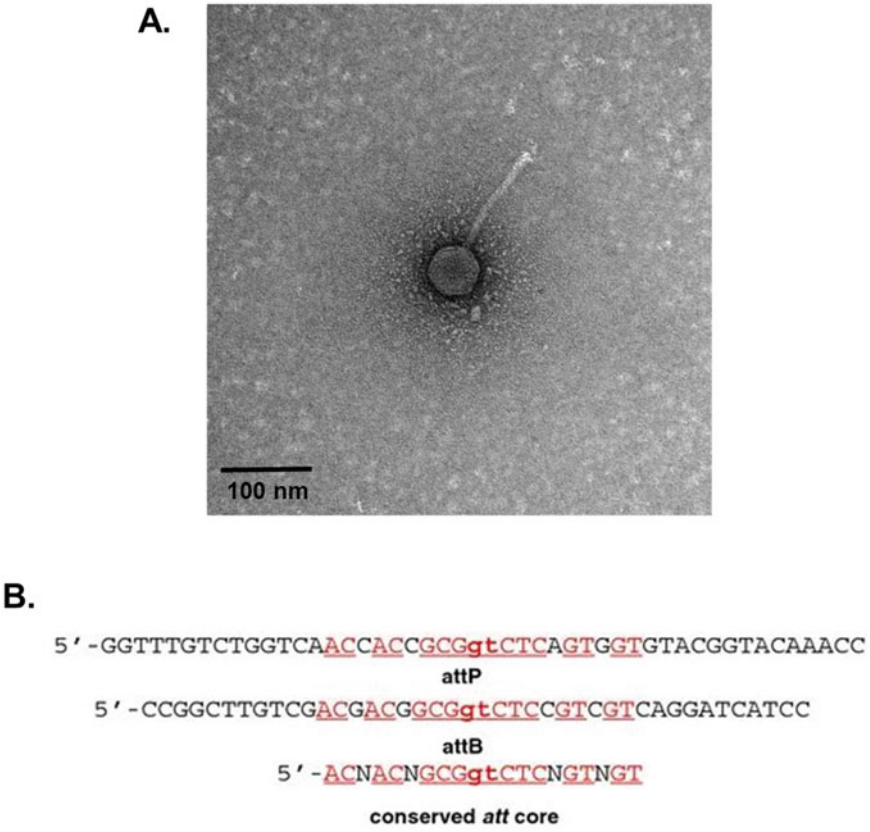

**Fig. 35.4** *Mycobacterium* phage Bxb1-*att* system. (**a**) *Mycobacterium* phage Bxb1. Direct Mag: 140,000×. Image obtained from (https://phagesdb.org/phages/Bxb1/) with permission. (**b**) Sequence of the 51 bp *attP* and 42 bp *attB* Bxb1 recognition sites, where the minimal required sequence is underlined and the two-nucleotide **gt** core region of crossover is in bold. Both *attB* and *attP* sites contain an 8-bp common core sequence (-GCGGTCTC-), within which strand exchange occurs (Kim et al. 2003). (Image **b** reproduced from Thomson, J.G., Chan, R., Smith, J. et al. (2012) *The Bxb1 recombination system demonstrates heritable transmission of site-specific excision in Arabidopsis*. BMC Biotechnol 12: 9(2012). The article is an open access article distributed under the terms of the Creative Commons CC BY license, which permits unrestricted use, distribution, and reproduction in any medium)

### 35.2.4 SSR Systems Used for Plant Research

Since SSR systems carry out recombination of two DNA fragments (recognition sites), researchers can manipulate eukaryotic genomes by incorporating SSR systems into genetic transformation events. Sauer and Henderson were first to demonstrate Cre protein promoting DNA recombination at two *loxP* sites stably residing in a mammalian chromosome (Sauer and Henderson 1989). Since that time, different SSR systems have been discovered and found functional in both mammalian and plant cells. *In planta*, Ow's group first provided evidence of Cre-*lox* SSR system functionality in tobacco cells (Dale and Ow 1991). Afterward, several SSR systems applications have been described for DNA deletion (such as SMG removal) or integration, either in nuclear genomes or plastid genomes in plants. These systems are Cre-*lox* (Dale and Ow 1991), R-*RS* (Onouchi et al. 1991), Gin-*gix* (Maeser and Kahmann 1991), FLP-*FRT* (Lyznik et al. 1993), λ-*att* (Suttie et al. 2008), HK022-*att* (Gottfried et al. 2005), ϕC31-*att* ((plastid genome, integration) Lutz et al. 2004; (nuclear genome, excision) Thomson et al. 2010), β-*six* (Grønlund et al. 2007), ParA-*MAR* ((nuclear genome, excision) Thomson et al. 2009; (nuclear genome, excision) Zhou et al. 2012; (plastid genome, excision) Shao et al. 2017), Bxb1-*att* ((nuclear genome, integration) Yau et al. 2011; (nuclear genome, excision) Thomson et al. 2012; (nuclear genome, excision) Blechl et al. 2012; (plastid genome, excision) Shao et al. 2014; (nuclear genome, integration) Hou et al. 2014; (nuclear genome, integration) Li et al. 2016), and CinH-*R2* ((nuclear genome, excision) Moon et al. 2011; (nuclear genome, excision) Zhou et al. 2012; (plastid genome, excision) Shao et al. 2017). A history of SSR system development for plant genome manipulation from the early days of SMG removal to more recent transgene stacking has been described in a review paper entitled "The Long Road to Recombinase-Mediated Plant Transformation" (Ow 2016).

*In planta*, a majority of publications demonstrate the use of SSR for SMG removal, but some studies reported use for integrating transgenes into a predetermined genomic location. SSR systems successfully used for transgene integration include Cre-*lox* (Albert et al. 1995; Vergunst et al. 1998; Pathak and Srivastava 2020), ϕC31-*att* (Lutz et al. 2004), R-*RS* (Nanto et al. 2005), FLP-*FRT* (Li et al. 2009, 2010; Nandy and Srivastava 2011, 2012), and Bxb1-*att* (Yau et al. 2011; Hou et al. 2014; Li et al. 2016). Integrated genes targeting the same chromosomal locus in different lines have demonstrated similar expression levels (Day et al. 2000; Nandy and Srivastava 2012; Hou et al. 2014).

### 35.2.5 Other Applications of SSR Technology

Beyond genome manipulation, SSR systems are also used for other applications. Three examples are (1) cloning tool, (2) DNA-assembly tool, and (3) a rewriteable digital data-recording device. SSR technology has served as a valuable *cloning tool* enabling DNA circuits to assemble on a vector efficiently, a technique called "recombinational cloning (RC)" (Hartley et al. 2000). A well-known case is the GATEWAY® technology using λ-*att* system to efficiently shuffle genes to expression systems for gene function studies (Liang et al. 2013). As a *DNA-assembly tool*, researchers have used ϕC31-*att* plus Cre-*lox* and *Streptomyces* phage ϕBT1 SSR systems to construct large transgenes, 150 kb and 400 kb, in vivo and in human minichromosome, respectively (Dafhnis-Calas et al. 2005; Xu et al. 2007). Xu et al. assembled a 400-kb transgene from 80-kb mouse DNA through five cycles of iterative SSR-mediated integration (Xu et al. 2007). ϕC31-*att* system was used in the serine integrase recombinational assembly (SIRA) method for rapid and effective assembly of a metabolic gene set involved in the bacterial carotenoid biosynthesis pathway. This paved the way for others to facilitate reconstitution of many genes involved in transgenic plant biosynthetic pathways. Random assembly of gene orders along these pathways allowed for evaluation of gene product outputs. This method allows researchers to conduct rapid pathway optimization (Colloms et al. 2014). The *rewritable digital data-recording device* harnesses the recombinase's unidirectional ability to "invert or flip" sequences. Bxb1 is being used by bioengineers to generate rewritable digital data stored in cells as part of research involving cancer and aging (Bonnet et al. 2012). Researchers reapply the recombinase to flip specific DNA sequences back and forth at will and visualize switch events through the use of GFP (green color) or RFP (red color) fluorescence. This technology may 1 day give scientists the ability to turn off cell division by stopping cell division cycles. The goal of switching off after reaching a certain threshold is prevention of multiple cancerous mutations (Bonnet et al. 2012).

SSR systems can also be used with novel genome-editing tools such as ZFN, TALEN, and CRISPR-Cas9 for precise gene integration/stacking at engineered sites or SMG deletion. Scientists in animal studies have combined the use of SSR and CRISPR-Cas technologies for genome manipulation, but few studies have emerged in the plant field (Yang et al. 2017). Srivastava (2019) recently described a gene stacking scheme using Cre-*lox* SSR system gene integration into a precise genomic sites and zinc finger nuclease (ZFN) to delete SMG in plants. However, the same group reported potential ZFN toxicity in rice. Inducible expression of CCR5-ZFN did not result in detectable excisions, although it transmitted stably to progeny (Pathak et al. 2019). Therefore, researchers should be careful in choosing an appropriate nuclease for use in any specific plant species.

## 35.3 Autoexcision of SMG from Potential Energy Crop

### 35.3.1 Autoexcision Mechanism

Autoexcision is an attractive method for simultaneous removal of several genes in a molecular cassette embedded in a genome. For example, it can be used to delete both the SMG and recombinase gene after their expression is no longer required. This accelerates the process of generating SMG-free transgenic plants, by removing the need for generating another generation of plants to segregate away the SMG and recombinase gene. The primary feature of autoexcision cassette design is placement of the recombinase gene under the control of an "inducible" promoter, while the selectable marker gene is under the control of a separate promoter placed between the two recombination sites (e.g., *loxP* site of Cre-*lox* system) in direct orientation. In this scenario, the gene of interest is inserted outside the recombination sites (Fig. 35.5a). Under controlled activation of the recombinase gene, both the recombinase gene and SMG are eliminated (Fig. 35.5a). The recombinase gene promoter can be inducible or tissue-specific. PCR can be used to evaluate an autoexcision event, by using primers designed outside of the two recombination

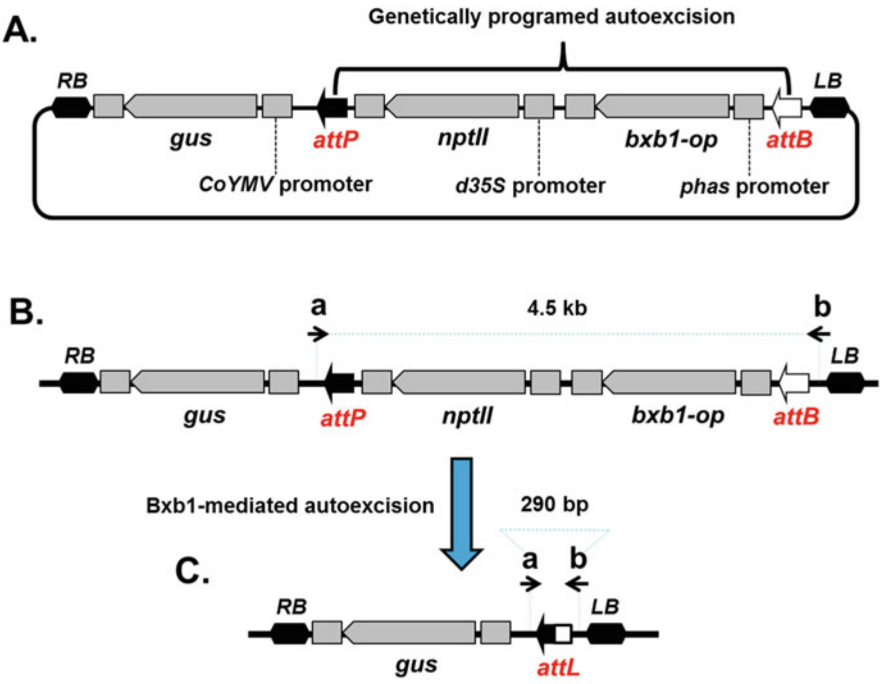

**Fig. 35.5** Plant binary vector used in this case study. (**a**) T-DNA structure of plant binary vector pRB140-Bxb1-op. T-DNA consists of β-glucuronidase (*gus*) gene driven by the CoYMV promoter and an autoexcision cassette designed to delete a selectable marker gene (*nptII*) and Bxb1 site-specific recombinase gene (*bxb1-op*, "op": gene codon optimized). *nptII* gene is under control of the double CaMV 35S promoter (d35S). Seed promoter *phas* is used to drive Bxb1 recombinase gene. (**b**) PCR is used to evaluate autoexcision events. Primers used for genotyping outside Bxb1 *attP* and *attB* sites are marked as **a** and **b**. (**c**) The T-DNA configuration generated after autoexcision of *attP* and *attB* flanked DNA fragment. PCR product of a 290-bp band is expected if autoexcision event occurs. *LB* T-DNA left border, *RB* T-DNA right border, *bxb1-op* codon-optimized *bxb1* recombinase gene, *nptII* neomycin phosphotransferase II gene, *gus* β-glucuronidase gene, *attL* footprint of a hybrid site after *attP* × *attB* recombination. Diagram not to scale

sites (Fig. 35.5b). Two examples of tissue-specific promoter are pollen promoter and seed promoter. With pollen promoter, autoexcision of unwanted DNA occurs in the pollen of transformed plants, preventing SMG escape through pollen. Inducible promoters can support regulation by presence of heat (Wang et al. 2005; Du et al. 2019), cold, or chemicals (Hare and Chua 2002).

### 35.3.2 Case Study: Bxb1-*att*-Mediated Autoexcision in Tobacco Plants

To assess the feasibility of using Bxb1-*att* system and a seed promoter for autoexcision in tobacco plant, a codon-optimized seed promoter is used to drive

Bxb1 integrase gene (Yau et al. 2012; Easterling 2014). A study construct is built and placed in *Agrobacterium* for tobacco plant genetic transformation. GUS-positive putative $T_0$ transgenic lines are grown in greenhouse soil to harvest $T_1$ seeds. $T_1$ seeds of each putative transgenic line are used for kanamycin selection to evaluate transgene copy numbers. Both $T_1$ seeds and $T_1$ plantlets are also used for autoexcision evaluation through junction PCR. Subsequent sequencing verification of PCR products is conducted to check for the presence of site-specific recombination footprint via the hybrid site *attL*.

### 35.3.2.1 Materials and Methods

#### Construction of Binary Vectors pRB140-Bxb1-op and *Agrobacterium* Strain

Principles and methods of molecular manipulation, such as plasmid isolation, restriction enzyme digestion, DNA fragment ligations, and transformation of *E. Coli*, are conducted as described by Green and Sambrook (2012) and manufacturer instructions. Binary vector pRB140-Bxb1-op (Fig. 35.6) was built for this study. Three plasmids were obtained before the construction of pRB140-Bxb1-op: (1) **pCambia2300** (CAMBIA, Canberra, Australia; https://cambia.org/),

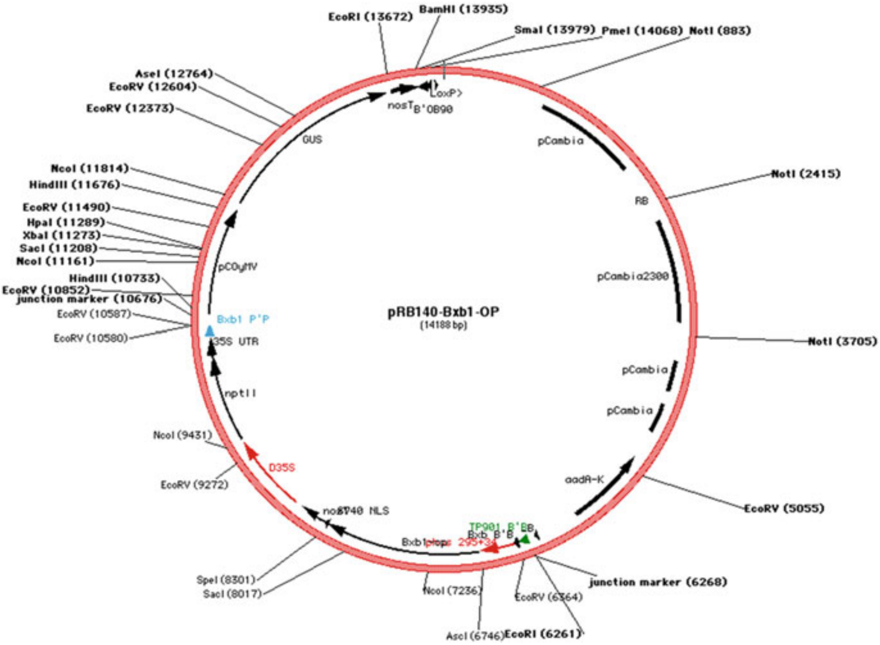

**Fig. 35.6** Final construct of pRB140-Bxb1-op vector for use in *Agrobacterium*-mediated tobacco genetic transformation in this study. Plant binary vector pCambia 2300 was used as backbone. Unique restriction sites are indicated. *d35S* double 35S promoter. *Bxb1-op* codon-optimized Bxb1 recombinase gene. *Bxb1 P′P* Bxb1 *attP* site. *Bxb1 B′B* Bxb1 *attB* site. *npII* plant selection marker for kanamycin resistance

(2) **pRB140** (a gift of Dr. Robert Blanvillain; https://www.researchgate.net/profile/Robert-Blanvillain/publications), and (3) **pUC57-phas-Bxb1-op** (customer-ordered and synthesized by GenScript USA Inc. (Piscataway, NJ, USA)). **pUC57-phas-Bxb1-op** contains a synthesized DNA fragment <TP901$_{attP}$-Bxb1$_{attP}$-Phas-Bxb1-op-T$_{nos}$- Bxb1$_{attB}$- TP901$_{attB}$ > as an insert, which was cloned into pUC57 backbone. Recombinase bxb1 gene is codon-optimized (Fig. 35.7). TP901$_{attP}$, TP901$_{attB}$, Bxb1$_{attP}$, and Bxb1$_{attB}$ were recognition sites for TP901-*att* and Bxb1-*att* site-specific recombination systems, respectively.

A seed-specific promoter has been chosen for the Bxb1 recombinase gene in this case study project. The phaseolin (*phas*) gene is native to the common bean plant, *Phaseolus vulgaris* (Bustos et al. 1991). In nature, it encodes the major seed storage protein, β-phaseolin. As a promoter, phas has proven sufficient for yielding high levels of seed-specific expression (Bustos et al. 1991; Odell et al. 1994; Van Der Geest et al. 1995). The 295-bp region incorporates into the vector proximal to the transcription start site (*−295phas*). The wild-type *bxb1* coding region was codon-optimized for adaptive expression in tobacco plants. A nuclear localization signal (NLS) added at Bxb1 recombinase gene terminus to facilitate nucleus entry for site-specific recombination to occur.

**Fig. 35.7** Side-by-side comparison of codon-optimized *bxb1* (Bxb1-op in Fig. 35.6) gene sequence with wild-type *bxb1* ORF. Codon-optimized *bxb1* is listed on each top line with Bxb1 ORF listed on each bottom line

**Construction Steps**

1. Construction of **pYW-Bxb1-op-10:** Restriction fragment d35S-*npt*II-$T_{nos}$ is obtained from **pCambia2300** vector by *Ase*I digestion. After *Ase*I cohesive ends blunted with large Klenow fragment (Cat. No. M0210S, New England Biolabs Inc., USA), fragment is sub-cloned into *Pme*I site between NOS terminus ($T_{nos}$) and $Bxb1_{attP}$ of **pUC57-phas-Bxb1-op** through blunt-end ligation resulting in plasmid **pYW-Bxb1-op-10**.
2. Constructing **pRB140-GUS**: *Spe*I-*Pst*I fragment from pRB140 containing *nptII* expression cassette was removed. *Spe*I and *Pst*I ends of pRB140 were blunted with Klenow and ligated together with T4-DNA ligase, resulting in plasmid pRB140-GUS.

   To avoid repeat use of d35S promoter in a vector, a *Commelina yellow mottle virus* (CoYMV) promoter was employed to drive d35S promoter. Multiple copies of d35S promoters with identical sequences in a T-DNA might induce promoter silencing in the plants. From a study, while CaMV 35S promoter is active in most cell types, the CoYMV promoter is primarily active at tobacco tissues such as the phloem, the phloem-associated cells, and the axial parenchyma of the roots, stems, leaves, and flowers (Medberry et al. 1992).
3. Constructing **pRB140-Bxb1-op**: *Hpa*I blunt-end fragment ($TP901_{attP}$-$Bxb1_{attP}$-Phas-Bxb1-op-$T_{nos}$-d35S-*npt*II-$T_{nos}$ $Bxb1_{attB}$- $TP901_{attB}$) was cut from plasmid pYW-Bxb1-op-10 and ligated to *Pml*I site of **pRB140-GUS** (backbone-LB-(*Pml*I)-CoYMV-GUS-$T_{nos}$-RB-backbone) to form the final plasmid: pRB140-Bxb1-op. Final pRB140-Bxb1-op vector was rechecked with different restriction enzyme digestions.

*Agrobacterium tumefaciens* LBA4404 is used to deliver binary vectors pRB140-Bxb1-op for transformation in this study. The vector electroporated into ElectroMax™ *Agrobacterium tumefaciens* LBA4404 competent cells (Cat. No. 18313–015, Invitrogen, USA) using an electroporator (Multiporator®, Eppendorf). Forty microliter LBA4404 competent cells and 3 μL vectors were mixed and transferred into a 1-mm-gap electroporation cuvette (Cat. No. 94000100–5, Eppendorf, USA). Electroporation was carried out using a manufacturer preloaded program designated for bacterial electroporation (2000 V, time constant: 5.0 ms). One mL LB liquid medium was added to electroporation cuvette and mixed with electroporated competent cells. Th mixture was then transferred to Falcon® 14-mL polypropylene round-bottom tube (Becton Dickinson Labware, USA) and incubated at 30 °C for 3 h with 225-rpm shaking. After 3 h, bacterial culture of 20 μL, 50 μL, or 100 μL spread onto LB + streptomycin (100 μg/mL) + kanamycin (50 μg/mL) plates. Streptomycin used to select *A. tumefaciens* LBA4404 cells disarmed Ti pAL4404, and kanamycin was used to select pC35. BNK.2. Plates were placed in a 30 °C incubator for 2–3 days to produce colonies. All antibiotics used in this research case study were sterilized with a sterile syringe filter containing a 0.2 μm cellulose acetate membrane (Cat. No. 28145–477, VWR, Batavia, IL, USA) (Fig. 35.7).

#### 35.3.2.2 Plant Materials and Tissue Culture Conditions

Wild-type tobacco (*Nicotiana tabacum* L.) cultivar "Petit Havana" mutant SR1 used to conduct genetic transformation through leaf disk transformation method. Wild-type tobacco seeds were sterilized with 70% ethanol for 2 min, followed by bleach (sodium hypochlorite) (30% (v/v), added drops of Triton-X 100) for 20 min, and washed thoroughly with sterile distilled water. Sterilized seeds were germinated on MS medium, containing MS mineral salts (Murashige and Skoog 1962; Cat. No. M524, *Phyto*Technology Lab), 3% (w/v) sucrose, 1× Gamborg's vitamin solution (Cat. No. G1019, Sigma-Aldrich), and 0.8% agar. In the present experiment, MS medium was supplemented with B5 vitamins (Gamborg et al. 1976), containing 100-fold thiamine concentration compared to original MS concentration. B5 vitamin-based medium has been reported to enhance growth and solasodine production in hairy root cultures of *Solanum khasianum* Clarke (Jacob and Malpathak 2005) and carrot (Yau and Wang 2012). Plates were sealed with a medical gas-permeable tape (Micropore™ surgical tape; 3 M Health Care, USA) and placed in a 25 °C growth chamber with 16-h photoperiod and 8-h dark.

#### 35.3.2.3 *Agrobacterium*-Mediated Genetic Transformation of Tobacco

Single colonies from plates were selected using a 15-cm sterile cotton-tipped applicator (Puritan Medical Products Company, Guilford, Maine, USA) and streaked on LB plates containing both streptomycin and kanamycin antibiotics. Plates were stored at 30 °C for 1 day to allow for colony growth. For tobacco genetic transformation, bacteria scraped from plates with a sterile inoculation loop and suspended in 35 mL transformation medium (MS mineral salts, 3% (w/v) sucrose, 1× Gamborg's vitamin solution, 3 μg/mL 6-benzylaminopurine hydrochloride (Cat. No. B5920, Sigma-Aldrich), and 100 μM Acetosyringone (AS) (Cat. No. D134406, Sigma-Aldrich)). Transformation medium adjusted to pH 5.8 with 0.1 N KOH or HCl and autoclaved at 121 °C for 20 min. AS was dissolved in 70% ethanol and added to cooled autoclaved medium.

Wild-type leaves were cut and placed in a sterile Petri dish. Each 1-cm-square leaf piece was cut in an ESCO Horizontal Airstream® Laminar flow hood (ESCO, USA).

After inoculation, cotyledons (on leftover stem) were placed abaxial on cocultivation medium (transformation medium solidified with 0.8% agar (Cat. No. A7921, Sigma)) for 3 days in dark, and subsequently transferred to selection medium ("transformation medium" + cefotaxime/carbenicillin (500 μg/mL) + kanamycin (100 μg/mL) and solidified with 0.8% agar). The mixture of 50% cefotaxime (Cat. No. C380, *Phyto*technology Lab., USA) and 50% carbenicillin (Cat. No. C346, *Phyto*technology Lab., USA) was used to remove *Agrobacterium*. Plates were sealed with a 3 M Micropore™ tape and placed in growth chamber with 16-h light/8-h dark photoperiod. Subculturing was carried out every 2 weeks.

Putative transgenic shoots 1 cm in length were cut at base from each explant and transferred to rooting medium (MS mineral salts, 3% (w/v) sucrose, 1× Gamborg's vitamin solution, 0.8% agar) supplemented with 100 μg/mL kanamycin and 400 μg/mL of mixture of cefotaxime and carbenicillin. Rooted plants were allowed to grow to 5 cm in Magenta® plant tissue boxes and transferred to soil.

#### 35.3.2.4 Kanamycin Selection of $T_1$ and $T_2$ Seedlings

$T_1$ seeds derived from kanamycin-resistant $T_0$ putative transgenic lines or $T_2$ seeds from $T_1$ transgenic lines were sterilized with ethanol and bleach and placed on germination medium (MS mineral salts, 3% (w/v) sucrose, 1× Gamborg's vitamin solution) supplemented with 100 μg/mL kanamycin and 200 μg/mL of mixture of cefotaxime and carbenicillin. Plates were placed in growth chamber with 16-h light/ 8-h dark photoperiod. Seedlings were displaying stunted growth, pale green to yellowish leaves, and inhibition of the main root extension considered susceptible to kanamycin, while seedlings with healthy green leaves and roots were considered kanamycin resistant.

#### 35.3.2.5 Histochemical GUS Assay

Putative transgenic lines and controls were tested for β-glucuronidase (GUS) expression as described by Jefferson et al. (1987). GUS was assayed in seeds by briefly crushing to allow GUS solution to pass through seed coat. Prepared seeds or leaf discs were placed in wells of 96-well plate containing GUS-staining solution (1 mM 5-bromo-4-chloro-3-indoxyl-β-D-glucuronide (X-gluc)) (Gold BioTechnology, Inc., St. Louis, MO, USA), 100 mM sodium phosphate buffer pH 7.0, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, and 0.1% Triton X-100). After a 10-min vacuum filtration, the plate was incubated overnight at 37 °C. To check GUS staining, the chlorophyll of the leaf tissue was removed by repeated washing in 70% ethanol. Chlorophyll interferes with observation of blue-colored stain. Stained leaf tissues were examined under a dissecting microscope and scored for blue coloration.

#### 35.3.2.6 Genomic DNA Isolation

Forty seeds or a portion (a quarter of the size of a 1.5-mL microcentrifuge) of each leaf were excised from putative transgenic plants or controls in Megenta® boxes harvested into 1.5-mL microcentrifuge tubes. Four hundred μL of grinding buffer (200 mM Tris-HCl, pH 5.7, 250 mM NaCl, 25 mM EDTA, and 0.5% SDS) was added to each tube, and the product was ground with a Kontes Pellet Pestle® (VWR, Batavia, IL, USA) driven by an overhead stirrer (Cat. No. 2572101 *IKA Works*, *Inc.*, USA). Ground samples were centrifuged for 5 min at maximum speed (16,800 × *g*) with an Eppendorf benchtop centrifuge (centrifuge model 5418). 300 μL of supernatant was transferred to a new microcentrifuge tube, and 300 μg/mL of isopropanol was added to precipitate genomic DNA. After inverting several times, the mixture was centrifuged for an additional 15 min. Supernatant was discarded, and 70% ethanol was added to wash DNA pellet. Ethanol was discarded after washing, and microcentrifuge tubes containing DNA samples were allowed to air-dry 20 min before being resuspended in 50 μL of $H_2O$. For seeds, further purification was performed with a QIAquick® PCR purification kit (Cat. No. 28104, Qiagen, Valencia, CA, USA). Concentrations of eluted DNA samples were measured using a NanoDrop2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

### 35.3.2.7 PCR Analysis

Isolated genomic DNA from $T_1$ seeds and $T_1$ or $T_2$ leaf tissues of putative transformants and wild-types was used for junction PCR analysis to identify site-specific excision. Primers were designed and purchased from Invitrogen (USA). A list of primers can be seen in Fig. 35.8. To detect excision event, primer **a** (“deletion 5”; forward primer): 5′- CTT TTT GCT TTT TTT GCC AAA GCT TTC TTC CG-3′; primer **b** (“CoYMV.rev-1”; reverse primer): 5′-TGG GGC TGA AGC TTG ATT TTT GTA CA-3′; or primer **c** (“CoYMV.rev-3”; reverse primer): 5′- AGG TGG TAA CTG AAG ATC AGG GAG ATT-3′ was used. Promega Go*Taq*® Flexi DNA polymerase kit was used for amplification. Each PCR reaction contains 3 μl (approximately 300 ng) of genomic DNA, 2 μL 2.5 mM dNTPs, 2 μL 25 mM $MgCl_2$, 5 μL 5× PCR buffer, 1 μL of each primer (10 μM), 0.12 μL polymerase, and autoclaved water for a total volume of 25 μL. Thermocycle program was used as an initial denaturation at 94 °C for 4 min, followed by 35 cycles of 94 °C (30 s), 65 °C (30 s), and 72 °C (1 min 20 s) and a final extension step at 72 °C for 2 min. A 290-bp PCR product is expected if primers **a** (“deletion 5”; forward primer) and **b** (“CoYMV.rev-1’; reverse primer) were used. A 500-bp PCR product is expected if primers **a** and **c** are used. For PCR amplification between promoter CoYMV and nptII gene, primer **a** (“deletion 5”; forward primer) and primer GUS-4 (reverse primer: 5-′-CGTAATGAGTGACCGCATCGAAACG-3′) were used, to produce a PCR band approximately 1.6 kb in size (see Fig. 35.8). To amplify *nptII* gene, specific primers nptII-F: 5′-ATGATTGAACAAGATGGATTG-3′ (forward primer) and

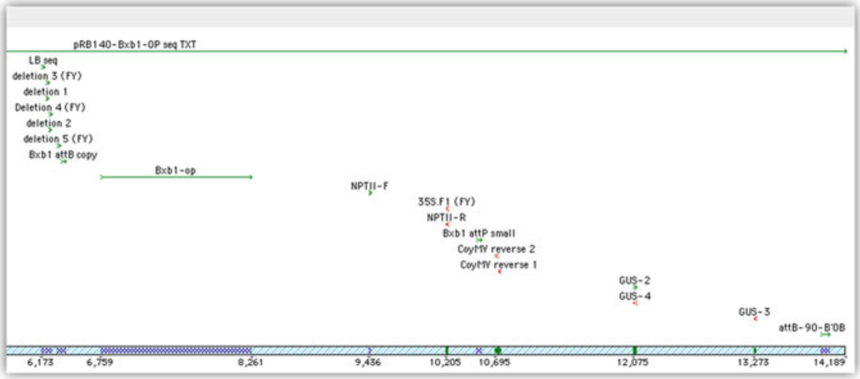

**Fig. 35.8** Map of primer loci in T-DNA of binary vector pRB140-Bxb1-op. Primers used to evaluate autoexcision events in transgenic tobacco plants. Forward primers (green arrow heads) or reverse primers (red arrow heads) are indicated with orientation of arrow heads. The map was constructed with a Sequencher® DNA sequence analysis software

nptII-R: 5′-GAAGAACTC GTCAAGAAGGCGAT-3′ (reverse primer) were designed and purchased from Invitrogen (USA).

All PCR performed on Eppendorf's MasterCycler Gradient® PCR machine (Eppendorf, USA). PCR products were separated on 1% TAE agarose gel (Cat. No. 820723, MP Biomedicals, USA) stained with ethidium bromide (Cat. No. E3050, Technova, USA). Gel was photographed with a GelDoc-It™ imaging system (Ultra-Violet Products LLC., USA).

#### 35.3.2.8 Gel Extraction and Sequencing

Bands of junction PCR product (290 bp) were excised from agarose gel and purified with QIAquick® Gel Extraction Kit (Qiagen) following manufacturer instructions. DNA purity and concentration were measured using a NanoDrop2000 spectrophotometer and directly followed by sequencing steps. Primers **a** ("deletion 5") and **b** ("CoYMV.rev-1") were used for sequencing. A mixture of 10 ng purified DNA fragment and 10 ng primer was placed in eight-strip PCR tubes and sent to Genewiz Inc. (South Plainfield, NJ, USA) for sequencing. These sequencing results was analyzed with Sequencher® software (Gene Codes Corporation, Ann Arbor, MI, USA; http://www.genecodes.com/) and Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology Information (NCBI).

### 35.3.3 Results

#### 35.3.3.1 $T_0$ Putative Transgenic Lines and GUS Staining

One hundred twelve $T_0$ putative transgenic plants were derived from genetic transformation (with binary vector pRB140-Bxb1-OP) of tobacco leaf disks obtained (Fig. 35.9a–e). Those plant lines were rooted in a rooting medium containing kanamycin (100 μg/mL) (Fig. 35.9f-h). Twenty-three (23) rooted plants were observed. Rooted plants measuring at least 5 cm were transferred to the soil in a greenhouse for acclimation and further growth (Fig. 35.9i). Greenhouse plants were then used for GUS staining. Plants negative for blue stain were discarded. Plants demonstrating no blue stain were considered either non-transgenic ("escapees" from kanamycin selection) or transgenic plants with *gus* gene silencing. Gene silencing caused by position effect is frequently reported in plant genetic transformation (Betts et al. 2019). While in soil, GUS activity were tested multiple times as part of the screening process using different leaves of each transgenic plant at different plant development states. This ensures that blue stains are due to bona fide gene insertion not contamination of residual *Agrobacterium* cells. Leaves of 12 lines of 23 rooted $T_0$ putative transgenic lines (≈52.2%) were stained blue (Fig. 35.9j). Of the 12 lines, 1 sterile line grew many buds but yielded no seeds. One line did not survive transplant to soil. Therefore, ten seed-producing lines moved forward for future analysis.

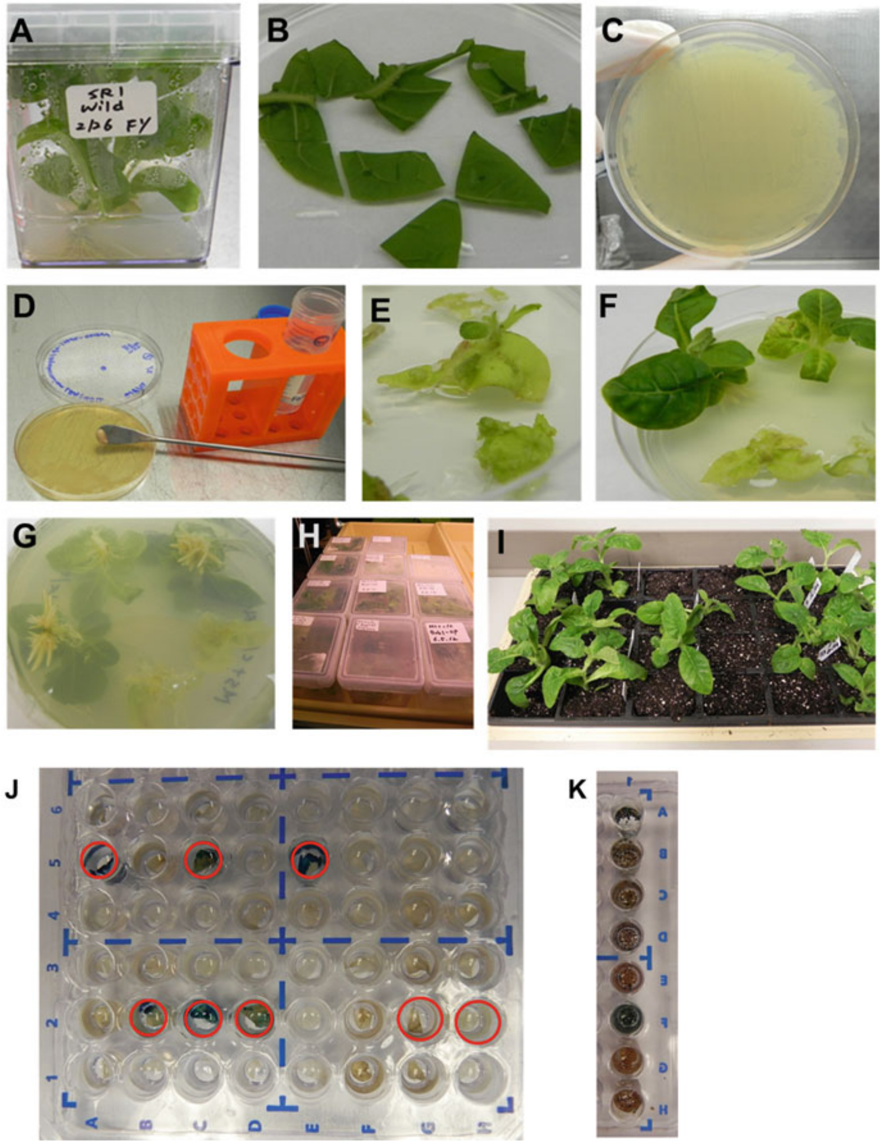

**Fig. 35.9** Putative $T_0$ tobacco transgenic lines. (**a**) Tobacco SR1 used for *Agrobacterium*-mediated transformation. (**b**) Leaf cut into 1 cm × 1 cm disks for disk-dip transformation. (**c**, **d**) *Agrobacterium tumefaciens* LBA4404. (**e**) Putative transgenic shoots on selection medium. (**f**–**h**) Putative $T_0$ transgenic lines in rooting medium containing 100 μg/mL kanamycin (in plates and in Magenta™ boxes). (**i**) Putative $T_0$ transgenic lines transferred to soil for ex vitro acclimatization. (**j**) Leaves of putative $T_0$ transgenic used for GUS staining in a 96-well titration plate. Leaves have been de-chlorophylled with ethanol. (**k**) GUS staining of $T_1$ seed derived from $T_0$ putative transgenic lines

#### 35.3.3.2 GUS Staining on $T_1$ Seeds

Ten fertile, GUS-positive plants grew to produce $T_1$ seeds that were immediately screened for GUS activity. For comparison, $T_1$ seeds from randomly selected *GUS-negative* $T_0$ greenhouse plants were also screened for GUS activity. The *gus* gene region, found outside *attB* and *attP* sites, contains its own CoYMV promoter undisturbed by the excision event. Blue GUS staining results were shown in figure (Fig. 35.9k).

#### 35.3.3.3 PCR Analysis for Autoexcision Events in $T_1$ Seeds

$T_1$ seeds from ten GUS-positive $T_0$ transgenic plants were harvested for PCR analysis to evaluate autoexcision events. Primers **a** ("deletion 5") and primer **b** ("CoYMV.rev-1") were used (Fig. 35.8). PCR product of 290 bp was observed (Fig. 35.10).

#### 35.3.3.4 Autoexcision Evaluation for $T_1$ Seedlings

To test for excision of recombinase and antibiotic-resistance coding regions embedded in transgenic tobacco genome, $T_1$ seeds were plated on selection medium containing kanamycin. Four of the ten GUS-positive lines showed ~3:1 ratio of resistant vs. susceptible to kanamycin. The remaining six lines also produced resistant seedlings, indicating *nptII* gene presence in $T_1$ generation (Fig. 35.11; Table 35.1).

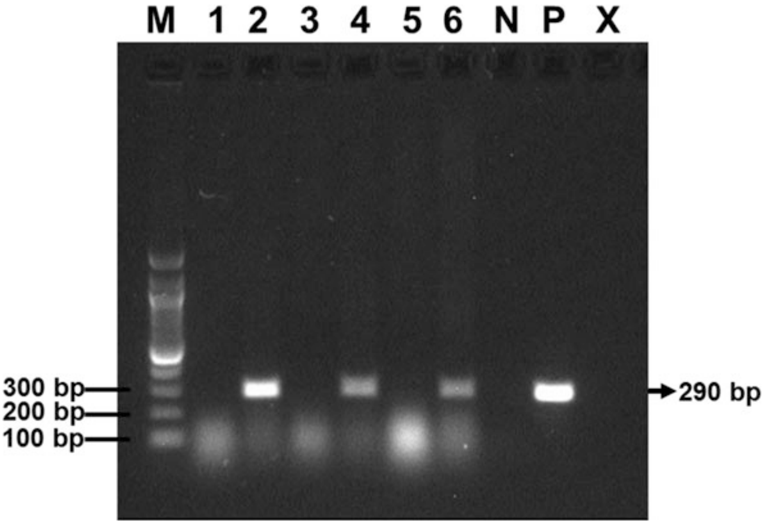

**Fig. 35.10** PCR results of gDNA isolated from $T_1$ seed derived from different $T_0$ putative transgenic plants. Lane M: 100-bp DNA markers. Lane 1: negative control (wild-type seed gDNA). Lanes 2–6: seed gDNA from different $T_0$ putative transgenic tobacco plants. Lane N: water. Lane P: positive control. Lane X: no PCR sample loaded. A 290-bp band is expected to be amplified. PCR products run on a 1.3% TAE gel containing ethidium bromide

**Fig. 35.11** $T_1$ seedlings derived from seeds of $T_0$ transgenic plant Bxb1-op #28 on selection medium (MS + kan (120 μg/mL)). Seedlings transformed with *nptII* gene survived kanamycin selection

**Table 35.1** $T_1$ seed of $T_0$ putative transgenic tobacco plants (GUS positive) screened on MS + kanamycin (120 μg/mL) (indicated in the table with "MS + K120") medium for progeny test. A 3:1 (resistant vs. susceptible) ratio was observed in some $T_0$ putative transgenic lines

| Line # BXB1-OP | [MS+K120] Resistant: susceptible | [MS only] | $T_1$ seed junction PCR | |
|---|---|---|---|---|
| 25 | 25:11 | 2:0 | Yes | $T_2$ |
| 28 | 93:31 | 51:0 | No | |
| 40 | 93:18 | 38:0 | Yes | $T_2$ |
| 51 | 82:28 | 60:0 | Yes Good | |
| 66 | 95:32 | 42:0 | Yes | |
| 68 | 42:08 | 30:0 | Yes Faint | |
| 70 | 55:18 | 37:0 | Yes Good | $T_2$ |
| 82 | 39:12 | 28:0 | No | |
| 96 | 18:06 | 16:0 | Yes Strong | $T_2$ |
| 98 | 47:13 | 30:0 | Yes Faint | |
| 99 | Sterile, many buds, no seeds | | | |

Next, $T_1$ seedlings of GUS-positive lines were used for autoexcision event evaluation. Two hundred ninety bp PCR product was observed when forward primer **a** ("deletion 5") and reverse primer **b** ("CoYMV.rev-1") were used for amplification (Fig. 35.13a). $T_1$ seedlings with 290-bp band were further checked with other primer pairs to see if DNA (transgenes in T-DNA) rearrangement occurs. PCR product of 500-bp was observed when forward primer **a** ("deletion 5") and reverse primer **c** ("CoYMV.rev-3") were used for amplification (Figs. 35.12 and 35.13b). PCR product of 1.6 kb was also observed when forward primer "CoYMV.rev-1" and reverse primer "GUS-4" were used for amplification, as expected. In the meantime,

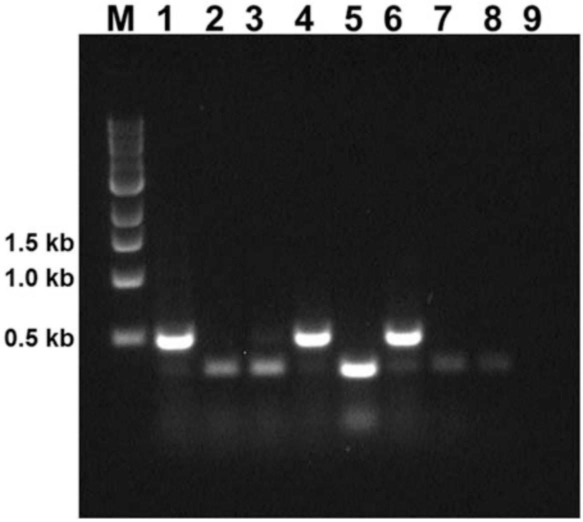

**Fig. 35.12** PCR evaluation of six $T_1$ seedlings derived from $T_0$ putative transgenic plant **Bxb1-op #96**. Lane M: 1-kb DNA size marker. Lanes 1–6: six $T_1$ individual plants (T1 Bxb1-op #96–1, #96–2, #96–3, #96–4, #96–7, #96–9). Lane 7: wild-type gDNA. Lane 8: negative control (water, instead of gDNA, was added to the PCR tube)

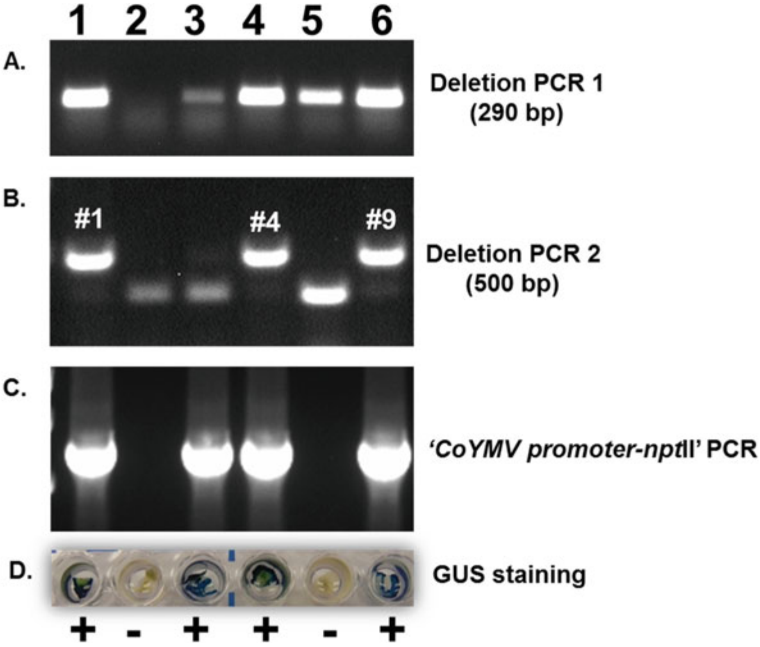

**Fig. 35.13** PCR evaluation of autoexcision event in six $T_1$ seedlings derived from $T_0$ putative transgenic plant Bxb1-op #96 (T1 Bxb1-op #96–1, #96–2, #96–3, #96–4, #96–7, #96–9). (**a**–**c**) Three different pairs of primers used for genotyping. (**d**) Leaf GUS staining of these six $T_1$ seedlings

primer pair nptII-F and CoYMV.rev-3 was used to test for *nptII* presence in some cells (Fig. 35.13c). Amplified bands indicate $T_1$ seedlings are chimeric for excision.

Two GUS-positive fertile lines in the studied $T_0$ group (ten lines) did not yield PCR results positive for excision in $T_1$ tissue. Therefore, only 8 of the original 23 lines rooted in the soil (34.7%) and tested positive for GUS demonstrated excision via junction PCR of $T_1$ tissue.

#### 35.3.3.5 Autoexcision Assay for $T_2$ Seedlings

An excision event examination of progeny lacking Bxb1 gene followed to determine if the genomic excision event occurred in germline tissue, indicating heritable transmission. From the $T_1$ plants testing positive for excision, four parental lines (#25, #40, #70, and #96) were chosen to propagate $T_2$ lines. Six descendants of these lines (#25–3, #25–5, #70–5, #96–1, #96–4, and #96–9) produced seeds in a timely manner, and produced seeds were plated on selection media. Four Bxb1-op $T_2$ lines (#25–5, #96–1, #96–4, and #96–9) appeared heterogeneous for excision phenotypically as many of their descendants remained resistant to the antibiotic kanamycin, indicating some continued presence of selectable marker. Three lines (#25–5, #96–1, and #96–9) maintained the 3:1 threshold by yielding resistant:susceptible ratios of 13:4, 27:9, and 21:8, respectively. Line #96–4 yielded a ratio of 30:0 resistant seedlings germinating on media containing kanamycin. Two Bxb1-op $T_2$ lines (#70–5 and #25–3) appeared homogenous for excision phenotypically and tested in triplicate yielding resistant to susceptible rates of 0:86 and 0:47, respectively (Fig. 35.14).

$T_2$ lines 70–5#3, 96–4#1, and 25–3#13 were used for excision event evaluation with junction PCR. Primer sets **a** and **b** were used to detect 290-bp band. Primers **a** and **c** were also used to detect 500-bp band. Both 290-bp and 500-bp bands were amplified and observed (Fig. 35.15a). These 290-bp and 500-bp bands were excised

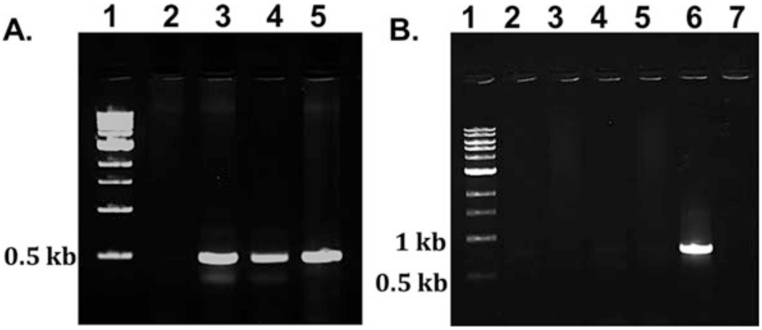

**Fig. 35.14** PCR amplification of $T_2$ lines 70–5#3, 96–4#1, and 25–3#13. (**a**) Gels showing junction PCR products from three $T_2$ lines. Lane 1: 1 kb DNA ladder. Lane 2: wild type. Lane 3: 70–5 #3. Lane 4: 96–4 #1. Lane 5: 25–3 #13. Bands (excision product) of 500-bp size amplified with primer **a** and **b**. (**b**) PCR amplification of *nptII* gene in $T_2$ lines mentioned in (**a**). An 890-bp PCR product indicates the presence of the gene. Wild type serves as negative control. Bxb1-op vector serves as positive control. All three putative transgenic lines tested show no band of 890 bp

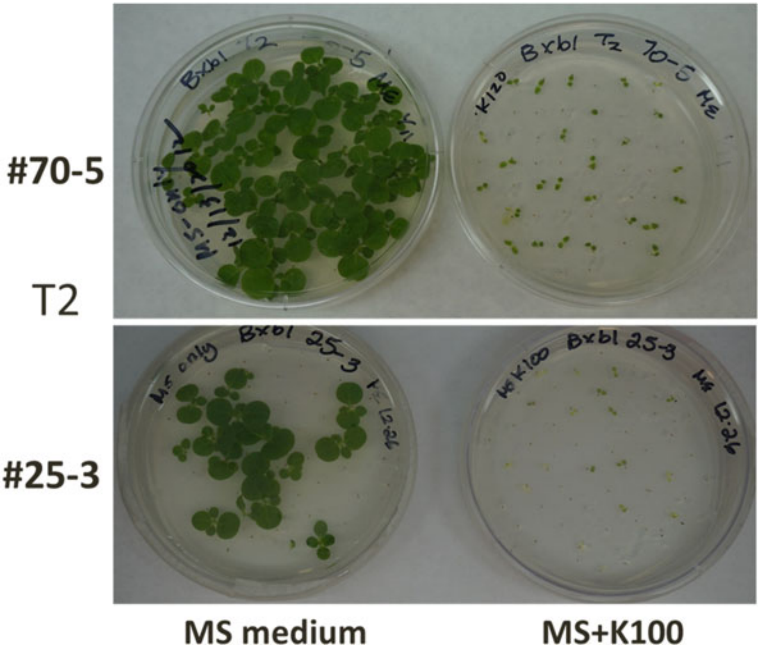

**Fig. 35.15** Autoexcision evaluation for $T_2$ seedlings. Seeds were plated on selection medium containing 120 μg/mL kanamycin. Two Bxb1-op $T_2$ lines (#70–5 and #25–3) appeared homogenous for excision phenotypically. All seedlings showed susceptibility to kanamycin

from gel, purified, sent for sequencing, and produced results consistent with excision. The three lines further underwent PCR screening using *nptII* primers nptII-F and nptII-R to detect the presence of antibiotic resistance gene. None of the lines tested showed bands for the resistance gene (Fig. 35.15b). These three $T_2$ lines also tested with nptII-F and CoYMV.rev-1 primers for successful gene excision. 1259 bp indicates the presence of DNA fragment. All five putative transgenic lines tested show no band at 1259 bp.

Seedling descendants of $T_2$ lines were screened with GUS staining in leaf tissue, yielding positive results for staining in sample batches. For example, #25–3 was tested in 15 different tissue samples with 12 positive results, which scored as 12/15. #25–5 yielded 15/20 positives, and #70–5 yielded 17/20. While three lines gave 100% positive results, #96–1 yielded 12/12, #96–4 yielded 15/15, and #96–9 yielded 12/12. The variation was consistent with the expectation of variety among high and low expression levels in leaf tissue.

#### 35.3.3.6 Sequencing Analysis for Autoexcision Events

290-bp bands were excised from electrophoresis TAE gels and gel-purified for sequencing. Sequence result revealed *attP* × *attB* recombination footprint, via hybrid site *attL* (Fig. 35.16). Hybrid site was observed, and DNA fragment between *attP* and *attB* sites was looped out and deleted. These results confirmed that *phas*

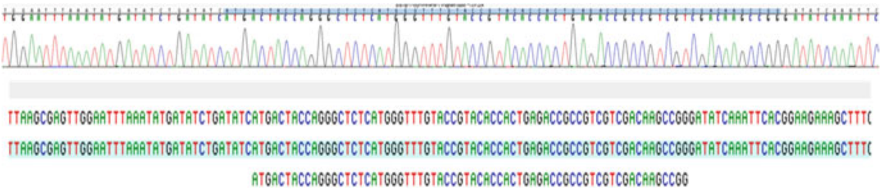

**Fig. 35.16** Sequence of a 290-bp PCR product representing the hybrid site *attL* footprint, after Bxb1 *attP* and *attB* site-specific recombination

seed promoter is capable of driving *bxb1* recombinase gene for autoexcision in tobacco plants.

## 35.3.4 Discussion

This research project case study investigates the Bxb1 recombination system as a potential tool for precise removal of transgenes such as SMG using a tissue-specific promoter. Following successful recombination, cassette containing both *bxb1* recombinase and *nptII* region was completely excised. Absence of these two genes would address concerns surrounding human consumption, and the potential transfer of these transgenes is capable of conferring antibiotic resistance to other organisms environmentally (Yau and Steward Jr 2013). Successful recombinase activity would result in a generation of transformed plants that do not contain the antibiotic resistance (*npt II*) gene or the Bxb1 gene.

This case study collected 112 $T_0$ putative transgenic tobacco lines. Twenty-three lines grew roots in the rooting medium with kanamycin and was transferred to the soil. Among these 23 lines, 12 lines tested GUS positive. However, one line was sterile and another did not survive soil transplant. Southern analysis has not been performed on these ten $T_0$ lines, leaving transgene copy numbers unknown in these lines. In theory, $T_0$ line harboring a single-copy transgene is more likely to produce progeny with a homogenous excision event.

Among the remaining ten lines, two lines did not demonstrate excision events in $T_1$ seedlings measured by junction PCR tests. This could be due to position effect, indicating T-DNA cassette inserted into a locus preventing effective expression of *bxb1* gene. Position effects impacting gene expression have been reported (Pérez-González and Caro 2019). Of the lines in soil, 8 of the original 23 lines, representing 34.7%, tested positive for GUS and excision by junction PCR in $T_1$ tissue. However, *nptII* gene was also detected in $T_1$ tissue, indicating $T_1$ plants were chimeric. Four of the remaining eight lines, #25, #40, #70, and #96, were used for further study on $T_2$ progeny. Six descendants of these lines (#25–3, #25–5, #70–5, #96–1, #96–4, and #96–9) produced seeds for kanamycin test. Among them, only two Bxb1-op $T_2$ lines (#70–5 and #25–3) appeared homogenous for excision phenotypically. In summary, results suggest seed promoter *phas* is capable of driving *bxb1* recombinase gene autoexcision in tobacco. These excision events were transmitted to the subsequent generation. Two independent lines with homogeneous excision events were obtained in $T_2$ generation.

Seed promoter have previously been used for SSR-mediated autoexcision. Moravcíková et al. (2008) used a strong, seed-specific *Arabidopsis thaliana* cruciferin C (CRUC) promoter for Cre-*lox*-mediated autoexcision. This promoter is active in seeds and silique but not in any other tissue type, including callus. However, the authors found promoter was leakage in early experimentation. Excision events were observed in callus cells experimentally. $T_0$ transgenic plants regenerated from calli were chimeric with excision events. $T_1$ plants derived from seeds of $T_0$ transgenic lines indicated10.2% of plants with complete removal of *nptII* gene with 86.4% chimera plants survived on kanamycin-containing medium. Autoexcision is effective in low numbers for these early generations of transgenic plants. However, repeated Cre activation in $T_2$ seeds produced more SMG-free $T_2$ plants in further study (Moravcíková et al. 2008).

Tissue-specific promoters provide a possibility for stronger *bxb1* recombinase expression. Another SSR-mediated autoexcision study using Bxb1-*att* SSR system is conducted in the monocot energy crop switchgrass (*Panicum virgatum* L.). This study incorporated a rice PS3 pollen promoter (Somleva et al. 2014). Authors report successful deletion of cassette containing recombinase and marker genes in switchgrass pollen through autoexcision, with efficacy range of 22–42%. In another study, pollen-specific LAT52 promoter from tomato was employed to control expression of CinH recombinase for autoexcision in tobacco (Moon et al. 2011). Autoexcision cassette flanked by two *Rs* recognition sites containing a green fluorescent protein (GFP) gene. A successful autoexcision event would delete the gene and remove GFP expression. The authors observed in CinH tobacco $T_1$ harboring single-copy transgene events, three independent lines exhibited less than 1% GFP-positive pollen, based on flow cytometry (FCM)-based pollen screening of 30,000 pollen grains. In the control event (CinH-Drec), GFP expression was observed in 70% of pollen (Moon et al. 2011). Root promoter approach offers an alternative for SSR-mediated autoexcision in plants.

Overexpression of recombinase causes host cytotoxicity or genotoxicity. Recombinase toxicity were reported in mammalian and plant cells (Loonstra et al. 2001; Coppoolse et al. 2003; Liu et al. 2009; Janbandhu et al. 2014). Loonstra et al. observed Cre expression causing numerous chromosomal aberrations and increasing sister chromatid exchange numbers in mammalian cells. They also found toxicity dependent on Cre activity level. Although Bxb1 toxicity has been reported in yeast (*Saccharomyces cerevisiae*) (Xu and Brown 2016), no abnormal plant phenotypes were observed in either model plants or wheat studies actively expressing Bxb1 integrase. In contrast, ϕC31-transformed *Arabidopsis* developed crinkle leaves (unpublished data), and Cre transgenic plants displayed abnormality (Coppoolse et al. 2003). Native docking sites in eukaryotic genomes can integrate transgene at unintended loci, leading to generation of unwanted GMOs. For example, Bi et al. (2013) reported theinsertion of a plasmid with ϕC31 *attB* site into pseudo *attP* sites in pig genome. In this study, four pseudo *attP* sites were identified, and two gave rise to increased integration rate of 33%. These unintended insertions might also lead to cell toxicity. It is prudent to remove the recombinase gene with the selectable marker gene once their purposed has been served. In this study, plants actively expressing Bxb1 transgene present phenotypically normal appearance and set seeds in a manner comparable to wild type, thereby demonstrating a lack of detrimental impacts from recombinase constitutive expression.

### 35.3.5 Conclusion and Future Perspective

Recombinase technology allows for precise levels of genomic integration through more sophisticated applications (Wang et al. 2011). This case study research project demonstrates Bxb1 recombinase successfully performing site-specific genome modification in *Nicotiana tabacum* L. Bxb1 produced conservative site-specific deletions of DNA in regions flanked by *attP* and *attB* within transformed plant genome. Homogenous genotype of excision obtained in $T_2$ generation, indicating that excised DNA heritably transmitted to next generation.

Tissue-specific promoters (e.g., seed or pollen promoter) drive recombinase for autoexcision, as well as other types of promoters that have been successfully studied. These include inducible promoters, heat shock response promoters, and chemically induced promoters. Du et al. (2019) employed heat shock response promoter (hsp70) for SSR-medicated autoexcision in maize to remove a DNA cassette containing selectable marker gene. Kleidon et al. (2020) used chemically induced recombination for autoexcision of a genetic cassette. This cassette contained both a negative selection gene and R-*RS* recombinase gene to produce selectable marker gene-free Cavendish banana (*Musa* spp.). Steroid-inducer molecule, dexamethasone (DEX), used to activate recombination activity. Tissue-specific promoters have an advantage over inducible promoters, because inducible promoters require an extra step for recombinase activation. Optimal conditions for applying inducers should be determined in advance, as inducers (i.e., chemicals) might have plant side effects or be impractical for large-scale production.

One disadvantage of using SSR technology for transgene integration is the requirement of embedding a landing pad in the target genome in advance. These docking sites, except for native pseudo-docking sites, must be genetically transformed into a genome to begin the process. For example, *attB* site of Bxb1 must first be embedded in the plant genome, and then a plasmid containing gene of interest (GOI) and *attP* site can integrate the *attB* docking site through site-specific recombination to bring in GOI. The genome embedding of *attB* site is random. They can be embedded in undesired sites. Emergence of revolutionary genome editing tool CRISPR-Cas system can change this. CRISPR-Cas system can first be used to deliver *attB* site to a desirable genomic locus X. Following characterization, this *attB* site will serve as a docking site for subsequent GOI integration. Of course, CRISPR-Cas alone can also insert the GOI at locus X through homologous recombination (HR) without SSR-system involvement with low efficiency. Differing optimization factors required to increase CRISPR-mediated HR efficiency.

One important concern for breeders with these recombination systems is freedom to operate (FTO) issue. Dupont's Cre-*lox* patent has expired, and patent application on Bxb1-*att*, previously filed by USDA, has been abandoned (Chen and Ow 2017). With this technology being free from patent licensing fees, breeders and researchers have more affordable tools at their disposal.

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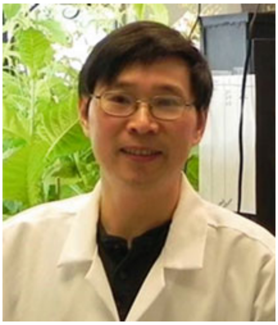

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# Microorganisms: An Eco-Friendly Tools for the Waste Management and Environmental Safety

# 36

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## Abstract

Microorganisms are ubiquitous in nature having multifunction affecting human life. Microbes play the significant role in maintenance of many natural and man-made compounds in the environment. Several microorganisms like bacteria, fungi, protozoa, and metazoan are adopted for the role in the management of waste by degrading the waste metabolites and converting into useful compost or organic fertilizers that can be used for organic farming. The use of organic fertilizers increases the fertility of soil and reduces the use of chemical fertilizers. In present scenario, organic farming is mostly being used in India or others countries because organic farming allows the use of organic fertilizers like biofertilizers, bio-compost, farmyard manure (FYM) and green manures, etc. All the compost don't contain the use of any chemical component but made by domestic, animal, and agriculture waste by the degradation process of several microorganisms. Some of the harmful or toxic pollutants could be degraded by several types of microbes and converted into nontoxic product; the process is known as bioremediation. The microbes which functions in bioremediation are *Pseudomonas putida*, *Dechloromonas aromatic*, *Methylibium petroleiphilum*, and *Alcanivorax borkumensis*. Bioremediation help to clean the contaminated soil and to remove pesticides, oil, fertilizers, and toxic chemicals such as arsenic from the environment. Microorganisms play an important role in recycling the nutrients by decomposing the waste metabolites like animal, domestic, and agriculture waste. Bacteria also help in the degradation of toxic pollutants and dead animals and recycle the nutrients back to Earth. Bacteria decompose the dead organisms so that the nutrients could be possible to recycle which helps to maintain the homeostasis in an ecosystem. Recycling of nutrients frees up nitrogen, potassium, and phosphorous which are the primary nutrients and important for plant health. In this chapter, we will discuss the role of microbes for waste management by focusing on bioremediation, biodegradation, FYM, biofertilizers, bio-compost, and aerobic and anaerobic waste management including the health hazards of industrial waste and their management. Organic industrial wastes might be discharged into the environment and cause serious environmental problems and health hazards to living organisms. Therefore the proper treatment of industrial waste is very essential so that we could preserve the environment and human safety.

## Keywords

Microorganisms · Waste · Nutrient recycling · Bioremediation

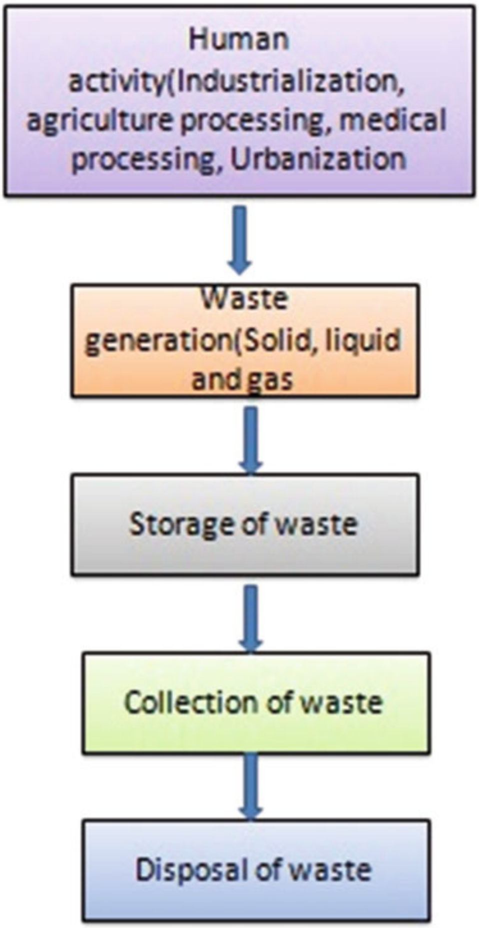
## 36.1 Introduction

Today's major concern is environmental protection and maintenance of its susceptibility. Constant release of harmful waste in the environment is caused by heavy industrialization and urbanization. This uncontrolled waste discharge causes a threat to mankind. Unscientific and ill management of waste has created a problem for ecology and environment. Waste generation is a big problem in Western countries and in developing countries like India. Developing countries are trying to hike their economy so they are being forced to increase industrialization. In India around 42 million tons/year of waste is generated (Surabhi et al. 2016). Treatment of waste generated requires more effective and green technologies. For this microorganisms are the suitable agent. Microorganisms act as magical bullets for the treatment and bioremediation of wastes. Nano-biotechnology when combined with microorganisms to clean the environment is called nano-bioremediation (Chaurasia et al. 2021). This nano-bioremediation is effectively used for the remediation of radioactive sites. The use of genetically modified organisms is common for remediating polluted sites.

Rapid urbanization and industrialization have put tremendous pressure on the environment (Dhankar et al. 2020). In India, rapid development of cities has a significant impact on the environment; if the rate of industrialization increases, the pollution will also increase unless scientific waste management is applied. There is vast difference in the process of waste management in developing and developed nations. Developing nations lack proper scientific collection and disposal mechanisms (Haris et al. 2020). So in developing nations, there is a requirement for developing guidelines for waste disposal mechanisms. Uncontrolled and unscientific duping of wastes has resulted in the overflowing of landfills which results in several environmental implications such as soil, air, and water pollution which ultimately results in global warming. For waste management an effective and environmentally safe system should be found out to properly implement waste disposal for a sustainable environment. Various popular methods of waste disposal are the incineration method, sanitary landfills, recycling, avoidance, and reduction (Saikat et al. 2019).

So, in today's scenario, the main problem is how to restore the environment sustainably. In recent times microorganisms have been found as an effective tool for waste management and for restoring the environment (Banerjee et al. 2018). Bioremediation, biodegradation, biocomposting, and biotransformation have been used to accumulate and degrade wastes effectively. A green algae *Cladophora* has the ability to accumulate toxic metals and has been effectively used for waste management (Maghraby and Hassan 2018). Microbes play an important role in the waste management. In today's time, nanobioremdiation is very popular; nanobioremediation nanoparticles are used for bioremediation. *Deinococcus radiodurans* (extremophilic bacteria) is used to remove the waste from radioactive sites in the USA (Brim et al. 2000; Varma et al. 2017). So, the role of microbes with different biotechnological implications can effectively treat waste disposal.

**Fig. 36.1** Process of waste generation



Waste is generated from human activity, and their unscientific and unplanned dumping has created the problem of pollution (Fig. 36.1). The whole environment is depleted due to the release of contaminates in the environment. Expansion of healthcare facilities, agriculture practices, and industrialization have led to the generation of large quantities of biomedical and agriculture and industrial waste, which have adverse effect on human and environmental health. Basically waste is of three types—solid, liquid, and gaseous (Hussain and Dhanker 2021) (Fig. 36.2). Basically the sources of wastes can be divided into four categories—industrial, municipal, biomedical, and electronic. Waste management is the storage collection and proper scientific disposal of waste materials. The main aim of waste management is to reduce the hazardous effects of waste materials. The four R theory of waste management is refuse, reduce, reuse, and recycle. The waste management theory in India depends upon the waste generation, storage, collection, transportation, recycling, treatment, and disposal.

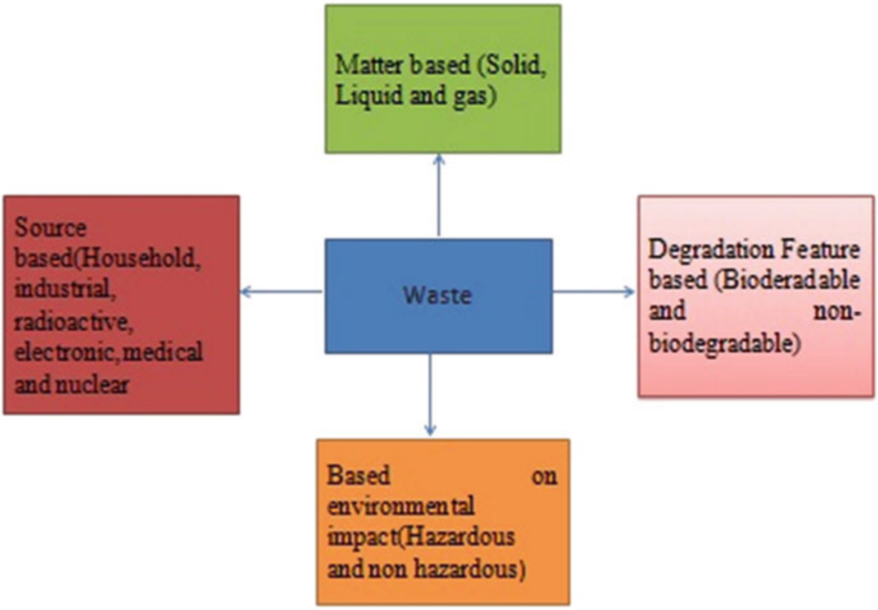


**Fig. 36.2** Waste categories

Microorganisms are widely distributed on Earth. They can easily grow in a wide range of environmental conditions because of their metabolic ability. They can easily breakdown many living and nonliving materials. Thus they help in the waste removal, and they are environment friendly too. Microorganisms play an important role in solving many environmental problems caused by human beings. Microorganisms are extensively used in genetic engineering, municipal waste treatment, industrial waste treatment, environmental bioremediation, etc. The disposal of liquid and solid wastes is the main issue for disposal (Maurya et al. 2020). High composition of nonbiodegradable wastes is the main issue regarding waste disposal. This problem can be solved by using microbes. So here we will describe the use of microorganisms in waste management.

## 36.2 The Role of Microorganisms for a Sustainable Eco-Friendly Environment

Microorganisms play an important role in the waste management; they decompose organic matter, reduce BOD, coagulate non-settleable colloidal soils, and stabilize organic matter. Microorganisms have the ability to convert organic matter into gaseous products. Chemical fertilizers, pesticides, herbicides, and others are frequently used for increasing agriculture production (Dhankar et al. 2021). But in

**Table 36.1** The role of microbes in degrading various types of pollutants

| S. no. | Waste to be degraded | Microorganism used |
|---|---|---|
| 1. | Microbes used for plastic degradation (Saikat et al. 2019) | *Corynebacterium* sp., *Pseudomonas* sp., *Arthrobacter globiformis*, *Bacillus* sp., *Rhodococcus ruber* C208, *Brevibacillus borstelensis* 707, *Rhodococcus ruber* C208, *Staphylococcus epidermidis*, *Bacillus cereus* C1, *Micrococcus* sp., *Vibrio* sp., *Arthrobacter* sp., *Pseudomonas* sp., *Rhodococcus rhodochrous* ATCC 29672. *Nocardia steroids* GK 911, *Bacillus mycoides, Pseudomonas aeruginosa*, *Pseudomonas putida*, *Bacillus subtilis*, *Serratia marcescens*, *Bacillus cereus*, *Pseudomonas aeruginosa*, *Streptococcus aureus*, *Micrococcus lylae*, *Pseudomonas* sp., *Micrococcus luteus*, *Bacillus subtilis*, *Streptococcus lactis*, *Proteus vulgaris*, *Streptomyces* KU5, *Streptomyces* KU1, *Streptomyces* KU6, *Acinetobacter calcoaceticus*, *A. gerneri, Chaetomium globosum*, *Aspergillus terreus*, *Curvularia senegalensis*, *Fusarium solani*, *Aspergillus niger*, *Penicillium* sp., *Chaetomium globosum*, *Pullularia pullulans*, *Fusarium* sp. AF4, *Aspergillus oryzae*, *Phanerochaete chrysosporium*, *Penicillium* sp., and *Aspergillus* sp. |
| 2. | Microbes used for xenobiotic degradation (Karigar and Rao 2011) | *Bacillus* sp., *Rhodococcus*, *Arthrobacter*, *Pseudomonas putida*, and *Mycobacterium* |
| 3. | Oil spill (crude oil, diesel oil) (Abatenh et al. 2017) | *Alcaligenes odorans*, *Bacillus subtilis*, *Corynebacterium propinquum*, *Pseudomonas aeruginosa*, *Bacillus cereus*, *Aspergillus niger*, *Candida glabrata*, *Candida krusei* and *Saccharomyces cerevisiae*, *B. brevis*, *P. aeruginosa KH6*, *B. licheniformis*, and *B. sphaericus* |
| 4. | Dye and paint (industrial dye, azo dye, vat dyes, oil-based paints) (Abatenh et al. 2017) | *B. subtilis strains NAP1*, *NAP2*, and *NAP4*; *Myrothecium roridum* IM 6482; *Pycnoporus sanguineous*; *Phanerochaete chrysosporium* and *Trametes trogii*; *Penicillium ochrochloron*; *Micrococcus luteus*; *Listeria denitrificans* and *Nocardia atlantica*; *Bacillus* spp. *ETL-2012*; *Pseudomonas aeruginosa*; *Bacillus pumilus* HKG212; *Exiguobacterium indicum*; *Exiguobacterium aurantiacums*; *Bacillus cereus* and *Acinetobacter baumanii*; *Bacillus firmus*; *Bacillus macerans*; *Staphylococcus aureus*; and *Klebsiella oxytoca* |
| 5. | Microbes used for heavy metal degradation (Abatenh et al. 2017) | *Saccharomyces cerevisiae*, *Cunninghamella elegans*, *Pseudomonas fluorescens* and *Pseudomonas aeruginosa*, *Lysinibacillus sphaericus* CBAM5, *Microbacterium profundi* |

(continued)

**Table 36.1** (continued)

| S. no. | Waste to be degraded | Microorganism used |
|---|---|---|
| | | strain Shh49T, *Aspergillus versicolor*, *A. fumigatus*, *Paecilomyces* sp., *Paecilomyces* sp., *Trichoderma* sp., *Microsporum* sp., *Cladosporium* sp., *Geobacter* spp., *Bacillus safensis* (JX126862) strain (PB-5 and RSA-4), *Pseudomonas aeruginosa*, *Aeromonas* sp., *Aerococcus* sp., *Rhodopseudomonas palustris* |
| 6. | Microbes used for pesticide degradation (Abatenh et al. 2017) | *Bacillus staphylococcus*, *Enterobacter*, *Pseudomonas putida*, *Acinetobacter* sp., *Arthrobacter* sp., *Acinetobacter* sp., *Pseudomonas* sp., *Enterobacter* sp., and *Photobacterium* sp. |
| 7. | Microbes used for hydrocarbon degradation (Abatenh et al. 2017) | *Penicillium chrysogenum*, *P. alcaligenes*, *P. mendocina* and *P. putida*, *P. veronii*, *Achromobacter*, *Flavobacterium*, *Acinetobacter*, *Pseudomonas putida*, *Phanerochaete chrysosporium*, *A. niger*, *A. fumigatus*, *F. solani* and *P. funiculosum*, *Coprinellus radians*, *Alcaligenes odorans*, *Bacillus subtilis*, *Corynebacterium propinquum*, *Pseudomonas aeruginosa*, *Tyromyces palustris*, *Gloeophyllum trabeum*, *Trametes versicolor*, *Candida viswanathii*, *Cyanobacteria* (green algae and diatoms), *and Bacillus licheniformis*, *Acinetobacter* sp., *Pseudomonas* sp., *Ralstonia sp.* and *Microbacterium* sp., *Gloeophyllum striatum*, *Acinetobacter* sp., *Pseudomonas* sp., *Ralstonia* sp., and *Microbacterium* sp. |

today's scenario, due to increased awareness regarding the harmful effects of these fertilizers, people are looking for environmentally safe products (Cai et al. 2013). The use of soil microbes is the best way to reduce the pesticide application in agriculture field (Mandal et al. 2021; Kumar et al. 2021). These microbes synthesize plant growth hormones, antibiotic, and siderophores and increase phosphate content of the soil (Vaxevanidou et al. 2015). Table 36.1 showed the potential of microbes against different types of pollutants for the development of sustainable environment.

### 36.2.1 The Role of Microorganisms as Biofertilizers

Microorganisms increase the availability of nutrients to plants, preventing soil erosion. Combined treatment of *Bacillus megaterium* and *Bacillus mucilaginous* increased the availability of P and K in soil (Hussain et al. 2020b). Fluorescent *Pseudomonas* strains have been found to be effective in increasing the *Vigna mungo* production in comparison to control. Microbes are eco-friendly, environmentally safe, and beneficial which can increase the crop production without harming the environment.

### 36.2.2 Heavy Metal Removal

After metal extraction the metal effluent is discharged which causes various environmental problems. Removal of metal ions from effluent discharge through physico-chemical method is called bioleaching, which is done by microorganisms. Physico-chemical techniques are costly, while bioleaching is a cheap and eco-friendly process. The process of metal removal depends upon the affinity between microbial cell and metal ion. *B. subtilis* and *Shewanella* and *Brevundimonas* spp. are reported to have the capacity to remove Ca, Mg, and Fe in the Nigel mining areas. Several strains of *Bacillus* have been reported to have the capacity to remove Pb, Cd, Cu, Ni, Co, Mn, Cr, and Zn (Haris et al. 2021).

### 36.2.3 Oil Spill Bioremediation

Oil spills pose a great threat to the ecosystem; the best way of this oil spill pollution is the use of microbes. The two approaches involved in the bioremediation of oil spills are bioaugmentation and biostimulation. Crude oil is very difficult to decompose. The success rate of oil spill degradation depends upon the physical, chemical, and biological conditions that enhance the rate of biodegradation.

### 36.2.4 Microbes and Natural Farming

In natural farming, mycorrhizae are propagated in plant roots. They are symbiotic in nature and convert complex substrates into simpler ones (Hussain et al. 2020a). The various advantages associated with natural farming are the following:

1. Cost-effective.
2. Without any environment and health side effect.
3. Higher yields.
4. Better quality.
5. Farmer friendly.
6. Zero waste emission.

### 36.2.5 Microorganisms and Biocomposting

In terms of disposal, agro-industrial wastes are the main problem. Open air burning of agriculture residues creates the emission of several air pollutants which impart a negative effect on human health and the environment. Discharging agro-waste directly into the river and lake can decrease the quality of water. Because of all these issues, there is a need to find efficient alternatives for waste management. The best alternative is composting in terms of low environmental impact and cost, and it generates valuable products for increasing soil fertility (Ahamd et al. 2019).

## 36.3 Microbes as Vital Additive for Solid Waste Management

Solid waste generation is a serious problem caused by urbanization, overpopulation, and industrialization. Indiscriminate disposal of wastes has created solid waste problem. Solid waste includes household wastes and commercial waste. It can be divided into three categories: degradable (paper, food waste, straw, and yard waste), partially degradable (wood and sludge), and nonbiodegradable (leather, plastics, metal, glass, electronics).The management strategies for solid waste include collection, transportation, processing, and disposal. In 2000, the Ministry of Environment, Forest and Climate Change has released the guidelines for the disposal of solid wastes. The present solid waste management practices in India include proper material recycling, reduction, and stabilization before the land filling (Bong et al. 2017). In India approximately 1,88,500 tonnes of waste are generated per year. The most common method used for waste management is land filling. In India, by 2047, about 1400 sq. km of land area will be required for land filling. Landfills generate a high amount of secondary pollutants such as leachate, greenhouse gases, etc. which are the main problems associated with landfill use. Among all the disposal methods, composting is the eco-friendly and economical method used for waste management. This method has several benefits like reduction in greenhouse gases, making organic matter and NPK content available. Composting can be defined as an aerobic, biochemical, and microbial process that breaks down the organic fractions into stable compounds called humus. Microbes used in composting utilize carbon and nitrogen source as the sole energy source and produce soil-enriching compost; this compost have significant amount of humic substances which act as soil amendment (Białobrzewski et al. 2015). The process of composting have three phases: initial mesophilic phase, thermophilic phase, and cooling phase characterized by microbial activity and decreased temperature. Composting is an oxygen-dependent process (aerobic), but anaerobic microorganisms like *Clostridium* sp. has been found effective for this process (Franke-Whittle et al. 2014; Bhatia et al. 2013).

**Factors Affecting Composting Process**

- C/N ratio.
- pH,
- Moisture content.
- Aeration/$O_2$ supply.
- Temperature.
- Particle size.
- Bulking agents.

Composting is a naturally occurring process, but an EM culture can increase the process of composting. Efficient microbial culture can enhance the degradation process. A major portion of solid wastes is organic (plant biomass, etc.). Limited number of microbes is capable of producing cellulase enzymes which can degrade the waste (Fig. 36.3). The potent cellulase-producing microbes include a few known potent cellulose-producing bacteria such as *Cellulomonas*, *Pseudomonas*, *Bacillus*

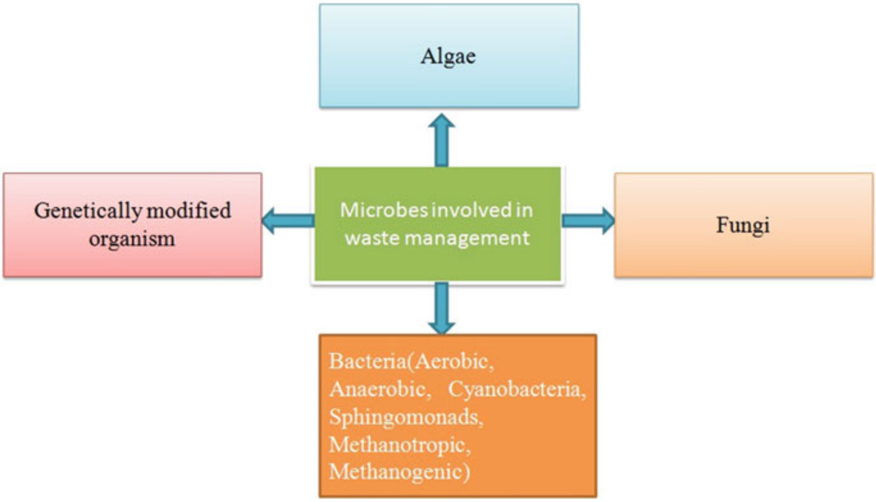


**Fig. 36.3** Microorganisms involved in waste management

spp., and *Thermophilic actinomycetes*. Likewise, fungal species *Aspergillus*, *Trichoderma*, *Sclerotium*, and white-rot fungi, the microbial additive to a compost mixture, affect the temperature profile and ammonia emissions, affecting thermophilic and mesophilic bacterial population increase in enzymatic activity which can reduce the emission of odors of mainly volatile organic compounds (VOCs) (Siddiqua et al. 2021). Microbes also decrease the time of operation in composting process. In a study conducted by Wei et al. (2019), it has been found that addition of actinomycetes in the compost results in the production of xylanase, cellulase, and lignin peroxidase and many other enzymes which accelerate the composting process. The use of *Actinomycetes* inoculums in dairy waste results in the increased rate of compost degradation and increase humic acid production (Zhao et al. 2017). In an experiment carried out by Manu et al. (2019), they inoculated microbial culture in a household waste and observed that the composting time reduced to about 30–36 days. In a composting experiment performed by Awasthi et al. (2015), feedstock material inoculated with *Phanerochaete chrysosporium*, *Trichoderma viride*, and *Pseudomonas aeruginosa* results in a shorten composting period. Municipal solid waste inoculated with mixed culture of *Bacillus casei*, *Candida rugopelliculosa*, *Lactobacillus buchneri*, *Trichoderma*, and white-rot fungi results in the reduced nitrogen loss and increased mineralization (Awasthi et al. 2016). SAM results were obtained by Varma and Kalamdhad (2015) by using *Phanerochaete chrysosporium* (white-rot fungus) during drum composting. In India, in urban areas for waste degradation, there are many waste management technologies. Among all the available technologies, composting with an effective microbial culture is the best choice. Nontoxicity of compost should be checked before use. With microbe addition in composting process, good results can be achieved.

## 36.4 Management of Domestic Waste

Domestic waste disposal is a burning issue for urban areas in any country. Countries without functioning waste disposal strategies face many health and economic issues. The waste disposal strategies include a sensible management of waste disposal including its segregation at the source; it should be divided into organic and inorganic waste category (Rousta et al. 2016). The process of waste management involves the following:

1. Collection.
2. Segregation.
3. Storage.
4. Transportation.
5. Processing.
6. Disposal.

### 36.4.1 Methods of Management

#### 36.4.1.1 Sanitary Landfills

The open dumping of solid wastes has been replaced by sanitary land filling. Sanitary landfills are natural and man-made depressions in which domestic waste are dumped and covered with a soil layer. As daily covering of waste by soil is done, issues associated with open land filling such as dirt, odor, flies, insects, and rodents are eliminated. The most commonly used method of waste disposal accounts for 80% of waste disposal. During closure landfills are covered with clay to protect it from rain. In comparison to dumps, landfills are filled up much faster. When landfill sites are full, they are converted into the playground and parks. In modern landfills vent pipes are provided to collect methane, which can be used to produce steam and electricity. This saves energy and lowers the atmospheric methane concentration; therefore, it reduces global warming. Pumps are also provided in the landfills to suck out leachate which is then transported to sewage plants.

#### 36.4.1.2 Composting

Domestic wastes can be composed in composting sites and sold as fertilizers. Composting is a process of biological decomposition and stabilization of organic substrates. The end product of composting is used as manure in agriculture. The factors affecting composting are C/N ratio, moisture, oxygen, and temperature (Miafodzyeva and Brandt 2013). The end product of compost is rich in nutrients and minerals which act as soil conditioner. About 60% water is required for the process of composting. Composting is of two types: aerobic and anaerobic. In aerobic process high temperature is produced, and odor-free compost is formed rapidly; in anaerobic process low temperature is formed and it is a slow process. It produces compost with odor.

#### 36.4.1.3 Vermicomposting

In vermicomposting process, biodegradable waste is converted into compost with the help of earthworms. Vermicomposting can be done at an orchard and farm.

#### 36.4.1.4 Biomethanation

It is an innovative technique used for treating waste; the end product formed is biogas and organic manure. For this process the waste should not be older than 24 h.

#### 36.4.1.5 Incineration

It is a technique which reduces the amount of landfill space. This process generates high energy. The main drawback associated with this technique is its high cost. This process destroys 99.99% organic compounds, converting them into $CO_2$ (water) and harmless gas. By this method around 80% of waste is degraded, and it also kills pathogenic organisms (Rousta et al. 2016).

#### 36.4.1.6 Fuel Pelletization

It is a process of production of fuel pellets from wastes. The process of fuel pelletization involves drying, removal of noncombustibles, grinding, mixing, and production of pallets from solid waste. Approximately 17–20 tons of pellets are produced from 100 tons of waste. These pellets can be used as fuel for heating plants and boilers (Rousta et al. 2017).

#### 36.4.1.7 Recycling

In India recycling is an unorganized sector. This sector consists of waste pickers, buyers, dealers, and wholesalers. The recycling sector is facing many problems like mixed disposal, toxic matter handling, accident risk, poor product design, and lack of market.

#### 36.4.1.8 Farmyard Manure

Farmyard manure (FYM) is the type of bulky organic manure which is prepared from the cow dung, urine-soaked litter of farm animals, and many other organic wastes and further goes through partial decomposition process by different microorganisms. It also nurtures the organisms living beneath the soil. It is oldest methods used for manure and more valuable organic fertilizer can be used by the farmers till now and also known as traditional manure. This manure is easily prepared. Earlier, the farmers incorporate FYM in their field when they were not able to afford synthetic fertilizers. FYM plays a vital role in improving the soil health which somehow helps in raising the productivity of the crop also.

### Composition of FYM

The well-rotten farmyard manure is composed of 0.5% N, 0.2% $P_2O_5$, 0.5% $K_2O$, 0.08% Na, 0.02% S, 0.004% Zn, 0.0003% copper, 0.007% Mn, and 0.45% Fe.

### Factors Affecting the Quality and Composition of FYM

1. *Types of animal*: The quality and composition of FYM also depends on the type of animals reared. The FYM prepared from the cow's dung and urine is good compared to other livestock animals.
2. *Bedding material used*: In best bedding process, the material used for improving the quality of FYM is the straw which is laid down on the floor because the straw reduced the wastage of urine because it contains near about percent of nitrogen and 1.35% of potassium.
3. *Type of fodder fed to cattle*: Superior fodder crop is fed to the cattle because it improves the quality of milk but also the quality and composition of FYM.
4. *Animal age*: As the age of animal increases, the quality of milk decreases, and similarly, the quality of FYM gets affected.
5. *Collection manner in shed*: In this, cow dung is collected from time to time because if the dung is still in the field, there will be wastage of litter and nutrients and they will get volatilize because cow urine contains nitrogen in the form of urea which results in leaching and volatilization.
6. *Storage in the pit*: The pit should be filled up to a height of 45–60 cm above the ground level and should be plastered with cow dung earth slurry, and shaped like a dome.

### Preparation Methods of FYM

Basically, there are three methods for the preparation of FYM.

(a) Pit Method.
  - Pits are made with dimensions 8 m × 2 m × 1 m.
  - The pits are to be filled layer wise which contains the cow dung and urine-soaked litter up to a certain height from the ground level.
  - After filling, the pit is plastered with mud paste or cow dung earth slurry.
  - For two adult animals, one pot is required.
  - The FYM is ready within 150–180 days after plastering.
  - Near about 10–12 tons of FYM are obtained per pit, and an animal gives 5–6 tons of FYM per year.

(b) Trench Method.
  - This method is also known as the Dr. C.N. Acharya method.
  - The dimensions of trenches are 6.5–8 m × 15–2.0 m × 1 m.
  - The mixture of cow dung and litter is placed in the trench and filled up to a height of 50–60 cm above the ground.
  - The trenches should be covered with mud paste or cow dung earth slurry.
  - The FYM is ready to be applied in the field within 150–160 days after plastering.

(c) Heap Method.
  - Commonly used method by farmers.
  - They sweep out every day, and the mixture of cow dung and litter is collected on a fixed place.
  - After 6–8 months, the well-rotten FYM is ready.
  - But in this method, there should be maximum nutrients losses because of many reasons, i.e., the heap is opened; leaching and volatilization is fast; and during rainfall, runoff also takes place, due to which its quality and composition both get reduced.

### How to Apply FYM in the Field

- FYM should be applied 15–20 days in the field before sowing.
- Well-rotten FYM should be applied, under composed manure if applied results in slow decomposition process.
- Optimum soil moisture is required at the time of application for better microbial activity; 2–5 tons/ha of FYM is applied on an average for most of the crops.

### Microorganisms Which Decompose the FYM

Basically, there are two types of decomposition. One is anaerobic decomposition (decomposition takes place without oxygen), and the other is aerobic decomposition (decomposition takes place with oxygen).

In these processes, bacteria, fungi, molds, protozoa, actinomycetes, and other saprophytic organisms feed upon decaying organic materials initially, while in the later stages of decomposition, mites, millipedes, centipedes, springtails, beetles, and earthworms further breakdown and enrich the manuring materials.

### Advantages of FYM

- It is a rich source of plant nutrients.
- It maintains soil microbial activity which further improves soil health.
- Nitrogen in a small amount is also being available to plant when the FYM decomposes.
- Soil fertility also improves.
- The soil's physical, chemical, and biological processes also get affected by the application of FYM.

### Disadvantages of FYM

- FYM takes more time to prepare as compared to other manures.
- It has slow decomposition rate.
- It should be applied in large quantity because the nutritive value is comparatively lower than other manures.
- For application it required more labor.

## 36.5 Bioremediation

Pollution from the agriculture, domestic waste, and industries generated toxic metals such as heavy metals which have effect on the inhabitant living in any community. Bioremediation strategy is a promising technology for solving environmental pollution due to the fact that it is environmentally friendly and cost-effective (Luka et al. 2018). Bioremediation involved the use of biological systems for the removal or reduction of contamination from the air, soil, and water. The process involves the use of organism obtained from the environment of interest or imported from other system and exposing it to a target contaminant so as to reduce or remove the toxic component. Therefore, bioremediation is the application of biosystems such as microbes, plants, and animals to reduce the potential toxicity of any contaminants in the environment by degrading, transforming, and immobilizing these undesirable substances to less harmful forms. Bioremediation relies on stimulating the growth of certain microbes that utilize contaminants like oil, solvents, and pesticides for sources of food and energy. These microbes convert contaminants into small amounts of water, as well as harmless gases like carbon dioxide (Endeshaw et al. 2017; Hussain et al. 2021).

Conditions that are unfavorable for bioremediation may be improved by adding "amendments" to the environment, such as molasses, vegetable oil, or simple air. In 1989, the Exxon Valdez oil tanker ran aground off the coast of Alaska; the tanker ended up spilling approximately 11 million gallons of oil. Around this same time, bioremediation was gaining traction as a viable option for oil cleanups. The EPA and Exxon Mobil Corporation (XOM) both began testing different compounds. Initial tests regarding the effectiveness of bioremediation looked promising. Between 1989 and 1990, more than 100,000 pounds of fertilizer was applied over more than 2000 applications to the affected areas. By mid-1992, the cleanup was considered complete, and the fertilizer had degraded nearly all the oil compounds (Leung 2004). Mushroom-forming fungi (mostly basidiomycetes) are among nature's most powerful decomposers, secreting strong extracellular enzymes due to their aggressive growth and biomass production. These enzymes include lignin peroxidases (LiP), manganese peroxidase (MnP), and laccase (Adenipekun and Lawal 2012). Bioremediation techniques are noninvasive, cost-effective, and environmentally friendly compared to their counterparts (physical and chemical methods of remediation). In addition, bioremediation conserves soil texture and characteristics. Although bioremediation has many advantages, the success of its application depends on complex factors, which are classified into two major groups, namely, nature of pollutant (degree of pollution, aggregate, and oxidation state of crude oil) and environmental conditions, (pH, moisture content, presence of alternate carbon sources, and microbes with degradation capability, soil property, and nutrient availability) (Naik and Duraphe 2012). Bioremediation strategies can be generally grouped into two, namely, ex situ and in situ methods.

Ex situ strategies comprise of bio-augmentation, bio-piling, bioreactors, composting, and land farming which involve the removal of materials by excavation, pumping, or dredging; ex situ allows greater process control, although there will be some disruption to the site. They also enable environmental conditions of contaminated material to be easily modified and monitored, leading to greater efficiency of treatment.

Concerning in situ technologies which include bioventing, biofilters, biostimulation and bioaugmentation, minimal disruption of sites, and elimination of handling costs, they usually require longer periods of treatment and extended monitoring. They can also be constrained by geological, hydrogeological, and other environmental factors, resulting in a low efficiency of contaminant removal. A great variety of organisms are potentially useful for bioremediation; in situ applications are limited to natives species as the introduction of exotic organisms represents an ecological risk, restricting possible interesting application (Nwogu et al. 2015).

There are so many factors affecting the rate of bioremediation which include energy sources, bioavailability, and bioactivity and biochemistry of the systems. Major factors affecting bioremediation are microbial growth until critical biomass is reached, mutation and horizontal gene transfer, enzyme induction, enrichment of the capable microbial populations, production of toxic metabolites, environmental depletion of preferential substrates, lack of nutrients, inhibitory environmental conditions, substrate with too low concentration of contaminants, chemical structure of contaminants, toxicity of contaminants, solubility of contaminants, biological aerobic versus anaerobic process, oxidation/reduction potential, availability of electron acceptors, microbial population present in the site, growth substrate versus cometabolism type of contaminants, concentration, alternate carbon source present, microbial interaction (competition, succession, and predation), physico-chemical bioavailability of pollutants (equilibrium sorption, irreversible sorption), incorporation into humic meters, mass transfer limitations, oxygen diffusion and solubility, diffusion of nutrients, and solubility/miscibility in/with water (Okparanma et al. 2011).

## 36.6 Wastewater Treatment

Nowadays lots of activities of man are mainly water dependent and therefore discharge “waste” into water. Some of the substances include body wastes (faces and urine of human and dairy industry), hair, hair shampoo, food scraps, fat, fabric conditioners, laundry powder, toilet paper, lots of chemicals, domestic and other detergent, household cleaners, dirt, and lots of microorganisms (including bacteria, viruses, fungi, and protozoa, e.g., *Vibrio cholera*, enterovirus, hepatitis A and E virus, *Candida albicans*, *Entamoeba histolytica*, *Giardia lamblia*) and sometimes including helminths (e.g., *Schisto somamansoni*, *Ascaris lumbricoides*), which are able to cause many diseases in people and damage the environment (Gaffney et al. 2015; Shah et al. 2015). These above microorganisms come in large quantities from faces of infected animals and humans (Donde 2017; Namratha et al. 2016). The total waste generated by the industries is estimated to be 591,000 tons/year (Hajjar 2012). Wastewater treatment process is a technology that resulted in contamination free reusable water and ensure a good public health and green and pollution-free environment that is the current need. In the current situation, water deficiency is a major problem in many countries, considering that recycling and reuse of water is the need of today. Many Arab and other country face water scarcity for this situation; they frequently use wastewater technologies and reuse the water (Bahadir et al. 2016). The main reuse of wastewater is in agriculture and other form. Now some company makes beer from wastewater. Handling of wastewater protects the environment to ensure public health, economic, and social welfare (Gaffney et al. 2015; Donde 2017; Namratha et al. 2016).

Wastewaters are classified mainly in two types: domestic and industrial wastewater. Preliminary treatment of wastewater includes the processes that allow the removal of debris and coarse biodegradable material from the waste stream and/or stabilize the wastewater by chemical addition. In wastewater treatment process, primary treatment mainly refers to a sedimentation process ahead of the main system. In domestic wastewater treatment, preliminary processes will remove approximately 25% of the organic load and other nonorganic solids. In industrial waste and wastewater treatment, preliminary or primary treatment may include flow equalization, pH adjustment, or chemical addition, which are compulsory steps to the entire treatment process (Tripathi and Hussain 2021). Table 36.2 lists the typical effluent levels by degree of treatment. This section of the manual will discuss the various types of preliminary and primary treatment processes available (Figs. 36.4 and 36.5).

There are several parts in wastewater treatments including screening and pumping, grit removal, primary settling, aeration/activated sludge, secondary settling, filtration, disinfection, and finally oxygen uptake steps.

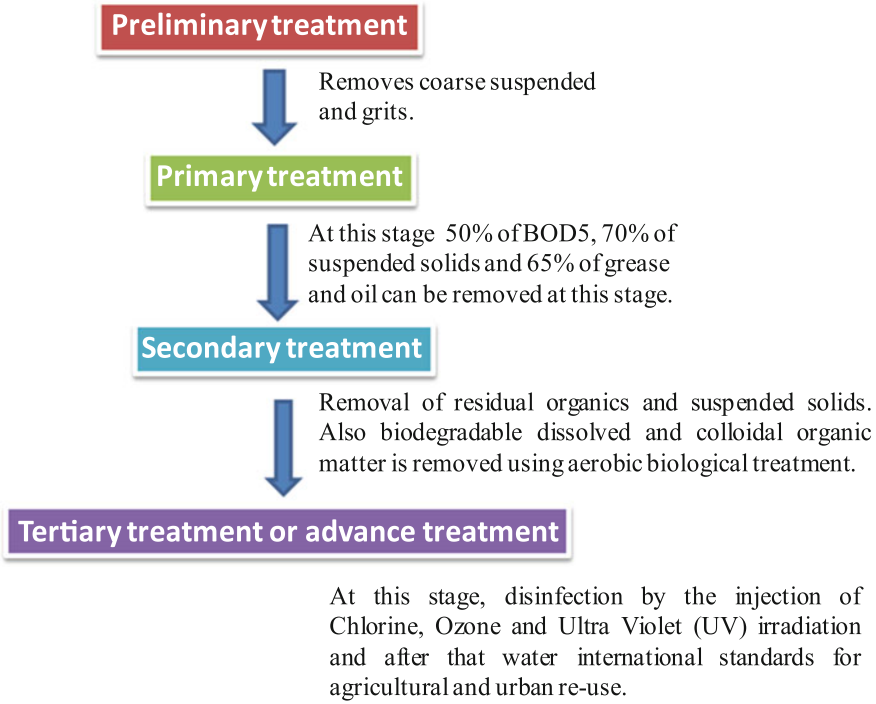
**These are the major steps involved in wastewater treatment:**
Step 1: Screening and pumping.
Step 2: Grit removal.
Step 3: Primary settling.
Step 4: Aeration/activated sludge.
Step 5: Secondary settling.
Step 6: Filtration.
Step 7: Disinfection (final treatment).
Step 8: Oxygen uptake.

## 36.7 Aerobic Treatment of Wastewater

Treatment of sewage water from industries or domestic becomes a very critical process in present days. An aerobic treatment of wastewater system is a type of small-scale sewage treatment system like septic tank system in which it uses an aerobic process for digestion. These systems are frequently used in rural areas where public sewer system are not present and may be used for a single residence or for a small group of homes (small town and village). The main reason for a successful aerobic wastewater treatment is low operation and maintenance costs and very easy

**Table 36.2** Typical effluent levels of principal domestic wastewater characteristics by degree of treatment (mg/L)

| Parameters considered for wastewater treatment | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Parameter | BOD (biochemical oxygen demand) | COD total (chemical oxygen demand) | COD soluble | Suspended Solids | Ammonia (as N) | Phosphate (as P) | pH (units) | Fecal coliform (no./100 mL) |
| Average Raw wastewater | 300 | 600 | | 300 | 25 | 20 | 7 | 1,000,000 |



Removes coarse suspended and grits.

At this stage 50% of BOD5, 70% of suspended solids and 65% of grease and oil can be removed at this stage.

Removal of residual organics and suspended solids. Also biodegradable dissolved and colloidal organic matter is removed using aerobic biological treatment.

At this stage, disinfection by the injection of Chlorine, Ozone and Ultra Violet (UV) irradiation and after that water international standards for agricultural and urban re-use.

**Fig. 36.4** Flow diagram shows the levels of treatment: primary, secondary, and tertiary

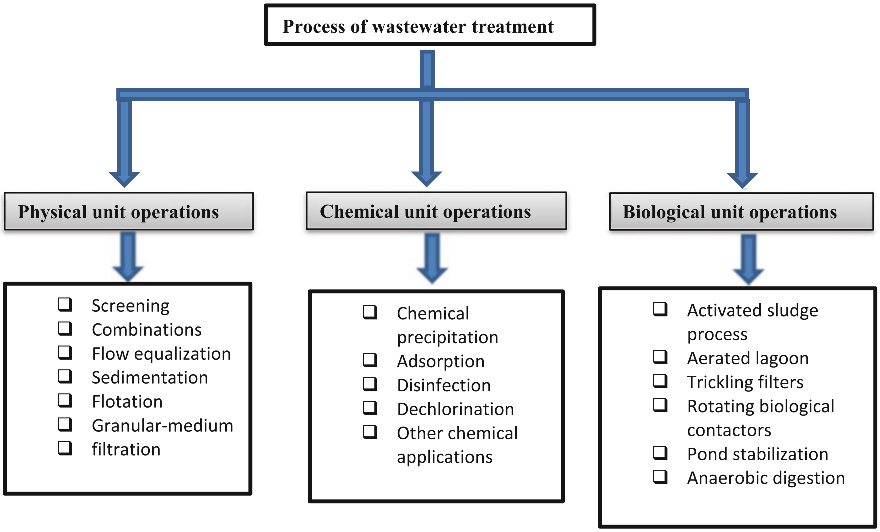


**Fig. 36.5** Flow diagram shows the process of wastewater treatment, all the major types including physical, chemical, and biological operations

to obtain, with well effectiveness and ability for degrading contaminants. In this chapter, the use of aerobic wastewater treatment process has been described in details and mentioned how to remove contaminants from wastewater (Tripathi and Hussain 2022a, b). This book chapter concludes that suspended growth bioreactors are very efficient at low organic loading rates for treating wastewaters. Most of the biofilm reactors have the same level of chemical oxygen demand (COD) removal. It is different from the traditional septic system; the aerobic treatment system produces a high-quality secondary effluent, which can be sterilized and used for surface irrigation purpose.

**The initial process of wastewater treatment are divided into four different stages:**

*Pre-treatment stage*: In this stage the large solid substances are removed.

*Aeration stage*: In this stage aerobic bacteria digest biological wastes.

*Settling stage*: This stage allows undigested solids to settle. This forms a sludge that must be periodically removed from the system.

*Disinfecting stage* (optional stage): It is where chlorine ($Cl_2$) or similar disinfectant is mixed with the water, to produce an antiseptic output. Sometimes UV disinfection is used, where the water is exposed to UV light.

## 36.7.1 Types of Aerobic Treatment Systems

Generally small-scale aerobic treatments systems are frequently used of the two designs. It is fixed film systems or continuous flow suspended growth aerobic systems (CFSGAS). The processes of pre-treatment and effluent handlings are similar for both types of systems and only differ in the aeration stage.

### 36.7.1.1 Fixed Film Systems

In this type of systems, a porous medium is used which provides a bed to support the biomass film that digests the waste material in the wastewater. Fixed film systems vary widely into two basic categories. The first type of system is where the media is moved relative to the wastewater, alternately immersing the film and exposing it to air, and the second type uses a stationary media and varies in wastewater flow, so the film is alternately submerged and exposed to air. The main drawback in both systems is that the biomass must be exposed to both wastewater and air for the aerobic digestion to occur. The film itself may be made of any suitable porous material, such as generally peat moss or plastic. Simple systems use stationary media and rely on intermittent, gravity-driven wastewater flow to provide periodic exposure to air and wastewater. A common moving media system is the rotating biological contactor (RBC), which uses disks rotating slowly on a horizontal shaft. Near about 40% of the disks are submerged at any given time, and the shaft rotates at the speed of 1–2 rpm (revolutions per minute).

#### 36.7.1.2 Continuous Flow Suspended Growth Aerobic Systems (CFSGAS)

These types of aerobic systems are designed in such a manner that allows continuous flow and do not provide a bed for a bacterial film, relying rather on bacteria suspended in the wastewater. Aerations are provided by an air pump, which pumps air through the aeration chamber, and for the addition of oxygen, constant stirring process of the wastewater is necessary; it is known as the oxygenation step. When excess biomass is contained in the wastewater compared with normal levels, then a medium to promote fixed film bacterial growth may be added to some systems designed to operate the situations.

#### 36.7.1.3 Retrofit or Portable Aerobic Systems

The use of this type of aerobic treatment is for the remediation of failing or failed anaerobic septic systems, by retrofitting an existing system with an aerobic feature. This class of product, known as aerobic remediation, is designed to remediate biologically failed and failing anaerobic distribution systems by significantly reducing the BOD5 and total suspended solids (TSS) of the effluent. The reduction of the BOD5 and TSS reverses the developed bio-mat. Further, effluent with high dissolved oxygen and aerobic bacteria flow to the distribution component and digest the bio-mat.

#### 36.7.1.4 Composting Toilets

These types of toilets are designed only for the purpose of toilet waste, and it is water-free toilets rather than the flush toilets attached with these types of aerobic treatment systems. These systems treat the waste as a moist solid, rather than a liquid suspension, and therefore separate urine from feces during treatment to maintain the moisture content in the system. *Clivus Multrum* (Latin for “inclined chamber”) is an example of a composting toilet, which consists of an inclined chamber that separates urine and feces and a fan to provide positive ventilation. Within the chamber, the urine and feces are decomposed and broken down, not only by aerobic bacteria, but also by fungi, arthropods, and sometimes also involve earthworms. In this treatment process, times are very long, with a minimum time between removals of solid waste of a year; in the treatment process, the actual volume of the solid waste is decreased by 90%; most of them are converted into water vapor and carbon dioxide ($CO_2$). Pathogens are eliminated from the waste by the long durations in inhospitable conditions in the treatment chamber.

## 36.8 Anaerobic Treatment of Industrial Wastewater

Anaerobic wastewater treatment is a type of biological process where microorganisms break down organic contaminant into their component in the absence of oxygen ($O_2$). There are lots of industrial waste materials treated by anaerobic treatment as wastewater from potato-processing industry, confectionery wastewaters, baker’s yeast wastewaters, corn-processing industry, opium alkaloid wastewaters, beverage (beer, malting, soft drinks, wine, fruit juices, and coffee), and others (chemical, pharmaceutical, sludge liquor, municipal sewage) (Brian and Tran 2011; Shah et al. 2015; Donde 2017). In a basic anaerobic treatment cycle, wastewater enters a bioreactor receptacle (Acharya et al. 2008; Ersahin et al. 2014; Zhang et al. 2009).

### 36.8.1 Anaerobic Degradation of Organic Polymers

The anaerobic degradation pathway of organic matter is a multistep process and run parallel and involved a lot of chemical reactions. This process of organic matter degradation proceeds in four successive stages: (1) hydrolysis, (2) acid genesis, (3) acetogenesis, and (4) methanogenesis.

The first anaerobic degradation starts from the hydrolysis of complex organic material to its simple monomer unit by the hydrolytic enzymes. The simpler organics are then fermented to organic acids and hydrogen ($H_2$) by the fermenting bacteria (acidogens). The volatile organic acids are broken down into acetic acid and hydrogen in the presence of acetogenic bacteria. *Archae methanogens* use hydrogen and acetic acid and finally produced methane gas. Methane production from acetic acid ($CH_3COOH$) and from hydrogen ($H_2$) and carbon dioxide ($CO_2$) is carried out by two important bacterial action *acetoclastic methanogens* and *hydrogenotrophic methanogens*. During methane production thermodynamic conditions play a key role. Therefore, accuracy of environmental conditions should be provided in order to carry out both *acetogenesis* and *methanogenesis* (Ersahin et al. 2014; Zhu et al. 2014).

### 36.8.2 Anaerobic Reactor Types

Different reactor configurations are used for the anaerobic treatment of industrial wastes. Among them, the most common types are discussed here and detailed. Most commonly used anaerobic reactor types are (1) completely mixed anaerobic digester, (2) upflow anaerobic sludge blanket (UASB reactor), (3) the anaerobic fluidized bed (AFB) or the expanded granular sludge bed (EGSB) reactor, and (4) upflow anaerobic filter (AF).

#### 36.8.2.1 Completely Mixed Anaerobic Digester

This type of digester is the basic anaerobic treatment system with an equal hydraulic retention time (HRT) and solid retention time (SRT). The range of this type of digester is 15–40 days in order to provide sufficient retention time for both operation and process stability. These types of digesters without recycling are more functional for wastes with maximum amount of solid concentrations (Tchobanoglous et al. 2003; Jensen and Gujarathi 2015). The main disadvantage of this type of system is its high volumetric loading rate; it is only obtained with all concentrated waste streams with a biodegradable chemical oxygen demand (COD) content, ranging between 8000 and 50,000 mg/L. However, much dilute waste streams are also found (Zhu et al. 2014). Thus, COD loading per unit volume may be very low with the detention times of this system which reduces cost-benefit of anaerobic treatment process. In this type of anaerobic digester, organic loading rate (OLR) range is between 1 and 5 kg COD/$m^3$ days (Shah et al. 2015; Tchobanoglous et al. 2003).

#### 36.8.2.2 Upflow Anaerobic Sludge Blanket Reactor (UASB Reactor)

This type of reactor is one of the most developed in anaerobic treatment process technology. Initially, it is used in the Netherlands and nowadays it is the most popular high-rate anaerobic system in the world with its wide applications to treat different types of wastewaters. The UASB process has been used widely in industrial wastewater treatment including paper and chemical industries, food processing, and slaughterhouse wastewater (Gaffney et al. 2015; Tchobanoglous et al. 2003). Influent flow distributed at the bottom of the UASB reactor travels in an upflow mode through the sludge blanket and passes out around the edges of a funnel which provides a greater area for the effluent with the reduction in the upflow velocity, enhancement in the solid retention in the reactor, and efficiency in solid separation from the outward-flowing wastewater. After several weeks, granules are formed naturally, and reactor operation consists primarily of a densely mixed population of bacteria, and these bacterial populations are responsible for the methane fermentation of substrates (Zhu et al. 2014). Good settling ability, low retention times, elimination of the packing material cost, and high biomass concentrations ranging 30,000–80,000 mg/L, high solid/liquid separation, and process at very high loading rates can be obtained by UASB methods. The main limitation of this type reactor is the wastewaters having high solid content which prevents the dense granular sludge development, and the design OLR is typically in the range between 4 and 15 kg COD/$m^3$ day (Tchobanoglous et al. 2003; Zhu et al. 2014).

#### 36.8.2.3 Anaerobic Fluidized Bed (AFB) and Expanded Granular Sludge Bed Reactors (EGSB)

This type of anaerobic reactor is comprised of small media, such as granular activated carbon or sand, to which bacteria attach. Good mass transfer results from the high flow rate around the particles, less clogging, and short circuiting due to large pore spaces formed through bed expansion. However, the difficulty is in developing strongly attached biofilm containing the correct blend of methanogens, detachment risks of microorganisms, negative effects of the dilution near the inlet as a result of high recycle rate and high energy costs. The main drawbacks of this system are high recycle rate/cost. The expanded granular sludge bed (EGSB) reactor is a modification of the AFB reactor with a small difference in the fluid's upward flow velocity. The upflow velocity is not as high as in the fluidized bed which results in partial bed fluidization (Zhu et al. 2014). The capacity of OLR of 10–50 kg COD/$m^3$ day can be applied in AFB-type reactors (Ozturk et al. 2017; Ersahin et al. 2014).

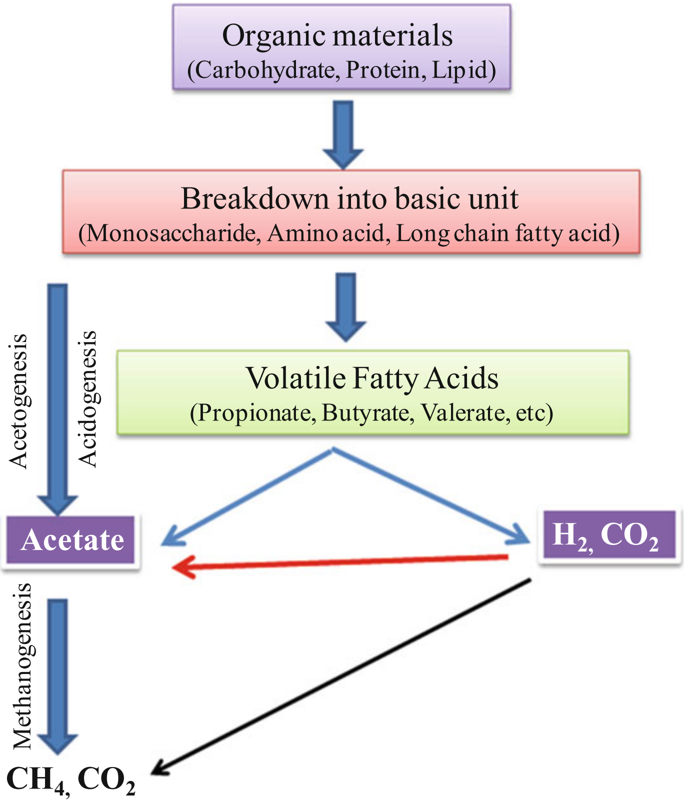


**Fig. 36.6** Steps involved in anaerobic digestion process

### 36.8.2.4 Anaerobic Filters (AF)

A wide application of the anaerobic filter (AF) has been in different food-processing, beverage, and chemical and pharmaceutical industries due to its high ability of biosolids retention. The main problem of this system is clogging by biosolids, influent suspended solids, and precipitated minerals. In this type of filter, both upflow and downflow packed bed processes can be observed. Prevention of methanogens (formation of methane gas ($CH_4$) found at the lower levels of the reactor from the toxicity of hydrogen sulfide ($H_2S$) is done by stripping sulfide in the upper part of the column. However separation of solids from the top by gas recirculation can easily be obtained in downflow systems in comparison to upflow systems. Here in this anaerobic process, higher risk is losing biosolids to the effluent in the downflow systems. The range of design OLR is between 8 and 16 kg COD/$m^3$ day which is more than tenfold higher than the design loading rates for aerobic processes (Zhu et al. 2014; Shah et al. 2015) (Fig. 36.6).

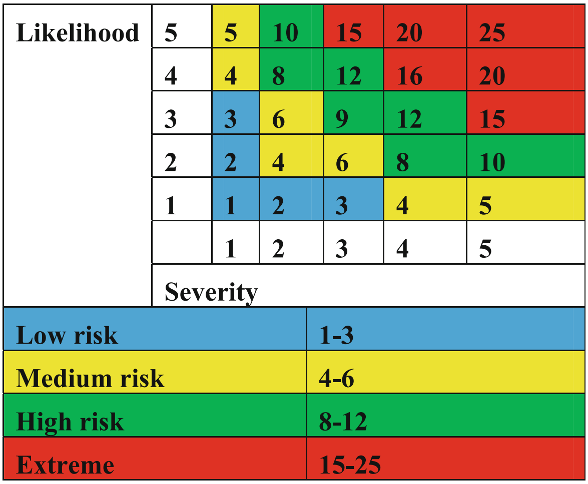
## 36.9 Environment Safety for Health Hazards from Industrial Wastewater

Hazard identification risk assessment (HIRA) is a method to identify hazard and risk factor that have the potential to cause harm and determine the appropriate ways to eliminate the hazard or control of the risk assessment. Physical, chemical, or psychological hazards responsible for the work-related injuries have impact on organizational productivity and profitability (Tripathi and Hussain 2021).

Bahn (2012) and Brown and Ithaca (2016) reported that the high hazard impacts in workplace often become the cause of work accident and occupational disease. Water treatment plant (WTP) has a key role in giving the society access to healthy clean water. In the progress of this, environmental and health safety aspects become the things to be concerned about. To work in the field of water treatment is considered dangerous, especially as it can lead to frequent deaths in confined spaces.

The steps include the following:

1. Hazard assessment: determining the risks and the risk designation (rating) associated to the hazard based on likelihood and severity.
2. Hazard control: controlling the hazards and the risks associated with the hazard.
3. Providing information, education, training, and supervision for employees affected by the hazards.
4. Review of the hazard assessment and control process.



| | | | | | | |
|---|---|---|---|---|---|---|
| **Likelihood** | **5** | **5** | **10** | **15** | **20** | **25** |
| | **4** | **4** | **8** | **12** | **16** | **20** |
| | **3** | **3** | **6** | **9** | **12** | **15** |
| | **2** | **2** | **4** | **6** | **8** | **10** |
| | **1** | **1** | **2** | **3** | **4** | **5** |
| | | **1** | **2** | **3** | **4** | **5** |
| | **Severity** | | | | | |

| | |
|---|---|
| **Low risk** | **1-3** |
| **Medium risk** | **4-6** |
| **High risk** | **8-12** |
| **Extreme** | **15-25** |

Rating the hazard plays important role to determine which hazard is the most serious and thus which hazard to control first. Priority is usually established by taking into account the severity and likelihood.

The following factors play an important role:

1. Severity of exposure: the impact when exposed to the hazard.
2. Likelihood that an incident will occur when exposed to the hazard.

The phase of risk identification is essential, because it puts the bases of the risk analysis. Risk can be calculated using the following formula:

$$\text{Risk}\,(\text{R}) = \text{Likelihood}\,(\text{L}) \times \text{Severity}\,(\text{S})$$

## 36.10 Factor Affecting Waste Management

Municipal solid waste management (MSWM) of developing countries' five typical problem areas can be identified: (1) inadequate service coverage, (2) operational inefficiencies of services, (3) limited utilization of recycling activities, (4) inadequate management of nonindustrial hazardous waste, and (5) inadequate landfill disposal.

Lack of advanced technology, facility for separation at source, strength of solid waste management policy and enforcement, environmental education and awareness, income status of individuals, and among others are factors affecting solid waste scenario. It was reported that education, income, and social status are important factors influencing per capita solid waste generation. Age, educational status, and amount charged for waste collection services had been identified as factors influencing solid waste management in highly populated cities.

## 36.11 Future Challenges

Core to the vision for waste management in India is the use of wastes as resources with increased value extraction, recycling, recovery, and reuse.

1. If solid waste management is to improve in India, then a strong and independent authority is needed to regulate waste. Strong waste regulations can drive innovation; without proper regulation improvements will not happen.
2. Information on future quantities and characterization of wastes is essential to determine the proper waste management and treatment option.
3. Public unawareness to littering and waste in streets is a major problem in India that severely effect on public health. Separating dry (inorganic) and wet (biodegradable) waste would have significant benefits and should be the responsibility of the waste producer.
4. A long-term waste management planning requires visionary project development by ULBs, the private sector, and NGOs. The roles and responsibilities to deliver sustainable systems need to be defined, with monitoring and evaluation to monitor progress.

There are a number of research institutes, organizations, NGOs, and private sector companies working on collaboration to SWM, and future waste management in India must involve appropriate method of different waste throughout the system.

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# Biochemical Effect of Nanoparticle-Treated Plant Extract on Water-Borne Pathogen: A Way Toward Future Technique for Water Purification

# 37

Gajra Garg and Sunita Rao

**Abstract**

Water is one of the imperative factors responsible for life on Earth. Improper garbage handling and sewage disposals can affect the availability of safe drinking water. A huge investment has been made by the government of India to provide safe drinking water. Chlorination is a popular method for water purification, which can cause several health issues like diarrhea and cholera among the populace. New technology is currently in demand which can result in the least side effects on health and sustainability of the ecosystem. In this context, we have explored the importance of nanoparticles like ZnO, CdS, $TiO_2$, and CuO which have gained popularity due to specific features and size-mass ratio. In the research article, the antimicrobial effect of these nanoparticles against bacterium *E. coli* (gram-negative bacteria) was explored. To evaluate the effects of the nanoparticles (NPs) on water-borne pathogens, water samples were collected for various microbial analyses. It was observed that nanoparticles ZnO and $TiO_2$ had shown inhibition by the prominent effect on *E. coli*. The observed effect could be due to metal ion released or cell wall-binding properties of the nanoparticles. We can conclude these nanoparticles can support water purification technology while minimizing the application of traditional methods.

**Keywords**

Nanoparticles · *Brassica juncea* · FT-IR · Antibiotics

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## 37.1 Introduction

Worldwide, there is a high risk of ailments due to the contaminated water. Unclean water is a noteworthy cause of fatalities in developing nations. Access to clean drinking water is also getting scarce day by day. It has been noticed by researchers that water contains a huge number of toxic materials, and conformist treatment frameworks, those are regularly not equipped for evacuating them legitimately, and particularly those are impervious to degradation by microorganisms. Every day worldwide numerous pharmaceuticals, personal care products, or added substances of industrial items are disposed of in wastewater (Rasheed et al. 2019; Yaqoob et al. 2020). Water-borne infections have been significantly reduced as a result of the deployment of purifying technologies (mostly chlorine). However, it is well known that the usage of disinfection by-products like chlorine, chlorine dioxide, or ozone is linked to the development of a variety of toxic by-products (e.g., trihalomethanes, halophenols, ketones, and aldehydes), some of which are highly mutagenic or carcinogenic (Ouyang et al. 2019; Khalil et al. 2020). Additionally, chlorination also influences the taste and odor of potable water. Therefore, technology is required for minimizing the discharge of harmful side products because of purification procedures. The utilization of nanotechnology at present could be one of the ways to deal with such problems.

Nanotechnology is a new field to create modern technology which consists of nanofiltration films which can be used to remove pathogens of size higher than 50–60 nm. Experiments have been performed by a few of the scientists by applying the carbon nanotube channels to take out bacterial and viral contamination, such as *Escherichia coli* or *Poliovirus sabin-1* (Srivastava et al. 2004; Brady-Estevez et al. 2008). $TiO_2$ nanoparticles have been applied for the sterilization against pathogens, for example, microbes, similar organisms, or other infection entities (Lee et al. 2018; Ali et al. 2019).

$TiO_2$ NPs may have the potential to reduce pathogenicity of the bacteria by interrupting with cell wall. The non-virulence of microorganisms and their growth were investigated using $TiO_2$ NPs under visible light (400–700 nm) via photoinactivation, which was found to be higher in gram-negative bacteria than gram-positive microbes and fungi. Sulfur- and nitrogen-coated $TiO_2$ nanoparticles were investigated for their enhanced antibacterial activity (Debnath et al. 2020). Surface adjustment of nanoparticles is particularly intriguing since it has demonstrated a raised antibacterial activity under visible light, making them appropriate material for the applicability under sunrays too. In addition, photo-Fenton reaction has been reported as a good treatment strategy as $Fe_3O_4$ reacts with pollutants and generates free radicals in the form of hydrogen peroxide (Wei et al. 2018; Rienzie et al. 2019). Silver nanoparticles had significantly higher antimicrobial effect against *Escherichia coli* MG1655 compared to larger polyvinylpyrrolidone-coated nanoparticles, while bulk silver nitrate was most effective as per the experiments done by Tajkarimi et al. (2014). Recently, carbon-based nanoparticles have been documented for antimicrobial properties in pathogen eradication. The current research article aims to identify the antimicrobial activity of different nanoparticles and future alternatives against existing processes such as chlorination. Here, all of the four synthesized ZnO, CdS, $TiO_2$, and CuO nanoparticles were investigated for the impact on water-borne pathogens.

## 37.2 Methodology

### 37.2.1 Characterization of Synthesized Nanoparticles

ZnO, CdS, $TiO_2$, and CuO nanoparticles were procured from Sigma-Aldrich. Nanoparticle characterization has been done by using ultraviolet-visible (UV-Vis) spectroscopy, X-ray diffraction (XRD), scanning electron microscopy (SEM), and transmission electron microscopy (TEM). To analyze the nanomaterials, UV-visible spectra obtained at high resolution using quartz cells with a 10-mm path length. The size, morphology, and chemical composition of solid powder samples of precursors were initially characterized using a field emission scanning electron microscopy at accelerating voltages of 15 kV. Specimens for the TEM studies was done by depositing a drop of aqueous suspension samples onto a 300 mesh Cu grid, coated with a carbon film. The chemical synthesis of nanoparticles had been firstly done but to maintain ideal conditions. The crystal structure of NPs was characterized by X-ray diffraction (XRD, Rigaku D/MAX-2200H/PC, Cu-Kα radiation). The morphology and particle size were observed using transmission electron microscope (TEM) JEOL JEM 1011 operating at 80 kV.

### 37.2.2 Nanoparticle Treatment to Plants

A nanoparticles suspension was made by dissolving the nanoparticles in deionized water and dispersed with the help of ultrasonic vibration (100 W, 40 kHz). Small magnetic bars were placed in the suspension to stir well to avoid the aggregation of NPs. Concentration of NPs in the supernatant of the suspension after centrifugation ($3000 \times g$ for 1 h) and filtration (0.7 mm glass filter) was determined with the help of a spectrophotometer. The treatment of the nanoparticles was given to plant *B. juncea* by hydroponic medium.

### 37.2.3 Isolation of Bacterial Species from Water Samples

Isolation of pathogenic bacteria is done from sewage water collected from the Department of Bioscience and Biotechnology, Banasthali University, Rajasthan, India. By using an inoculation loop, the samples were streaked for the growth of isolated colonies on nutrient agar. Then the plates were incubated at 37 °C for 24 h. After that the colonies grown on the plates were examined for their morphology, and the same colonies were used for Gram's staining for proper discrimination of cell wall of bacteria.

### 37.2.4 Characterization of Bacterial Species

The isolated bacterial culture was further identified and then characterized using standard microbiology methods' results as shown in Table 37.1.

### 37.2.5 Assessment of the Defensive Nature of Bacteria Against Various Nanoparticles

To evaluate the susceptibility of *E. coli* against nanoparticles, variable methods of evaluation were applied and repeated thrice.

### 37.2.6 Method 1: Bacterial Growth in the Presence of Nanoparticles

First of all, the growth of the *E. coli* was obtained, by reviving the 100 mL Luria Broth by inoculating 2000 μL of the overnight-grown culture with final concentration as 0, 0.01, 0.5, and 1% nanoparticles with respect to bacterial culture. The bacteria were grown in aerobic conditions at 36 °C for 24 h in an incubator shaker. To check the optimal titer of bacteria, absorbance was recorded at 600 nm every 20 min, till the culture has attained the stationary phase noticed by no change in absorbance.

### 37.2.7 Method 2: Bacterial Kinetics in the Presence of Nanoparticle in Suspension Medium

In the second step, the culture media was aliquoted and resuspended in distilled water, till it reaches the desired concentration of 6.3log CFU/mL. The final concentration of the *E. coli* suspension was made in 100 mL distilled water. Different concentrations of ZnO, $TiO_2$ NPs (0.01, 0.5, and 0.1%) were made by the addition of bacterial suspensions in the incubator shaker at 4 °C for 48 h. Absorbance was measured to get the optimum results. Aliquot of 1000 μL of the growth mixtures (water + bacterial cells + nanoparticles) was sampled every 2 h. The number of

**Table 37.1** Water sample collected from the Department of Bioscience and Biotechnology, Banasthali University, has been analyzed for the confirmation of *E. coli* by the following tests

| Test performed | Positive/negative |
|---|---|
| Indole | ++ |
| Methyl Red | – |
| Citrate utilization | – |
| Coagulase | – |
| Catalase | + |
| Oxidase | – |

"+" symbol shows the positive response of bacterial culture for the given test, and "–"symbol represents the negative result for the given test method bacterial cells was estimated by measuring the optical density (OD) at 600 nm. The OD values were converted into the *E. coli* concentration as logCFU/mL (Sondi and Salopek-Sondi 2004).

### 37.2.8 Test the Nanoparticles in Agar Medium

In the third step, the same bacterial strain was grown on a semisolid nutrient media supplemented with 0.12% glucose and 2% agar (control plates), adding 1% nanoparticles (treated plates). Bacterial cells were grown at 30 °C for 48 h. Later, the plates were visually examined and bacterial colonies were counted. The data obtained in all tests was compared to the control plates.

### 37.2.9 Preparation of Extracts from Treated Plants

Fresh leaves and root samples were collected and rinsed with running tap water and later on by distilled water to remove the traces of undesirable visible NPs. The samples were then chopped finely by a new blade and kept for drying in shade. One gm sample was weighed precisely and transferred into beakers with 10 mL distilled water in each which is then boiled for 20 min at 100 °C; the obtained extract was then filtered thrice by using Whatman No. 1 filter paper to remove plant debris and to get clear solution which was then refrigerated (4 °C) in 250 mL Erlenmeyer flasks for further experiments. In each and every step of the experiment, sterile conditions were maintained for the effectiveness and accuracy in results without contamination.

### 37.2.10 Antimicrobial Activity of *B. juncea* Treated with Nanoparticles

The antibacterial activity due to NPs was estimated by using disc diffusion method and well diffusion method. The bacterial cultures were swabbed on these plates. The sterile discs were dipped in treated plant extract solution (10 mg/mL) and placed on the nutrient agar plate and incubated at 37 °C for 24 h. In the well diffusion method, wells were formed, and the diameter of zones of inhibition for control, NPs, and antibiotics were measured. The experiments were repeated thrice and mean values of diameter were documented.

## 37.3 Result and Discussion

### 37.3.1 Characterization of Nanoparticles

Before experimental analysis, the morphology and size of the nanoparticles were characterized, which is an essential step as all properties strongly depend on physico-chemical characteristics of NPs. The TEM image of the ZnO NPs revealed the diameter of NPs with a spherical, truncated, and uneven nature with an average size of approximately 68 ± 1 nm (Fig. 37.1). Similarly, $TiO_2$ too had a spherical shape observed by TEM with a size of 49 ± 1 nm (Fig. 37.2).

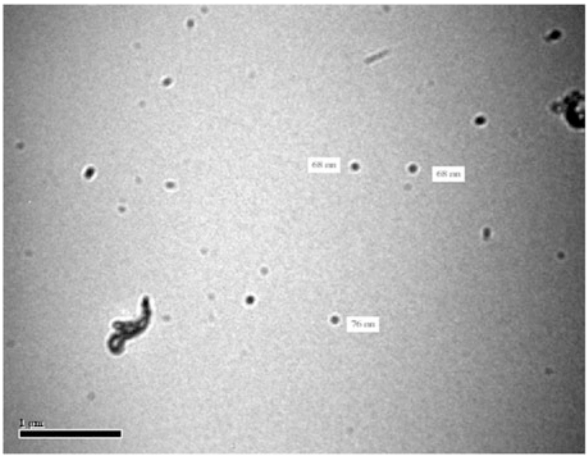

**Fig. 37.1** Characterization of ZnO NPs by transmission electron microscope

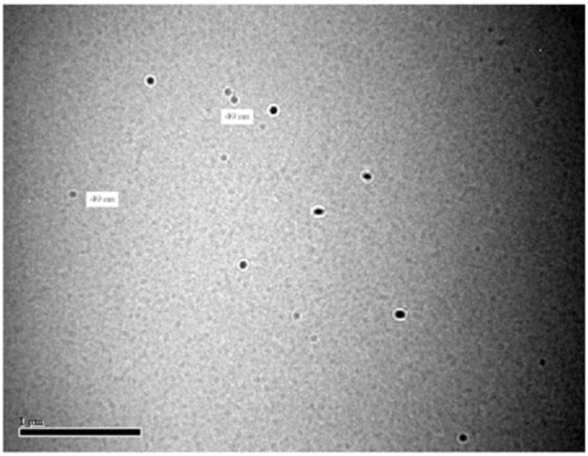

**Fig. 37.2** Characterization of $TiO_2$ NPs by transmission electron microscope

### 37.3.2 Effect of Nanoparticles on Water-Borne Pathogen

For the evaluation of the effect of the four (ZnO, CdS, $TiO_2$, CuO NPs) different types of NPs on water-borne pathogens, water samples were collected, and different microbiological analyses have been done as listed in Table 37.1. *E. coli* was isolated on the basis of tests performed.

The first experiment has been indicating the testing of different concentrations of NPs in the broth culture of *E. coli*. The absorbance of the medium was compared to calculate the difference in the number of bacterial colonies after the effect of nanoparticles. In Fig. 37.3 the effect of different concentrations of NPs at concentrations of 0.01, 0.5, and 1% on the growth of *E. coli* has been clearly shown, and it was noticed that CuO and CdS NPs have no effect on the growth of bacteria, therefore, exempted for further studies. Only ZnO and $TiO_2$ NPs have been further studied for the evaluation of antimicrobial properties.

In the next step, the assessment of the number of viable *E. coli* cells grown in the presence of ZnO and $TiO_2$ NPs (1% solution) was estimated at 4 °C for different contact time intervals. Present result shows the decline in the number of *E. coli* cells from 6.3 logCFU/mL to undetectable levels after 12 days. The given solution of the nanomaterials to the bacterial culture has shown the decreased survival rate after 2 days as compared to that of 12-day experiment for control group. Figure 37.4 represents the number of viable *E. coli* cells in contact with 1% nanoparticles suspended in water at 4 °C for different time intervals. On contrary, when *E. coli* culture was suspended in water treated with ZnO and $TiO_2$ NPs, it was observed that the number of microbial cells declined up to almost zero after 24 h. These results are indicating toward the fact that ZnO and $TiO_2$ NPs have high antibacterial efficiency against *E. coli*.

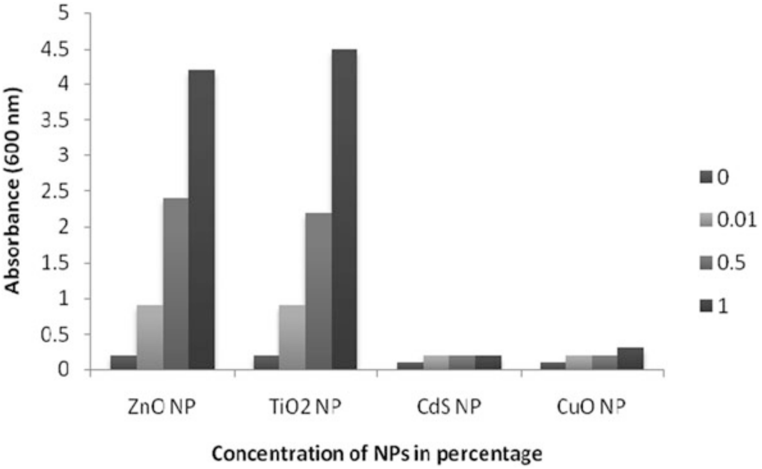

**Fig. 37.3** Effect of four different nanoparticles (ZnO, $TiO_2$, CdS, CuO) at four different concentrations (0, 0.01, 0.5, 1%) on the bacterial titer

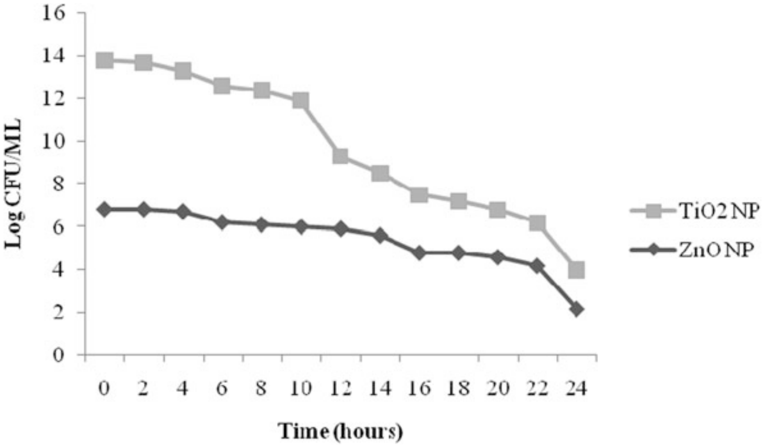

**Fig. 37.4** Growth kinetics of bacterium *E. coli* in the presence of two different ($TiO_2$ and ZnO) nanoparticles

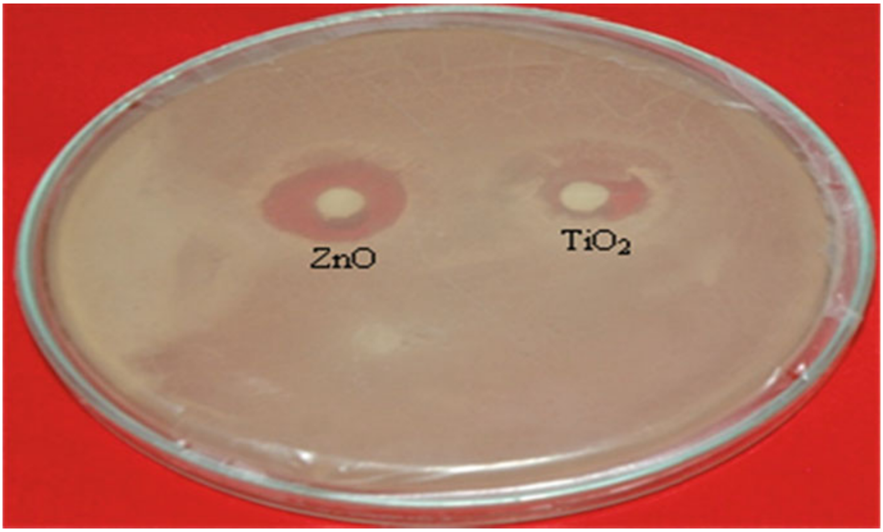

**Fig. 37.5** Effect of ZnO and $TiO_2$ NPs on *E.coli* by disc diffusion method

Later, *E. coli* was cultured with sterile disc dipped in 1% each (ZnO, $TiO_2$ NPs). Distinct bacterial colonies were observed at $10^5$ times dilution. The colonies were counted by using a Quebec colony counter. In Fig. 37.5, we can observe clear zone formation by 1% nanoparticle solution against *E. coli*.

The antimicrobial activity of various concentrations of ZnO and $TiO_2$ NPs was examined during the analysis process. Growth inhibition result was observed when the bacterial cells were incubated with nanoparticles dispersed in the liquid and solid culture suspension. The quantitative examination of bacterial activity was estimated by using the survival ratio which was calculated using the number of viable cells that had formed colonies on the nutrient agar plates. Data collected so far can illustrate well that the advancement in available formulations could be made with the biologically stabilized ZnO and $TiO_2$ NPs, which later can be added for the cure of the infection caused by *E. coli* bacteria.

### 37.3.3 Confirmation of Nanoparticle Uptake

The next step was the confirmation of nanoparticle uptake in plant *B. juncea*, which has been done by SEM analysis, and plants showing the visible uptake of NPs which were found as shining dots were chosen for further study; as shown in Figs. 37.6 and 37.7, there is clear indication of ZnO and $TiO_2$ NPs. Further, this was confirmed by FT-IR analysis (Fig. 37.8) of plant extract and finally used for evaluation of antibacterial effect against *E. coli*.

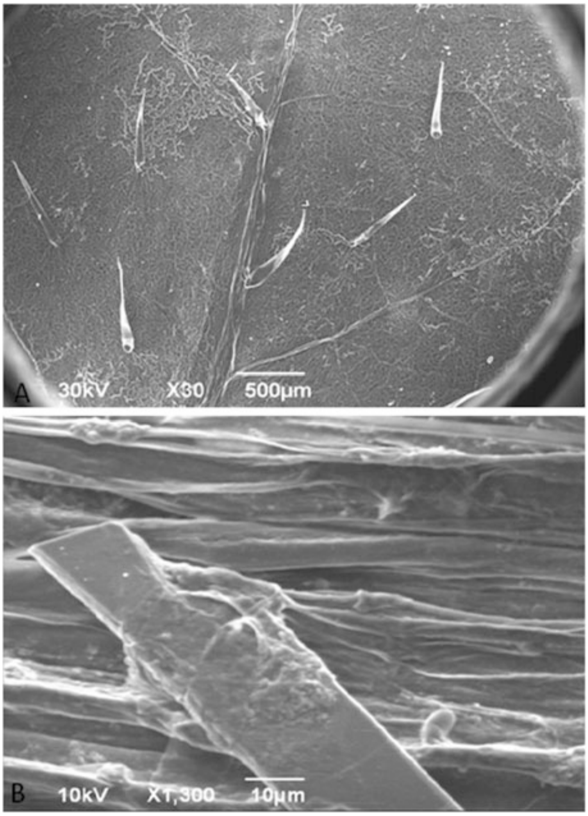

**Fig. 37.6** (**a**) Nanoparticle uptake in the plant leaf treated with ZnO NPs. (**b**) Presence of ZnO NPs at the surface of root tissues of plant

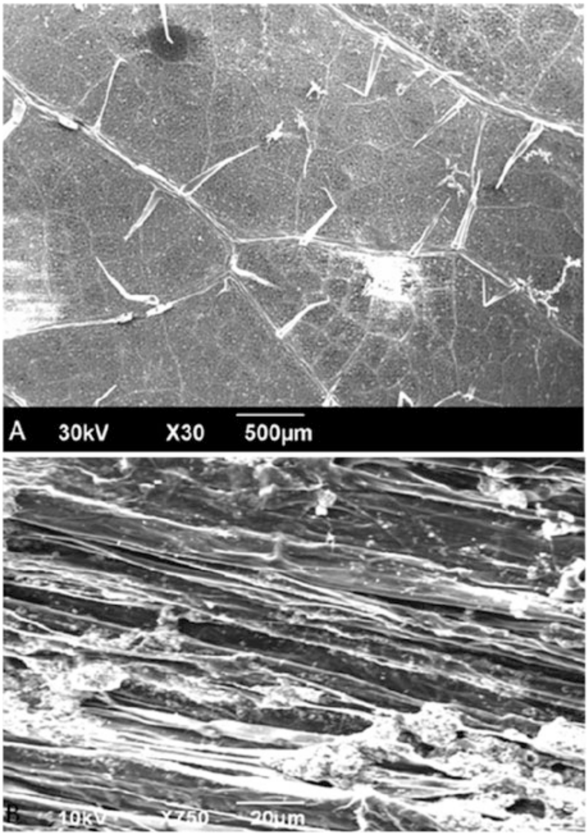

**Fig. 37.7** (**a**) Nanoparticle uptake in the plant leaf treated with $TiO_2$ NPs. (**b**) Presence of $TiO_2$ NPs in the root tissues of plant

### 37.3.4 Antibacterial Effect of Nanoparticle-Treated *B. juncea* Plant Extract

When antibacterial activity of antibiotics and NPs has been estimated, it was observed that as the percentage of zinc oxide nanoparticles has been increased in the culture medium, there was inclined zone of inhibition evaluated by using disc diffusion method as shown in Table 37.2 (Fig. 37.9).

It is observed that when the extract was tested by well diffusion method at different concentrations of Zinc oxide nanparticle, result has been shown in Fig. 37.9 which shows (b) the antibacterial effect of root extract of *B. juncea* at four different concentrations (1) 0.01%, (2) 0.02%, (3) 0.1%, and (4) 0.5% ZnO NPs with diameter of inhibition zone, i.e., 20.0 ± 0.8 and 36.0 ± 0.9 mm in 0.1% and 0.5%, respectively.

Similarly, $TiO_2$ NPs were evaluated against *E. coli* by using two different methods as shown in Fig. 37.10a which was showing the antibacterial effect of $TiO_2$ NPs at two different concentrations of (1) 0.5% and (2) 1% synthesized in the laboratory; (b) the antibiotic sensitivity test of *E. coli* against penicillin, ampicillin,

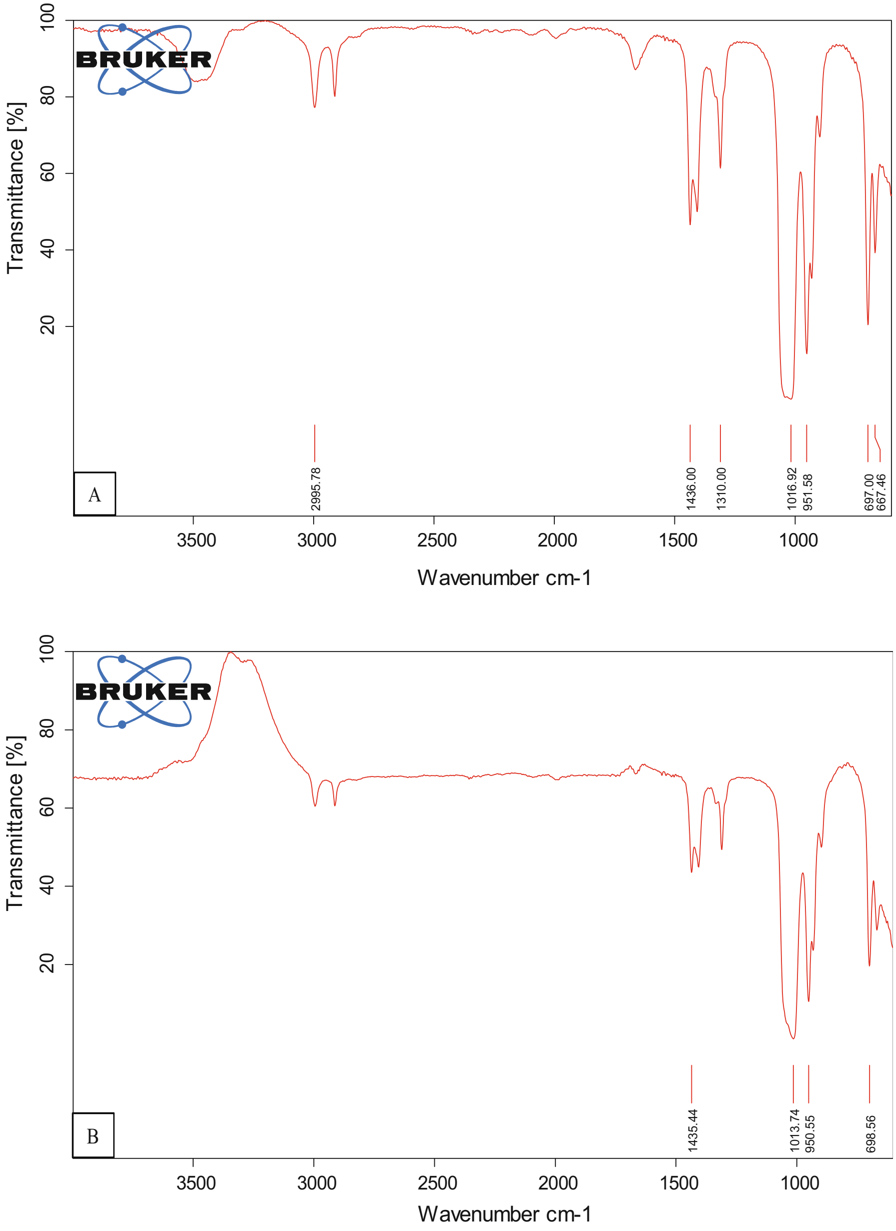

**Fig. 37.8** FT-IR spectrum of plant samples of a 15-day-old *B. juncea* exposed to (**a**) ZnO NPs and (**b**) $TiO_2$ NPs for 96 h by hydroponics mode

**Table 37.2** Zone of inhibition shown by the antibiotics and nanoparticles

| Nanoparticle/antibiotics (%) | Diameter of zone (mm) |
|---|---|
| Streptomycin (0.5) | 14.1 ± 0.10 |
| Ampicillin (0.5) | 12.2 ± 0.09 |
| Penicillin (0.5) | 20.0 ± 0.10 |
| ZnO NPs (0.01) | 15.1 ± 0.10 |
| ZnO NPs (0.02) | 15.4 ± 0.20 |
| ZnO NPs (0.5) | 18.0 ± 0.01 |
| ZnO NPs (1) | 20.0 ± 0.01 |
| $TiO_2$ NPs (0.5) | 26.0 ± 1.0 |
| $TiO_2$ NPs (1) | 28.00 ± 0.5 |

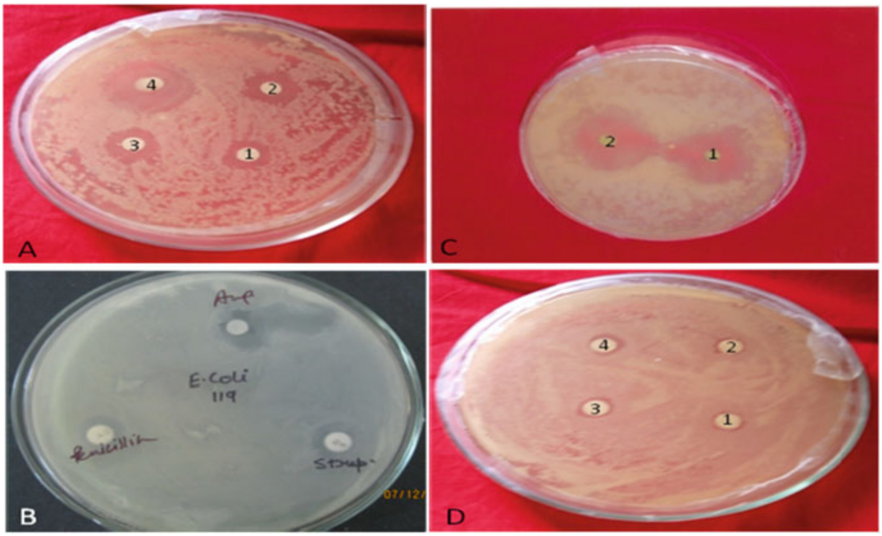

**Fig. 37.9** (**a**) Antibacterial effect by disc diffusion method at four different concentrations of (1) 0.01%, (2) 0.02%, (3) 0.5%, and (4) 1% ZnO NPs. (**b**) Antibiotic sensitivity test of *E. coli* against penicillin, ampicillin, and streptomycin. (**c**) Antibacterial effect of root extract of *B. juncea* at two different concentrations and (**d**) leaf extract of *B. juncea* at four different concentrations of ZnO NPs

and streptomycin; and (c) the antibacterial effect of leaf extract of *B. juncea* at two different concentrations (1) 0.5% and (2) 1% $TiO_2$ NPs with zone of inhibition diameter of 26 mm and 28 mm. Figure 37.10d was showing the antibacterial effect of the root extract of *B. juncea* at three different concentrations (1) 0.01%, (2) 0.02%, and (3) 0.5% of $TiO_2$ NPs. The results have been concisely represented in Table 37.2.

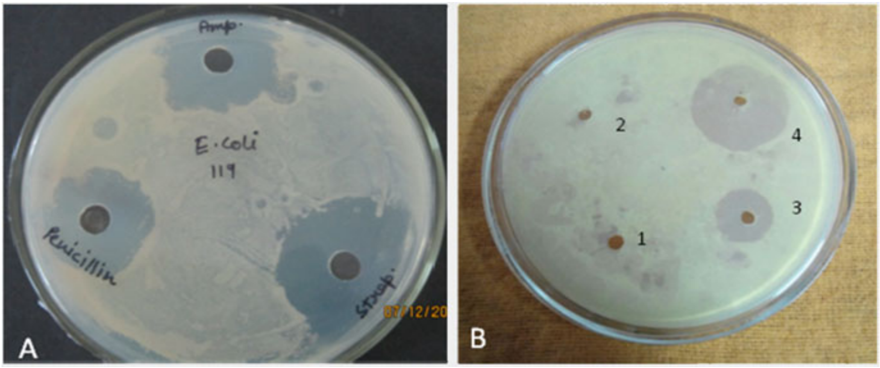

**Fig. 37.10** (**a**) Well diffusion method for antibiotic sensitivity test of *E. coli* against penicillin, ampicillin, and streptomycin. (**b**) Antibacterial effect of root extracts of *B. juncea* at four different concentrations of ZnO NPs

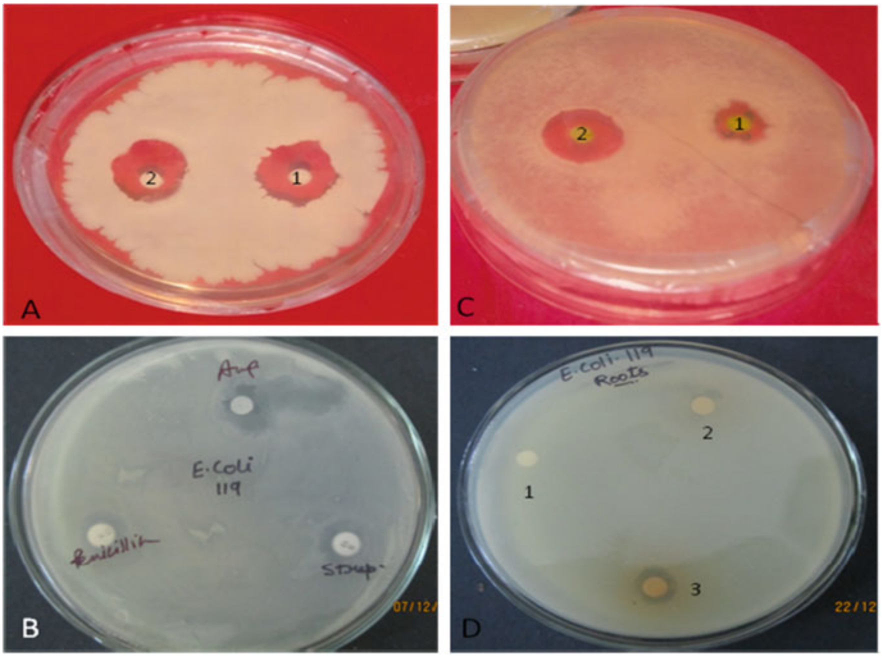

**Fig. 37.11** (**a**) Antibacterial effect of $TiO_2$ NPs at two different concentrations of (1) 0.5% and (2) 1% synthesized in laboratory. (**b**) Antibiotic sensitivity test of *E. coli* against penicillin, ampicillin, and streptomycin. (**c**) Antibacterial effect of leaf extract of *B. juncea* at two different concentrations. (**d**) Antibacterial effect of root extract at three different concentrations of $TiO_2$ NPs

## 37.4 Conclusion

Antibiotics have been used for more than 50 years to treat various communicable as well as noncommunicable diseases. But the antibiotic resistance acquired by the microorganisms came as a major pitfall for the medical industry. In recent times, the usage of nanoparticles are being explored as a capable alternative for antimicrobial agents due to the fact that none of the other researchers, at the present time, are displaying the potential of microbes to develop resistance against nanoparticles.

Remarkable antimicrobial properties of zinc oxide and titanium dioxide nanoparticles are allowing the scientific community for various applications. The method employed for synthesis has remarkable advantages as it is very cheap and its reaction is environment friendly. So, the particular research concerning the interaction of nanoparticles with cells generated new ideas to be used in medical application. In this context, nanoparticles were prepared by using a chemical method, which includes the formation of metal and further few reactive oxygen species. Their accumulation in the bacterial membrane causes damage to the cell.

When the effect of nanoparticles has been tested against the water-borne pathogen (*E. coli*), there was a positive response shown by plant extracts treated with ZnO and $TiO_2$ nanoparticles. It has been observed that the nanoparticles attached to the surface of the bacteria was due to surface charge; bacterial surface was negative, while nanoparticles were positive at the pH studied. The potential of ZnO NPs within the cell wall, resulting in destructing bacterial cell integrity (Brayner et al. 2006; Zhang et al. 2007; Jalal et al. 2010), liberation of antimicrobial ions mainly $Zn^{2+}$ ions on cell wall (Kasemets et al. 2009; Brunner et al. 2006; Li et al. 2011), and formation of reactive oxygen species (Kasemets et al. 2009). However, the toxicity mechanism varies in various media as the species of dissolved Zn may change according to the medium components besides the physicochemical properties of ZnO NPs (Sirelkhatim et al. 2015). Recent researches done by Siddiqi et al. (2018) have also proved the fact that ZnO NPs have various fantastic properties and can be safely utilized as antimicrobial agents. Similarly, Tajkarimi et al. (2014) has also shown the requirement of potential antimicrobial agents which could be significantly applicable for global health problems.

Since nanoparticles are showing its direct action is mainly by ionic action on the bacterial cell wall, without the need to penetrate inside the cells, most of the resistance mechanisms seen with antibiotics are irrelevant. This raises the hope that nanoparticles would be less prone than antibiotics to promote resistance in bacteria. Conclusion can be drawn that these extracts can be applied for various water purification treatments and in medicinal applications to reduce the use of antibiotics.

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# Modern Waste Management

# 38

Yoshiharu Mitoma and Cristian Simion

## Abstract

Nowadays, human communities produce enormous quantities of various wastes, the management of which has evolved from simple dumping to mixed forms of recycling. In a consumer's society, waste management has posed a pregnant problem for which several solutions have arisen. The scope of this chapter is to present those solutions, from minimization and prevention (including the right pricing of waste disposal, the promotion of efficient uses of resources in the production processes, and the implementation of the 3Rs both in homes and businesses), efficient and selective recycling (enhancing recycling infrastructure to maximize resources recovery), volume reduction and subsequent waste-to-energy transformation (gas, heat, and electricity), to smart landfill use (by minimizing waste to landfills and by simultaneously maximizing landfill lifespan and safety). An understanding of the theoretical tools and practical means can indicate what future solution can be found for targeted zero-waste solutions in a smart waste management scenario. Moreover, waste management in the pandemic COVID-19 era presents new challenges that must be overcome.

## Keywords

3Rs · Life cycle assessment (LCA) · Waste management · Landfill · Zero waste index

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## 38.1 Introduction

Human societies have produced waste since the ancient Stone Age. In fact, wastes have been used by archeologists and paleontologists to study habits of the first humans (Havlicek and Kuka 2017). Humankind has long produced wastes in markedly larger quantities than all other species, mainly because human adaptation and later transformation of the environment is associated with needs for artifacts, tools, weapons, etc. The main anthropogenic categories of waste are similar among historical periods: kitchen waste, biological waste, construction, mining and industrial wastes, wastewater, and mixed and other wastes. Once human civilizations began to settle, the amounts of wastes grew exponentially along with population growth. However, that was no mere period of reuse (a concept resembling that of "recycling"). Raw materials were scarce. Transformed products (metal tools, weapons, clothes, etc.) were even scarcer. An incipient concept of waste management appeared along with larger cities, e.g., the Greek *polis*, between 5000 and 2500 years ago, when magistrates from these cities (Athens, Thebes, Knossos) ruled that wastes must be buried at least a few miles from the city. In that respect, antique societies, from China to the Roman Empire, fared much better than later societies, in which simple dumping in a river or dumping directly on a street was the primary method for discarding waste. Diseases and infections such as the endemic plagues that devastated Europe and Asia during the Middle Ages were a direct result of these practices. As a result of the early industrialization period, alongside sustained urban growth of large population centers, the accumulation of wastes caused rapid deterioration of the general quality of urban life, especially in the domain of sanitation.

Only in the late 1800s did the first signals of the creation of systems to manage wastes appear in Western Europe: the so-called dust-yards in England or the introduction of waste bins in France (*poubelle*[1]). After the 1870s, the first incinerators for wastes were introduced in England: the "destructor furnaces" designed by Alfred Fryer. Soon after, incinerators could be found in all major cities of Western Europe and North America. In that respect, however, the "civilized" societies were preceded by ancient Mayans. Indeed, Mayan society had a strict rule that once a month, all members of a city would gather, carry their wastes, and burn them at a special dumping ground (Wilson 1976).

It is during this same period, the late 1870s, that the first concepts of reuse and recycling appeared, even if under the form of a widely used expression, even today: *one man's trash is another man's treasure*. What were the steps that humankind took at the dawn of waste management? What will be the next steps that humankind will have to take to achieve the goal of zero waste?

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[1] Named after Eugène-René Poubelle (1831–1907), a French politician and diplomat who introduced waste containers in Paris and who made their use compulsory.

## 38.2 Recycling, Selective Collection, and Life Cycle Assessment

In the second half of the twentieth century, the accumulation of anthropogenic wastes of various types has urged modern societies to find solutions. The complexity of the problem, involving economic, technical, social, and environmental dimensions, delayed the formation of a consensus on a solution for a few years, but soon material recycling formula appeared as a savior. If, however, the solution must specifically relate to environmental issues, and if the main objective of materials recycling is to minimize overall resource consumption and environmental impact, then the problem of waste should be approached from a life cycle perspective to minimize the costs of waste accumulation (Bjorklund and Finnveden 2005).

A large component of municipal solid waste is generated in households, along with restaurants and food service industries. As a direct result of increased population, of urbanization, and of economic development, challenges have arisen for the service sector and municipal authorities in the form of increased quantities of municipal solid wastes and growing concerns for its treatment. Particularly, health and environmental implications related to solid waste management are mounting in their urgency. Indeed, because of high associated costs and the lack of understanding of all factors affecting the different stages of solid waste management, municipal solid waste treatment stands as an important issue for cities in both emerging and in consolidated economies. Generally, a direct connection exists between population size, economic development, and the rate of urbanization, all of which will engender a higher rate of municipal solid waste production, in addition to change in its composition and technologies for its treatment.

Life cycle assessment (LCA), introduced by the International Standard Organization (ISO) in 1997, examines environmental aspects and potential effects throughout a product's life, from raw material acquisition through production and use, up to final disposal, from "cradle-to-grave." It is instrumental in understanding the life cycle of physical materials. A key feature is that similar materials provide similar functions. In fact, LCA can be used also to analyze services, including waste management systems.

Because major challenges are posed for large municipalities by the collection, recycling, treatment, and disposal of increasing quantities of solid waste and wastewater, LCA can be used as a tool for choosing the best waste management system, or for adopting strategic decisions related to resource use priority. For example, various scenarios can be analyzed to select the best options in terms of costs and benefits, environmental safety, etc. (Cherubini et al. 2009). Among these scenarios, the most commonly compared are landfills without biogas utilization, landfills with biogas combustion to generate electricity, sorting plants that split the inorganic waste fraction (for producing electricity via refuse derived fuels) from an organic waste fraction (for producing biogas via anaerobic digestion), and direct incineration of waste. Analyses are usually performed in terms of global and local emissions, total material demand, total energy requirements, and ecological footprints. Reliable results typically indicate landfill systems as the worst waste management options. They also show considerable environmental savings at a global scale achieved from energy recycling. Furthermore, waste treatment finalized to energy recovery provides energy output that can meet up to 20% of a large city's electricity consumption. Several studies conducted around the globe have produced concurrent results for Finland and China (Liikanen et al. 2017), Iran (Nabavi-Pelesaraei et al. 2017), Italy (Cherubini et al. 2009; Ripa et al. 2017), China (Song et al. 2013), Brazil (Goulart Coelho and Lange 2018; Ibanez-Fores et al. 2018), and other countries.

However, the number of parameters that must be considered when using LCA as an analytical tool can be arduous because of the extremely large amount of data needed for acquisition to ascertain the best scenario properly. Finnish researchers (Liikanen et al. 2017) proposed that, for diminishing the LCA study workload, one solution would be to use default or generic data instead of directly acquired data and case-specific data recorded from the field.

Nevertheless, all studies concurrently showed that mixed municipal solid waste imposes the main burdens for proper treatment through mechanical biological treatment, landfill, or waste-to-energy. Furthermore, LCA permits determination of vulnerable points along the waste management chain, which allows sensitivity analysis to test potential improvements of a particular scenario and provide deep understanding of costs and benefits for waste management at a local scale. However, LCA results are strongly dependent on the context or local specificities of the solid waste management strategies. Developing such strategies is a complex task that depends on numerous parameters that are specific to each site, but LCA analysis can model local conditions and incorporate these approximations during assessment of the environmental performance of a particular scenario for solid waste management systems. A general conclusion shows that the performance of a particular scenario is strongly linked to energy and material recovery efficiency. To improve the environmental efficiency of a treatment plant, a certain need exists for optimized materials recovery through increased automation of selection processes and prioritization of thermal energy, electricity, and biogas production.

The European Union, through its directive 2006/12/EC on waste, has proposed a four-point hierarchical system of waste management regulation: (1) reduction of solid waste production, (2) recovery of material, (3) recovery of energy, and (4) landfill disposal. To improve each point of this system, a preliminary step in an LCA analysis is to establish a life cycle inventory (LCI). The LCI is aimed at identifying and quantifying any environmental intervention related to the system, resulting in a list of environmental inputs and outputs. One such intervention is the source separation of solid waste. Indeed, if first-generation solid waste treatment plants collected complex mixtures of wastes and performed fractionated separation in the plant, a more effective approach appears to be the collection of solid waste selectively, with source separation depending on residents' or individuals' activities.

Several studies logically concluded that better LCA performance was obtained in scenarios with high separate collection rates (Goulart Coelho and Lange 2018). However, education, awareness, and self-discipline in the area of waste and waste management are increasingly important from a global perspective of resource management. A Brazilian study demonstrated that despite efforts made to implement a selective collection program, growth is slow. There is still clearly a great deal of room for improvement (Ibanez-Fores et al. 2018).

Usually, the solid wastes are collected and transported to the treatment plant, where they are sorted and the respective fractions are processed as needed. It is readily apparent that selective collection is highly beneficial for overall treatment.

The first two steps of the process were recently reviewed from an LCA assessment perspective (Perez et al. 2020). According to these authors, pre-collection and collection and transport systems differ. A large city should have both fixed and mobile "drop-off points" located in specific areas of the cities. At those special spots, collection of every MSW fraction, including hazardous or toxic materials, such as oils, paints, electric, and electronic wastes, can take place. The pre-collection and collection and transport systems are classifiable into conventional and pneumatic systems. Conventional systems are (1) door-to-door, (2) curbside, and (3) drop-off points. These conventional systems use bags and household containers or bins, the contents of which are collected by trucks for transfer to treatment centers. They are used most widely because of their low cost, their flexible capacity, flexible location, and flexible collection systems. Different collection options are available depending on the proximity of the waste generating point: households, restaurants, different enterprises, etc. The main options are the following.

1. Door-to-door systems: Waste is collected at the point of generation, actually "at the resident's door," remaining on a public road for a very short period. Such systems have different bagging or domestic bins. With bagging, the waste generated by the public is deposited into bags or similar receptacle that are left at the doors of the residences or at places on the street set aside for this purpose, or near buildings in the case of block housing. Domestic bins are left outside for a short time during the specified collection times. These bins tend to have a small capacity (normally rear-loading, with a volume of 80–360 L) because they are stored inside the residence and are taken outside only during the specified collection time. The system is versatile. It allows selective collection through, for example, differently colored bags for materials of different types, such as paper, plastic, metal, glass, and compostable materials.
2. Curbside systems: The waste is deposited in large-capacity containers permanently located on the sidewalk or near the curb. The containers can be traditional surface containers or underground containers located below ground level with the entire system hermetically closed with a metal cover, thereby provides sealing against water and dust. Underground containers reduce the visual effect and odors. However, they increase the initial investment and maintenance costs. Surface containers can be rear-loading (800–1100 L volume), side-loading (2000–3500 L), top-loading (2500–3000 L), or front-loading (largest capacity). They might also be suitable for selective collection, with different colors and imprints for their purposes.
3. Drop-off point systems: Containers for various separately collected fractions are grouped in the same place. Regarding curbside systems, surface or underground containers are useful. The collection and transport stages involve the use of

> heavy-duty vehicles of different capacities, depending on the type of container to be collected, the volume of waste generated, the population density, and the urban characteristics of each part of the city. In the case of door-to-door systems, low-capacity curbside containerization, and low-capacity drop-off points, the collection vehicles are usually rear-loading (with typical capacity of 5–24 $m^3$). For high-capacity drop-off points, which normally use side-loaded, top-loaded, or underground containers, trucks with side-loading or top-loading are used (with typical capacity of 25 $m^3$).

The pneumatic system was designed to facilitate the transfer of wastes from collecting systems to trucks, to “take the waste to the lorry, not the lorry to the waste.” It consists of a network of fixed drop-off boxes, optimally distributed where residents deposit the generated waste. Those boxes are located curbside, in building walls, or at the entrances to the buildings or dwellings. The boxes are linked to an underground network of pipes through which the waste is transported to a collection station using pneumatic suction.

Mobile and fixed systems might be used depending on the element used to generate the air stream which transports the waste deposited in the boxes. Generally speaking, by mode of air streams, the waste bags are transported to the designated collection points located at the periphery, where the collecting trucks are stationed. Consequently, collecting vehicles need not travel through residential areas. There are systems in which the collecting vehicle itself can generate air suction. An alternative system is one in which waste bags deposited in the boxes are suctioned by air streams generated by turbo-extractor fans to a collecting station. Once there, the different fractions are deposited in specific containers with large volumes, where they are compacted before being sent to the recovery, composting, or disposal plant.

The pneumatic systems present some important benefits: (a) elimination of both containers and trucks from streets, which avoids noise during the collection and emission of air pollutants that influence local air quality; (b) odor reduction; (c) possible integration of collection inside the dwellings or buildings; and (d) possible application in areas of difficult access, narrow streets, and pedestrian areas. The main shortcomings of the pneumatic system are associated with its investment costs, electricity consumption in the operation stage, and the need to undertake work during installation and in the case of failure.

An LCA analysis-based comparison of two systems, conventional and pneumatic, in terms of selective collection demonstrated that the environmental effects from pneumatic systems are greater than those from conventional systems (Perez et al. 2020). This fact is attributable to energy consumption, extraction of raw materials, and equipment manufacturing, especially pipelines. For conventional systems, underground installations have a higher effect than surface containerization systems (Perez et al. 2020).

Several studies conducted independently in several countries related to municipal solid waste source-separated collection have presented several similar conclusions (Tai et al. 2011; Agovino et al. 2016). For instance, improved legislation, coordination mechanisms, and public education are problematic issues that must be addressed

(Tai et al. 2011). Countries that have promulgated environmental laws and policies to discourage the use of landfilling and to facilitate reuse-recycling-recovery schemes for waste tend to exhibit slightly higher levels of public participation in recycling activities. Resident participation in ecological associations and voluntary activities generally exerts a positive effect on separate collection. However, the motivational aspect in choosing to do voluntary work differs from that which drives a person to join ecological associations. Therefore, the effect of participation in voluntary activities is often weaker once a transition from a medium-low to a high separate collection state occurs (Agovino et al. 2016). Another interesting result is that larger cities, and to a greater extent metropolitan areas, generally perform worse than smaller towns.

Although LCA analyses can be reliable for ascertaining the best waste management system, they are subject to important sources of uncertainty of diverse origins. To improve the reliability of LCA results, those uncertainties must be addressed systematically and quantitatively (Clavreul et al. 2012). In actuality, several mathematical and statistical approaches have been developed particularly for waste management, such as applications of optimization modeling, multi-objective approaches, multicriteria decision analyses, and artificial neural networks.

## 38.3 Mathematical Models

Larger cities have concomitantly more complex situations and exponentially increasing numbers of data types. Therefore, numerous mathematical applications must be adapted or even created for LCA analyses to be as accurate and relevant as possible.

Among these numerical methods that were applied, artificial neural network (e.g., model with 4–5–5-11) structures are extremely useful structures (Nabavi-Pelesaraei et al. 2017). Another approach was made using the Eco-indicator’99 method [9]. This method is a multistep, fully aggregated method leading to a result of a single number. The overall method is an example of a damage-oriented or end-point approach. Sensitivity analysis conducted through this method identifies sensitive parameters: for example, whether a small change in an input parameter (such as the recycling rate) would induce a large change in the impact category.

A comprehensive review of mathematical approaches has been published recently (Singh 2019). Recent development of new software technologies and the internet along with the introduction of gradually more compact and dependable hardware products have presented the ability to deal accurately with the panoply of problems raised by modern waste management systems, with their procedures, more easily than costly and tedious field experiments. In the past, researchers developed adaptable numerical models to evaluate environmental issues related to urban ecosystems.

These models offer vital support for the proper management of waste disposal difficulties. An examination of the literature revealed that optimization models are usually used to find an answer of “what is the best” under an explicit arrangement of conditions, whereas simulation models were usually helpful to get an answer to "what if?" because of their predictive capability. A range of techniques has been used in optimization models for solid waste management with diverse focus and objectives. For example, linear programming has been used to reduce aggregate system expenditure and to incorporate optimal methods of waste management in municipalities, whereas nonlinear programming has been applied to maximize profit and minimize waste. Fuzzy method programming has been used to minimize disparities among individual e-waste items. Stochastic programming has been employed to minimize costs of waste flows and capacity expansion, whereas hybrid models have used various modeling techniques to achieving several objectives. Goal programming-based optimization models have been used widely to handle several conflicting objectives in municipal waste management. The analysis also revealed that the single-objective optimization models were used to obtain a novel ideal solution, whereas multi-objective issues engender several compromised arrangements. Furthermore, the analysis showed that the artificial neural network-based models have been used widely for dealing with suitable forecasts for solid waste production rates (Singh 2019).

## 38.4 Waste Management Systems and the Zero Waste Target

Waste management is the collection, transport, processing, recycling or disposal, and monitoring of waste materials. A typical waste management system comprises collection, transportation, pre-treatment, processing, and final abatement of residues. Waste management systems consist of the whole set of activities related to handling, treating, disposing, or recycling of waste materials.

A 2016 report described an analysis of municipal solid waste management systems of 172 countries, serving a collective population of 3.37 billion, indicating that humankind generates around 1.47 billion tons (436 kg/cap/year) of municipal solid waste annually. Moreover, waste generation is increasing over time. Projections for 2050 were 4.16 billion tons, almost three times more than today (Zaman 2016). Some common waste sources are domestic wastes, commercial wastes, ash, animal wastes, biomedical wastes, construction wastes, industrial solid wastes, sewer wastes, biodegradable wastes, nonbiodegradable wastes, and hazardous wastes.

Various strategies for waste management have been recently reviewed comprehensively by Ghodrat et al. (Das et al. 2019). Those strategies represent the very fundament of each waste management system.

Waste management systems are divisible into six categories: (1) landfilling, (2) composting, (3) material recovery, (4) anaerobic digestion, (5) incineration, and (6) refuse-derived or solid residue fuels. Another classification of integrated waste management concepts comprises (1) source reduction, (2) reuse, (3) recycling, (4) landfill and gas-to-energy conversion, and (5) waste-to-energy conversion. The systems have their own characteristics with widely diverse waste-to-energy technologies applied at numerous plants worldwide. According to the definition given in 2013 by the World Energy Council, waste-to-energy technologies are any waste treatment process that creates any form of energy, such as electricity, heat, or transportation fuel, from a waste source (World Energy Resources: Waste to Energy 2016 Report).

The role of sustainable waste management is to reduce the amount of waste that is discharged into the environment by reducing the amount of generated waste. Wastes polluting the environment and threatening human health require public education. Educating personnel and educated (and aware) residents will also improve the efficiency of waste management systems and minimize their possible health and environmental risks. Improper management of wastes can engender severe health threats as a result of fire, explosion, or contamination of air, soil, and water. Similarly, improper waste management and disposal pose threats to those living in nearby communities. They can result in costly cleanups. Moreover, the overall environmental input of wastes depends on the so-called upstream materials: each kilogram of waste is associated with 71 kg of upstream materials, resulting in only a 5% contribution to end-of-life waste (Zaman 2016). Nevertheless, material recovery from waste would markedly diminish "upstream" environmental burdens. For that reason, waste elimination from all processes has led to a zero waste philosophy. The "zero waste" concept, as defined in 2013 by the Zero Waste International Alliance is "designing and managing products and processes systematically to eliminate their waste and materials, conserve and recover all resources and not burn or bury them." Under that concept, a system must be designed to eradicate waste production instead of devising methods to eliminate it. In other words, wastes must not be regarded as a burden, but as a potential raw material.

Actual waste management systems are designated mostly to diminish end-of-line waste quantity, but especially to optimize their final transformations, either by recycling, by recovery of useful compounds, or by transformation to energy and the disposal of what cannot be used in any other way. However, as preceding example illustrates, when a more environmentally friendly collection process imposes the highest burden on the environment, how beneficial are waste management systems either for the environment or from an economic perspective? Generally, low-income up to middle-income countries are using unsanitary waste management systems such as open dumping and landfills. High-income countries rely on sanitary landfill and incineration. Formal waste recycling services are only available in high-income countries with a recycling rate of 22% and very low recycling rates (less than 3%) for countries from other income groups. Even targets set by the EU are difficult to reach for most European countries, especially those in parts of Eastern Europe. Regarding the actual rate of waste generation versus smart waste management, humankind will continue to have growth in the amount of anthropogenically produced wastes up through 2100. Only then, and only if targets are reached during the proposed time, and especially if technological progress produces proper treatment methods, will there be a descending trend. The author used the "zero waste index," which measures the performance of a waste management system by accounting for the potential amount of virgin material that can be offset by recovering resources, either materials or energy, from waste. The zero waste index tool considers the economy of energy and water, as well as the decreased greenhouse gas emissions, by offsetting virgin materials and recovering energy from waste. The zero waste index (ZWI) of the world in the 2016 study was measured as 0.12, which means that the current waste management system potentially offsets only 12% of the total virgin material substitution potential from waste. The ZWI measures the potentiality of virgin materials to be offset by waste management systems. Presumably, the amount of material recovered from waste would obviate extraction of that amount of material and eventually save and avoid the energy, greenhouse gases, and water use during the extraction process. Therefore, the zero waste index measures the material substitution efficiency (in kilograms, kg) and energy (in kilowatt hours, kWh), greenhouse gases (in carbon dioxide equivalent, $CO_2e$), and water savings (liter, L) of the waste management systems. The ZWI is calculated using the following equation.

$$\mathrm{ZWI} = \frac{\sum_{1}^{n} \mathrm{MSW}_{ij} \times \mathrm{SF}_{ij}}{\sum_{1}^{n} \mathrm{MSW}_{i}}$$

Therein, the following variables are used:

$\mathrm{MSW}_{ij}$ = amount of waste stream $i$ ($i = 1, 2, 3\ldots n$ = paper, plastic, metal, etc.) managed by the system $j$ ($j = 1, 2, 3\ldots n$ = amount of waste avoided, recycled, treated, etc.)

$\mathrm{SF}_{ij}$ = substitution factor for the amount of waste stream $i$ ($i = 1, 2, 3\ldots n$ = paper, plastic, metal, etc.) managed by the system $j$ ($j = 1, 2, 3\ldots n$ 1/4 amount of waste avoided, recycled, treated, etc.)

$\mathrm{MSW}_{i}$ = total amount of municipal solid waste managed ($i = 1, 2, 3\ldots n$ = paper, plastic, metal, etc.)

The author also presented a conversion table for material substitution efficiency, which is calculated using a material substitution factor. Using this calculation, the author measured a global zero waste index of 0.118, which gave the resource substitution efficiency of the global waste management systems. Consequently, the potential average savings were around 51 kg of materials, 219 kWh of electricity, 48 kg of $CO_2$ equivalent emissions, and 38 L of water, all per person, from resource recovery activities.

Overall, the study highlighted that the global overall resource recovery and waste management performance are remarkably low. The study also underscored the need to improve waste management performance. Regional and transboundary waste management strategies must necessarily involve all countries, international organizations (United Nations, World Bank, OECD, European Union, etc.), business organizations, and local communities (Zaman 2016).

Therefore, existing waste management systems with proven efficiency have been implemented, and new one must be elaborated. One aspect that will never be emphasized sufficiently is that larger urban areas have more complex data streams. Indeed, there will be more challenges than simply collecting households', restaurants', enterprises', and other buildings' weekly garbage and emptying streets' garbage bins. Amounts of municipal solid wastes generated during special events must be included along with those of larger cities, for which the numbers of sports, cultural festivals, and other events tend to grow.

Consequently, numerous studies have specifically examined the sustainability of waste management programs in large urban areas. The usual challenges of waste generation, inadequate waste collection, transport, treatment, and disposal processes are strongly dependent on the degree of development of a country. Recommendations made by these studies are usually similar: (1) switch from open dumps to eco-friendly waste recycling technologies; (2) minimize odor from leachate from landfills using scientific and traditional or indigenous methods; (3) devise proper pathways for plastic waste management (e.g., for reduction of movement of plastic into bodies of water); (4) formulate proper legal instruments such as acts, decrees, and ordinances; and (5) offer rewards for residents and organizations who resist nonscientific waste disposal, thereby encouraging sustainability, to support the tenability of legal instruments (Das et al. 2019). How easy or difficult would implementing these changes be? That depends strongly on the degree of economic development, on education, and on legislation.

The first aspect to be addressed is the economic aspect, especially because of poor economic growth, unequal distribution of wealth among people, sociocultural limitations, local and international influences, and lack of effective national policies. Waste collection and transport have been observed to incur approximately 70% of total management costs of systems (Das et al. 2019). Recycling and selective collection are expected to reduce these costs. Moreover, using various processes by which solid wastes can be exploited to generate benefits through transformation to energy or by composting, costs will be reduced further, rendering the waste management system as a profitable one. The costs for collecting and transporting solid wastes can be approximated easily, considering vehicle operations and maintenance costs per hour, daily working hours, salary rates of the vehicle driver per hour, number of workers per collection vehicle, salary rates, and back-up rates of collecting workers, fringe benefit rates, miscellaneous costs involved, and a yearly discount rate. Considering the so-called demand and supply curve applied to solid wastes, the "green economy" concept was introduced by the United Nations Environmental Program in 2008 and served to identify specific spheres in which public investments would be able to lead the way to achieving this concept using the so-called green stimulus packages. This idea compelled many governments to adopt a sustainable waste management program, endorsing the sustainability of a waste management system, and thus allowing that waste generation is minimized and that recycling and recovery are favored. Consequently, only a meager amount of waste reaches disposal. It markedly reduces the economic load. According to the World Bank, economically developed countries spent 46 billion USD in 2017 to manage the generated solid waste. The cost of solid waste management is projected to rise to around 150 billion USD by 2025 based on the present economic scenario (Das et al. 2019).

The waste sector can make important identifiable contributions to the economy.

- Through recovery, waste could provide cost-effective, high-quality secondary materials, thereby reducing expenditures on production of primary materials.
- New enterprises can emphasize creation of more jobs, providing low-cost, easily accessible energy, and reducing costs for waste remediation.
- Proposing new, cost-effective waste management strategies can emphasize reducing, recycling, and recovering materials effectively,
- Encouraging new investments in the waste sector can develop a durable infrastructure and can provide affordable services to society.

With low waste transfer to disposal areas or by reusing and recycling technological interventions for transforming it, the avoidance costs associated with environmental degradation and greenhouse gas emissions would decrease considerably. The use of a waste-to-energy alternative approach would engage people through jobs and allow recovery of materials and energy, making a lower carbon footprint attainable (Das et al. 2019).

Because most traditional waste management pathways are highly cost-intensive, they become less feasible in less economically developed or economically developing countries (Marshall and Farahbakhsh 2013). Through the green economy mentioned above, definite steps have been made to implement waste management systems in economically developing countries, but much work remains. One important related aspect is reduction of the costs of implementing such a program: a point which is also valid for economically developed countries. Because most solid waste in an economically developing country comprises organic materials, a readily apparent solution would be composting: microbe-assisted decomposition of organic waste materials in particular vessels for the production of humus-rich manure. It is a highly popular technology worldwide, even in economically developed countries, mainly because of the rapid decomposition of waste materials, improvement of end products (topsoil), and biological sustainability. Moreover, it does not necessitate high investment.

Usually, a simple solid waste management system follows the “three R” policy to reduce, reuse, and recycle. This is done already in some way or other in economically developing countries where people, because of necessity, throw away less and reuse materials to a great degree. Recycling is still difficult. Nevertheless, a fourth R, “recovery,” has been added to the strategic framework of solid waste management.

A second possibility is more expensive because it requires some initial investment. It consists of energy recovery technologies. According to the 2016 report of the International Energy Agency, the global demand for energy is increasing rapidly. In fact, the situation is more pressing in economically developing countries. The report predicts marginal declines in oil and gas demand, while waste-to-energy generation is expected to increase 54% by the end of 2030. Waste-to-energy represents an alternative to usual methods of generating energy by diminishing the amount of solid waste in the same time. Some studies consider waste-to-energy a cleaner mode of energy generation because it can help avoid the emission of $CO_2$ from fossil fuels and $CH_4$ from landfills. By contrast, combustion of waste generates $CO_2$, biogasification generates $CH_4$, and both processes generate a wide spectrum of gases.

Solid wastes comprise 66% of biodegradable components, which make them useful to recover $CO_2$-neutral energy while reducing dependence on fossil fuels (Das et al. 2019). The total $CO_2$ budget from waste-to-energy units is generally expressed as a $CO_2$ load and $CO_2$ savings. The $CO_2$ load involves direct emissions from the unit, whereas $CO_2$ savings account for materials recovered at the end of the process. From a global perspective, North America, Asia, and Europe represent ca. 54% of $CO_2$ emissions but operate approximately 90% of waste-to-energy capabilities. Because of the scientific management of waste-to-energy installations, Europe, North America, and Asia (especially Japan, Korea, and China) have managed to realize a $CO_2$ economy, whereas South America, owning just 5.4% of global waste-to-energy units, emits ca. 36% of the total $CO_2$ emission amounts.

The two main classes of waste-to-energy conversion are thermochemical and biochemical conversions. Examples of thermochemical conversion are incineration, gasification, combustion, pyrolysis, carbonization, and mechanical extraction. The main biochemical conversion processes are anaerobic digestion and fermentation. Each has its benefits and shortcomings.

- Incineration. Direct burning of solid waste with a sufficient oxygen supply produces heat energy and ash residues. It necessitates special furnaces with stokers and fluidized beds.
- Combustion. The direct burning of solid wastes is usually done directly in a landfill without any specific device.
- Pyrolysis. This thermal decomposition of organic materials at high temperatures in an oxygen-limited environment is conducted in special reactors.
- Gasification. Thermal decomposition in the presence of limited oxygen yields a mixture of both combustible and noncombustible gases. Gasification of different types is chosen based on the type of gasifier, such as counter-current fixed bed gasifier, cocurrent fixed bed gasifier, fluidized bed gasifier, entailed flow gasifier, and plasma gasifier.
- Hydrothermal carbonization. Thermal conversion conduced at ca. 180–350 °C in a water-based environment can convert solid waste into carbonaceous residues.
- Mechanical extraction. A technique is used for mechanical pressing of organic wastes such as seeds, pulp, and peels, which are at 170–190 °C for oil production. The produced oil is subsequently trans-esterified to obtain fuel oil.
- Anaerobic digestion (aka bio-methanation). This process uses specific reactors (biogas digesters), with the organic fraction of solid wastes fed in $O_2$-devoid conditions to produce biogas ($CH_4$ and $CO_2$) and sludge.
- Fermentation. Bioethanol can be produced using various microbial or bacterial agents.

Several reports of recent years have described various biotechnological processes that present suitable pathways for the production of several enzymes or chemicals from solid wastes, on an industrial scale. For example, butanol is obtainable from municipal solid wastes through the acetone-butanol-ethanol process using *Clostridium beijerinckii*, whereas several enzymes such as amylases, pectinases, and proteases from solid wastes are obtainable through microbe-assisted production.

| Process | Output | Secondary | Advantages | Disadvantages |
|---|---|---|---|---|
| Incineration | Heat | Ash | High energy efficiency<br>Noiseless<br>Odorless | Particulate emissions<br>SOx and NOx emissions<br>Dioxin production, cost-intensive |
| Combustion | Heat | Ash | No costs | Extremely harmful for the environment |
| Pyrolysis | Gases ($CO_2$, $H_2$, $CH_4$, etc.)<br>Wax, tar, bio-oil | Char | Fuel gases, pyrolysis oil (additive for diesel)<br>Modular characteristics of the pyrolysis<br>Units<br>Low air pollution | Low net energy output with high moisture content<br>Production of toxic gases such as HCN, polyacrylonitriles, high oil viscosity<br>Need for pre-treatment of waste<br>Cost-intensive |
| Gasification | Syngas ($CO + H_2$) | Vitreous slag | Slag can be used as a filling material in road construction<br>$H_2SO_4$ is obtainable from S impurities | Huge coal requirement<br>Production of toxic polyhalogenated organic compounds (dioxins, PCBs)<br>High installation cost |
| Hydrothermal carbonization | Hydro-char | Oils<br>Chemical-rich<br>Processed water | Easy operation with moisture-rich waste substances<br>Exothermic<br>Low energy requirements | High toxicity of processing water<br>Cost-intensive |
| Mechanical extraction | Oil<br>Particle board | Press residues | Alternative fuel generation<br>Low environmental degradation potential | Cost-intensive<br>High skill and handling requirements<br>Not tenable in rural areas, low oil output |
| Anaerobic digestion | Biogas | Sludge/ slurry | Low costs | Gas emissions |
| Fermentation | Ethanol, $CO_2$ | Bio-solids | Low costs | Gas emissions |

Unfortunately, solid waste is far from being a homogeneous material that can be regarded as the starting material of the entire process of sorting, recovering, recycling, and waste-to-energy and waste-to-compost transformations. The composition of municipal solid wastes can vary widely within a country, even more between countries, influenced by geographical location, industrial activities, food habits, lifestyle and living standards, energy sources and consumption, and weather and climate. Wastes from economically developing countries have higher organic components, but economically developed countries produce diverse wastes with larger shares of metals, plastics, and paper. The fractions of organic component in wastes differ moderately among economically developed countries such as the USA (24%), EU (34%), and Japan (40%), being composed mostly of food waste, agricultural residues, and paper wastes. In an economically developing country, organic wastes correspond to 60–70% of the total amount of waste. Different technologies and waste management infrastructure must be considered depending on different waste compositions.

Wastes will be segregated primarily into biodegradable and nonbiodegradable fractions. The nonbiodegradable fraction can include various subcategories such as plastics, glasses, and construction debris. Each category or subcategory can then be included in a transformation process.

Therefore, it is imperative to undertake thorough monitoring of municipal solid wastes to have an efficient sorting process. For that reason, prior sorting is imperative, enhancing the performance of selective collection processes and, simultaneously, diminishing sorting process costs. Segregation of wastes seems easy on paper, but in fact it raises numerous problems, among which those related to health issues are not the least. To alleviate such difficulties, smart recycling technologies such as computer-programmed robotic machines can intervene. Solving health issues can greatly increase costs of sorting. Nevertheless, for several waste management systems, the introduction of artificial intelligence can be effective because the segregation efficiency can be greatly enhanced, by obtaining more homogeneous categories in a much shorter time.

The use of modern technologies has improved waste management systems considerably. Spatial and data communication technologies can be used for site selection, planning, management, cost estimation, optimization, long-range communications, monitoring of waste movements from source to sink, and generation of authentic data for additional management decision-making. Moreover, identification and data acquisition technologies for bin and driver tracking can improve process optimization, sorting, and recycling, in addition to moisture, energy, and odor measurements.

Therefore, a need exists to develop new strategies to approach waste management systems to achieve the targets proposed for 2050. Those strategies are aimed at increasing the quality of life and at changing the way that residents contemplate difficulties related to their own wastes. Those strategies are expected to reduce the effects of several processes on the environment through products, processes, and corporate policies using applicable green sustainable resources and environmental management systems such as ISO 14001, EMAS, as well as numerous activities (prevention, reduction, reusing, etc.) while simultaneously measuring their effects on society. In a series of papers, Antonis Zorpas laid the foundations for the establishment of such strategies (Zorpas 2020).

Along with increased complexity of waste management systems, there are more powerful tools to elucidate them and to identify solutions for newly arisen difficulties. Such tools could serve to underpin a circular economy. Indeed, the continuous growth of the Earth's population, the mutual development of all societies, and the continuous rise of standards of living impose onerous degrees of stress on all natural resources. Wastes stopped being an end-product of human activities, instead coming to represent a by-product along all supply chains for all products that humans need. The environmental and socioeconomic effects have become almost overwhelming.

Humankind requires rapid assessment of the situation and rapid identification of a solution to correct this situation before it becomes detrimental to all future generations. One means of reducing these environmental and socioeconomic effects is through adoption of a circular economy. A circular economy could be the tool to resolve resource, waste, and emission challenges confronting all societies from every continent by creating a production-to-consumption supply chain that is inclusive, restorative, regenerative, and environmentally benign (Avraamidou et al. 2020). How could this be achieved? A circular economy proposes to keep products, components, and materials at their highest utility and value, with minimal to nonexistent waste, at all times.

In recent years, discussions, debates, and negotiations have explored and assessed circular economy package implementation, the European Green Deal, and adoption of UNDPGs to boost Europe in a low carbon economy and to achieve climate neutrality by 2050. At the same time, some slow progress has taken place in the development of effective waste management strategies and measures for the proper treatment and disposal of waste (Zorpas 2020).

With a history of nearly half-a-century (according to a technical report of the Ellen MacArthur Foundation, published in 2013: Towards the Circular Economy: Economic and Business Rationale for an Accelerated Transition, the concept appeared in the early 1970s), the circular economy concept relies on three simple principles: (a) design out wastes, (b) build resilience through diversity, and (c) rely on energy from renewable sources. Despite being a rather simple concept, theorists proposed more than 100 various definitions (Avraamidou et al. 2020), but the one able to represent a closely circular economy was given by Saidani et al. 2019): "a circular economy is an economic system that replaces the end-of-life concept with reducing, alternatively reusing, recycling and recovering materials in production/distribution and consumption processes. It operates at the micro-level (products, companies, consumers), meso-level (eco-industrial parks) and macro-level (city, region, nation and beyond), with the aim to accomplish sustainable development, thereby simultaneously creating environmental quality, economic prosperity and social equity, to the benefit of current and future generations."

There can be an assessment of an organic or an inorganic life cycle. Moreover, its sustainability can be studied, but however the scope and steps of a circular economy are chosen, its characteristics are expected to be the same, allowing one to take proper measures whatever the situation (Avraamidou et al. 2020). Those characteristics are as follows:

1. *Reduction of material losses and residuals:* Waste and pollutant minimization through the recovery and recycling of materials and products.
2. *Reduction of input and use of natural resources:* Reduction of stresses posed on natural resources through the efficient use of natural resources (e.g., water, land, and raw materials).
3. *Increase in the share of renewable resources and energy:* Replacement of nonrenewable resources with renewable ones; limiting the use of virgin materials.
4. *Reduction of emission levels:* Reduction in direct and indirect emissions and pollutants.
5. *Increase the value durability of products:* Extension of product lifetimes through the redesign of products and high-quality recycling.

Referring strictly to waste management systems, can a circular economy be a reliable tool? According to Fan et al., it certainly can be because correct waste management is an important part of the transformation from a linear economy to a circular economy (Fan et al. 2019). For assessing the effects of a system or a supply chain on waste generation, several approaches are useful: life cycle assessment, input-output models, and consumer lifestyle approaches. However, again, the same problem arose. The complexity of collected data from countless sources can support real-time prediction until the gradual introduction of the "Internet of Things" (IoT) concept into waste management systems. Indeed, waste management deals with widely diverse issues from waste segregation to efficient and selective collection, treatment and product distribution to close the loop for waste-to-wealth, volume reduction, waste-to-energy processes, etc. Through IoT, a new concept slowly appeared—that of a smart city, with IoT waste management systems that paved the way toward the once-elusive zero waste target. Consequently, the transition from waste management to sustainable waste management, integrated waste management, and subsequently to smart waste management occurred in less than a decade.

The work of the authors described above suggested several directions for future research that will draw waste nearer to being regarded as a "resource."

1. In all the proposed environmentally sustainable methodologies and strategies, the necessary investment and data availability are common hindrances to putting the suggestions or potential solutions into practice.
2. Real-time data collection with the aid of IoT can further demonstrate reliability and improve the credibility of the proposed framework.
3. Stakeholder involvement is also a linchpin of practical solutions and implementation.

Integration of the "smart" concept into waste management can improve environmental performance considerably through smart sensors and real-time monitoring (Fan et al. 2019).

## 38.5 Smart Waste Management and Smart Cities: Internet of Things

Actually, IoT describes the network of physical objects—"things"—that are embedded with sensors, software, and other technologies for connecting and exchanging data with other devices and systems over the internet. The devices which can then be connected are extremely various, from kitchen appliances, cars, thermostats to baby monitors but also sophisticated industrial tools. With more than ten billion devices connected to IoT, experts expect this number to double by 2025.

Using low-cost computing, the cloud, big data, analytics, and mobile technologies, physical objects can share and collect data with minimal human intervention. Under these conditions, digital systems can record, monitor, and adjust each interaction among all connected things in a real connection between physical and the digital domains. That being said, how can IoT help sustainable waste management become smart waste management as part of a smart city?

The smart city concept is applicable to an urban area for which huge amounts of data have been collected through electronic methods of different types and sensors of various types. Interpretation of these data allows the city to manage all its services, resources, and various assets while improving operations of all types including health, education, law enforcement, transportation, electricity, heat, postal services, and of course, waste management. Another definition of a smart city is given by the European Union, for whom smart cities are a system in which people interact, using energy, materials, services, and waste to stimulate economic development and to improve the quality of life.

The smart city concept was made possible by remarkable progress in the field of information and communication technologies: They include radiofrequency identification (RFID), sensors and actuators, wireless mobile communication technologies, embedded systems, and cloud computing technologies. These technologies allow city leadership to have closer contacts with the urban community, optimizing the efficiency of all operations and services that improve the standard of living of all city residents. At the same time, all these data passing through information and communication technologies permit improvement of the city's infrastructure, thereby enhancing the performance and quality of all urban services, reducing costs and resource consumption (which were precisely the goals of a circular economy).

Why are cities so important compared to rural areas that are paramount for production of food resources? Although cities occupy around 2% of the Earth's surface, they accommodate half of the global population. According to the United Nations Organization, they will be home to up to 80% by 2050. Today, they are responsible for 80% of greenhouse gas emissions, consuming 75% of the global energy output (Eremia et al. 2017). In the next few years, cities hosting more than ten million residents, the so-called megacities, are expected to double. The United Nations' Department of Economic and Social Affairs emphasized that "sustainable urbanization is key to successful development." Sustainable urbanization is definable as smart urbanization based on innovation and on the collection, interpretation, and distribution of data via communications technology. Using information and communication technology, the goal of a smart city is to improve its livability, workability, and sustainability. A city that continuously monitors all its infrastructure, such as transportation, communication, health, education, police, and mail, will be able to mobilize and allocate all of its resources: natural, human, equipment, buildings, roads, tunnels, subway, airports, water, and power. Thereby, it can improve security and plan in advance the preventive or maintenance operations to maximize its services for its residents.

Consequently, smart city technologies allow city officials to interact directly with the community but also directly with its residents. Smart city applications are being developed to manage urban flows and to allow for real-time responses, being already prepared to respond to usual or unusual challenges (such as cultural or sport events), the ultimate goal being to increase the residents' welfare. One important aspect for a city's residents' welfare is its waste management system.

In smart cities, the effective management of wastes is a crucially important challenge for the environment that IoT is intended to address. Waste management covers all activities necessary for monitoring the waste generated in a city, from its beginning, when residents start filling their bin, through collection, transportation, and arrival at its final accommodation, which can be the landfill, incineration, or recycling (Pardini et al. 2020). The worldwide growth of waste management systems is estimated to have an annual growth rate of 6.2% through 2023, with greater growth in the Asia-Pacific region. In Europe, this sector grew by more than 30% in 2016. Growth is expected to continue to accelerate because of the presence of advanced infrastructure and the high demand of several related sectors (Pardini et al. 2019).

Most cities around the world are not smart cities. They are usual cities where wastes are collected indiscriminately at fixed dates, transported to a processing plant or, as is in most cases, to a landfill. The entire process is being paid for by city residents. How can this simple and humble system improve a smart city with respect to the demands of a circular economy and those of a smart waste management system?

1. Through recycling, reuse, and recovery programs, one can be highly improved through selective collection. Those programs are destined to obtain positive value from waste by recovery of precious metals from e-wastes, plastics, paper, metals, and other materials.
2. Through waste-to-energy or waste-to-compost programs, it is possible to obtain positive value from biodegradable wastes that can serve as energy precursors, biogas, biofuels, or by simple transformation in organic fertilizers. Again, selective collection is a paramount concern.

All collection and transportation are made by garbage trucks that have a regular circulation pattern. However, the quantity of waste collected is not the same on all days. Consequently, there is an unnecessary expense of fuel, with consequent emissions of greenhouse gases. It would be better for each truck to make the trip to the bin to pick it up when the bin is at full capacity. That is where IoT intervenes. It is the scope for every major city around the world to make a transition to a smart city that possesses smart waste management.

Indeed, without a smart waste management system, waste generated by urban activities, industrial or domestic, will provoke health issues and will harm the environment. Understanding how and where wastes are generated, what resources are available and how, and what environmental conditions exist for each city are the pillars on which a smart waste management system is built (Pardini et al. 2019).

Compared with economically developed countries, residents of underdeveloped countries are more severely affected by unsustainably managed waste. In such countries, garbage is often disposed of inappropriately, being thrown directly on the street, in adjacent streams of water, or burned in open pits. These practices present severe consequences for human and environmental health and safety. Improperly managed waste can serve as a rich source of disease and can contribute to global climate change through the generation of greenhouse gases. It even promotes urban violence through degradation of urban environments. Proper waste management is necessary for the construction of sustainable and habitable cities, but it poses a challenge for many economically developing countries and cities. Effective waste management often becomes costly, compromising municipal budgets. Operating this municipal service requires an integrated system that is efficient and sustainable (Pardini et al. 2020).

The possible practical solutions for smart waste management systems have been reviewed to a considerable degree in the recent past. What all proposed solutions had in common were general strategies and the use of smart bins.

The general strategy can be summarized as follows: using sensors and radiofrequency identification and actuators in the process of monitoring identification; this set is divided into three phases: (1) planning and execution of waste collection using solutions of routing in trucks with dynamic adaptation of routes according to restrictions introduced; (2) transport to a specific place according to the type of waste; and (3) recycling of waste that can be reused (Pardini et al. 2019).

The same papers described above provided reviews of the reference models available for IoT architecture as well as an algorithm that is useful in a smart waste management system. They were implemented in several large urban areas around the world and are expected to be implemented in the future in increasingly large cities: predictions are that by 2025 there will be around 100 such smart cities, mostly in the Northern Hemisphere.

Learning from the experiences of those cities which have already implemented a smart waste system will help each community newly aspiring to smart city status to make the best decision about which smart waste management system is best suited in terms of local conditions, population, climate, etc.

As described earlier, various collection systems exist, among which a choice can be made, but most promising is selective collection in smart bins. Transportation also offers different solutions, from pneumatic pickup or systems based on underground containers to those which use trucks with loads of different types (side or rear).

The use of smart bins is expected to eliminate the costs of unnecessary trips to almost empty containers and failure to meet demands to empty those which are filled to capacity or overflowing. Real data transfer from the detection device in the smart bin will allow not only knowledge of which bin needs collection but also correlated to real data on transportation, to set an optimal route from point A to B. As described above, selective collection greatly improves the entire process. For a fixed point of selective collection, the residents' responsibility is to dispose of their waste selectively. However, household wastes pose an altogether different set of circumstances. They are very often mixtures of wastes of all types, both organic and inorganic, rendering waste processing difficult. In most cities where waste processing (starting from door-to-door collection) is a service paid for by residents, most users tend to be reluctant to sort wastes before collection. The implementation of such a system is usually slow, depending on funding, education, legislation, and people's general involvement.

One possible solution would be for companies that are implementing such smart waste management systems to forego asking for money for collection of household wastes and instead buy it from residents if it is already sorted by categories. These companies would lose a source of income, but there will be beneficial economies related to transportation, municipal funding, and incomes from products derived from waste processing.

### 38.5.1 Singapore

Among successful examples of a smart city with a smart waste management system, Singapore is at or near the top of the list: Long a leader in both technology and successful urbanization strategies, Singapore was ranked first in the Global Smart City Performance Index in 2017. The index graded cities on how well technology improved resident life in four areas: mobility, health, safety, and productivity.

In the smart waste management system area, Singapore began experimenting in 2016 with smart, solar-powered rubbish bins that served as internet hotspots and which came equipped with fill-level sensors. Each bin was also equipped with a compactor, which gave it eight times the capacity of a normal rubbish container. Singapore was the first smart city to implement the intelligent rubbish bin concept. Today, Singapore has more of these smart public waste bins, which greatly aid in the transition from classical waste management to smart waste management. In Singapore, public waste collectors (PWCs) are appointed by the National Environment Agency (NEA). Typically, the collection service will travel a fixed route with a routine schedule and empty trash irrespective of whether it has reached its maximum capacity or not. This manual method of collection is inefficient. Moreover, it lacks the visibility required for effective waste management. By implementing IoT and advanced technologies, waste management practices can be made more efficient and can reduce costs. The municipality of Singapore opted for three directions: (1) tracking waste bins and compactors; (2) optimizing the fleet's resources, and (3) improving driver management.

Implementing sensors to track location, capacity (real-time weight or fill level), operating incidents, and collection status enable predictive analysis and allow for rescheduling and optimization of routes for more efficient use of resources. They are expected to improve customer experience and to enhance overall public hygiene and health.

With accurate GPS tracking, a real-time information dataset of driver routes and vehicle records can be obtained at any time. Tapping on this information will allow optimization of driver routes, yielding better fuel efficiency and reduced costs, as well as reduced emissions. The information database can also generate easy-to-read reports so that quick decisions during crucial moments can be made on the spot.

By monitoring driver behavior and by understanding driver behavior patterns, unsafe driver habits are identifiable early on; road accidents are preventable. Furthermore, greater transparency of driver activities is likely to make drivers more accountable for their road driving behavior. This transparency makes employee management easier and reduces the rate of accidents in the long run, thereby leading to reduced costs and higher productivity.

### 38.5.2 Barcelona

Best recognized for its globally renowned soccer team, Barcelona was an early adopter of a smart approach to urban development. High-tech improvements that are apparent throughout Barcelona offer a strong template for various other cities looking to improve their technological infrastructure similarly. Barcelona has implemented a sensor system for drivers that guide them to open parking spots. Embedded underneath the asphalt, sensors can identify available parking spaces and notify drivers. The program has reduced emissions and congestion by directing drivers to vacant parking spaces.

LED-based lighting systems, along with sensor networks, have replaced streetlights across Barcelona. This lighting system is more energy-efficient. It reduces heat produced by old lamps, thereby leading to cost savings for the city. This solution dealt with the problem of street lighting being used ineffectively in a manner that is quite harmful to the environment. Assisted by sensors, the lighting system receives information related to the environment: pollution, humidity, temperature, the presence of people, and noise.

Before its smart commitment, Barcelona spent 1.5 billion Euros over 4 years to manage waste and recycling. Today, however, the city has committed itself to reducing these costs through home-based recycling and fill-level sensors in waste bins. Now, the Barcelona residents dispose of their household wastes into smart bins assisted by a smart waste disposal system. These smart bins use a vacuum to suck the waste into underground storage. This process reduces odors from trash waiting to be collected, as well as noise pollution from collection vehicles. It also enables the city to detect the level of waste from various origins and optimizes the collection of waste, which decreases both the resources and time necessary for this service. Waste incineration is used later to produce energy for heating systems.

### 38.5.3 Seattle

Home to giant companies such as Microsoft and Amazon, Seattle was bound to become a smart city. The city of Seattle's first comprehensive smart waste management plan was approved and adopted in 2013. Since then it is updated every few years. Innovative thinking reduced emissions and favors recycling. For example, in the construction-demolition sector, companies must provide on-site recycling facilities. Commercial entities can manage their recyclables as the like, but all other sectors must comply with a flow control requirement, which states that all waste generated in the city must be managed by the city's system. The city has two state-of-the-art transfer stations that process all collected wastes. These stations are equipped with software systems and other technologies to help the city track recycling, waste, organic materials, and other data. In 2016, Seattle had a 58% recycling rate. It is aiming for 70% by 2022. To attain that number, Seattle organized the "Think Green Reuse and Recycle Challenge" each year, in which the region that reduces their garbage the most will win $5000 to give to the community nonprofit (s) of their choice, whereas neighborhood regions that encourage their neighbors to reduce their garbage, while recycling and composting more can win up to $15,000 to give to the community nonprofit(s) organization of their choice.

### 38.5.4 Seoul

At one point in recent history, Seoul, the most populated city in South Korea, had an aggravated problem because of the amount of its generated wastes, in addition to its small number of public litter bins. Similarly to other large cities, it obliged collection of litter bin contents extremely often. The bins were rapidly filled. Waste accumulated even on top of these bins. However, since the introduction of solar-powered compacting bins that can, as in Singapore, store up to four times more waste before having to be emptied, the city of Seoul reduced waste collection costs by 83% and eliminated difficulties posed by overflowing bins. The bins have also enabled the frequency of the waste collection tasks to be reduced by 66%. Recycling rates have increased by 46%. This increased the capacity by 700%, which in turn reduced waste collection by 85%. The system guarantees that the bins will only be collected when they are full, thereby requiring fewer collection visits and improving the traffic flow within the city. The ultimate result is cleaner and safer streets. Traffic reduction because of fewer collection visits helps reduce carbon dioxide and other emissions. In turn, the reduced traffic raises public awareness about the use of renewable energies. Furthermore, the recycling bins have led to reduction in the production of food waste by around 10% in the capital. Some food waste containers also have a special function of converting organic waste into compost. This material is then used in community gardens. Alternatively, it is donated to farms. Finally, by virtue of the use of solar energy, these bins can charge themselves and broadcast the amount of litter they contain by electronic transmission. Therefore, their status can be checked from a mobile phone.

Seoul also has a selective collection program using variously colored garbage bags: white for domestic wastes, yellow for food wastes, orange for business wastes, blue for public wastes, and gunny bags for construction debris and glass wastes. Moreover, Seoul has a strong program for engaging its residents and for fighting antisocial behavior for illegal dumping of wastes.

### 38.5.5 Toronto

The city of Toronto has a long-term, 30–50-year integrated waste management strategy that includes both circular economy and zero waste components. The city fleet consumes compressed natural gas. Moreover, an ample program exists for multiple recycling. Toronto has two anaerobic digestion facilities for its organics. It uses biogas from those facilities to fuel its fleet. Toronto also launched an application through which Torontonians can readily access information related to recycling, collection schedule, drop-off points, and other areas of interest.

From the experiences of these cities and others such as San Francisco, Tokyo, Amsterdam, Atlanta, London, Melbourne, Philadelphia, Minneapolis, and Santander, the key steps are selective collection, compaction, transportation, and processing. Processing is easy if wastes are already sorted. Processing represents a mode of making profits because the transformed no-cost starting materials can be sold with amortization of processing costs. IoT largely facilitates the first three steps, allowing both economies in city budgets and increased efficiency of recycling and recovery of wastes, with fewer emissions along the way.

There are over 150 specialized, high-tech waste bins. Among the most are those sold by a French company: Smartup cities are equipped with fill-level sensors and detectors with wireless connectivity that allows for accurate planning, reporting, and optimization. Smart bins and smart containers that have fill-level ultrasonic sensors can provide complete remote visibility over any type of container and help plan the collection routes accordingly.

Seoul's smart bins were created by the South Korean startup Ecube Labs. Waste compactors read a waste bin's fill-level in real time using sensors and then trigger automatic compaction, which increases the bin's capacity by 5–8 times. Moreover, the bin is solar-powered.

Australia-based Smartsensor monitors fullness and temperature levels of each bin and provides maintenance alerts based on customer requests. During fill-level measuring, built-in sensors measure the current temperature inside a container and transmit this information to a server together with other measured data. In the case of sudden temperature changes (e.g., through a fire), the system automatically informs operators via text or E-mail alerts.

The US-based company, EvoEco, developed the *EvoBin*, which displays responsive messages to a user. It is triggered by sensors inside depending on the type of waste that has been thrown in it. They are innovative waste bins that come equipped with screens, dashboards, and other interactive systems that capture the users' attention. For example, they can include elements of a game, educate how to sort waste correctly, and motivate users every time they throw their waste away in the bins.

The Slovakian company Sensoneo combines ultrasonic sensors (single, double, quatro, and microsensors) that monitor waste in real time with sophisticated software (smart analytics, smart route planning, and smart waste management system). The system provides cities with data-driven decisions for optimization of waste collection routes, frequencies, and vehicle loads.

An important benefit of these smart bins is that they are self-powered by solar panels. These panels harness solar energy and use sensors for continual compaction of the deposited waste, thereby increasing the capacity by up to 700% and reducing waste collection by up to 85%.

The “smart bin” communicates information related to fill levels and ensures collection only when the bin is full. The fewer collection visits reduce congestion and traffic interruptions, resulting also in cleaner and safer streets. Traffic reduction because of fewer collection visits helps reduce carbon dioxide and other emissions. The “smart bins” are standardized so that they can be emptied using existing equipment.

From a social perspective, the social benefits of “smart bins,” aside from their economic and environmental advantages, are interesting. They raise public awareness about using renewable energy, improve street sanitation, encourage recycling, collect and analyze area-specific data on waste volumes for better planning, and increase WiFi coverage via their function as a free public WiFi hotspot.

Whereas these cities and other technologically innovative regions are leading the way in smart development, any municipality can take advantage of IoT technology to conserve funds, improve services, and tackle environment issues. It is clear that any economically developed country having the drive, the means, and the infrastructure can start to implement smart waste systems. Legislation can be adopted and plans drawn.

Implementation of smart waste systems in economically developing countries would be inhibited or prevented by several factors such as urbanization, inequality, economic growth, cultural and socioeconomic aspects, policy, governance and institutional issues, education, lack of involvement of residents, and international influences.

## 38.6 Waste Management System in the COVID-19 Pandemic

The recent COVID-19 pandemic has drawn attention to a special category of waste: healthcare-related wastes. According to the World Health Organization, of the total amount of waste generated by healthcare activities, about 85% is general, nonhazardous waste; the remaining 15% is considered hazardous material that might be infectious, toxic, or radioactive.

Waste and by-products cover a broad range of materials, as the following list illustrates.

- Infectious waste: waste contaminated with blood or other bodily fluids (e.g., from discarded diagnostic samples), cultures and stocks of infectious agents from laboratory work (e.g., waste from autopsies and infected animals from laboratories), or waste from patients with infections (e.g., swabs, bandages, and disposable medical devices).
- Pathological waste: human tissues, organs or fluids, body parts, or contaminated animal carcasses.
- Sharps waste: syringes, needles, disposable scalpels and blades, etc.
- Chemical waste: solvents and reagents used for laboratory preparations, disinfectants and heavy metals contained in medical devices (e.g., mercury in broken thermometers) and batteries, and similar materials.
- Pharmaceutical waste: expired, unused, and contaminated drugs and vaccines.
- Cytotoxic waste: waste containing substances with genotoxic properties (i.e., highly hazardous substances that are mutagenic, teratogenic, or carcinogenic), such as cytotoxic drugs used in cancer treatment and their metabolites.
- Radioactive waste: such as products contaminated by radionuclides including radioactive diagnostic material or radiotherapeutic materials.
- Nonhazardous or general waste: wastes that pose no particular biological, chemical, radioactive, or physical hazard.

The major sources of healthcare waste are hospitals and other health facilities, laboratories and research centers, mortuary and autopsy centers, animal research and testing laboratories, blood banks and collection services, and nursing homes for elderly people.

High-income countries generate, on average, up to 0.5 kg of hazardous waste per hospital bed per day, whereas low-income countries generate, on average, 0.2 kg. However, healthcare wastes are often not separated into hazardous or nonhazardous wastes in low-income countries, making the real quantity of hazardous waste much higher.

When the SARS-Cov-2 virus was first contracted by humans and started to spread worldwide, one question was how it would affect waste management systems (Naughton 2020). COVID-19 social distancing measures led to business, university, and school closures that have reduced waste generation but which have shifted some waste generation to households. Furthermore, COVID-19 caused many people to buy items and food online, which yielded more packaging waste. In many countries, people hoarded food and items, which generated some waste from spoilage. However, COVID-19 led to mass unemployment which decreased spending capacity and, most likely, decreased waste generation among those unemployed people. Moreover, an increase of medical waste and personal protective equipment (PPE) related to COVID-19 has occurred, but a decrease in other healthcare wastes has also occurred.

Many recycling facilities had to close or reduce their capacity to protect workers and thereby divert their items to landfills. The principal cause was, of course, the long half-life of the virus on materials such as waste containers, bags, and in wastewater and its possible transmission via contaminated waste surfaces and aerosols from wastewater systems (Nghiem et al. 2020). Indeed, fomites (objects or materials which are likely to carry infection, such as clothes, utensils, and furniture) are recognized as key vehicles for the spread of other infectious human viruses (e.g., noroviruses) during outbreaks. Numerous studies have demonstrated that SARS-Cov-2 and other coronaviruses can remain active on surfaces of several types for a few hours to several days. The survival on the various surfaces, such as metals, paper, plastics, and clothes, reportedly extends such that materials originating from households for which occupants have been tested positive and who have remained in quarantine are potential virus carriers. Their wastes should therefore be regarded as clinical wastes and should be treated as such through incineration (Nghiem et al. 2020). A delay of at least 3 days, the maximum life span of the virus on plastics and metals, should be respected for collecting potentially contaminated wastes.

After almost a year of the COVID-19 pandemic, some patterns have emerged (Fan et al. 2021).

- Manual sorting and recycling have been restricted because of safety precautions. Landfilling became a choice method of disposal.
- Depending on social habits, increases or decreases in household waste amounts occurred around the globe.
- Amounts of medical wastes clearly increased, principally because of protective equipment.
- A circular economy based on recycling and reuse has been affected.
- Changes were recorded in waste distribution, composition, and amount, as well as in the frequency of waste collection.

People who usually ate at restaurants started to eat at home. Therefore, more household wastes were generated. People who usually ate at home started to be more selective with their food, producing less waste. Lower incomes in many households led to less shopping and therefore less wastes. More online shopping brings supplementary wrapping materials, carton boxes, etc., and therefore more wastes. Many more such assumptions might be made. Nevertheless, it is clear that the current pandemic has disrupted seemingly perfect waste management systems based on circular economy concepts. Humankind has been forced in many cases to revert to landfilling and incineration, with important implications for the environment and greenhouse gas emissions.

One sensible advice would be that temporary storage capacities, clean and secured, should be prepared in order to gather wastes collected for recovery, in anticipation of any disruption in waste processing (such as the lack of dedicated disposal or incineration capacity). It is therefore paramount that wastes (especially medical wastes) are safely stored temporarily until the capacity issue is solved. Storage should consider the use of sealed containers in secured areas where access is limited only to authorized personnel. The inner and outer surfaces of the containers should be treated with a suitable disinfectant. The containers should be stored locally. In emergency situation, industrial incinerators or even cement kilns could be requisitioned in order to incinerate the excess wastes.

Another aspect is that the pandemic has increased the production and consumption of personal and medical equipment, often single-use and containing resources like plastics, cotton, metals, and even electronic components. These healthcare wastes should be treated as any infectious waste: being deposited in infectious wastes bins (properly labelled or color-coded), periodically collected in leak-proof bags or puncture-proof containers, and transported to clean storage areas (that are protected from elements, pests, and the public). These healthcare wastes could be incinerated, but their larger volume (compared to pre-pandemic period) would generate larger amounts of greenhouse gases and ashes. Larger capacities for steam treatment should be provided (30 psi saturated steam for 30 min is enough to kill a wide range of pathogens, including SARS-Cov-2 virus). In that regard, wastes from healthcare cleaning facilities should be treated the same as medical wastes.

Repercussions of the COVID-19 pandemic on municipal waste management (including medical and healthcare wastes) has been recently sensitively assessed (Kulkarni and Anantharama 2020). The study highlighted the potential risks of SARS-CoV-2 transmission in usual waste management practices and reported the risk-mitigating approaches to municipal solid wastes management such as decentralized waste management and its integration within the existing systems.

The crisis can nevertheless serve as an opportunity to revise and improve the existing waste management system. Entrepreneurship opportunities abound for developing sustainable resource and waste management technologies (Neumeyer et al. 2020).

## 38.7 Conclusions

Major cities worldwide have or will evolve sooner or later toward smart waste management systems, based on several concepts such as a circular economy, life cycle assessment, and IoT. The benefits and implications are multiple, both for environment preservation and human welfare. However, even in economically developed countries, progress is slow. For economically developing countries, the process is at least strenuous.

A cornerstone of the process is pre-separation of wastes: most of the Earth's population seems reluctant to do it. Some measures have been taken in various cities, predominantly in the so-called smart cities, among them being lower costs for lower amounts of wastes or preferential tariffs for those who have sorted their wastes. Because most companies that deal with waste management usually declare profits, a solution for the future would be to buy wastes from residents on the condition that it is already segregated and gaining profit from recycling, reusing, recovering various materials, and transformation of wastes into profitable resources such as energy, biogas, biofuels, and even simple compost.

The COVID-19 pandemic has demonstrated that even the best plans can have flaws and that even smart cities must have alternative waste management systems for such periods. One clear conclusion is that nations have to be prepared for the future, and in that aspects both short-term (as in emergency response plan) and long-term (as in recovery plan) action plans must be devised and resources prepared to take action.

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# Health Aspects of Indoor Environmental Quality

# 39

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## Abstract

Indoor environmental quality (IEQ) refers to the environmental quality inside the building that affects the comfort and health of those who occupy it. It determines the indoor air quality in terms of thermal comfort, lighting condition, odor, noise pollution, etc. Building occupants are exposed to poor environmental quality due to different types of contaminants generated from cleaning products, building materials, paints, construction activities, cigarette smoke, and perfumes. Poor lighting, inefficient ventilation, humidity, and thermal discomfort trigger sick building syndrome which leads to poor health condition, resulting in low productivity and absenteeism in educational institutes and offices. This problem is gaining attention of scientists and engineers in both developed and developing countries. This chapter focuses on the need of IEQ inside the buildings and factors affecting it.

## Keywords

Indoor environmental quality · Air quality · Thermal comfort · Health · Buildings

## 39.1 Introduction

IEQ is expressed in term of occupants' health determined by environmental aspects like indoor air quality (IAQ), thermal comfort, lighting, and acoustical quality. In relation to the building occupants' health, the World Health Organization (WHO)

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states that health is a state of complete physical, mental, and social well-being and not merely the absence of disease or infirmity (WHO 1984). IEQ does not consider psychological effects and individual and physical reasons such as age, diseases, and degradation of parts of the human body as these are hard to determine.

The quality of environment inside the building is determined by many factors like temperature, humidity, lighting quality, odor, ventilation efficiency, meteorological parameters, acoustic conditions, and furniture and space layout. The indoor environment is the result of the interactions of many factors—the building's location, area, climate, construction methods and materials, renovations, occupant activities, and furnishings. IEQ has impact on health, comfort, safety, and productivity of building occupants. The impact of poor IEQ is also governed by these factors. However, the type of pollutants, duration of exposure, and vulnerability of an individual also play a significant role in affecting human health.

### 39.1.1 The Need for Good Indoor Environmental Quality

People spend about 90% of their time indoors, majorly in buildings, which are constructed to protect occupants against any unfavorable outside conditions and to provide them with healthy, safe, and comfortable conditions for their activities. Temperatures that are too high or too low, continuous high noise levels, or excessively bright or inadequate lightening levels increase the stress level in the human body, thus creating health issues like sleep disorder, digestive problems, and memory and concentration impairment. Understanding the need of ensuring good IEQ, building construction and renovation are changing today in order to create a high-quality indoor environment. They are becoming more improved to meet the people's needs, for example, educational buildings. These are places where young and adult people stay for a long period of time daily. Studying is a very intensive and complicated process which requires a lot of mental work. It is difficult to concentrate in a classroom where there is poor IAQ and unacceptable thermal and acoustical conditions. Moreover, harmful and unhealthy conditions due to dust, mites, malodors from toilets, insufficient lighting, etc. can be a reason of health issues such as allergic and asthmatic reactions and sick building syndrome complaints such as eye or nose and throat irritation. Young children are more vulnerable to the health problems. Therefore, poor IEQ may be considered as one of the factors which is responsible for the decrease in productivity of students and frequent absenteeism.

The healthy and comfortable indoor conditions heavily depend on the design and operation of the ventilation system as well as its maintenance because it flushes out indoor-generated air contaminants that would otherwise build up to unhealthy concentrations inside the building. The existing ventilation systems in most of the old buildings are natural exhaust ventilation and do not comply with current standards. Thus, it is becoming an actual and serious problem which needs to be solved. According to several studies, the improvement of the indoor environment increases productivity. If the money invested in designing, operation, and maintenance of the building can result in decreased complaints of health issues and increased productivity, then this investment is highly cost-effective. It is the most efficient method to assure comfort, productivity, and healthy conditions to occupants.

## 39.2 Parameters Affecting Indoor Environmental Quality

### 39.2.1 Indoor Air Quality (IAQ)

As per the American Society of Heating, Refrigeration, and Air Conditioning Engineers (ASHRAE), "IAQ is defined as the air in which there are no known contaminants at harmful concentrations and with which a substantial majority (usually 80%) of the people exposed do not express dissatisfaction". IAQ is a major concern to businesses, building, schools, managers, workers, and residents because it can impact the health and comfort of the building occupants. By controlling indoor pollutants, we can help reduce the risk of indoor health concerns. The health issues caused due to indoor air pollutants may be noticed soon after exposure or later. IAQ depends on various factors like indoor sources and emission of pollutants, nature or toxicity of pollutants infiltrations from outdoor air, ventilation design and maintenance, humidity, and occupant perceptions. The effects of poor IAQ can be enhanced by other psychological factors such as physical, mental, and emotional reactions of an exposed individual.

IAP describes the generation and transport of pollutants inside a variety of interior environments in which people live and work. These pollutants may be particles or gases that occur in the air inside building, exposure to which may have an adverse effect on the health of their occupants. Airborne particles are important sources of indoor pollution. The principal sources of these particles are cooking, smoking, and re-suspension of the previously settled particles. Indoor particulate pollutant concentration and characteristics are important in determination of total exposure, because individuals spend a large fraction of time indoors, and so continuous exposure to these contaminants can constitute a potential health hazard.

Indoor pollutants come primarily from combustion of liquefied petroleum gas (LPG) and bio-fuels like wood, charcoal, dung cake, and agricultural crop residues. Smoke from combustion of solid fuels contains a large number of potentially hazardous pollutants like particulates, CO, benzo[a]pyrene, formaldehyde, nitrogen dioxide ($NO_2$), polycyclic organic compounds, and metals such as arsenic. Domestic cooking is an important source of IAP and is associated with significant morbidity and mortality (Kaul et al. 2017).

#### 39.2.1.1 Present Status in India

The level of air pollution in Indian cities is getting worse today because of unbridled industrialization, unprecedented growth in the number of vehicles, huge energy consumption, and indiscriminate burning of waste. The developed cities are fast becoming gas chambers, and their citizens are forced to breathe polluted air.

Numerous studies done across the world and in India indicate that the people living in these severely polluted cities are exposed to a deadly cocktail of gaseous, particulate, and photochemical pollutants (Gupta et al. 2002). The concentration of the pollutants like carbon monoxide (CO), oxides of nitrogen ($NO_x$), oxides of sulfur ($SO_x$), respirable suspended particulate matter (RSPM), lead, benzene, polycyclic aromatic hydrocarbons (PAH), and ozone is higher than the desired levels set by the Environmental Protection Agency (EPA), WHO, and other health regulating agencies in most of the cities (USEPA 2013). Exposure to air pollutants results in adverse effects on human health ranging from acute to chronic.

Many people associate air pollution only with urban outdoor environment, but some of the highest concentrations of pollutants occur in the indoor environment. According to the WHO, indoor air pollution (IAP) from solid fuel ranks fourth among risks to human health in developing countries and ranks still higher in India. Other than malnutrition and lack of safe sanitation and drinking water, IAP is a major factor affecting human health in India. Studies have shown that daily particulate dose to the urban Indian women from domestic cooking is comparable with the dose resulting from outdoor particulate exposure (Varghese et al. 2005).

#### 39.2.1.2 Factors Determining Health Effects of Indoor Air Pollutants

The major factors that govern the effects of exposure to indoor pollutants on human system are as follows.

1. Concentration and characteristics of pollutants (emission rate, toxicity, etc.) released in a confined space, for example, cooking. The magnitude of pollution depends on the location of the kitchen, fuel type, cooking practices, duration of cooking, and efficiency of ventilation.
2. Duration of time spent in polluted air.
3. Presence of sensitive persons like children, elderly, and asthmatics.
4. Ventilation mechanisms removing pollutants from the indoor air.

Thus, it is not only enough to prepare an inventory of all the sources and their emission rates, but it is equally essential to understand the atmospheric conditions like wind forces and thermal stability of the environment or the ventilation in the indoor spaces that determine the concentration of pollutants to which an individual could be exposed. Besides, the duration for which that concentration persists in the inhaled air determines the total exposure.

The effects of principal indoor air pollutants have been listed in Table 39.1, which summarizes the acute as well as chronic problems related to indoor air contamination due to a host of pollutants.

### 39.2.2 Thermal Comfort

Thermal comfort is an important aspect of residential buildings other than the usual safety and security aspects. Thermal comfort is a subjective response, defined as that

**Table 39.1** Summary of indoor air pollutants and their effects

| Pollutant | Source | Mechanism | Potential health effects |
|---|---|---|---|
| $PM_{10}$ and $PM_{2.5}$ | • Environmental tobacco smoke (ETS)<br>• Re-suspension<br>• Condensation of vapors<br>• LPG, solid fuel combustion products<br>• Gas particle conversions | • Absorption of toxins like PAH<br>• PM-$NO_x$ synergistics | • Wheezing, exacerbation of asthma<br>• Respiratory infections<br>• COPD<br>• Exacerbation of COPD<br>• Excess mortality, including from cardiovascular disease<br>• Lung cancer (Delfino et al. 2005) |
| Carbon monoxide | • Fuel combustion (charcoal briquettes, firewood, coal, etc.)<br>• Indoor tobacco smoking<br>• Use of generators during power cuts<br>• Entry of outdoor vehicle exhausts | • Binding with hemoglobin (Hb) to produce COHb<br>• Interference with intracellular oxidation | • Low birth weight (fetal COHb 2–10%, or higher)<br>• Increase in perinatal deaths<br>• COHb levels between 50 and 60% can result in fainting and convulsions, while higher exposures can lead to coma and death<br>• Chronic exposure (at 10–30% COHb) often produces symptoms that are easily misdiagnosed or overlooked, such as headache, fatigue, dizziness, and nausea |
| Benzo[a] pyrene | • Coal and biomass smoke | • Carcinogenic | • Lung cancer<br>• Cancer of the mouth, nasopharynx, and larynx |
| Formaldehyde | • Insulation, particleboard, furnishing, and environmental tobacco smoke | • Nasopharyngeal and airways irritation<br>• Increased allergic sensitization | • Increased susceptibility to infections<br>• May lead to asthma |
| Nitrogen dioxide | | • Methemoglobin formation<br>• Combine with water in the lungs to form nitric acid ($HNO_3$) and nitrous acid (HONO) which react with lipids and proteins | • Acute exposure increases bronchial reactivity (Dennekamp et al. 2001)<br>• Long-term exposure to 50–100 $\mu g/m^3$ $NO_2$ may significantly affect respiratory and immunity systems of children and sensitive adults<br>• Wheezing and exacerbation of asthma<br>• Respiratory infections |

(continued)

**Table 39.1** (continued)

| Pollutant | Source | Mechanism | Potential health effects |
|---|---|---|---|
| | | | • FEV reduction in children<br>• Anxiety, hypertension<br>• Death in extreme cases |
| Sulfur dioxide | • Combustion of high-sulfur coal<br>• Outdoor air | • Acute exposure increases bronchial reactivity<br>• Longer term: Difficult to dissociate from particulate effects | • Wheezing and exacerbation of asthma<br>• Exacerbation of COPD, CVD |
| Biomass smoke (component uncertain) | | • Absorption of toxins into lens, leading to oxidative changes | • Cataract |

condition of the mind which expresses satisfaction with the thermal environment. It has been identified that when 80% of the building occupants are satisfied with the surrounding thermal conditions, only then thermal environment is satisfactory (ASHRAE 1992). Thermal comfort conditions are a very important factor in deciding the building sustainability, worker productivity, health, and nature of the occupants' living (Singh 1996). Excessively hot or cold environments can affect motor and cognitive behavior of individuals. Extremely hot conditions can lead to loss of performance capacity and slow production output, while excessively cold environments have effect on manual agility and sometimes are associated with pain. As per ASHRAE Standard 55 (2013), the recommended temperature ranges perceived as "comfortable" are 22.8 to 26.1 °C in the summer and 20.0 to 23.6 °C in winters. The factors that are responsible for the change in thermal comfort inside a building are air temperature, metabolic rate of occupants, radiant temperature, humidity, and air speed. In modern buildings good ventilation has become more imperative for health, aesthetics, and thermal comfort (Kaul and Parik 2020).

### 39.2.3 Relative Humidity

Humidity refers to the presence of water vapor in the air and affects how warm the air feels to us. Relative humidity levels below 25% are associated with increased discomfort and drying of the mucous membranes and skin, which can lead to chapping and irritation. Low relative humidity also increases static electricity, which causes discomfort and can hinder the operation of computers and paper processing equipment. On the other hand, high humidity levels can result in condensation within the building structure and on interior or exterior surfaces. Too high relative humidity can contribute to the growth and spread of biological contaminants, and people think it feels "sticky." In a humid environment, the comfort and attentiveness of an individual may be significantly reduced, and his productivity can be hampered. As per ASHRAE Standard 55, indoor humidity levels should be maintained between 30 and 65% for optimum comfort.

### 39.2.4 Lighting Conditions

Visual comfort is one of the physical criteria for the building occupants to identify their comfort level in indoor environment, thus achieve good IEQ. Visual comfort is achieved when the illuminance levels (lux) are maintained in good conditions. Daylight can be efficiently used to maximize occupant comfort and to conserve energy. While designing windows to optimize occupant comfort, one must keep in mind the amount of daylight needed and type of activity in the room and select materials with recommended surface reflectance and matte finish. Proper lighting condition enhances the learning experience in school and colleges. Also the productivity of workers during intricate work like designing, painting, etc. can be substantially increase by providing adequate lighting condition. The desirable limit for lighting for different requirements is prescribed in the Bureau of Indian Standards (BIS) code IS 3646 (1992).

### 39.2.5 Noise Intensity

Environmental noise pollution, a form of air pollution, is a threat to health and well-being. It is more severe and widespread than ever before, and it will continue to increase in magnitude and severity because of population growth, urbanization, and the associated growth in the use of increasingly powerful, varied, and highly mobile sources of noise. When a sound appears in an unpleasant, frustrating, or annoying way, it can affect the IEQ within those spaces. Whereas, indoor noise generated due to mechanical ventilation system emits high sounds that disturb the occupants in the building. In addition, the noise from the residents, office activities, and machine used inside the building such as computer, air conditioner, etc. also increase the intensity of the noise in the building. Noise produces direct and cumulative adverse effects that impair health and that degrade residential, social, working, and learning environments with corresponding real (economic) and intangible (well-being) losses. The WHO has recognized seven categories of adversative health effects of noise pollution on humans such as hearing impairment, interference with spoken communication, sleep disturbances, cardiovascular disturbances, disturbances in mental health, impaired task performance, and negative social behavior and annoyance reactions (Goines and Hagler 2007). As per Indian standards, the permissible limit for indoor noise is 55 dBA in daytime and 45 dBA in nighttime (CPCB 2000). The noise standards are documented separately for industrial/commercial, residential, and silence zones.

### 39.2.6 Volatile Organic Compound

Volatile organic compounds (VOCs) have low boiling points, usually less than 100 °C, and therefore evaporate readily and are emitted as gases from certain solids or liquids. Concentrations of many VOCs are consistently higher indoors (up to ten times higher) than outdoors. Organic chemicals are widely used as ingredients in household products. Paints, fuels, varnishes, and wax all contain organic solvents, as do many cleanings, disinfecting, cosmetic, degreasing, and hobby products. All these products can release organic compounds while we are using them and, to some degree, when they are stored. Some examples of sources of VOCs are building materials like carpets, adhesives, paints, and vinyl floors and home and personal care products like air fresheners and moth balls and can also be generated through cooking (frying), dry cleaning, newspapers, photocopiers, smoking, etc. Breathing low levels of VOCs for long periods of time may increase some people's risk of health problems. Several studies suggest that exposure to VOCs may make symptoms worse in people who have asthma or are particularly sensitive to chemicals and acute diseases like headaches, nausea, dizziness, etc. the IAQ index provides the following ratings to VOC levels: $<1.0$ ppm good or "green," 1.0 to $<10$ ppm marginal or "yellow," 10 ppm and higher poor or "red."

### 39.2.7 Ventilation and Carbon Dioxide Levels

Ventilation is the intentional introduction of outside air into a space. Ventilation (mechanical or natural) is mainly used to control indoor air quality by diluting and displacing indoor pollutants; it can also be used for purposes of thermal comfort or dehumidification when the introduction of outside air will help to achieve desired indoor psychometric conditions. In many instances, ventilation for indoor air quality is simultaneously beneficial for the control of thermal comfort. The rate of ventilation by natural means through windows or other openings depends on direction and velocity of wind outside, sizes and disposition of openings, and convection effects. The rate at which outdoor air replaces indoor air is known as air exchange rate and is a measure of the number of times the complete volume of inside air is replaced with fresh outside air per hour.

Ventilation guidelines are based upon the minimum ventilation rate required to maintain acceptable levels of bioeffluents. Using $CO_2$ as an indicator of ventilation, ASHRAE has divided $CO_2$ levels as their effect on indoor environment and occupants as $<1000$ good, 1000 to 1200 marginal, 1200 to 5000 poor, and less than 5000 dangerous. Exhaust and chimney are an effective way to flush out the accumulated pollutants in an indoor space. Inadequate ventilation substantially increases particle and $NO_x$ concentration during cooking period in a kitchen. Forced ventilation has been found to be more efficient than natural ventilation. Studies show that exhaust fan and chimney both are effective in the removal of coarse particles. However, efficiency of chimney is found to be limited for high particle concentration (during frying), because of its low air exchange rate which is not a limitation of an exhaust fan. Also chimney has limited capacity to flush out fine particles and gaseous pollutants. It is important to design the ventilation system of an existing building considering the strengths and limitation of both exhaust fan and chimney (Kaul 2011).

### 39.2.8 Odor

Odor is defined as the perception of smell. Unlike conventional air pollutants, odor has distinctly different characteristics, which, to an extent, can be compared with noise pollution. IEQ is affected not only due to conventional pollutants but also due to unpleasant odor. Odor affects human beings in several ways. Strong, unpleasant, or offensive smells can interfere with a person's enjoyment of life especially if the exposure is frequent and/or persistent. Offensiveness, duration of exposure to odor, frequency of odor occurrence, tolerance, and expectation of the receptor are the major factors relevant to perceived odor nuisance. In indoor environment the odor is mainly produced from dirty toilets, rotten food, paints, cigarette smoking, and garbage and from outdoor activities. Studies document discomfort of people due to the use of room fresheners, perfumes, vaporizers, phenyl (toilet cleaning agent), hair oil, etc. Few people also are sensitive to odor emitted during cooking eggs and meat and also fumes from heating mustard oil. These problems get aggravated if the exposure occurs in a confined space like AC train compartments, restaurants, etc. It is important to develop and design effective ways to overcome the problem of odor which can be fitting to the sociodemographics of a site. CPCB India has a complete manual to address the extent of the problem and its measurement, control, and regulating aspects (CPCB 2008).

## 39.3 Prevention Strategies

Ensuring IEQ in indoor spaces is very important for maintaining a healthy lifestyle. It deals with various aspects that govern the well-being, productivity, and comfort of the occupant in an indoor environment.

- General ventilation is one of the most effective methods of improving environmental conditions in the buildings. Detailed design guidelines for natural ventilation are available in the National Building Code of India, 2005. The standards of general ventilation are recommended/based on maintenance of required oxygen, carbon dioxide, and other air quality levels and for the control of body odors.
- Following good cooking practices like switching on mechanical ventilation immediately after the stove is switched on, minimum use of oil (especially during frying), and taking breaks during prolonged cooking (when oil is heated alone).
- Follow the standard practices of building design, renovation, construction, operation, and maintenance.

- Reduce the dependence of electrical lightning and equipments such as elevator, air conditioner, and fans.
- Use fragrance free and low VOC cleaning products.
- Creating a window and door opening protocol to maintain enough air flow.
- Avoid using material and products, which contain harmful and toxic chemicals, for example, benzene, formaldehyde, etc.
- Maintain thermal comfort in such a way that it can be a suitable indoor environment.
- Adopt building materials in such a way that it can improve both room and building acoustics. The use of furnishings and noise barriers should be encouraged.
- Indoor air purifiers can also be considered.
- Plants can also be used to enhance the quality of indoor air. They can play a significant role in reducing the harmful effects of sick building syndrome. The plants used commonly are spider plants (Chlorophytum), bamboo palms (Chamaedorea), English ivy (*Hedera helix*), peace lilies (Spathiphyllum), etc.

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# 40 Innovation Elements in the Sustainable Production of Indigenous Coffee in the Amazon

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## Abstract

The main objective of the study is to understand the main elements of innovation that enable the sustainable development of coffee in an indigenous reserve in the Amazon. The research is qualitative in nature with a case study strategy. It has a descriptive and exploratory approach using the triangulation method for data analysis. The institutional and economic development theories were adopted as a theoretical basis to support the interpretation of the innovation scenario directed toward sustainable development. As a result, it was found that the production of this commodity is carried out based on the triple bottom line. The best practices of management in the indigenous reserve were directed to the concept of sustainable development. The production is improved qualitatively with the innovation of processes, technologies, and improvement of social relations with institutions capable of potentiating coffee production from the perspective of economic growth and possible consolidation of the export market.

## Keywords

Innovation · Sustainable production · Indigenous coffee · Amazon

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## 40.1 Introduction

According to Lustosa (2002), sustainable regional development is a modern and recommended strategy for government actions, mainly to overcome poverty in some regions or social groups. Thus, the search for models that integrate the social, economic, and environmental aspects are essential to the new development paradigm focused on the sustainability of the means of production.

The relevant role of the indigenous peoples, object of this study, is in fine harmony with the search for sustainable development. These peoples have the tacit knowledge of the basic rules of the forest ecosystem and the biological cycles of nature, which when they fit with new processes and technologies adapted to the way of life and the logic of the environment allow to generate innovations directed to local sustainable development.

Since the first contact with the white man in 1969, the Paiter-Surui tribe, located in the Sete de Setembro Indigenous Territory (SSIL) in Rondônia state, has seen its way of life and culture altered by modernity (PARMSRN 2010). The closer relationship with non-indigenous peoples, nongovernmental organizations (NGOs), educational institutions, and government agencies allowed these traditional peoples of the Amazon to adopt better practices for agricultural activity, in addition to implementing procedures that improved productivity and reduced the costs of their economic activities (RIPCFS 2014).

These modernization processes that took place over the years at SSIL encompassed a way of exploring new ideas to solve problems arising from the planting of coffee, which allowed the generation of successful results essential to sustain competitiveness and the generation of wealth for this new economic reality of the indigenous peoples of that region. In this context, the question that directs this research is: What are the elements of innovation used or required that provide sustainable development in the Sete de Setembro Indigenous Territory, located in the municipality of Cacoal, Rondônia State?

To answer this question, the institutional theories and the economic development theory were adopted as the theoretical basis to support the interpretation of the innovation scenario directed toward sustainable development. This study focused on understanding the main elements of innovation that enables the sustainable development of the practices adopted in the Sete de Setembro Indigenous Territory and established its direction through the following specific objectives: characterize coffee farming in the indigenous reserve (1), identify the main factors of sustainable production (2), and describe the structure of innovation for sustainable development (3).

The body of the article is divided into five sections: the first presents the structure of the research; the second is the theoretical framework with a brief contextualization of the institutional and economic development theories, aggregated to approaches on innovation, sustainable development, sustainability, and sustainable production, for later analysis of selected data. The third section presents the methodology using the case study strategy. The fourth section addresses the results of the research and is fragmented into three topics: characterization of coffee growing in the Sete de

Setembro Indigenous Territory; identification of the main factors of sustainable production; and description of the structure of innovation for sustainable development in the indigenous reserve. And the fifth section deals with the final considerations with recommendations and limitations of the research.

## 40.2 Theoretical Background

Institutional theory comprises a set of theoretical constructs derived mainly from economics, sociology, and political science (Vailatti et al. 2017). In the scope of political science, for Fonseca (2015), developmentalism becomes a guide for action by ideologies, since it incorporates in its concept the very values pursued, a state action that is not restricted to the economic area (political means, ends, and institutional), but extending it to education, culture, public health, social laws, and environment, among others.

In the scope of the new institutional economy, according to North (1990), institutions can be understood as entities created to structure and regulate political, economic, and social interactions, i.e., they are the “rules of the game” whose composition is made of formal institutions (laws and regulations) and informal institutions (values, social norms, and customs). Considering the aspects of sociology, Pereira (2012) addresses that institutional theory facilitates the understanding of implicit patterns and the diversity existing in organizations, demonstrating flexibility and comprehensiveness in their applicability to understand complex social phenomena and how organizational structures and actions become legitimated with consequence in the results planned by organizations.

For Cavalcante (2014), there are three institutional dimensions or meanings conferred to the term institution, found in specialized literature: (1) institutions as rules of the game; (2) institutions as mental models; and (3) institutions as organizations. Complementarily, Salles and Ribeiro (2012) clarify that institutions and their changes, underlying the process of economic and social development, are interconnected and interdependent phenomena; therefore, they are important tools for the promotion of sustainable development, and on the environmental side, they are at the basis of economic instruments for regulating the use of goods and services derived from the environment. Institutional, technological, and social changes must move simultaneously and articulately in the direction of economic growth, a fact historically complex to achieve (Conceição 2012).

In 1911, Joseph Alois Schumpeter published his classic work *Theory of Economic Development* (TDE), which established the difference between a static economic system and an economic system for the development process, centered on the concept of innovation. In his thesis he defined innovation as creative destruction to describe the process of economic development. For Schumpeter (1961), the economic development process is related to endogenous and discontinuous changes in the production of goods and services. And through continuous changes, there is economic growth, but development occurs in situations without exogenous interference. The development applies only when there is a qualitative change in the system, and capitalism offers, in a natural way, incentives to change (innovation) led by the owner. The innovation process, according to Schumpeter (1997), includes the following cases: introduction of a new product; introduction of a new production method; opening of a new market; and obtaining a new source of resources and the establishment of a new organizational structure. Thus, for innovation to occur, the participation of two elements is essential: the entrepreneur (entrepreneur—innovator), seen as a transforming agent, and the credit as a means to finance innovation or mobilize production factors (Schumpeter 1997). Thus, a new format for the development of the economic system, according to Souza (2012), can be established at various times, different technological and institutional groupings.

### 40.2.1 Contextualization of the Concepts of Innovation

Innovation, according to Dosi (1988), can be understood as the search and discovery, development, imitation, experimentation, and adoption of new products, new production processes, and new organizational configurations, that is, new combinations of resources, people, ideas, knowledge, and/or technologies. For Karlsson and Tavassoli (2016), innovation can be conceived as the transformation of ideas, information, and knowledge to increase competitiveness and sustained competitive advantage. It is, in general, an indispensable component for organizational strategies.

Four types of innovation have been established by the Organization for Economic Cooperation and Development (OECD), Table 40.1, which combined can generate new innovation strategies (Karlsson and Tavassoli 2016).

Regarding innovation, according to Prajogo and Ahmed (2006), as a fundamental factor for the creation of new products and services, it can be used to restructure industries by redefining the rules of competition, which is by creating or dismissing old markets. They add that human factors (people and social practices) are largely responsible for organizational success.

For Barbieri et al. (2010), constant innovation is not enough; it is necessary to use sustainable innovation, considering the three dimensions of sustainability: (1) the social dimension addresses the social impacts of innovations on human communities, both internal and external to the organization; (2) the environmental dimension is concerned with the environmental impacts caused by the use of natural resources and emissions of pollutants; and (3) the economic dimension relates to economic efficiency, as a way to perpetuate itself in the market.

In 2012, the OECD report “The Future of Eco-Innovation: The Role of Business Models in Green Transformation” presented innovative ways to promote economic activity in order to develop policies to revitalize industrial activity, labor markets, and competitiveness while addressing global environmental challenges such as climate change and scarcity of natural resources. Thus, the OECD (2012) sought to quickly and more widely disseminate sustainable innovation or eco-innovation, in some countries known as green innovation. This mechanism is adopted to create economic and environmental value, as a way to leverage improvements to the environment, as well as economic and social improvements.

**Table 40.1** Types of innovation

| Product innovation | Process innovation | Marketing innovation | Organizational innovation |
|---|---|---|---|
| A good or service, significantly improved in relation to its characteristics or intended uses, including relevant improvements in technological specifications, components and materials, incorporation of software, ease of use or other functional characteristics of the product | To implement a new or significantly improved production or delivery method through relevant changes in techniques, equipment and/or software | Implement a new method involving significant changes in product design or packaging, product layout or pricing strategy, to meet customer needs. Opening new markets or a new product position in the market | Implement a new method in business practices, workplace planning or external relations, aimed at increasing organizational performance and reducing administrative and transaction costs, improving productivity and job satisfaction |

Source: elaborated by the authors based on Karlsson and Tavassoli (2016)

Horbach et al. (2012) state that eco-innovation is related to the production, application, or exploitation of a good, service, production process, organizational structure, and business management seen as unprecedented for the organization or user, resulting in the reduction of environmental risk, pollution, and the negative impacts of resource use (including energy use) compared to relevant alternatives. For the authors, there are four determinants of eco-innovation, as follows: regulation, market factors, technology, and characteristics specific to the organization. Regulations, when well designed, promote innovation, resource productivity, and competitiveness of organizations, in addition to being determinants for the use of clean technologies. The market is formed by the demands of consumers, current or potential, and competing organizations. The technology allows the use of environmental management systems, allowing organizations to adapt to the requirements of environmental and market regulations. Finally, the determinant of the organization comprises, for example, the mechanism of knowledge transfer and participation in networks, being essential for generating sustainable innovations (Horbach et al. 2012).

### 40.2.2 Description of the Environment of Sustainable Development and Sustainability with Sustainable Production

The focal point of the discussions on sustainability and the expansion of environmental and social awareness resulted from the finding of a growing reduction in the availability of natural resources, which consequently impacts on the survival of living beings on the planet, leading to a growing demand for transparency regarding the environmental impact of organizations and, therefore, for measures to minimize this impact (WCED 1987). It was at the 1972 Stockholm Conference (UN Conference on the Human Environment) that the need to change the way we live on our planet was understood. In 1987, the United Nations Brundtland Commission defined sustainability as "meeting the needs of the present without compromising the ability of future generations to meet their own needs." (WCED 1987). The United Nations Conference on Environment and Development (UNCED), also known as the 'Earth Summit', was held in Rio de Janeiro, Brazil, from 3-14 June 1992. The 'Earth Summit' concluded that the concept of sustainable development was an attainable goal for all the people of the world. The Sustainable Development Goals form the framework for improving the lives of populations around the world and mitigating the hazardous man-made effects of climate change. SDG 13: Climate Action, calls for integrating measures to prevent climate change within development frameworks. SDG 14: Life Below Water, and SDG 15: Life on Land, also call for more sustainable practices in using the earth's natural resources.

In this context, in 1990, John Elkington formulated the triple bottom line concept—the tripod of sustainability, also known as 3P (people, planet, and profit—an English expressions for people, planet, and profit, respectively). For Elkington (1998), the economic aspect is related to the creation of viable ventures, attractive to investors; the environmental aspect aims to analyze the interaction of processes with the environment without causing it permanent damage; and the social aspect is related to the concern with the establishment of fair actions for workers, partners, and society. Thus, when there is an intersection between two pillars, it results in a viable process, and the intersection of the three pillars results in the achievement of sustainability.

Sustainable development (SD) can be understood as an attempt to formulate a program of integration of different spheres of human activity, previously seen in a disaggregated manner (Pawlowski 2008). From Pawlowski's (2008) perspective, SD has beyond the ecological, social, and economic spheres—the spheres: moral, technical, and legal (aggregated to the economic sphere), as shown in Table 40.2.

Feil and Schreiber (2017) distinguish the concepts of sustainable development and sustainability. The term sustainable, according to the authors, originated from the deterioration between global ecology and economic development, is related to the solution of the scarcity of natural resources linked to energy issues and natural resources, aiming at a future for both resources and the human race. With regard to sustainability, they clarify that it is directed toward mutual balance, covering the environmental, economic, and social aspects and needs evaluation with indicators and indexes in order to obtain quality and ownership of the global system. They complement that sustainable development aims at economic growth without environmental aggression and has a long-term vision for future generations. And it needs a mutual balance between the environmental, economic, and social aspects, with a proposal of change in the behavior of humanity, that is, a strategy used in the long term to improve the quality of life (welfare) of society.

As far as sustainability is concerned, the discussion on aspects related to sustainable production is primordial. Silveira and Ferraz (2004) clarify that studies on the sustainability of agriculture need to present long-term viability, being fundamental the integration of environmental, economic, and social aspects, which make it

**Table 40.2** Dimensions of sustainable development

| Moral | Ecological | Social | Economic and legal | Technical |
|---|---|---|---|---|
| Ethical principle of the DS, a sense of environmental obligation which holds that man must act with caution so that the effects of his actions are compatible with the continuity of human life | Related to nature preservation, impact reduction and pollution | Relationship between man and the environment, customs, traditions, culture, spirituality, and interpersonal relationships, where one must analyze the actions that can cause damage to collective welfare | They serve to define a policy of environmental protection and to create economic instruments that stimulate the development of sustainable innovations | Development of new technologies that enable process improvement and minimization of impacts on the environment |

Source: elaborated by the authors based on second Pawlowski (2008)

possible to optimize the use of nonrenewable natural resources, allowing socioeconomic improvements to the population. From the point of view of Arco-Verde (2008), sustainable production is related to food security and the social and economic well-being of rural producers, besides being related to the control of the scarcity of financial resources and the conservation of natural resources.

Certain agricultural production practices can be considered appropriate for sustainable production, according to Giordano (2005), such as reforesting deforested areas, maintaining native forest areas, conserving and replanting native plant species, maintaining permanent preservation areas, prohibiting predatory hunting and instituting hunting and fishing areas where possible, prohibiting and rigorously supervising the cutting of native forests, establishing crop rotation, correct and localized use of defensives, selecting defensives that are less aggressive to the environment and humans, restoring organic material to the soil, and introducing organic practices and environmental education. Thus, sustainability is related to the conservation of soil, water, and other natural resources, in order to minimize the degradation of the environment, therefore, “must be technically appropriate, economically viable and socially accepted” (Giordano 2005).

Sachs (2017) states that the ecological cycle can be understood as a true model for the economic system. And it is not just a matter of “safeguarding natural ecosystems at all costs, but of conceiving of man-made artificial systems as true ecosystems and making sure that they are inserted into general ecological cycles so as not to alter the latter” (Sachs 2017, p. 82).

Thus, one can conceive the reach of sustainability, through an intense effort of innovation in product, process, management, and business model, which can be achieved with targeted actions such as elimination of burning, biological pest control, organic fertilization, new devices in machinery, and agricultural implements to avoid soil compaction and preserve terrestrial biodiversity (Barbieri et al. 2010).

## 40.3 Methodology

This research has a qualitative approach of an applied nature, with an exploratory-descriptive objective, allowing the adoption of the case study strategy. For Gil (2009) some purposes are defined for case study: (1) to explore events of lived reality whose limits are not clearly defined; (2) to safeguard the unitary character of the object; (3) to describe the conjuncture of the investigation; (4) to formulate hypotheses or develop theories; and (5) to explain the causal variables of a given phenomenon in complex situations that do not allow the use of surveys and experiments.

This type of research allows the understanding of a complex reality, according to Yin (2010), through the articulation of a series of methods of data collection and sources. This study used semi-structured interviews, nonparticipating observation, and documentary research. Therefore, it is based on multiple evidences using the previous development of theoretical propositions to guide data collection and analysis through triangulation (Yin 2010). The empirical part of this study describes the situations observed, confronting them with the theory in a manner restricted to the organization researched.

In short, the qualitative studies have as fundamental concern the analysis of the empirical world in its natural environment, valuing the direct and prolonged contact of the researcher with the environment and the situation being studied (Godoy 1995). Thus, the meaning, in this type of research, plays an important role, as the researcher absorbs the various perspectives and their relationships to abstract information from the object studied (Yin 2010).

### 40.3.1 Case Selection: Research Subjects

For Eisenhardt (1989), an important stage of the research is the selection of cases, in which the choice of the population defines the set of entities from which the research sample will be drawn. For this research, the subjects of the interview were selected intentionally, taking into account the objective of the research to identify the elements of innovation aimed at the sustainable development of indigenous coffee growing. The actors reached were the village chiefs in Indigenous Land Sete de Setembro (SSIL) and representatives of government institutions working in the study region through semi-structured interviews from 23–27 May 2018, as listed in Table 40.3.

The selected governmental institutions act and interact with the populations in the study area; for this reason the interviews were carried out with the objective of understanding how these organizations are acting to encourage the development of sustainable innovations that meet the demands of the indigenous population.

**Table 40.3** Actors interviewed

| Interview code | Number of interviewees | Description |
|---|---|---|
| HEVL 1, 2, 3 | 3 | Heads of Villages at SSIL |
| TSSA | 1 | Technician of the State Secretariat of Agriculture (Seagri) |
| TBARC | 1 | Technician of the Brazilian Agricultural Research Company (Embrapa) |
| TBREC | 1 | Technician of the Brazilian Rural Extension Company of the state of Rondônia (Emater-RO) |

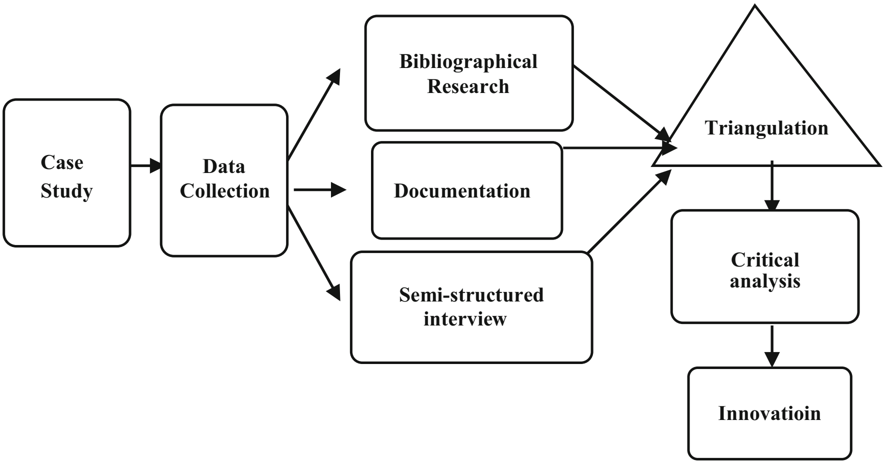


**Fig. 40.1** Methodological operational diagram

### 40.3.2 Data Collection and Analysis

For the data collection procedure, bibliographic and documental surveys (manuals, projects, and reports) were carried out, and, later, semi-structured interviews with the selected actors were applied (Fig. 40.1 made by authors).

The dimensions and categories in Table 40.4 were elaborated in order to provide a direction for data collection. The data treatment involved the triangulation procedure, using multiple sources and theoretical perspectives, with the purpose of analyzing the occurrence of convergence or divergence of information, which enabled the data treatment, in line with what Yin (2010) proposes. The interviews were directed to individuals who were familiar with the activity of coffee farming, as a way to obtain concise answers to the analysis of data, and thus achieve the objectives of the research.

After data collection, the interviews were transcribed and analyzed, along with institutional records and annotations from direct observations. The observations made are clear and detailed descriptions through the records and impressions

**Table 40.4** Data analysis structure

| Dimension | Category | Source |
|---|---|---|
| Social (people) | Moral | Elkington (1998), Pawlowski (2008), Barbieri et al. (2010) and Karlsson and Tavassoli (2016) |
| | Social | |
| Economic (Profit) | Economic and legal | |
| | Technical | |
| Environmental (Planet) | Ecological | |

about the dimensions established from analysis, thus meeting the criteria of validity and reliability, aiming to highlight the aspects of innovation that intervene in the sustainable development of coffee growing, based on the proposed theoretical-empirical structure.

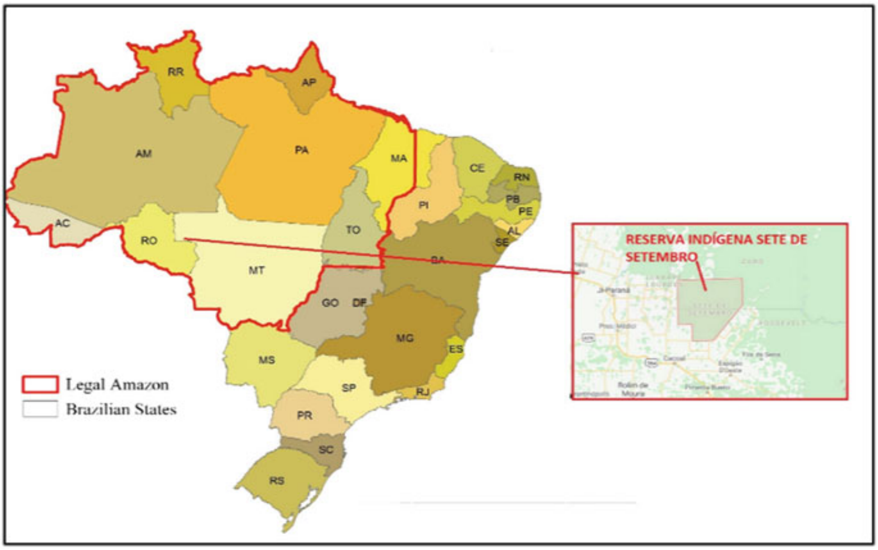
### 40.3.3 Area of Study

The Sete de Setembro Indigenous Land (SSIL) is located in the Brazilian Legal Amazon, Map 40.1, with approximately 248.146,9286 hectares of territorial extension, shelters most of the Indians of the Surui tribe, of linguistic origin of the Tupi Mondé, who call themselves Paiter, which means "we ourselves" or "real people," composed of 215 families, being 26 villages distributed along the SSIL (PARMSRN 2010).

The state of Rondônia is home to 22 villages, and the other 4 villages are located in the state of Mato Grosso. This territorial distribution has generated strong commercial and social relations within the state of Rondônia, especially in the municipality of Cacoal, where most of the Paiter-Suruí population is concentrated (PARMSRN 2010).

## 40.4 Results and Discussion

This section will be subdivided into three topics: characterizing indigenous coffee farming and identifying the main factors of sustainable production and describing the structure of innovation for sustainable development in Indigenous Land Sete de Setembro. The actors interviewed are identified by codes, according to Table 40.3 of the methodology.



**Map 40.1** Location of Sete de Setembro Indigenous Land (reserve)

### 40.4.1 Characterization of Coffee Growing in the Indigenous Land Sete de Setembro

When the demarcation and withdrawal of settlers from traditional indigenous lands in the Amazon region took place in the 1980s, the Paiter-Suruí community appropriated the coffee plantations abandoned by non-indigenous people, initially used for tribal subsistence (PARMSRN 2010). Due to the high humidity in the region, according to HEVL 1, there was a proliferation of pests, which damaged the fruit, causing the abandonment of plantations, and only in 2012, a little over 5 years ago, the planting of coffee resumed.

The SSIL is located in the region of strong pressure of deforestation, known as the "Arc of Deforestation," due to the invasions that took place in that area for illegal logging, after denunciation by indigenous leaders occurred the resumption of land ownership by the owners of the land (RIPCFS 2014).

According to HEVL 1, 5 years ago, this area devastated by illegal logging was selected to begin planting coffee and considers that the number of coffee-producing families in the village is increasing. For this reason, information from TMAT, the indigenous with the objective of improving the quality and production of coffee crops, requested technical assistance from Emater-RO, which offers guidance specifically focused on coffee culture; these activities are developed with the unconditional support of FUNAI (National Indian Foundation). According to TBARC, the coffee species are productive, provided they are adapted to each climate condition. This allows the cultivation system to be resistant to pests and diseases, these being some of the main components of the sustainability of coffee farming.

The Indigenous Land Sete de Setembro, according to HEVL 1, was the first indigenous village to develop a Surui Forest Carbon Project (PCFS), the main focus was the maintenance of the forest and at the same time enable the raising of funds to invest in agricultural activities of SSIL. The idea of carbon sequestration, according to HEVL 2, is part of the actions of conservation, protection, and sustainability, provided for in the SSIL Management Plan. This plan was realized in 2000, which includes the planning for the next 50 years of SSIL. This plan presents the need to create programs: culture, institutional strengthening, health, education, and environment. Thus, the idealization of the environmental incentive initiative emerged, with a view to promoting activities of protection, supervision, sustainable production, and improvement of local capacity.

In 2007, according to HEVL 1, through the Metareilá Association of the Paiter-Suruí Indigenous People, the following institutions were invited to support the construction of a carbon stock initiative: Ethno-environmental Defense Association (Kanindé), Amazon Conservation Team (ACT-Brazil), Forest Trends, Brazilian Fund for Biodiversity (Funbio), and the Amazon Conservation and Sustainable Development Institute (IDESAM). It was the first REDD+ (reducing emissions from deforestation and degradation) mechanism project on indigenous lands in Brazil.

The PCFS is recognized as a national and international reference, as reported by HEVL 1. The concepts and methodological construction are used by Federal Government agencies, such as FUNAI and the Ministry of Environment (MMA), and by other project developers in Brazil and worldwide to enable REDD+ (RIPCFS 2014). According to the data from the MMA (2016), REDD+ is an instrument developed under the United Nations Convention on Climate Change (UNFCCC) to financially reward developing countries for their results related to REDD+ activities: (1) reduction of emissions from deforestation; (2) reduction of emissions from forest degradation; (3) conservation of forest carbon stocks; (4) sustainable management of forests; and (5) increase of forest carbon stocks.

The first financial contribution from the sale of credits generated during the 2009–2012 period that occurred at the end of 2013, according to RIPCFS (2014) data. Thus, the project managed to sell the credits generated during the first monitoring period, which enabled the beginning of the implementation of its activities. According to HEVL 1, coordinator of the Metareilá Association, the payment for environmental services, especially the sale of carbon credits, represents a new and promising alternative for the Paiter-Suruí people, ensuring the investment in improvements for other activities carried out at SSIL.

This topic addresses aspects presented by Feil and Schreiber (2017), a vision of sustainable development with a long-term vision in relation to future generations, seeking economic growth without environmental aggression. It covers environmental, economic, and social aspects in mutual balance, with proposal of PCFS, because it seeks to improve the quality of life (welfare) of the people of SSIL.

### 40.4.2 Identification of the Main Factors of Sustainable Production

According to TBREC, EMATER has been developing the Collective Technical Assistance Method (MATEC), which consists of attending, in a joint manner, a specific group, seeking to solve the information needs for the coffee culture. This method has contributed to the satisfactory production results and the quality of the coffee obtained in the village. Likewise, TBARC notes that increasing the profitability of the producer is a way to ensure their permanence in the activity, reaching another important aspect for the sustainability of the production of grains, since there will be financial return, consequently improving the quality of life of indigenous people who depend on this product to survive. For TBREC, due to the scarcity of resources and the absence of new areas for planting, it is necessary to adopt a more rational coffee farming.

At SSIL, according to TSSA, 4 villages with a total of 20 families are now participating in the Emater and Seagri coffee plantation project, totaling an area of 50 hectares with genetically improved plants for conilon coffee production. According to TSSA and TBREC, through agroecology techniques, Seagri and Emater develop a pilot project for organic coffee production at SSIL. In the view of TBREC, the Paiter-Suruí can be considered, as a single family, the largest coffee producers in the state of Rondônia and one of the largest in the country.

According to TBARC, harvesting is done only for selected mature beans; this procedure takes place between the months of May and July. The drying process of the beans is carried out naturally, following EMBRAPA’s guidelines with the use of suspended terreiro. This procedure, according to TBARC, allows the evidence of chemical components, which highlights the flavor and aroma of the grains naturally. In addition, the coffee harvested outside the ideal point will demand more beans and, when processed improperly, will have defects that depreciate the physical and sensory quality, with negative effects on price.

According to HEVL 2, the volume and scale of production is small, but in 2017, 80 bags of coffee were sold to Switzerland. The production of coffee is usually directed to the market in the municipality of Cacoal in Rondonia State, where it also benefits. Part of the coffee produced is processed in the municipality of Cacoal in the pillar machine provided by FUNAI to the Metareilá Association of the Surui Indigenous People. The other part is sold directly by the indigenous families to the processing companies. The income from the sale of this product is used in products such as clothing, tools, and food for the tribe.

In the village Nabecob Abalakiba, according to HEVL 3, there are currently 18 families of the Paiter-Surui people who participate in the PAA—Federal Government Food Acquisition Program—selling products grown by these indigenous people, such as bananas, coffee, cassava, and nuts. According to TSSA, this is a stimulus to family agriculture, under sustainable development parameters, generating a monthly financial contribution for these indigenous families of approximately 3000 to 6000 reais.

The village also produces other foods for its own consumption, such as peanuts, almonds, potatoes, oranges, and mandarins. For HEVL 3, the organization acquired from the cooperative has improved the quality of their food, as well as improving prices, as they are able to avoid the middlemen, as the intermediaries of agricultural products are known, who previously took most of the profits from indigenous production.

This topic corroborates Giordano's (2005) approach on sustainable production, considering the model developed within SSIL that seeks to reconcile the use of technology for the production of organic coffee, aiming to minimize degradation to the environment, which makes the proposal technically appropriate, economically viable, and socially accepted.

## 40.4.3 Description of the Innovation Framework for Sustainable Development in the Indigenous Land Sete de Setembro

In line with what Barbieri et al. (2010) said, the innovations present in this study are sustainable, because they consider the three dimensions of sustainability: (1) the social dimension, the actions dealt with aim at minimizing impacts on the culture of indigenous communities within and outside the social organization; (2) the environmental dimension, the aspects found in SSIL address the proper use of natural resources and reduction of impacts by pollutants; and (3) the economic dimension, the projects developed are related to economic efficiency in order to ensure the perpetuation of ethnicity. This corroborates Schumpeter's (1961) theory, since the process of economic development in the region of study is related to endogenous and discontinuous changes in the production of goods and services, which generate innovation.

Based on the dimensions selected for analysis; social, economic, and environmental and observing documentary data; and interviews conducted, this section presents the structure of innovation for sustainable development at SSIL.

### 40.4.3.1 Social Dimension Analysis

The leaders of the Surui people are articulated and make partnerships and maintain contact with different spheres of society. One of the projects renewed in 2012 was with the company Google, where the village leaders can record their stories and make them available on the website, through geographical labels; the content is updated on the cultural map. This allows the permanence of the cultural and ethnic valorization of these peoples, with the use of technology to record the history of SSIL.

Another important point to be mentioned refers to the role of indigenous leaders, who seek the valorization of local culture, not in keeping it intact, but interacting in a positive way with the society around them, demystifying prejudices and disseminating the cultural heritage. To this end, information presented by HEVL3, the Paiter A Soe Museum (Paiter Things) was created in 2016 with the support of the Gabgirey Association and FUNAI, a space to promote actions related to material and immaterial culture, rescue practices that have been lost through contact, and value the culture of these peoples. For the indigenous leaders, this museum directs the community to reflect on the relationship between past and present ways of life, analyzing the main changes that have occurred and seeking alternatives for sociocultural survival.

In October 2017, according to HEVL 3, the Paiter Indigenous Production and Development Cooperative (COOPAITER) was created, which allows the commercialization of food without the intervention of third parties.

Several partnerships have already been realized with the innovative vision of SSIL leaders, such as the use of technology, among them: in 2008, Google Earth Outreach (the company's responsible area for social projects) provided cell phones and laptops equipped with data programs capable of supplying the tribe with information about the forest; a group of 30 Indians were trained to monitor the limits of Surui lands with the help of this equipment, learned to film and post videos on YouTube, and used the tools of Google Earth in forest surveillance.

These aspects corroborate Pereira's (2012) approach, in which institutional theory facilitates the understanding of implicit patterns and the diversity existing in the organizations; this aspect can be observed in the creation of the museum to foster material and immaterial culture, thus allowing the understanding of social phenomena and their structures and actions. Furthermore, the arguments of Salles and Ribeiro (2012) are expressed in the actions of creation of Coopaiter and through the use of technology for monitoring land, as well as for cultural and ethnic valorization of these peoples, confirming that the process of economic and social development are interconnected and interdependent phenomena and important tools for promoting sustainable development.

#### 40.4.3.2 Analysis of the Economic Dimension

In the economic aspect, according to the documentation raised, the Paiter-Suruí have a plan of the Paiter Surui Fund, designed by the Brazilian Biodiversities Fund (Funbio), being a financial mechanism to manage the resources destined to the implantation of the Indigenous Land Sete de Setembro Ethno-Environmental Management Plan. This financial mechanism presents robust innovations in strengthening indigenous autonomy. It is a solution for financial planning and resource management required for efficient governance of indigenous territory.

According to the Implementation Report of the Surui Forest Carbon Project (RIPCFS) of 2014, the PCFS aims to contribute to the conservation of biodiversity, improvement in the quality of life of communities, maintenance of watersheds, recovery of degraded areas, and reforestation and strengthening of indigenous culture, with the intention of being a long-term financing mechanism to subsidize one of the pillars of the sustainable management plan of indigenous communities. Other projects are developed within SSIL, for example, the tourist activity employs part of the Indians as guides, receptionists, and cooks, in addition to having the profit of the activity applied in benefits to the community, such as construction of community centers, solar energy, and sanitation works.

The legal aspect is represented by the SSIL Management Plan, which includes the governance structure, providing for the identification of the instances and actors responsible for carrying out control activities, in addition to defining the roles—attributions and obligations—of each instance and actors.

The method adopted for the business practices of SSIL aims to increase the performance of production, reducing administrative and transaction costs, increasing productivity and satisfaction of people involved in coffee production and other activities.

Regarding the technical aspect, the pilot project with government agencies (Seagri, Emater, and Embrapa), for organic coffee production, brings with it the call for sustainability, since it works on the selective collection of beans, natural drying, and activity performed by indigenous people. The factors of organizational innovation allow the addition of value to the product and make it possible to win a Green Seal project for the product and open doors to the international market. For the specialists of Emater and Embrapa, the programs of genetic improvement of coffee have as objective the increase of productivity and better quality of the drink and, with the development of cultivars resistant to plagues and diseases, avoid the excessive use of defensives.

SSIL has an ethno-zoning plan, through which land management planning is carried out, bringing the Paiter-Suruí proposal for land use, cultural values, and traditions. It presents the division of the indigenous land in areas of action, thus established: Paiterey Karah Katap, Cultural Zone; Palah at ah, Sacred Zone; Gakorap ah, Hunting Zone; Morip ey Pãyah, Fishing Zone; Garah Alawata, Forest Zone for Extraction; Garah Iter, Integral Protection Zone; Sodoy Karah, Production Zone; and Garah Pine Wah, Recovery Zone.

This topic presents characteristics that corroborate the innovation proposals established by Karlsson and Tavassoli (2016), that is, the processes developed within SSIL designed the transformation of ideas, information, and knowledge, increasing competitiveness and ensuring a sustained advantage of the activities developed within the villages. Moreover, it reaffirms the arguments of Schumpeter (1997) that to make innovation happen the participation of two elements is essential, the entrepreneur, in this case the indigenous chiefs, as agents of transformation, and the credit of the FUNBIO and PCFS that enabled the financing of innovation and mobilization of production factors.

#### 40.4.3.3 Environmental Dimension Analysis

In the ecological sphere, according to HEVL 3, it can be mentioned that, in the past, the extraction of wood was the only source of sustenance for the Indians; however, the change in the vision of the leaders allowed the forces to be directed toward the modality of sustainable agriculture of items such as coffee, Brazil nuts, and, especially, bananas. Another point refers to the partnership with national and international institutions, which made it possible to modify the economic structure of SSIL. The HEVL 2 exemplifies with the work performed by the NGO Strategic Fund for Conservation, which allowed the tribe to create an ecotourism plan for the region. In this project, visitors stay in typical dwellings and follow the day-to-day life of the Indians and their cuisine and culture.

The Indigenous Land Sete de Setembro Management Plan, according to HEVL 1, foresees the planning for the implementation of the Paiterey Program for environmental management, establishing procedures and guidelines for the forwarding of sociocultural demands and the responsible use of natural resources, in order to enable the generation of the necessary benefits, the appreciation of culture, and the conservation of the environment.

As demonstrated by the data contained in the Implementation Report of the Surui Forest Carbon Project, the indigenous leaders recognize the importance of preserving the Amazon Forest, where all the dimensions of sustainability and sustainable development must be met. It should be emphasized that these leaders do not fear the use of more modern tools such as Google, laptops, cell phones, and GPS to ensure the survival of its people and the ecosystem where they are inserted. The village chiefs (HEVL 1, 2, and 3) emphasize that the use of technology has improved communication, monitor threats to the forest, and disseminate the plan to manage the wealth of the forest and preserve the Surui culture. As an example, HEVL 1 reports that, with the help of technological innovation, it was possible to monitor hundreds of trucks with illegally harvested timber from indigenous territory and thus provide denunciation to the competent agencies that set up task forces to seize machinery, equipment, and loggers that were acting illegally in the region. In the devastated area, according to HEVL 2, they currently plant coffee and other cultures that help the survival of indigenous peoples.

In this topic, it is possible to confirm the approach presented by Horbach et al. (2012), as the research data present the use of the following determinants of eco-innovation: regulation (SSIL management plan) and marketing factors (the Surui Forest Carbon Project, in addition to others mentioned above, such as the green seal). The company's main objectives are to promote the use of technology (use of satellite monitoring, Google Earth, and audio and video equipment) and specific characteristics of the organization (the transfer of knowledge using YouTube channels, and the establishment of procedures and guidelines for sociocultural demands and use of natural resources). It is also in conformity with the theoretical proposal presented by Pawlowski (2008) when formulating the integration of different spheres of human activity (moral, ecological, social, economic and legal, and technical) in activities developed at SSIL, as can be observed in the aspects mentioned above in the research, being explicit the role of leadership for the structuring of sustainable development in the region where they live.

## 40.5 Conclusions

Coffee production, by the Paiter-Suruí tribe in the Amazon, is based on the triple bottom line, which guarantees the survival of indigenous peoples, the preservation of the environment, and the permanence of this heritage for future generations. Based on the findings of the research, the use of the best management practices for sustainable development adopted in the indigenous reserve is perceived. Agriculture and the sale of carbon are the main sources of income in SSIL. These people improve production qualitatively with innovation of their processes, use technologies, and improve their social and commercial relations with institutions capable of potentializing the production of coffee under the perspective of economic growth, through domestic sales and possible consolidation of exports.

This research was limited to the vision of village leaders, who practice coffee farming; no research was conducted seeking the perception of other members of the tribe, which enables future research in order to quantitatively assess sustainable development indicators for the aspects presented here. Moreover, although technological innovation has brought changes in the customs of the tribe, in the social, environmental, and economic areas, there is a rapid adaptation and use of various elements of innovation, among which, it should be noted the promotion of the culture of innovation and the support of the high summit of the tribe for innovation; the establishment of goals, objectives, strategies, and vision favoring creativity and innovation; clear mechanisms for rewarding and recognizing the efforts of the indigenous people; established rules favorable to the process of broad innovation; adopting the culture of change and innovation; and encouraging partnerships for the development of best practices to be adopted that enable the sustainable development of activities carried out in the village.

Finally, that distorted vision that Indians live from hunting and fishing, and walk seminudes, is no longer part of the reality of the Paiter-Suruí people, who show to society that it is possible to live in the midst of nature, preserving it, and at the same time make use of technologies, in addition to increasing the productivity and income of the community, aiming at the well-being and perpetuation of their species and the other species present in the ecosystem that are parts. Showing that it is possible to innovate efficiently in economic terms and with social and environmental responsibility.

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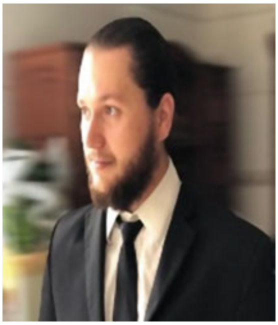

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# Appendix

This timely book is a compilation of edited articles by 105 distinguished international scientists discussing the different trends of Environmental Bioremediation in the given three different parts of the book. Scientists have been developing environmental bioremediation as a solution to environmental problems. This book covers a wide range of emerging bioremediation techniques for the remediation of industrial wastes and contaminants, including nano-bioremediation, phytoremediation, arbuscular mycorrhizal fungi-assisted bioremediation, and environmental health and risk assessments of innovative technologies. In addition, the book contains up-to-date information as well as future research directions in the field of industrial waste bioremediation. This book describes the scope and applications of bio/phytoremediation technologies and especially focuses on the associated eco-environmental concerns, field studies, sustainability issues, and prospects. The book also examines the feasibility of environmentally friendly and sustainable bio/phytoremediation technologies to remediate contaminated sites, as well as future directions in the field of bioremediation for environmental sustainability. Students, researchers, scientists, and professionals in the fields of microbiology and biotechnology, bio (chemical) engineers, environmental researchers, eco-toxicology, environmental remediation, and waste managers who desire to work in these fields would find this book valuable.

This timely book is a compilation of edited articles by distinguished international scientists discussing the different trends of environmental bioremediation in the given three different parts of the book. Offering a comprehensive, state-of-the-art overview of current and future aspects of environmental biotechnology, this book appeals to policy makers, universities, and research institutions.

S. Arora et al. (eds.), *Biotechnological Innovations for Environmental Bioremediation*, https://doi.org/10.1007/978-981-16-9001-3