Sanket J. Joshi
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Hemen Sarma *Editors*

Biotechnology for Sustainable Environment

Springer

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Preface

Over the past century, humankind has seen tremendous growth in all aspects, be it socioeconomic or scientific, and thus providing much comfortable life, and made it virtually possible to live in a “world without border.” However, we achieved it at a cost, and our environment suffered the most! Better life expectancy and opportunities lead to increased population and extensive industrialization, urbanization, cutting forests, overexploitation of agricultural lands, soil erosion, increased salinities and scarcity of potable-clean water, frequent uncontrolled use and release/accumulation of recalcitrant toxic chemicals, pollution of three spheres of life (air, water, and land), and other catastrophes. Although at a slower pace, mother nature takes care of recycling those resources (such as agricultural resources) and also remediating and rejuvenating the environment. However, currently environmental pollution beyond natural healing is a matter of grave concern! Efficient and effective environmentally friendly approaches are the need of the hour, which can be implemented for sustainable environmental bioremediation. Plant- or microorganism-based biotechnological processes are some of the promising environmentally friendly techniques which can benefit humanity by either utilizing it as such, or their metabolites enabling the degradation of toxic chemicals and/or biotransformation to harmless or useful intermediates and end products. It could play a significant role in bioremediation of such contaminated sites with persistent toxic compounds, heavy metals, pesticides, PCBs, PAHs, and different types of industrial waste in an environmentally friendly manner. The bioconversion of such agro-industrial solid or liquid wastes to industrially useful products is already being exploited as an attractive option, for both environmental remediation and economy.

This book ‘Biotechnology for Sustainable Environment’ highlights recent advancements in such a vast topic, through contribution from experts demonstrating different applications in day-to-day life, both existing and newly emerging technologies, and thought provoking approaches from different parts of the world, potential future prospects associated with some frontier development of biotechnological research related to the environment. Experts from India, Serbia, Algeria, Tunisia, China, Egypt, France, Poland, Iran, Spain, The Netherlands, Norway, and Oman, authored chapters in the current book. Introductory chapters cover the role of environmental biotechnology for sustainable future, a possible role of methanogenic archaea, and chitosan coatings for sustainable development of the environment. Two chapters covered the possible bacterial degradation of bisphenol-A, and current state and future prospects of microbial degradation of marine plastics. Issues associated with heavy metal and mine deposits are covered by chapters on the mechanism and pretreatment effect of fungal biomass on the removal of heavy metals; metal bioremediation, mechanisms, kinetics, and the role of marine bacteria in the bioremediation; biofilm-associated metal bioremediation; phytoremediation of mine waste disposal sites; metallicolous plants associated with amendments and selected bacterial consortia, to stabilize highly polymetallic contaminated mine deposits; and bioindication of heavy metal contamination by mushrooms and mosses in a highly industrialized environment. Two chapters covered issues of polycyclic aromatic hydrocarbons toxicity and bioremediation approaches, including biogenic nanoparticles-based strategies of "Nano-Bioremediation." The remaining chapters covered value-added products from agroindustry by-product: bagasse; bio-prospecting of fruits waste for exopolysaccharide production by bacteria; plant growth-promoting rhizobacteria as bioinoculants for plant growth; and microbial and enzymatic bioconversion of tannery wastes: progress toward a circular economy in the leather industry. With the stated objective of our book, we believe that it will be able to address potential applications and challenges in this particular area. We hope the chapters of this book will be novel to readers and can be readily adopted as references for newer and further research. Moreover, since this book contains information related to different applications, we assume that international readers, especially students and researchers, will also find this book valuable for reference purposes.

Last but not least, the editors are thankful to all the researchers, expert academicians, and leading scientists whose contributions as authors and reviewers have enriched this book. We also express our deep sense of gratitude to our family members, for their kind understanding and unconditional support during the course of such scholarly academic activities. We all strived to make sure that this book is free from any misleading or erroneous information, and any such mistakes are completely unintentional, and pardon us. We are also thankful to Springer Nature for giving us this opportunity, and especially the editorial support team members, Ms. Aakanksha Tyagi and Ms. Vaishnavi Venkatesh, for their relentless support throughout the publishing process. We would also like to sincerely thank our universities for extending the facilities and encouragement for such activities. We thank them from the core of our heart.

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Contents

1 **Environmental Biotechnology: Toward a Sustainable Future** 1
Sajjad Ghahari, Somayeh Ghahari, Saeid Ghahari, Ghorban Ali Nematzadeh, and Hemen Sarma

2 **The Mystery of Methanogenic Archaea for Sustainable Development of Environment** 33
Mythili Ravichandran, Kasi Murugan, Sharmila Devi Natarajan, and Sumathi Samiappan

3 **Chitosan Coating Biotechnology for Sustainable Environment** 63
Amr Sayed Emam Ismail

4 **Bacterial Biodegradation of Bisphenol A (BPA)** 95
Snehal Ingale, Kajal Patel, Hemen Sarma, and Sanket J. Joshi

5 **Microbial Degradation of Marine Plastics: Current State and Future Prospects** 111
Annika Vaksmaa, Victor Hernando-Morales, Emna Zeghal, and Helge Niemann

6 **Mechanism and Pretreatment Effect of Fungal Biomass on the Removal of Heavy Metals** 155
Aparna B. Gunjal

7 **Metal Bioremediation, Mechanisms, Kinetics and Role of Marine Bacteria in the Bioremediation Technology** 173
Kinjal H. Upadhyay, Avni M. Vaishnav, Devayani R. Tipre, and Shailesh R. Dave

8 **Biofilm-Associated Metal Bioremediation** 201
Amrita Jasu, Dibyajit Lahiri, Moupriya Nag, and Rina Rani Ray

9 **Phytoremediation of Mine Waste Disposal Sites: Current State of Knowledge and Examples of Good Practice** 223
Ksenija Jakovljević, Dragana Ranđelović, and Tomica Mišljenović

10 Metallicolous Plants Associated to Amendments and Selected Bacterial Consortia, to Stabilize Highly Polymetallic Contaminated Mine Deposits 251
Manhattan Lebrun, Romain Nandillon, Florie Miard, Domenico Morabito, and Sylvain Bourgerie

11 Bioindication of Heavy Metals Contamination by Mushrooms and Mosses in Highly Industrialized Environment 271
Marek Pająk and Marcin Pietrzykowski

12 Polycyclic Aromatic Hydrocarbons: Toxicity and Bioremediation Approaches 289
Djaber Tazdaït and Rym Salah-Tazdaït

13 Biogenic Nanoparticles and Strategies of Nano-bioremediation to Remediate PAHs for a Sustainable Future 317
Punniyakotti Parthipan, Chandar Prakash, Dhandapani Perumal, Punniyakotti Elumalai, Aruliah Rajasekar, and Liang Cheng

14 Value-Added Products from Agroindustry By-product: Bagasse . . . 339
Aparna B. Gunjal

15 Bio-prospecting of Fruits Waste for Exopolysaccharide Production by Bacteria 353
Avni M. Vaishnav, Kinjal H. Upadhyay, Devayani R. Tipre, and Shailesh R. Dave

16 Plant Growth Promoting Rhizobacteria as Bioinoculants for Plant Growth 373
Aparna B. Gunjal and Balasaheb P. Kapadnis

17 Microbial and Enzymatic Bioconversion of Tannery Wastes: Progress Toward a Circular Economy in the Leather Industry 387
Rania Ouertani, Mouna Mahjoubi, Habib Chouchane, Ameur Cherif, and Mohamed Neifar

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Sanket J. Joshi is a Deputy Director, Oil & Gas Research Center, and an Application Specialist, Oil & Gas Science at Sultan Qaboos University, Oman. Dr. Joshi has 16 years of academic teaching and research experience, and 4 years of industrial R&D experience, in India and Oman. While working in Indian pharma companies, he undertook several turnkey projects (both upstream and downstream) on antibiotics (β-lactams, macrolides, glycopeptides), antidiabetic drug (for type II diabetes), and Co-enzyme Q10. His current research interests encompass: Energy (In-situ/ex-situ microbial enhanced light/heavy oil recovery; chemical enhanced oil recovery; biofuels); Microbial products (biosurfactants, biopolymers, R&D and scale-up); and Environmental bioremediation. Those research projects are multidisciplinary, involving both biotechnology and engineering disciplines. He serves as an Academic/Associate/Guest Editor for some of the highly reputed journals.

Arvind Deshmukh has served several academic institutes with a career spanning more than 37 years. During those years he served as the Head of Microbiology department, board member of University Teaching and Research, Board of Studies in Microbiology, Academic Council, District Environment Committee, formed by Govt. of Maharashtra (2000–2006), Examination Committee, Research & recognition Committee in Microbiology and Biotechnology, Chairman, Board of studies in Microbiology, and several other important posts in various colleges and Universities across Maharashtra, India. He was awarded as a fellow of Biotech Research Society of India, in 2005. Along with other experts, he formed Microbiologists Society, India, a registered NGO, and is currently serving as President of the society. Currently, he tirelessly provides guidance to Microbiology students throughout India and abroad for free, engages subject experts over several topics, provides lectures and trainings.

Hemen Sarma is currently senior Assistant Professor at Nanda Nath Saikia College, affiliated to Dibrugarh University, Assam, India, and has been working at this institute since 2012. His research focuses on plant-microbiome interactions, biosurfactants, sustainable remediation, CRISPR/cas9 gene editing and nanobiotechnology. He has a patent and over 50 publications, including conference papers and book chapters. He received numerous awards, distinctions and fellowships, including the DBT-Overseas Associateship [2015–16] and the DBT-Research Associateship [2011–12], the IISc Research Associateship [2009] and the UGC Dr. DS Kothari Postdoctoral Fellowship Awards [2009–10]. In 2017–18, Dr. Sarma joined the Visitor Professor Fellowship Program as an affiliate of the Department of Chemistry and Biochemistry, University of Texas at El Paso, USA.

1 Environmental Biotechnology: Toward a Sustainable Future

Sajjad Ghahari, Somayeh Ghahari, Saeid Ghahari,
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Abstract

The environment is vital to human life, and organisms require it to survive. The sustainability of the physical environment indicates to what extent a society is prosperous and whether everyone is well off. The disposal of hazardous substances is a major challenge every day. The best way to sustain the environment is to minimize waste generation and maintain a harmonious balance between esthetics and health. Biological aspects of environmental sustainability are explored through a review of available biotechnological tools. Environmental biotechnology is enabling major advances in remediation technologies that will assist in minimizing the potential release of waste from industrial facilities. This technology will also produce products useful in efforts to prevent the release of waste. Biotechnology offers tremendous potential to transform harmful waste and pollutants into useful by-products using microbial communities or just by allowing microbes and plants to degrade these wastes as harmless metabolites.

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Environmental contamination · Environmental sustainability · Disposal of toxic substances · Environmental biotechnology

1.1 Introduction to Environmental Biotechnology

Environment can be defined as a combination of biological and physical factors that interact with each other. Environmental science is the study of all things on Earth and the various substances that exist within them (Fig. 1.1). Changes to an environmental attribute will result in human health being negatively affected indirectly, that is, not just suffering the effects, but indirectly experiencing the results (Singh 2017). In 1991, the term "biotechnology" was used by Karl Rekey to describe this concept of development (Fahmideh et al. 2014). Environmental biotechnology is concerned with the creation of products and services that benefit people through the use of biological systems or that provide technical support for them. Furthermore, it has a significant impact on a number of technologies, including manufacturing, food products, protection of the environment, agriculture, pharmaceuticals, and resource protection.

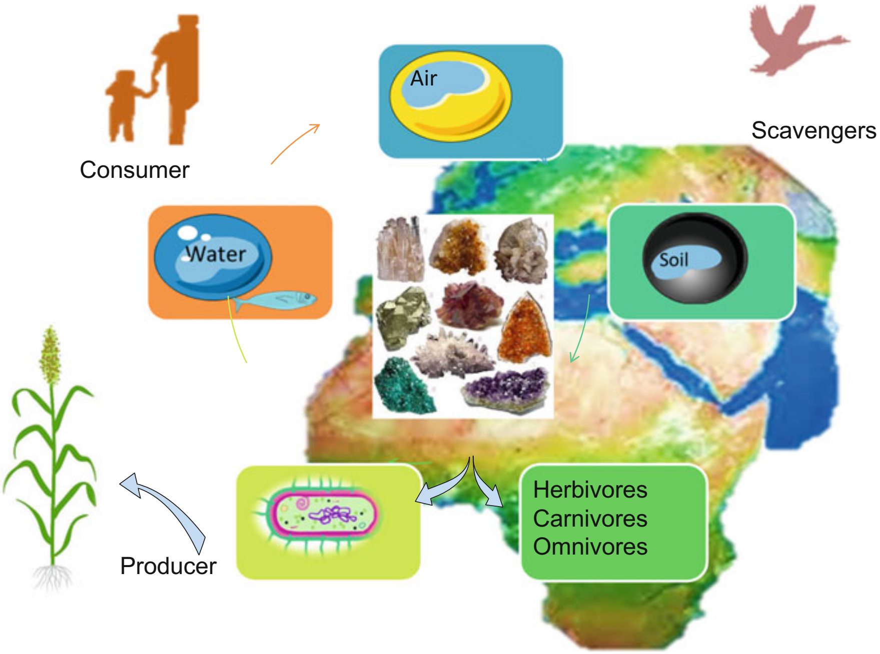

Fig. 1.1 The surrounding environment and its constituents

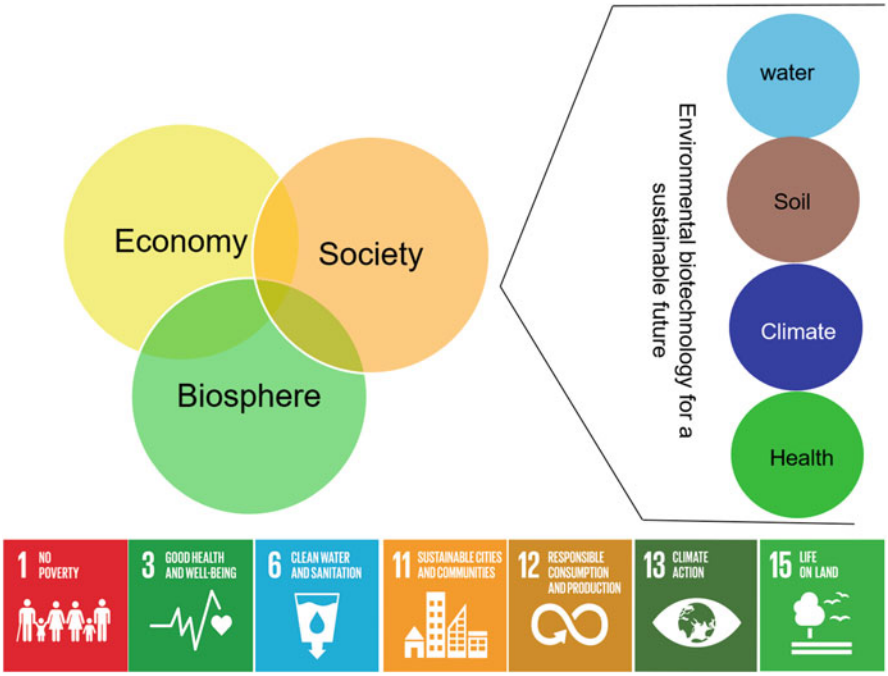

Fig. 1.2 Environmental biotechnology's role in the sustainable future

At the beginning of the twenty-first century, scientists viewed environmental biotechnology as the most important technology for sustainable environmental conservation. Environmental biotechnology, such as the use of living organisms to clean up polluted environments, can be used for long-term environmental conservation and management. Environmental biotechnology involves the recycling and reuse of products, the development of renewable energy plants, the collection of recycled materials and other ways of preserving the environment. Biotechnology is used in many different aspects of our everyday lives, including environmental protection, conservation of energy, agriculture, waste management, and environmental monitoring. Biotechnology, as shown in Fig. 1.2, can serve as one of many reasons for integrating environmental protection into sustainable development.

Environmental biotechnology brings significant improvements to new solutions for soil and water restoration that are contaminated with persistent chemicals. This technology also plays an immense role in the monitoring, detection, and prevention of pollution. The conservation of resources by recycling hazardous waste into beneficial by-products is one of the primary objectives of environmental biotechnology. Potential microbial and plant species have been involved in the management of these environmental crises. As a recommendation, researchers suggest the use of bio-cleaning agents, plant microbes, especially where we need to remove unwanted contaminants from soil, drains, and surfaces. In addition, environmental biotechnology has been used in various industries for the production of high-value chemicals and products, resulting in high economic and environmental profits. This technology not only helps to improve the product, but is also environmentally sustainable compared to traditional processes (Singh 2017).

1.2 Worldwide Environmental Problems

The environment in which we live is essential to our well-being, because everything that surrounds us in the course of our lives is collectively referred to as our environment. Both living things and nonliving substances are included in ecosystems. The human race has literally transformed and expanded its food sources in a very short time. As a result, entire ecosystems have changed. Global environmental problems include pollution, global warming, ozone depletion, acid rain, depletion of natural resources, overpopulation, waste disposal, deforestation, and loss of biodiversity. Many of these processes are the result of unsustainable consumption of natural resources. These processes are slowly but extremely damaging our environment. One of the main impacts of the burning of fossil fuels is the release of large amounts of carbon dioxide and other greenhouse gasses into the atmosphere. The result is global pollution problems, the changing climate of the earth, and the potential for ozone holes to change. Environmental problems, including the loss of forests, damage water bodies and their ecosystems due to acid rain, over-exploitation of natural resources, and the mass extinction of animal species due to habitat destruction, are linked to environmental problems worldwide. Furthermore, as demographic changes, industrialization, deforestation, and loss of agricultural land continue to increase, environmental concerns are growing. Urbanization is expanding and the global economy is rising dramatically. This has negative consequences. The health impact of the environment is profound and deteriorates due to contaminated food and hazardous water; it can lead to disease if it is not properly managed. Poor sanitation and pollution, as well as global climate change, pose serious health risks as well (Sarma et al. 2019a). With the global environmental issues mentioned above, our planet is likely to face a serious environmental crisis. Future disasters are expected to occur as a result of the current environmental degradation. This will also require the competent authorities and/or nations, as a matter of urgency, to lay down appropriate legislation to help find appropriate solutions to the problems and to make people aware of the safe use of natural resources (Singh and Singh 2017).

1.2.1 Environmental Contamination

Environmental pollution is a global issue identified by Singh and Singh (2017). This happens in unsustainable activities, resulting in an excessive loss of life. Anthropogenic activity is believed to be the main causes of contamination. Pollutions are also classified into different classes (see Table 1.1).

Table 1.1 Different sources of environmental pollutants

Pollutants	Type	Effects on health
Dust, chlorofluorocarbons (CFCs), ozone, peroxy acyl nitrates (PANs), oxidant (NO, NO_2, N_2O_4, SO, SO_2), smoke	Air	Individuals with diseases like chronic and acute respiratory failures, heart problems
Significant quantities of pollutants have been released by airplanes, motor vehicles, and transport systems	Noise	Heart disorders, depression, cognitive diseases (developmental disabilities), kidney problems, nausea, vision loss, and rashes
Radioactive isotopes (^{210}Po, ^{210}Pb, ^{230}Th, ^{232}Th, ^{226}Ra, ^{232}U, ^{238}Pu, ^{237}Np, ^{241}Am, and others)	Radioactive	Cancer, cardiovascular disease, damage to certain types of DNA
Pesticides, petroleum, and polynuclear aromatic hydrocarbons, heavy metals, and metalloids	Soil	Cancers, kidney disease, lower intelligence, neurological damage, bone and skeletal health problems are caused by pollution
Detergents, industrial and domestic wastewater, pesticides, pathogens, synthetic fertilizers, wastewater, etc.	Water	Diseases caused by microbes, cardiovascular disease, cancer, gastrointestinal disease, neurological disease, and respiratory disease

1.2.2 Global Warming

Global warming refers to the gradual rise in the earth's average temperature. A rise in global climate change refers to a steady increase in the earth's average temperature. Global warming is a worldwide phenomenon caused by the increase of the amount of greenhouse gasses in the atmosphere, such as carbon monoxide, carbon dioxide, and methane. As a result, the average temperature of the earth is increasing. In the last 10 years, the temperature of the earth has increased, and this is not the case in the last 8000 years, which has become relatively stable. In the past 10 years, temperatures have increased by around 0.3° per year (Singh 2017). The Ministry of Earth Science reports that the mean Indian temperature rose to around 0.7 °C during 1901–2018, mainly due to greenhouse gases.

1.2.3 The Depletion of the Ozone Layer

Present in the atmosphere, the ozone layer protects us against harmful ultraviolet rays. It is becoming depleted by human activities, which lead to a growing global concern. By reaching the whole amount of the ultraviolet radiation, the sky will be destroyed, and life on earth will end. The most broadly recognized as the ozone layer is the stratospheric ozone because of absorbing more than 99% of the UV light (Sivasakthivel and Reddy 2011).

1.2.4 Acid Rain

Acid rain has been a threat to the environment since the nineteenth century. "Acid rain" is the atmospheric deposition of acidic components that affect the environment, such as rain, snow, particulate matter, vapor, and gases. Acid rain was first observed and described by Robert Angus Smith, an English chemist (1852). Smith's pioneering observations have linked industrial emissions to a number of harmful environmental effects. Acid rain caused by the emission of sulfur dioxide and nitrogen dioxide from various sources to the atmosphere and dissolved in atmospheric water, forming acids when rain falls (Burns et al. 2016).

1.2.5 Depletion of Natural Resources

Natural resources that exist in the environment, such as minerals, energy, water, air, and soil, are considered vital or beneficial to human beings. All products come from natural resources for human use (Aquilas et al. 2019). By taking steps to reduce the usage of natural resources to the minimal possible, one can help preserve the earth's climate and mitigate the problems of climate change. With a little bit of knowledge about the usage of natural resources, people will be able to make a reduction on their carbon footprint. Problems such as a decline in natural resources, insecurity in the world, and a troublesome situation are the most pressing issues for this period.

1.2.6 Overpopulation

Human overpopulation has become an issue as it impacts the environment. Some of the impacts we have today are global warming as well as habitat loss, environmental pollution, intensive agriculture practices, along with the utilization of limited natural resources such as arable land, fresh water, and fossil fuels. Population has become an unintended liability for India. India will surpass China as the most populated country in the world by as early as 2024. Population growth also causes negative economic and environmental effects ranging from over-farming, deforestation, water contamination, and global climate change.

1.2.7 Waste Disposal

Waste refers to goods that are not main products, according to the United Nations Statistics Division (UNSD) report; and the original customer no longer requires it for processing, production, or the purposes of consumption hoping to throw it away. Several processes may be brought to an end when raw supplies are processed or extracted into products (intermediate or final). Once the final products have been used, human activities can produce a wide range of waste, including solid waste (home waste) generated in urban areas, radioactive waste, wastewater in the form of industrial sewage, effluent, and surface run-off (Farraji et al. 2020). Littering adversely affects the environment and damages the ecosystems. Waste in many urban areas is often buried in holes in the ground, sometimes in old quarries or specially dug quarries. Some waste will eventually rot, but not all, and may become smelly or release gasses that contribute to the greenhouse effect.

1.2.8 Deforestation

Tree species absorb carbon dioxide and release oxygen during photosynthesis. Carbon dioxide emitted by plants is also used in photosynthesis. The plant stores the carbon, helping it grow. The cutting and burning of trees releases the carbon they store. Deforestation and degradation of forests are therefore the main source of global warming. Global warming is caused by deforestation, which is a major threat to people around the world (Chakravarty et al. 2012).

1.2.9 Loss of Biodiversity

Our natural environment contains numerous species, from aquatic species to riverine species, and to the forest areas that surround us. The term ecological niche refers to how species interact with their environment and how they adapt over time (Cardinale et al. 2012). An ecological niche describes the range of conditions necessary for the species to thrive, as well as describing how it fits into the ecosystem. Extinction of various species, a population, an area, etc. has a direct connection to the consequences of environmental damage and habitat degradation. The theory behind biodiversity understanding is a dynamic linkage between millions of lifeforms on earth. The UN report warned that over one million species could be extinct worldwide in the next few decades. This mass extinction of all life on earth puts the planet at risk. These mass extinctions were responsible for losses of between 60 and 95% of all species. This takes millions of years to recover from the disaster.

1.3 Bioremediation

In contrast to using costly synthetic processes to remove contaminants, bioremediation is an attractive alternative. This uses biological organisms that naturally exist on toxic compounds to convert them into less toxic or nontoxic compounds (Sarma et al. 2017). In particular, many natural organisms, plants, and earthworms are required to do work, such as removing or detoxifying various chemicals and waste in a manner that is environmentally friendly. Scientists have identified a specific native microbe present in a polluted environment that can solve the problem at hand and restore the area (Mishra et al. 2019). The environmental factors influence the bioremediation process. For example, the change in soil type, pH, temperature, nutrients, and oxygen may affect the bioremediation process. Inspired by microbes, it can be said that they are able to transform inorganic compounds into inert materials via gut transfer or adsorption (Sarma et al. 2016; Sarma and Prasad 2018). According to Chandra et al. (2011) it is impossible to dispose metals unlike natural organic contaminants. Therefore, they must be eliminated or conversed into other stable products. Microorganisms can be used to bioremediate heavy metals through a variety of processes, including sorption, accumulation, leaching, mineralization, and transformation (Lloyd and Lovley 2001).

Some metal-resistant bacteria are capable of tolerating metal exposure by accumulating metal ions in a less toxic state (Ramírez-Díaz et al. 2008). Additional mechanisms have been evolved by microorganisms to protect themselves from heavy metal toxicity, including the oxidation of heavy metals, the uptake of heavy metals, and methylation. Many bacteria are reported to have the ability to detoxify harmful metals, such as *Arthrobacter* sp., *Pseudomonas* sp., *Alcaligenes* sp., *Bacillus* sp., *Flavobacterium* sp., *Azotobacter* sp., *Microbacterium* sp., *Corynebacterium* sp., *Rhodococcus* sp., *Nocardia* sp., *Methylosinus* sp., *Ganoderma applanatum*, *Aspergillus niger*, *Stereum hirsutum*, *Rhizopus arrhizus*, and *Pleurotus ostreatus* (Megharaj et al. 2003).

1.3.1 Nano-Bioremediation Technologies for Sustainable Environment

The use of nanoparticles to accelerate the process of bioremediation is known as "*nano-bioremediation*." Nanoparticles (NPs) may be metallic or nonmetallic and may have different forms. Previous researchers have already used iron NPs to clean the environment (Tratnyek and Johnson 2006; Sarma et al. 2018). As a photocatalyst, Zn NPs are capable of degrading various dye (El-Kemary et al. 2010). Silver and gold NPs are used in many different industries. Researchers have recently studied the ability of gold and copper nanoparticles to degrade organic dye molecules (Davis et al. 2017). In bioremediation, biologically synthesised nanoparticles derived from plant extracts, fungi, and bacteria are used. When applied to environmental contaminants, these biogenic nanoparticles produced very promising results. Thus according various studies, bioremediation of pollutants using biosynthetic nanoparticles is promising and sustainable method of environmental cleanup.

1.4 Biotechnology to Control and Clear Air Pollution

The expansion of industrial centers has negative social effects in urban areas as they have brought about many air pollution concerns (Grommen and Verstraete 2002). Now, odor dispersal is a problem that is serious and has a large environmental impact far beyond that of the immediate area (Brooks 1998). The disposal of contaminants in the environment leads to a variety of issues including the ozone accumulation in the environment, the depletion of ozone layer, formation of greenhouse gasses, and photochemical reactions, which threaten ecosystems, plants, as well as humans and animal life. Absorption (surface or deep), oxidation (both catalytic and thermal), condensation, and incineration are common procedures taken for controlling gaseous pollutants (Bidar et al. 2007). The techniques are expensive and have sometimes led to some undesired consequences, such as when a primary contaminant creates a secondary contaminant (Kümmerer 2004). In recent years, biological approaches have become very popular because they consume less energy while not generating toxic by-products. As the biological treatment methods used to remediate the contaminants vary, biofilters and trickling filters have become the most common (Lezcano et al. 2011). Biofilters can generally function in the appropriate temperature and pressure conditions. Historically, filters have been reported to be the best for removing gaseous pollutants (Marchiol et al. 2004). Biofilters are typically successful only at low levels of organic contaminants. They are classified as column reactors and filled with absorbent material (Marchand et al. 2010). Materials with pores in them can absorb gasses. Activated carbon, wood chips, compost, coal, and a blend of organic nutrients are the most widely used "filler" ingredients (Macek et al. 2000).

1.4.1 Control Methods of Odor and Volatile Organic Compounds (VOCs)

To develop an appropriate controlling technique for odors and VOCs, a physical, chemical, and thermodynamic approach is required. Figure 1.3 describes primary technologies used to regulate VOC, H_2S, and odor. The approaches are described based on the nature of each control technology and the various physical, chemical, or biological characteristics of each method (Revah and Morgan-Sagastume 2005).

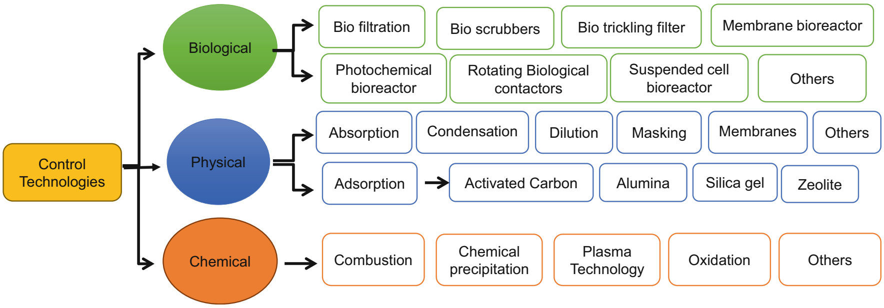

Fig. 1.3 Three primary technologies used to regulate odor and VOC

1.5 Soil Management and Contamination

Over the last two decades, the increase in soil contamination has caused damage to the environment and to living organisms. Soil pollution is caused by both natural processes and human activities. The former includes floods, tsunamis, and volcanic eruptions, and the latter includes organic chemicals, metals (trace and heavy metals), and radioactive waste. Numerous chemicals, including pesticides, crude oil, and its derivatives and polymers, can be categorized in this list. Radioactive waste generation involves the generation of electricity from radioactive sources and from other nuclear technologies used in research and medicines. These are dangerous additives that remain in the environment for a long time after they accumulate to concentrations that are potentially harmful to plants and animals at higher levels of the food chain. Many of these compounds are believed to cause cancer, premature births, and mutations. It is necessary to develop tools to assess the potential risks of human contact with chemicals and to identify the levels of contaminants needed to protect the environment (Koul and Taak 2018). Soil quality monitoring is a complicated and expensive operation, as there are no readily available indicators. The alarming environmental degradation situation necessitates that researchers develop quick, low-cost methods for treating polluted soil (Koul and Taak 2018). As of now, there is no single soil restoration process capable of restoring and remediating environmental degradation without any residual effects (Koul and Taak 2018).

1.5.1 Sources of Soil Pollution

There are various sources of pollution in the soil, and they are originates many different places are shown in Table 1.2.

1.5.2 The Available Options for the Integrated Management of Contaminated Soils

Today, environmental scientists have used a range of solutions available for the remediation or cleaning of contaminated areas. Measurements of concentrations of pollutants, field experiments, and GIS-based technologies are very common, for example. Using this tool, the concentration of heavy metals can be measured (Shankar 2017). Chemical, physical, and biological methods are proposed to remove heavy metals from the environment. But the price difference and the rate of failure are problems across all technologies. Integrated processes are therefore becoming increasingly popular due to their effectiveness in the various environmental matrices. Integrated process is a fusion of two strategies aimed at achieving a synergistic effort to eliminate pollutants.

Table 1.2 Various sources of soil pollution and their examples

Various source of soil pollution	Examples	Reference
Coal ash	Coal ash, found in residential, commercial, and industrial areas, as well as in industrial procedures (e.g., ore smelting), is at high risk for environmental impact	Shankar (2017)
Sewage	Various pollutants also enter waterways like industrial effluents and domestic sewage illegally discharged directly into rivers	Snyder (2005)
Pesticides and herbicides	Perhaps the most well-known compounds found in pesticides are 2,4,5-T, 2,4-D, Paraquat, DDT, and Aldrin	Apitz (2008)
Heavy metals	Cobalt, cadmium, chromium, iron, copper, mercury, molybdenum, manganese, nickel, tin, lead, zinc	Ashraf et al. (2014)
War agents	Mustard gas and anthrax are deadly biological agents used as a bioweapon	Shankar (2017)

1.5.2.1 Controlling Pollutant Entry into the Soil

Paper, plastics, metals, glassware, organic material, oil products, and sewage sludge can be recycled to prevent unnecessary soil pollution. Industrial waste needs to be properly treated at source. Integrated waste management methods should be implemented (Saha et al. 2010). The soil is the largest “global sink” of environmental pollution. There are several factors that contaminate the soil. There is an urgent need to stop soil pollution in such a way that the soil remains healthy and productive. Risk assessment associated with pesticide application, contaminated soil, sewage sludge amendment, and other human actions that cause soil contamination is a complex issue that needs to be carefully addressed for a sustainable future.

1.5.2.2 Use of Physical and Chemical Means to Decontaminate Soil

Different physical treatments include soil substitution and thermal desorption (von Lindern et al. 2016; Koul and Taak 2018). Soil spading, soil substitution, and import are the three kinds of soil substitution. The efficiency of different physical processes varies according to soil properties such as soil form, size, soil particle dispersal, moisture, clay, humus content, hydrophobic and magnetic characteristics of the soil particles.

- When the soil-clay or slit content is greater than 30–50%.
- When heavily bound metals are present in the soil.
- When humus levels are high.
- When soil or rock particles have similar properties to those of metal bearing particles.

Chemical methods involve the addition of many chemicals to water, soil, and sludge to eliminate the pollutants from them. There are a number of different soil remediation methods, including chemical leaching, electro-kinetics, vitrification, and some others. These methods have been tested on site and are quick and efficient.

The efficacy of a chemical process determines how well it works. Physical separation methods are less beneficial than chemical treatments. A wide range of heavy metals can be treated in chemical methods; adsorbed heavy metals can be extracted; very fine-grained soil can also be treated; and extracted heavy metals can be recovered by various other techniques. Soil washing with nontoxic or degradable chemicals may be the most effective soil remediation technique due to lesser hazards. There are, however, drawbacks to the use of chemical analysis. In addition to their large-scale application, the cost of treatment may be affected by chemical agents; by-products of chemical reagents could be harmful to soil microbes as well as soil constituents; the extraction of contaminants from highly polluted sludge is difficult to operate; the treated soil may be used for re-vegetation in different instances due to its modified chemical, physical, and bi-vegetation. The chemicals used in these tests also affect other environmental chemicals that may affect the ecosystem. Physical and chemical treatment options should be used in an integrated manner to compensate for limitations, establish a sustainable, cost-effective, and eco-friendly strategic plan for soil remediation (Koul and Taak 2018).

1.5.2.3 Soil Contaminants Bioremediation

As a comprehensive method for addressing soils contaminated with substances, bioremediation has attracted more attention from researchers due to its potential improvement as an eco-friendly and sustainable method to decontaminate soil structure using microorganisms and plant species (Mulligan et al. 2001; Sarma and Prasad 2015, 2016, 2018; Sarma et al. 2019a). The bioremediation strategies evaluated are shown in Table 1.3.

1.6 Effective Treatment of Wastewater

There are no more fundamental and essential natural resources than water on earth (Min et al. 2012). Despite various efforts to prevent the destruction of water resources, human impacts on the natural water cycle are catastrophic and require novel approaches to avoid pollution of water resources (Delplanque et al. 2013). When liquid- or water-based waste is disposed of from home, industry, public, and industrial facilities, wastewater is formed. The main objectives of wastewater treatment methods may include: the reduction of biodegradable organic content (Sarma et al. 2019c; Sarma and Lee 2018), the elimination of heavy metals, the elimination or reduction of phosphorus and nitrogen compounds, the elimination or inactivation of microorganisms and pathogenic particles (Ghosh and Singh 2005).

1.6.1 Choice of Methods for Wastewater Treatment

The treatment process occurs at a wastewater treatment plant (WWTP) or sewage treatment plant (STP), also known as a water resource recovery facility (WRRF) or sewage treatment plant (STP). During the treatment process, the wastewater

Table 1.3 Bioremediation strategies and their significance

Bioremediation strategies	Significance	Reference
Earthworm-assisted bioremediation	Earthworms can be used for bioremediation as they use organic contaminants in a more efficient manner. Earthworms have been shown to improve the soil quality and fertility of field crops by aerating and enriching bioturbed soils. Earthworms are able to delay the binding of organic pollutants to soil, initially release soil bound pollutants for subsequent degradation, and encourage and disperse organic degrading contaminants	Hickman and Reid (2008)
Mycorrhiza-assisted bioremediation	Mycorrhiza is a symbiosis between the plant and the fungus. This helps the plant to remove toxins from its soil. Thus, along with mycorrhizae, phytoremediation is a viable alternative remediation technique and should be further studied	Chibuike (2013)
Enzyme-assisted bioremediation	Enzyme-assisted biodegradation uses enzymes to reduce or eliminate hazardous substances at contaminated sites	Rao et al. (2010)
Plant growth promoting rhizobacteria-assisted bioremediation	Bioremediation through plant growth promoting rhizobacteria is shown to be more eco-friendly and cost-effective than physical and chemical remediation to prevent heavy metal toxicity	Zhuang et al. (2007)
Biosurfactant-assisted biodegradation of organic compounds	Microorganisms break down oil by dissolving and emulsifying it, while its solubility rate is the limiting factor for the biodegradation of organic compounds. Biosurfactants improve the degradability of oil by improving the solubility of oil	Bustamante et al. (2012)
Biochar-assisted bioremediation	Biochar improves the efficiency of removing pollutants and increasing carbon sequestration. It also improves the quality of soil management, lowers greenhouse gas emissions, and limits soil erosion	Oni et al. (2019) and Sarma et al. (2019b)
Nanomaterial-assisted bioremediation	Nanotechnology is a particular focus area, as studies have shown that it can be used in the remediation of toxic environmental contaminants. Nanoparticles derived from biological sources have been shown to be promising against a variety of	Kumar and Gopinath (2017) and Rizwan and Ahmed (2018)

(continued)

Table 1.3 (continued)

Bioremediation strategies	Significance	Reference
	environmental pollutants. Research has shown that bioremediation with biosynthetic nanoparticles can be a sustainable approach	
Plant-assisted bioremediation	Plant-assisted bioremediation or phyto-assisted bioremediation has a synergistic effect between plant root systems and natural microbes (fungi and bacteria) on the remediation of environmentally contaminated soil, sediment, or water. It can be used efficiently in a contaminated environment	Ancona et al. (2017)

pollutants are removed, converted, or broken down. The selection of the treatment method reflects the capacity of the treatment plant, the quality of the wastewater treatment plant, the costs of the treatment plant, and the proximity of the treatment plant. Water treatment facilities fall into a number of categories based on capacity (Islam et al. 2019; Asthana et al. 2017).

1.6.1.1 Small-Scale Wastewater Treatment

Wastewater from communities and households is treated in small wastewater treatment units, which are in the form of disposal units on-site treatment and others.

1.6.1.2 Large-Scale Wastewater Treatment

Water treatment from large sectors of our society is carried out in big water plant that is established for the treatment of human household waste. Wastewater is collected from various sources, and by being sent to a central treatment facility, it is processed.

1.6.2 Biological Approach to Wastewater Treatment

Biological treatment involves the treatment of waste water via the use of microorganisms such as fungi, algae, or bacteria to treat the water, following the degradation of contaminants by microorganisms such as fungi, algae, or bacteria. In order to convert the dissolved organic matter into a dense biomass that can be kept separate from the treated waste water by sedimentation. In fact, dissolved organic matter is used as food by microorganisms themselves. Among the biological methods of wastewater treatment are: (1) Aerobic wastewater bioremediation (aeration lagoons, oxidation ponds, aerobic bioreactors, percolation or filtration filters, activated sludge, biological filters, biological nutrient removal, rotating biological contactors) and anaerobic treatment (anaerobic lagoons, anaerobic bioreactors); (2) phytoremediation processes (Sumner et al. 2015).

1.7 Biotechnology Application to Industrial Sustainability

Human activities, such as urbanization, crude oil, coal mining, agriculture and fisheries, timber harvesting, food production, urban growth, and habitat restoration, have a profound impact on the environment and quality of life (Sharma et al. 2018). These natural resources are prone to depletion. Sustainable natural resource conservation is a process that involves the rational use, skilled management, and preservation of the natural environment in its entirety. Integrated environmental education can provide knowledge about natural resource management that is applicable to sustainable resource management. Moreover, it is essential that the volume of man-made garbage be reduced where possible. Green technology development appears to be a viable option for reducing the environmental effects of industrial byproducts while also improving quality of life (Rogers et al. 2005). The previous study provides information on how biotechnology was used by different industrial companies to reduce the costs and the ecological consequences of their manufacturing activities. The following section is a summary of some of the current research (Rogers et al. 2005).

1.7.1 Fine Chemicals

Biochemie (Austria/Germany), a subsidiary of Novartis, has developed an enzyme-catalyzed procedure for producing antibiotic cephalosporin, which has been enhanced by the genetic modification of the microorganisms that produce it. In contrast to the normal chemical method, the waste solvent generated by the enzymatic process that needs to be incinerated is 100 times less, which reduces both the cost of production and ecological impact. Hoffmann La-Roche (Germany) is currently developing the metabolism of a microorganism to produce vitamin B2 in just one-step method instead of the conventional six-step methods. Consequently, the use of nonrenewable raw materials decreased by 75%, the release of volatile organic compounds to water and air decreased by 50%, and operating costs decreased by 50%.

1.7.2 Intermediate Chemicals

Numerous biotechnological methods have been established at considerable cost and been introduced in the chemical industry, especially in the fine medicinal and petrochemical segments. However, they can be used and modified more economically to produce less valuable products, such as intermediate chemicals used to synthesize other or chemicals or plastics. For example, *S*-chloropropionic acid is being used to synthesize specific herbicides. *Pseudomonas* sp. was used to generate pure S-chloropropionic acid by the British company Mitia Limited. Productivity is fourfold increased by the use of advanced fermentation methods. Similarly, the optimization of performance through genetic modification has resulted in a fivefold increase in efficiency. The use of this technology lowers the cost of production, with less waste by-products.

The Japanese company, Mitsubishi Rayon, produces acrylamide used to generate acrylic polymers. Here, sulfuric acid and copper catalysts in high temperatures are used in the conventional chemical process for the production of acrylonitrile acrylamide. Also, the company launched a bioprocess using nitrile hydratase, a natural enzyme, to catalyze acrylonitrile into acrylamide. The yield and efficiency of this enzyme have been increased by the genetic modification of the microorganism that naturally produces the enzyme. This enzyme-catalyzed method uses 80% less energy and produces more acrylamide compared to traditional chemical processes.

1.7.3 Polymers

Titanium- or tin-based catalysts are conventionally used with inorganic acid and solvents for the production of special polyesters at high temperatures (200 °C). In a bioprocess developed by the British company *Baxenden Chemicals*, the enzyme lipase extracted from the yeast *Candida antarctica* was used to catalyze polymerization at a significantly lower temperature (60 °C). Subsequently, the costs related to enzyme production were reduced through genetic engineering by transferring the lipase gene to the *E. coli* bacterium. In the enzyme-catalyzed polymerization technique, the use of inorganic acids and organic solvents is not required in comparison with the conventional method, which saves at least 2000 megawatts of energy. In addition, the polymer chain length of the bioprocessed polymer is more uniform, implying that it can melt at a lower temperature than conventional polyester, adding to its advantage as a hot-melt adhesive. Consequently, the use of enzyme-based biotechnology is economically and environmentally beneficial.

1.7.4 Food Processing

In food processing, large amounts of water are often used, generating large amounts of organic waste. Bioprocessing methods may reduce wastewater and the production of organic waste. For instance, Pasfrost (the Netherlands) has established a biological treatment plant for vegetable processing, which has reduced water use by 50%, resulting in significant cost savings. In addition, Cereol, a German company, has developed a new technique based on an enzyme for the purification of vegetable oil after extraction. Biotechnology is now being used to produce more cost-effective and environment-friendly food constituents, flavours, aromas, and food additives.

1.7.5 Fiber Processing

Large amounts of water, energy, and chemicals are used for the whitening and treatment of natural fibers for textiles and paper processing. Some of associated environmental effects and such input-related costs may be reduced by the use of enzymes. As an example, the Windel Company (the Netherlands) used an enzymatic technique to reduce the energy and the time required to wash H_2O_2 bleach from textiles prior to dyeing. Due to the use of the enzyme, both the temperature and the amount of the second wash were reduced from 80–95 °C to 30–40 °C, resulting in 9–14% more energy savings, 17–18% more water savings. This technique is very much taken into account in the current textile industry. Canadian company (Domtar) used the enzyme xylanase produced by another Canadian company (Iogen Corporation) as an assisting agent for wood pulp in the production of paper. This new method of whitening is called biobleaching. Because of enzyme utilization, wood pulp lignin is degraded, which has led to a saving of 10–15% in the consumption of chlorine dioxide in order to achieve an optimum level of brightness.

1.7.6 Biotechnology Can Create a Source of Renewable Energy

Instances of biotechnological uses in energy relate to two main energy parts: (1) fossil fuels and (2) renewable energy. Iogen Corporation (Canada) has developed a method that uses cellulose enzymes to optimize the transformation of cellulose into fermentable sugar. In addition, the yield and action of cellulose enzymes have been optimized by the use of biotechnological approaches.

The microbial fuel cell (MFC) system makes use of an active microorganism as a biocatalyst in an anaerobic anode section for producing bioelectricity (Rahimnejad et al. 2015). The MFC has several advantages, including low cost, simple compact design, and renewable energy production, all of which are considered promising in environmental management technology. MFCs can convert the waste to electricity, providing an environmentally friendly and sustainable solution to the degradation of pollutants (Zhang et al. 2016). MFCs may be used in the wastewater treatment section to treat domestic sewage, livestock wastewater, industrial wastewater, metal ion removal and recovery, and antibiotic removal. Sediment microbial fuel cells (SMFCs) can degrade organic pollutants in sediments, immobilize phosphorus in sediments, remove nitrogen from sediments, and remove heavy metal from sediments. In addition to that MFCs can be used to remove organic compounds (Polyaromatic compounds and chlorinated hydrocarbons), metals, and nonmetallic inorganic pollutants for groundwater (such as nitrate). MFCs are also a promising method for sludge treatment and solid waste management (Wu et al. 2020).

1.8 Microorganisms in the Environment

Microbial communities have played a key role in balancing ecosystem work, biotic and abiotic stress mitigation, earth's environmental balance, nutrient cycles, bioconversion of complex animal and plant residues, and bioremediation and mineralization of soil contaminants. They lead to soil fertility and development as well as plant growth through complex and critical, yet ongoing and extraordinary processes. The microbiome is holistically important for the biogenesis of the earth. Microbial abilities related to the reuse of carbon, nitrogen, greenhouse gasses, and minerals are important to environmental processes. They sequester, solubilize, fix, mobilize, mineralize, transform, neutralize, accumulate, recycle minerals and nutrients to improve agroecosystems and improve plant health and soil fertility. Microbial communities have a vital role to play in reducing diseases, promoting plant growth, and inducing resistance due to their outstanding function (Singh et al. 2019).

1.8.1 Bio-Inputs for Global Sustainability

Bio-inputs, such as plants, bacteria, and fungi, can be modified using recombinant technology and genetic engineering and can be used as biostimulants, biofertilizers, biocontrollers, biostabilizers, contaminant biodegraders, crop protection, and soil fertility enhancers (de Salamone et al. 2019). Furthermore, bio-inputs are generally referred to as biotechnological inputs, which are used for both widespread organic farming and agriculture. Bio-inputs are categorized by the Food and Agriculture Organization (FAO) as bio-insecticide, biorepellent, biostimulant, biofungicide, biofertilizer, or inoculant according to their performance and functions. Their use as an alternative to the use of conventional agrochemicals in agriculture is increasing globally (de Salamone et al. 2019).

1.8.2 Antibiotics Are Used to Protect Plants

Antibiotics are drugs used to treat bacterial infections in humans and animals, but they are also widely used in plant protection. According to Duffy et al. (2003), they inhibit the growth of microorganisms at low concentrations through blockage or suppression of several metabolic activities. Antibiotics have been manufactured using PGPRs due to the beneficial biocontrol mechanism demonstrated by these PGPRs for a variety of phytopathogens (Glick et al. 2007). Antibiotic agents can be categorized into six groups based on the results of a study carried out by Haas and Défago (2005), considering their ability to control plants root diseases: phloroglucinols, phenazines, lipopeptides, pyoluteorin cyclic pyrrolnitrin, and hydrogen cyanide (volatile anti-toxin). For example, wheat plant disease caused by *Gaeumannomyces graminis*, can be controlled by 2,4-diacetylphloroglucinol produced by *Pseudomonas* sp. (de Souza et al. 2003). Furthermore, *pseudomonads* produce phenazines antibiotics that are active against phytopathogens such as

Gaeumannomyces graminis and *Fusarium oxysporum* (Chin-A-Woeng et al. 2003). Since the 1950s, antibiotics have been used to control a variety of bacterial diseases that affect fruit, vegetable, and ornamental plants. Today, the most frequently used antibiotics on plants are oxytetracycline and streptomycin.

1.9 Further Biotechnological Aspects

1.9.1 Eco-Friendly Fuels

Global energy consumption is gradually increasing, necessitating the exploration of alternative ecofriendly and less expensive energy resources such as bioethanol, biodiesel, biogas, synthetic fuel, and hydrogen. Generally energy is of two types: (1) renewable resources, which are the naturally discovered, meeting about 16% of the universal energy demand (Obama 2017); (2) nonrenewable resources, such as petroleum, coal, and fossil fuels, all satisfying more than 80% of universal energy demand (Agarwal 2007). Due to growing needs and usage, the capacity of the nonrenewable resources is retreating since they are basically used in industrial sectors, transportation, and energy generation sections, which are the main reasons the environment gets contaminated. As a result, biofuels are gaining public and scientific attention as having less of an impact on the environment (Lund 2007). Coal and other fossil fuels contain significant amounts of polyaromatic hydrocarbons. These toxic compounds are emitted by combustions and pollute the environment (Fattah et al. 2013). Alternatively, biofuels have a positive effect on people helath and environment (Arshad et al. 2018). The aim of introducing various biofuels is to reduce the fossil energy consumption and lowering the outflow of ozone-depleting substances in the environment.

1.9.1.1 Biofuel Sources

Given the growing demand for energy, the restrictions on the use of fossil fuels, and the reduced environmental effects of fossil fuels, various attempts were made to produce renewable energy sources, such as biofuel. Biofuels generated from biomass sources are the main supply to replace fossil fuels and now account for 11–14% of the total globally consumed energy (Megharaj et al. 2011; Sivrikaya et al. 2011). Maize, wood waste, agricultural waste, sugar cane, oil residues (for example, cooking oil and chicken fat), vegetable and herbal oil, non-food products (e.g., algal oil), and fresh vegetable oil (e.g., soya oil) are typically the best biofuel resources. Wood chips or grass can produce fuel from remaining products, such as ethanol or kitchen oil, since they are environmentally compatible. Biofuels are characterized based on economic, social, and environmental considerations. According to Tahir et al. (2019), modern biofuels are recognized as second-generation (2G), third-generation (3G), and fourth-generation (4G) eco-fuels. Modern eco-fuels and examples of them are shown in Table 1.4.

Table 1.4 Modern eco-fuels and examples

Modern eco-fuels	Examples	Reference
2G eco-fuel	Urban solid waste, lignocellulose feedstocks, lesquerella oil, *Jatropha curcas*, *Pongamia glabra*, timber, grass, and plant residues.	Azad et al. (2016) and Tahir et al. (2019)
3G eco-fuel	Algal biomass	Tahir et al. (2019)
4G eco-fuels	Fuels derived from the air through new methods like biochemistry, geosynthetic, as well as petroleum hydroprocessing	Tahir et al. (2019)

1.10 Biopesticides

Excessive use of synthetic pesticides in plant protection (fungicides, herbicides, insecticides) leads to pollution of agro-ecosystems. Pesticides cause genetic alterations in bird and animal populations and are harmful due to their persistence in nature, contamination of water, soil and food chains. They have been present in the environment for many years as recalcitrant xenobiotics (Sarma and Joshi 2020). For example, DDT (1,1,1-trichloro-2,2 bis [P chlorophenyl] ethane), widely used for nearly 50 years since the 1930s, and extremely persistent in the environment. DDT residue has been detected in all life support systems. Therefore, the development of eco-friendly alternatives should be seriously considered to reduce the environmental impact of chemical pesticides. The use of biopesticides was one of the promising alternatives.

Biopesticides are effective tools for the production of crops for sustainable agriculture. Typically, they are less average environmental impacts compared to the deadly, conventional chemical pesticides. Biopesticides solve a variety of problems, such as pest resistance, environmental and human health effects.

1.10.1 Microbial Pesticides

The active component of a microbial pesticide is a natural or genetically engineered microorganism. Fungi, bacteria, and nematodes are among the most significant types of microorganisms identified. Whereas each active individual microbial component has specific target pests, microbial pesticides can control different types of pests. Some fungi, for example, can kill certain insects, while others can control certain weeds. *Trichoderma* (biofungicides), the strains *Bacillus thuringiensis* (bioinsecticides), and *Phytophthora* are the most commonly used microbial pesticides (bioherbicides).

1.10.2 Biochemical Pesticides

Compounds such as pheromones or plant extracts, and fatty acids, naturally present and capable of killing pests non-toxically, can be considered as biochemical pesticides (Ghahari et al. 2015, 2017, 2018; Hadadi et al. 2020).

1.10.2.1 Benefits of Biochemical Pesticides

Biopesticides are nonpathogenic and nontoxic to nontarget organisms, not directly affecting animals, parasitoids and predators. Biopesticide residues are safe and nonhazardous all the time, even close to the time of harvesting. No risky residues remain in food, feed, and fiber. They have short shelf-life and limited field persistence and are quickly decomposed because of their biodegradability, making them safer for humans and the environment. Biopesticides are usually products and by-products of organisms that occur naturally, such as animals, plants, and microorganisms (bacteria, viruses, and fungi). Biopesticides are effective in lower or smaller concentrations or quantities. It is assumed that pests will not develop resistance to biopesticides. The international market for biopesticides is on the rise (Essiedu et al. 2020).

1.10.2.2 Limitations of Biochemical Pesticides

Even though research proves that the use of biopesticides is effective against a wide variety of crop pests, biopesticides are not yet well established on the market. The commercialization of botanical pesticides highly depends on the availability of large quantities of plant resources and the cultivation of the plants. To date, the source plants are cultivated for other purposes, such as food or medicine. In addition, the cultivation of plants for the production of botanical pesticides need a vast land, which is therefore highly competitive with arable land for the production of food. Besides, given the high rate of biodegradability, biological pesticides have a short shelf-life. As far as the specificity of a microbial pesticide is concerned, only a portion of the pests available in the field can be controlled by microbes. Other types of pests present in treated areas may not be controlled by them, which may result in continuous damage. The efficiency of microbial pesticides is reduced by UV light, heat, and desiccation; the delivery system of such pesticides needs attention. In addition, the need for specific formulations and storage processes results in a short shelf-life, which in turn increases the cost of production. The discovery of active substances and scientific research on formulation and delivery will improve the commercialization and use of biopesticides. It seems necessary to promote the commercialization of biopesticides by making available biological pesticides to farmers, particularly in developing countries. However, regulating processes to improve the registration of low-risk substances can develop the exploitation and market accessibility of biopesticides (Essiedu et al. 2020).

1.11 Biofertilizers

Biofertilizers play a crucial role in refining crop quality and productivity in modern agriculture (Mahanty et al. 2017). Generally believed as biostimulants, any or all of the biological mixtures added to seeds, soil, or plants are intended to improve the efficiency of nutrient absorption and crop quality and activate stress tolerance (du Jardin 2015). They are, in fact, colonizing the rhizosphere of plants to support plant development (Malusá and Vassilev 2014; Vessey 2003). Bioinoculants based in *Rhizobia* have been actively used in agriculture for the last decades, as reported by Arora et al. (2017). According to the estimated results of the BBC (2014) study, more than 150 microbes based products have been used for agricultural purposes, while the total share of biofertilizers in the global fertilizer market is 5%. Various types of biofertilizers are currently used in agriculture: nitrogen fixers, phosphate solubilizers, potash solubilizers, etc. Together with nitrogen fixers (*Azotobacter, Cyanobacteria, Rhizobium, and Azospirillum*), phosphate solubilizers (*Bacillus megaterium* and *Pseudomonas* sp.), have been widely used in agriculture (Mishra and Dash 2014) among all biofertilizers. In addition, biofertilizers rich in K, S, and Zn are being developed as major bio-inoculants to correct plant deficiency diseases (Khatibi 2011; Shaikh and Saraf 2017). Biofertilizers increase crop yields by up to 10–40% by improving the intake of various nutrients, such as amino acids, proteins, nitrogen, and vitamins (Bhardwaj et al. 2014).

1.11.1 Microbial Biofertilizers

Microbial biofertilizers are substances containing the living or dormant cells of microorganisms. When applied to the seeds, plant surface, or soil of a host plant, biofertilizers increase the nutrients available to the plant by colonising the plant's rhizosphere. Unlike chemical fertilizers, microbial biofertilizers are also more accessible to small-scale farmers. The main classes of microbes used for the preparation of microbial biofertilizers are fungi, bacteria, and cyanobacteria, most of which have an interactive relation with plants (Thomas and Singh 2019).

1.12 Bioleaching

Minerals are a vital part of the country's economic development and therefore need to be extracted and recovered. Bioleaching has become an increasing and effective biotechnology field, a method by which microorganisms are used to extract and recover precious and fundamental metals from primary ores and concentrates (Rawlings and Johnson 2007). The discovery of bacterium *Thiobacillus ferrooxidans* (now *Acidithiobacillus ferrooxidans*) in the mid-1940s and later its findings in the role of bacteria of bioleaching (of copper) set the stage for systematic investigation of bioleaching.

1.12.1 Bioleaching Uses

Bioleaching is primarily used to mine metals from electronic waste (Hoque and Philip 2011), metal waste (Hoque and Philip 2011), fly ashes (Mahajan et al. 2017), waste from mines (Mahajan et al. 2017), polluted sediments (Mahajan et al. 2017), and waste from batteries (Kang et al. 2010).

1.12.2 Mechanism of Bioleaching

Bioleaching is based on the solubilization of metals by biological oxidation or on a complex reaction from various sources (Borja et al. 2016). A model that involved two mechanisms was proposed in explaining the microbial metal solubilization of sulfide minerals. Metal sulfides can be oxidized by microorganisms through a direct mechanism in which electrons are directly obtained from the reduced minerals. Here, the cells must be attached to the mineral surface where close contact is required. The adsorption of cells to suspended mineral particles occurs within a few minutes or hours. The oxidation of the reduced metals is mediated by ferric (III) ions, another mechanism called the "indirect" mechanism, which is formed by microbial oxidation of ferrous iron present in the minerals. Ferric iron acts as an oxidant and can oxidize metal sulfides and is reduced to ferrous iron, which in turn can be microbially oxidized. In this case, iron acts as an electron carrier. It has been suggested that iron oxidation does not require direct physical contact. In many instances, it has been concluded that the "direct" mechanism dominates the "indirect" mechanism, mostly because the direct mechanism involves direct physical contact with the mineral surfaces of the bacteria. It has been proven that *T. ferrooxidans* is easily attached to the surface of the metal sulfide-S. In the case of Cu_2S, the electron transfer from sulfide-S or cuprous copper involves Fe (II) bound in the envelope and the cell exopolymer. It has been noted that there appear to be limited sites on a metal sulfide particle for bacterial attachment. As a result, once maximum attachment has been achieved, further multiplication of the attached cells should result in the transfer of one of the two daughter cells of each dividing bacterium to the bulk phase. However, the attachment of microorganisms to surfaces does not constitute an indication of the existence of a direct mechanism. In order to indicate the significance of bacterial adhesion to mineral surfaces, the term contact leaching has been formed. The following equations describe the direct and indirect mechanisms of pyrite oxidation (Mishra et al. 2005).

Direct:

$$2\,\mathrm{FeS_2} + 3.5\,\mathrm{O_2} + \mathrm{H_2O} \overset{\text{Thiobacillus}}{\rightarrow} \mathrm{Fe^{2+}} + 2\,\mathrm{H^+} + 2\,\mathrm{SO_4^{2-}}$$
$$2\,\mathrm{Fe^{2+}} + 0.5\,\mathrm{O_2} + 2\,\mathrm{H^+} \rightarrow 2\,\mathrm{Fe^{3+}} + \mathrm{H_2O}$$

Indirect:

$$\mathrm{FeS_2} + 14\,\mathrm{Fe}^{3+} + 8\,\mathrm{H_2O} \overset{\text{Thiobacillus}}{\rightarrow} 15\,\mathrm{Fe}^{2+} + 16\,\mathrm{H}^{+} + 2\,\mathrm{SO}_4^{2-}$$
$$\mathrm{MS} + 2\,\mathrm{Fe}^{3+} \rightarrow \mathrm{M}^{2+} + \mathrm{S}^{0} + 2\,\mathrm{Fe}^{2+}$$
$$\mathrm{S}^{0} + 1.5\,\mathrm{O_2} + \mathrm{H_2O} \rightarrow 2\mathrm{H}^{+} + \mathrm{SO}_4^{2-}$$

The model of direct and indirect leaching of metals is, however, still under discussion.

1.13 Bioplastic

The generation of plastics from synthetic polymers has created several biological issues because they are nonbiodegradable (Kikuchi and Tanaka 2012). The production of new materials such as biomaterials using fats, proteins, sugars, fibers, and other natural ingredients extracted from plants would prevent the use of synthetic polymers for plastic generation. Bioplastics are plastics made from renewable biomass, such as vegetable fats and oils, corn starch, straw, woodchips, sawdust, and recycled food waste.

1.13.1 Merits of Bioplastics Over Conventional Plastics

1.13.1.1 Biodegradable

Bioplastics require a shorter time to degrade when thrown as rubbish in comparison with conventional plastics. It does not, therefore, require any recycling (Sinan 2020). Bioplastics are usually made from sugar derivatives such as starch, cellulose, and lactic acid. Common plastics, such as fossil-fuel plastics (also known as petrobased polymers), are derived from petroleum or natural gas and thus easily degraded.

1.13.1.2 Eco-Friendly

In the production and burning process of bioplastics, less emissions of carbon and greenhouse gases are observed compared with conventional plastics (Sinan 2020). Compared to conventional plastics, bioplastics are completely safe because they are constructed using natural sources, so they have no chemicals or poisons (Sinan 2020). Bioplastics require less than half of the energy needed for production as compared to conventional plastics (Sinan 2020). Bioplastics are produced from agricultural raw materials compared to conventional plastics, so there is no shortage of raw materials (Sinan 2020). Plastic is the main contaminant of the environment (Windsor et al. 2019). The accumulation of petroleum-based plastics is a chief source of environmental contamination, and its harmful effects destroy the ecosystem balance. Thus, bioplastics are an eco sustainable solution to this problem. These biopolymers may degraded aerobically or anaerobically into water, biomass, and carbon dioxide (Sinan 2020). In the process of biodegradation, microorganisms convert plastics into carbon dioxide, compost, and water. These biodegradable polymers can be utilized in numerous fields, such as automotive, medicine, agriculture, packaging, and controlled release of drugs. Researchers are working globally to develop bio-based polymers from different waste materials (Sinan 2020).

1.14 Conclusion

Technological progress is often seen as a challenge to sustainable development. That is because it creates a high level of uncertainty and a need for more government regulation. Indeed, we may face an even greater challenge to sustainability in the coming years. For example, the technologies that we are currently using may not be able to solve our sustainability problems in the near future. It is clear that all advancements in biotechnology depend on sufficient laboratory and institutional support. It is hoped that this new material production technique can be environmentally friendly without damaging the environment.

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The Mystery of Methanogenic Archaea for Sustainable Development of Environment

2

Mythili Ravichandran, Kasi Murugan, Sharmila Devi Natarajan, and
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Abstract

Methanogenic archaea are an environmentally enthralling proposed candidate. It has created an innovative thrust in the research world. In recent times, methanogens have been a striking paradigm and significant present-day representatives among the Archael kingdom. It has unique physical, ecological attributes with microbiological credits, which contrast from other microbial groups. In the present situation, it works viably on the carbon dioxide sequestration; it plays a pivotal part in the overall management of the carbon cycle. Principally, the emergence of methane from water muds was recorded by Alessandro Volta (1776). This discovery created an elegant and widespread interest among methanogens. Besides, it laid the sound framework for methanogenic study and the potential production of methane-based fuel. This chapter revealed out a broader understanding of the microbiological and applied aspects of methanogens. Compared to all the others, the in-depth study of

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methanogenic archaea provides tremendous opportunities in various fields, and those contents are also summed up in this chapter. It also centers on the recent advances of methanogenic biogas, focusing on viable and sustainable biofuel production.

Keywords

Archaebacteria · Methanogens · Carbon cycle · Biofuel

2.1 Introduction

Methanogenic archaea produce valuable biofuel through the anaerobic digestion-based renewable energy technology. In nature, the biogas ($CH_4 + CO_2$) is produced by anaerobic degradation of organic substances or the biomass by microbial communities. The biogas system of anaerobic digestion recycles bio-solids, livestock manure, and solid waste organic materials. Methanogens recycle almost all organic material through anaerobic digestion and hence into the valuable biogas typically. However, the pure culture of methanogen was obtained only during 1947 by Schnellen. Methanogens have a position in the Euryarchaeota kingdom under the Archaeal domain (Woese et al. 1990; Ferry 2010). The methanogenic pathway seized the researcher's curiosity for several decades. They are in the limelight among anaerobes since they developed a range of molecular strategies for survival under anoxic, harsh, and seemingly drastic surroundings. In the past decades, methanogens have been considered candidates of selection among archaea because of their bizarre biochemical and genetic properties (Claus and Konig 2010). Since they are the eccentric group of communities that produces a significant measure of methane in the earth, the search for sustainable biofuel production and the greenhouse gas abatement approaches makes them most attractive. Moreover, the methanogenic archaea's active participation in biofuel production and global carbon cycle neutralization leads to the wide acknowledgment and recognition of them as economically and environmentally viable organisms. They are the sole biogenic methane-producing organisms on earth through anaerobic digestion or biogas fermentation. They are the best models for better understanding and forecasts of climate change, extreme environments, and nitrogen fixation.

This chapter attempts to briefly address the general microbial and technical aspects of methanogens in this present contest. Ultimately, this chapter aims to discuss the cutting-edge science behind the exploration of methanogens by refreshing and updating their microbiological, taxonomical, and ecological traits. Moreover, the methanogenic sequestration, biofuel production, and the scientific community's new environmental and economic demands are also reviewed. Besides, methanogens' superiority over other sequestration candidates in resolving sound environmental issues due to their different metabolic activities have also discoursed.

2.2 Microbiological Facets of Methanogens

2.2.1 Archaebacteria

Archaebacteria are an extraordinary fraction of organisms that are distinct from all others at the elevated level. These organisms are positioned at the Archaeal kingdom of cellular living forms (Woese and Fox 1977; Woese et al. 1990), which scientists missed for quite a long time. They are a well diverse and abundant group of prokaryotes that can survive in extreme environments. The name archaebacteria mirrors their delightful status of being the oldest known bacteria. Moreover, they evolved the get-go throughout the entire existence of the globe. There is no much change since that time (Woese and Fox 1977). Bergey's manual categorizes the archaebacteria in Division IV of prokaryotes. Though intricate, a large part of the cultivable and very much reviewed types of Archaea have been identified. The Archaeal domain presently has the following taxonomic phyla based on 16S rDNA analysis like Crenarchaeota, Euryarchaeota, Thaumarchaeota, Nanoarchaeota, Aigarchaeota, Parvarchaeota, and Bathyarchaeota (Zuo et al. 2015). However, most methanogens cultivated are successfully placed in the kingdom of Euryarchaeota (Angel et al. 2011). Conversely, some of them are located within the Crenarchaeota (Nicol et al. 2003), Bathyarchaeota (Evans et al. 2015), and Verstraetearchaeota kingdoms (Vanwonterghem et al. 2016).

2.2.2 Definitive Characteristics of Methanogenic Archaea

Carl Woese and Ralph Wolfe both initially revealed that the methanogens were only abstractedly related candidates other than bacteria. In the carbon cycle-based anaerobic ecosystem, these methanogens are considered a decisive part; hence, their characterization is fundamental for the comprehensible understanding of anaerobic ecology (Jablonski et al. 2015). Methanogenic archaea are ancient primeval bacterial forms of the earth. They are very slow-growing, obligate anaerobic (Garcia 1990; Liu and Whitman 2008), chemolithoautotrophic, or chemolithoheterotrophic, and methane offering unicarbonotrophic (Woese et al. 1990; Boone et al. 1993) unicellular, prokaryotic forms. They are one of the most significant, distinct, phylogenetically unusual, and well-analyzed groups within the domain archaea. All methanogens are obligate lithotrophs. As their name implies, methanogens make energy and generate methane as a by-product of their united catabolic specialization pathway of methanogenesis, a unifying hallmark of all of them. They contribute nearly all biogenic methane on earth (Balch et al. 1979; Ferry and Kastead 2007; Meeres 2009). Notably, methanogens are called the thermodynamic edge of life (Buan 2018).

2.2.3 Anaerobiosis

The methanogens are strict anaerobes. They are compassionate toward oxygen. They are killed by the presence of minute levels of oxygen concentration as 0.18 mg/L of soluble oxygen. They are growing at redox potentials below −300 mV (Vance 1997). Hungate (1969) expressed that the oxygen concentration potential of methanogens is 10–56 mol/L. Interestingly, recent research studies delivered evidence of the methane paradox that is the active methanogens present in oxic environments (Angle et al. 2017).

Before the 1970s, the researchers believed that the cytochromes are not significant for methanogenesis. After examining cytochromes in methanogens (Kuhn et al. 1979), they were divided into two metabolic assemblies based on their presence (Thauer et al. 2008; Mayer and Muller 2014). The Methanosarcinales, Methanobacteriales, Methanococcales, and Methanomicrobiales are the cytochrome possessing orders. They can utilize H_2/H_2 with formate/secondary alcohols as an electron donor and CO_2 as the electron acceptor, which got reduced to methane. The orders such as Methanobacteriales, Methanococcales, Methanomicrobiales, and Methanopyralles lack cytochromes (Thauer et al. 2008) and methanophenazine. They can use $H_2{}^+CO_2$ or formate as an electron donor. However, some of them possess membrane-bound cytochromes. They have the ability to use electron donors such as acetate, methanol, or methylamines. During ATP synthesis, the cytochrome-free methanogens use Na_+ current over the membrane. However, the cytochromes possessing methanogens have additional coupling sections. They can translocate protons. Concurrently, they use Na^+ and H^+ for ATP synthesis (Schlegel and Muller 2013). Recently, *Methanosarcina acetivorans* became a dynamic model microbe for the cytochrome-based improvement study on extracellular electron transfer in Archaea (Holmes et al. 2019). These findings are directly linked pieces of evidence for the progress of various environmental adaptation criteria among methanogenic communities.

2.2.4 A Diminutive Historical View of Methanogenic Archaea

Methanogens are the most primordial and ancient organisms on the earth. During the Archaean era, they would have involved in regulating climate through the transformation mechanism of greenhouse gas such as carbon dioxide into methane by way of their natural absorption mechanism (Ueno et al. 2006). The Volta's experiment was conducted by Italian physicist Alessandro Volta (1776) on Lago Maggiore which lead to the exploration and isolation of methane. Later, many scientists ignite findings and making light on the methanogenesis pathway. It makes milestones on the germination of methanogenic archaeal research. Later Hungate (1950) extensively developed many anaerobic techniques, which were subsequently modified by many forthcoming intellectuals.

2.2.5 Habitat

Methanogens exhibit extensive distribution in nature. The significant division of the earth's biosphere holds large and numerous oxygen-free anoxic habitats (Ferry 1992). Although the metabolically limited group, it shows extreme diversity of conditions. Methanogens are found in almost every conceivable anoxic habitat. Abundantly, they were found in electron acceptors (O_2, SO_4^{2-}, NO_3^{-}, and Fe_3^{+}) confining habitats like sediments (pond, lake, river, and sea) (Issazadeh et al. 2013), flooded and waterlogged soils, wetlands (bogs, peatlands, swamps, and marshes), landfills, anaerobic digesters, sewage, activated sludge, and hydrocarbon-contaminated soil (Watanabe et al. 2002). In addition, they were also isolated from Tundra areas, geothermal springs/hydrothermal vents (Jeanthon et al. 1999), hypersaline environment, certain groundwater aquifers, terrestrial arthropods gut (Hackstein and Stumm 1994), gastrointestinal tracts of human, rumen, and insects (Parmar et al. 2015), avian species (chicken, goose, turkey feces) (Saengkerdsub et al. 2007), arctic tundra, permafrost soil (Wagner et al. 2013), submerged soils, rice paddies (Sakai et al. 2007), the heartwood of trees (Liu and Whitman 2008), living trees wet wood region (Zeikus and Ward 1974), and other places where organic material is decomposing under strongly anaerobic conditions. Diverse methanogens in boreal peatlands (Sizova et al. 2003) and permafrost sediments were also reported. However, recent research studies also report their existence in many aerated upland soils, including desert soils. These mentioned habitats directly accentuate the methanogen's ability of metabolic adaptation to diverse anoxic ecosystems.

2.2.6 Methanogenic Phylogeny

Methanogens arranged in the Archaeal domain are phylogenetically mixed fractions (Liu and Whitman 2008). Freshly, the methanogens are branched into seven orders (Ferry and Kastead 2007) viz. Methanobacteriales, Methanococcales, Methanomicrobiales, Methanosarcinales, Methanopyrales, Methanocellales, and Methanoplasmatales (Thermoplasmatales) (Paul et al. 2012). It encompasses 150 characterized species. Still, the list extends as new species discovery work proceeds (Jablonski et al. 2015). Currently, 120 species of methanogens are described. Among them, hydrogenotrophs, acetotrophs, and methylotrophs make up 77%, 14%, and 28%, respectively. In methylotrophs, ten species are strict methylotrophs. Just 3% of the species employ H_2 to diminish methanol to methane (Garcia 1990). These phylogenetic, evolutionary signals such as unique complex energy metabolism-based positioning of methanogens among extreme taxonomic branches create exciting avenues for the ecologists and phylogenists toward an in-depth branching study on the ecological profile of methanogens.

Based on the phylogenetic search, all the orders are labeled as class I, class II, and class III (Fig. 2.1). Chiefly, class I and II methanogens were bestowing prevailing physiological and metabolic traits. Methanopyrales, Methanococcales, and Methanobacteriales are placed under class I, and Methanomicrobiales come under

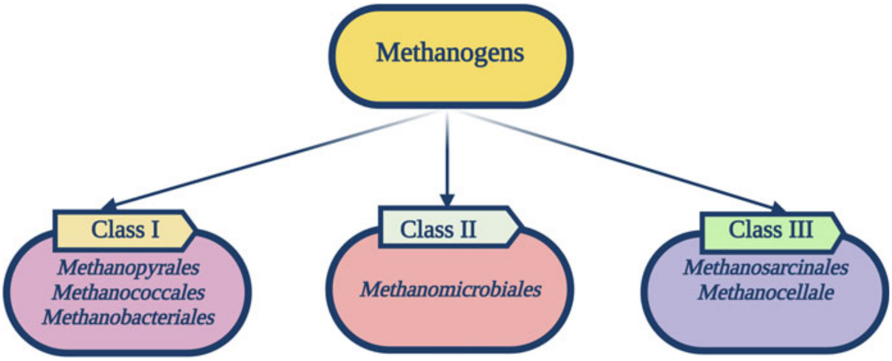

Fig. 2.1 Schematic depiction of classification of methanogens based on comparative genome analysis

class II. Methanosarcinales and Methanocellales are placed under class III (Bapteste et al. 2005; Sakai et al. 2008; Anderson et al. 2009). Class II methanogens expose strong adaptation than class I methanogens in oxic habitats (Lyu and Lu 2018).

2.2.6.1 Methanobacteriales

The order Methanobacteriales is very short lancet-shaped cocci to long filamentous rods and non-motile. Their rigid cell wall has pseudomurein with C20 and C40 isopranyl ethers. The pseudomurein makes Gram stain to be positive, and some as Gram variable. Mostly they are hydrogenotrophs. It can utilize hydrogen, formate, CO, or secondary alcohol as electron donors for its methanogenesis except for the Methanosphaera. The cell membrane contains caldarchaeol and myo-inositol lipids. It includes the two families Methanobacteriaceae and Methanothermaceae, based on 16S rDNA sequence (Whitman et al. 2001; Karakashev et al. 2005; Bonin and Boone 2006).

2.2.6.2 Methanococcales

Methanococcales are holding a high fraction of genetic diversity. They are Gram-negative coccoid/ irregular cocci. Most of them are motile. All are autotrophs and require sea salts for their culture. They are halophilic, and most are chemolithotrophic. It can utilize hydrogen or formate as electron donors for its metabolism. Cell envelopes are covered with a protein cell wall or S-layer. The cellular lipids range from archaeol, caldarchaeol, and macrocyclic archaeol. Their most optimum growth temperature ranges from 35 to 88 °C (Whitman et al. 2001; Whitman and Jeanthon 2006). It is composed of two families, the Methanocaldococcaceae as well as Methanococcaceae, and four genera. The Methanocaldococcaceae covers two hyperthermophilic genera, Methanocaldococcus and Methanotorris. The Methanococcaceae embraces the Methanococcus (mesophiles) and the Methanothermococcus (extreme thermophiles) (Karakashev et al. 2005; Bonin and Boone 2006; Liu 2010).

2.2.6.3 Methanomicrobiales

These slightly studied Methanomicrobiales comprise morphologically varied shapes such as cocci, rod, plate, or spiral organisms. They may be Gram-positive or Gram-negative, and motile or non-motile. They are widely distributed in many environments. They utilize H_2/CO_2 for metabolism, whereas some of them also use formate or secondary alcohols. However, they cannot use acetate or methylated C-1 compounds. Their cell walls have a glycoprotein S-layer with an exterior sheath. It includes three families and 11 genera. Family Methanomicrobiaceae are Gram-negative cocci or slightly curved straight rods. Family Methanosarcinales are large, Gram-positive, and spherical-to-pleomorphic. It can frequently form packets of various sizes, non-motile, and mesophiles to thermophiles (Whitman et al. 2001; Karakashev et al. 2005; Bonin and Boone 2006).

2.2.6.4 Methanosarcinales

Methanosarcinales have a distinct morphological appearance like cocci, pseudosarcinae, and sheathed rods with an S-layer. It consists of three families, Methanosarcinaceae, Methanosaetaceae, and Methermicoccaceae. Methanosarcinaceae contains six genera, and Methanosaetaceae includes some genus of obligate acetotrophs (Boone et al. 1993; Galand et al. 2004; Karakashev et al. 2005). In this order, all methanogens can use methylated compounds without hydrogen and formate (Whitman et al. 2001). Methanosaeta (Methanothrix) members use only acetate for methanogenesis. The Methanobacteriales and Methanosarcinales orders were mostly accounted for by the planktonic methanogenic community. The substrate versatility of this order can support diverse metabolic adaptations (Crevecoeur et al. 2016).

2.2.6.5 Methanopyrales

Methanopyrales are the most profoundly branching methanogen. Currently, it comprises only a single species, viz. *Methanopyrus kandleri* (Euzeby 2011). It is classified under the family of Methanopyraceae. They are hyperthermophilic, obligate hydrogenotrophic, rod-shaped organisms having a protein S-layer surrounded pseudomurein cell wall. They can survive 84–110 °C, a temperature found in marine hydrothermal systems. It uses H_2/CO_2 for its metabolism (Whitman et al. 2001; Karakashev et al. 2005). 2,3-Di-*O*-phytanyl-sn-glycerol and 2,3-di-*O*-geranylgeranyl-sn-glycerol lipids are present in them (Oren 2014).

2.2.6.6 Methanocellales

Methanocellales (methane-producing cell) was first identified in the soil of the rice field. It plays a pivotal part in paddy field methane emanations (Sakai et al. 2008; Lu and Lu 2012). It was primarily proposed and isolated from Italian rice crop soil. Its pure culture was first obtained from Japanese rice field soil (Sakai et al. 2010). They are unique for their slow growth, fastidious culture conditions, tolerance of oxygen stress (Angel et al. 2011; Yuan et al. 2011; Lu and Lu 2012), and adjustment to low H_2 partial pressure (Sakai et al. 2009). It includes the species *Methanocella paludicola* (Sakai et al. 2007). Beforehand, this order was acknowledged as Rice

Cluster 1 (RC-1). Cells are rod-shaped, Gram-negative, mesophilic, non-motile, and hydrogenotrophic. *Methanocella arvoryzae* species was a recent addition (Sakai et al. 2010). In the year 2012, Lu and Lu identified a *Methanocella conradii* from Chinese rice field soil. It has an optimum growth temperature of 50–55 °C. It uses H_2/CO_2 for CH_4 production.

2.2.6.7 Methanoplasmatales (Thermoplasmatales)

Methanoplasmatales was first proposed by Paul et al. (2012), which are currently known as Methanomassiliicoccales. They use methylamines or methylamines $^{+}H_2$ (or di- and mono-methylamines) for methanogenesis. Owing to this, they are perceived as obligate methyl respiring methylotrophic methanogens (Buan 2018). Many strains are identified, such as *Methanomassiliicoccus luminyensis*, "*Candidatus Methanomethylophilus alvus*." Both are strict hydrogen-dependent methylotrophs of human feces (Borrel et al. 2012; Dridi et al. 2012). Amazingly, the largest methanogenic euryarchaeon genome from *M. luminyensis* (Gorlas et al. 2012) was identified and classified.

2.3 Morphological, Ecological, and Biological View of Methanogenic Archaea

Methane-forming archaea have a phylogenetically distinctive 16S rDNA gene sequence than others. They also have peculiar characteristic cell wall components, cell walls, and unique membrane lipids. The ecological aspect highlights their combined interplay of other anaerobes with the controllability of physical and chemical factors for their function.

2.3.1 Cell Shape, Motility, and Gas Vesicles

The microscopic wet mount preparations generally reveal the morphological characteristics of methanogens. Methanogens show diverse cell morphology, including size and shapes like rods (individual/pairs/narrow, curved rods/spirillum-shaped/short/very short/long filamentous rods), regular and irregular cocci, coccoid, highly irregular coccoid, spirilla, sarcina, lancet, plate, angular plate, rounded shape and long thin chains, long thicker filaments, clusters of rounded cells, spheres, filaments, long unusual flattened shaped, multiple forms, irregular, unusual flattened plates, and aggregate as clusters (Zeikus 1977; Cuzin et al. 2001; Wagner et al. 2013; Weng et al. 2015; Gunsalus et al. 2016). Owing to the presence of flagella, methanogens are motile. The Methanococcales and Methanomicrobiales and the genera *Methanospirillum, Methanogenium, Methanolobus*, and *Methanomicrobium* (Jones et al. 1987; Gunsalus et al. 2016) exhibit motility. Moreover, methanogens are used to improve their positions in flotation habitats with the aid of gas vesicles. In addition, gas vesicles were explored in some mesophilic *Methanosarcina* strains and some thermophilic *Methanothrix* strains (Mah et al. 1977; Zinder et al. 1987; Kamagata and Mikami 1991).

2.3.2 Gram Reaction

Supreme methanogen strains are Gram-negative based on their broader sections of cell wall structural pattern. They lack the peptidoglycan layer. It has been disrupted during the staining regimen. Based on the investigation, if Gram stain preparation of a mixed microbial population containing methanogen and bacteria were analyzed, the methanogens exhibit Gram-negative reaction whereas the bacteria exhibit Gram-positive nature. Some of the limitations have occurred owing to the appearance of a type of peptidoglycan known as pseudomurein. It can make them Gram-positive (Todar 2009; Cuzin et al. 2001; Dridi et al. 2012; Gunsalus et al. 2016).

2.3.3 Methanogens as Syntrophs

Methanogens are commonly free-living. However, few cases act as syntrophs in the intestine of the rumen, arthropods, and vertebrates during interspecies hydrogen transfer. However, it is widespread among rumen protozoa and anaerobic ciliates. Besides, it can be associated intracellularly and extracellularly (Stams and Plugge 2009). Nevertheless, occasionally, it can also relate to anaerobic bacteria, fungi, and protozoa (Sharp et al. 1998; Joblin et al. 2002; Thauer et al. 2008). The protozoa harbor methanogens based on the fermentative type of *Clostridium*-type metabolism. They were reported in a few amoebaean flagellate species (Fenchel and Finlay 2010). The potential symbiotic betterment of methanogens with other species is used to understand methanogens' useful functional analysis.

2.3.4 Cell Envelope, Lipid Composition, and Antibiotic Resistance

Methanogens maintain a distinctive range of cell envelope formats. It is always simple and rigid, seldom nonflexible, with a lack of peptidoglycan (Albers and Meyer 2011). The methanogenic cell envelope was separated into three layers based on their cell wall components, viz. pseudomurein, protein or glycoprotein, and heteropolysaccharide layers (Sirohi et al. 2010). The pseudomurein is analogous to the peptidoglycan layer of bacteria. Some of them have a glycoprotein S-layer for their cell wall. It can provide support to the cell (Whitman et al. 2006). The *Methanobrevibacte*r and *Methanosphaera* lack S-layer. Besides, some of the methanogen cell envelopes consist of various layers of polymers. The *Methanobrevibacter* and *Methanobacterium* have L-talosaminuronic acid, and the *Methanosarcina* has methanochondroitin. The *Methanosarcina* can produce cell aggregates. It can synthesize a cell wall polymer, methanochondroitin (Kreisl and Kandler 1986; Todar 2009; Albers and Meyer 2011). The *Methanosarcina* possesses a heteropolysaccharide, and *Methanomicrobium* holds a protein layer (Balch et al. 1979).

Methanogens hold unique biochemical structures. It has a diverse class of core lipids by distinct polar associations (Garcia et al. 2000). However, it is chiefly glycerol ethers. The membrane lipids connect the alkyl chains to the glycerol using phytane or biphytate. Archaeal polar lipids consist of a core lipid added to the polar head group. The archaeal core lipids are saturated isopranoid chains attached via ether bonds to the glycerol 2,3-Sn carbon states (Sprott 2011). Novel core lipids identified from many methanogens such as caldarchaeol, allyl ether-type core lipids, digeranylgeranylglycerol, unsaturated archaeol, unsaturated hydroxy archaeol-based phospholipids, and polar lipids (Hafenbradl et al. 1993; Grather and Arigoni 1995; Sprott et al. 1997; Morii et al. 1998; Nicol et al. 2003). Ether lipids of methanogens act as a chemotaxonomic biomarker. In addition, they are also used in environmental research.

Methanogens are commonly insensitive to antibiotics such as penicillin G, D-cycloserine, cephalosporin C, vancomycin, and its derivatives. The deviations in the transformation between archaea and bacteria also cause antibiotics sensitivity for kanamycin (Whitman et al. 2006). Rifampicin influences the level of RNA polymerase and affects some methanogens. However, it inhibits all the tested eubacteria. This unusual trait has been used to isolate and purify methanogenic populations (Garcia et al. 2000).

2.4 Growth Parameters

The methanogens are found to extremely diverse in nature and are found well-habituated in all habitats. They present many unusual niches, and hence, their metabolism alters consequently. They survive at all temperatures and differ from freezing and boiling; pH varies from acidic to alkaline, and salinities cover from freshwater to saturated brine. The study of their growth parameters exhibits a versatile platform for the generation of functionally amplified methanogenic counterparts.

2.4.1 Temperature, pH, Pressure, and Salinity

Methanogens adopt them and survive in extreme conditions. It allows exploration of them as workhorses in many multifactorial stress conditions. The methanogens are incredibly diverse, ranging from psychrophilic, mesophilic, thermophilic, and even hyperthermophilic species (Lu and Lu 2012; Wagner et al. 2013). They are able to grow from 0 to 122 °C (Jablonski et al. 2015). They also comprise distinct acidophilic to alkaliphilic and halophilic to nonhalophilic organisms. They are found at temperature ranges from 20 °C to 98 °C (Boone et al. 1993). However, methanogenic pathways are operating in extensive temperature ranges from 0 to 122 °C (Cavicchioli 2006; Takai et al. 2008). Thermophilic habitats, including hot springs, hydrothermal vents, and solfatara, are also major parts of vibrant methanogenesis. In the total population, thermophiles cover 25% only. The methanogens too thrive energy from cold Antarctic lakes, marine sediments, inside rocks, located miles below located subsurfaces, and in extreme pH, salinity, and nutrient limitation areas. Based on the temperature tolerance range, like narrow or wide, the psychrophilic methanogens are classified as stenopsychrophiles (thrive only in a narrow temperature range) and eurypsychrophiles (thrive at elevated level) (Taubner et al. 2015). Many of them grow better within pH from 6 to 8 (Jones et al. 1987). Besides, they can exist in extreme pH environments.

Methanogens are enthralling candidates using their capability to tolerate and to grow in pressurized environments. Generally, the methanogens survive at a pressure range from below 20 MPa to above 300 KPa (Taubner et al. 2015). Based on this adaptation criterion, methanogens are cultivated under multifactorial stress conditions. They display enormous diversity in their growth salinity requirement. They are found in the salinities ranging from freshwater to hypersaline. They need a minimum of 1 mM Na^+ for active bioenergetics of its metabolism (Muller et al. 1987; Kaesler and Schonheit 1989). Some methanogens grow well up to 3 M salt concentrations (Zhilina and Zawarzin 1990). Recent research studies focus on their adaptations, which caused metabolic changes since they directly link microbial ecological studies of interspecies competition or symbiosis.

2.4.2 Substrate Range

Methanogens always depend on others for their substrates, which they convert into methane. Generally, methanogens utilize one or two substrates. The *Methanosarcina* showed limitations for their substrate utility. They show the ability to use seven substrates (Garcia 1990; Ferry 1993). *Methanosaeta*, the strict acetoclastic methanogen, uses only acetate (Smith and Mah 1980; Hedderich and Whitman 2006). Many different kinds of substrates are utilized during methane production, such as carbon dioxide, acetate, formate, and methyl substrates. Overall, 70% of the total global methane is from acetic acid, whereas the leftover 30% is derived from hydrogen and carbon dioxide (Conrad et al. 2011). The following equations depict the methanogenic methane generation from carbon dioxide, acetate, and methyl group substrates.

$CO_2 + 4H_2O$
$CH_4 + 2H_2O$ (from carbon dioxide)

$4CH_3OH \rightarrow 3CH_4 + CO_2 + 2H_2O$ (from acetate)

$CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^-$

$CH_3OH + H_2 \rightarrow CH_4 + H_2O$ (from methyl group)

Methanogens produce methane by three different pathways, such as aceticlastic, carbon dioxide reducing, and methylotrophic methanogenesis. It can diversify in the range of substrate, carbon compound used, and the source of reducing potential

(Bapteste et al. 2005). Based on their substrate requirement, methanogens were placed into three main nutritional categories (Liu and Whitman 2008).

2.4.2.1 Acetoclastic Methanogens

They are acetotrophic/acetate-splitting or acetate-fermenting methanogens, which use acetate as the dominant substrate for their 70–75% biogas formation (Zinder 1993; Garcia et al. 2000). These methanogens produce methane through the aceticlastic methanogenesis pathway, by which they cleave acetate into two parts: one carbon is used for methane formation, whereas the other is used during CO_2 formation. Nearly nine species of these methanogens generate methane utilizing the acetate's methyl group as their carbon source. Acetate's carboxyl carbon oxidation produces electrons of acetate reduction (Hedderich and Whitman 2006). The order Methanosarcinales is called acetoclastic methanogens since it uses acetate as the substrate for its methanogenesis. Methanosaetaceae uses only acetate as their substrate, but the other member Methanosarcinaceae is a versatile one, which can use other than acetates like CO_2, carbon monoxide, and methylated C1 compounds (Kendall and Boone 2006). Among the members of this order, the *Methanothrix* sp. displays a higher attraction of acetate than *Methanosarcina* sp. (Smith and Mah 1980). Besides, it performs an effective part in the digestion process of low concentration acetate sludge.

2.4.2.2 Hydrogenotrophic Methanogens

These methanogens are non-aceticlastic/CO_2-reducing/H_2-oxidizing/H_2 gas-consuming methanogens. Hydrogenotrophs are the earth's common inhabitants, found in all methanogenic orders except Methanomassiliicoccales (Vanwonterghem et al. 2016). These methanogens produce methane through the carbon dioxide reducing methanogenesis pathway. It uses hydrogen and CO_2 as the primary carbon source for methane production. Some of them use formate for methane formation (Garcia et al. 2000; Liu and Whitman 2008). Some of them also utilize secondary alcohols like 2-propanols, 2-butanol, cyclopentanol, and ethanol (Hedderich and Whitman 2006). However, hydrogen oxidation acts as powerful energy generating and terminal electron removing mechanism (Balch et al. 1979). These use an electron as an energy-conserving step in its metabolism that is distinct from other methylotrophic methanogens.

2.4.2.3 Methylotrophic Methanogens

These methanogens apply the disproportionation of methanol and methylamine for methane production. It is one of the relatively versatile nutritional groups. The substrates like H_2 and CO_2, acetate, methyl-group containing compounds such as methanol, methylamines (monomethyl amines, dimethylamine, trimethylamine, tetramethylammonium), methyl sulfide, dimethyl sulfides, methanethiol, and carbon monoxide are used for methane production. H_2 is utilized as an outside electron donor (Garcia et al. 2000). Besides, electrons are used for reducing these substrates rising from the oxidation of another methyl group (Hedderich and Whitman 2006). The orders Methanosarcinales, Methanobacteriales, and Methanomassiliicoccales are classified under these levels. It can also be categorized based on cytochromes' presence or need (Vanwonterghem et al. 2016).

Despite this, several additional criteria are also used for their classification. They include morphology, motility, nutritional spectrum, growth rates, growth conditions, metabolic end-products, Gram staining, susceptibility to lysis, antigenic fingerprinting, lipid analysis, distribution of polyamines, nucleic acid hybridization, G + C content of the DNA, 16S rRNA sequencing, and sequence analysis (Boone and Whitman 1988). According to these standards, significant orders, families, genera, and many species have been defined (Boone et al. 1993).

The proper understanding of the answers for following long-lasting fundamental questions about CH_4 biocatalysts, viz. (1) source of electrons, (2) natural community or synthetic co-culture controlling strategies, (3) genetic traceability, (4) CH_4-conversion potential with other processes like nitrogen fixation will address the industrial application as well as innovation challenges of methanogens (Kalyuzhnaya et al. 2020).

2.5 Bioeconomy-Based Technologies for Environmental Sustainability

Methanogens are the best bio-inspired economists, which plays a significant bioeconomy-based ecological role globally. This is believed to be the most appropriate time for methanogenic research to maintain the carbon cycle and reduce global warming. It acts as the environmental signaling engineer for climate change. Moreover, it will give a unique view of bioeconomy development in developed as well as developing countries. However, it has been an attractive, unique, unusual metabolism-performing candidate, which helps to understand better the thermodynamics and bioenergetics, evolution and biodiversity, and microbial interactions. Also, it gives wings to our sustainability through bioeconomy-based ecofriendly environmental approaches. The methanogenic archaea give tremendous applications for our environmental sustainability, as shown in Fig. 2.2.

2.5.1 Bio-Based Carbon Dioxide Capture, Sequestration, Utilization, and Conversion (CCSUC) Technology

The continuous emission of greenhouse gas (GHG), predominantly CO_2 in the atmosphere, becomes a significant concern and also gaining much attention on the environmental agenda nowadays. So many lines of evidence clearly show the tragical emission of atmospheric CO_2 into the environment. The emission of CO_2 in the environment is a primary reason for global warming (Singh and Dhar 2019). It also causes human nutrition threats (Myers et al. 2014), ocean acidification (Caldeira and Wickett 2003), sea level increase, modification of earth ecology, and global weather patterns. They result from mainly anthropogenic activities (Mistry et al. 2018) and pollutions of the fossil fuel burning, thermal power plants flue gases,

Fig. 2.2 Schematic description of applications of methanogens for environmental sustainability

various industrial applications, and vehicle emissions. Now, the world is facing an intrinsic environmental responsibility with a view first to control and reduce global warming. Hence, CO_2 emissions from anthropogenic activity reduction are necessary. The concentration of atmospheric CO_2 has been rising to a great extent since record-keeping began. A recent survey indicates that human activities' CO_2 generation level increases up to 20 Gt/year. With status-quo, this concentration will rise to 1020 ppmv by the turn of 2100 at an average.

The IPCC (intergovernmental panel on climate change) assessed that the global mean surface temperature would go up by 1.5–50 °C. It leads to unexpected vegetation changes, melting of ice in North Pole and mountains, global warming and warning, modification of overall weather and rainfall patterns, alteration of earth-atmosphere ecological balance, ocean acidification, and rising water level in the sea all over the world. It also steers to a loss of food production by up to 15% due to climate change. The effects are realized and have forced us to reduce the greenhouse gases in the environment (Van De Wal et al. 2011). It is equally essential to undertake CO_2 sequestration. CCSUC of CO_2 is a powerful new, safe, and environmental managing method developed to balance our environment's carbon cycle (Lackner 2003). Different options are being thought of for CO_2 capture. These include mostly the chemical and physical processes. Developed countries have initiated some programs in this direction. However, a breakthrough approach does not yet come. Physical, chemical, and biological methods need to be developed, tested, and applied for the sequestration of CO_2. Now, biological means are also being considered to find out the technological options for CO_2 sequestration. In biological processes, photosynthesis, autotrophic metabolism, and methane formation are possible. Higher plants' photosynthesis is one beneficial route. However, it has constraints like time and space.

The most promising route seeks the use of beneficial microbes. Microorganisms have a tremendous capacity to convert CO_2 to other useful metabolites in the absence or the presence of negligible oxygen. The biological sequestration employing methanogenesis is applicable in a variety of CO_2 emitting sources. It is the most promising emerging technology. In this process, carbonic anhydrase (CA) plays a vital role in having the atmospheric CO_2 into carbonate, a soluble source of carbon for microorganisms, further metabolized to produce useful carbon compounds (Smith and Ferry 2000). The formation of methane from CO_2 is one such process that produces valuable biofuel-methane and finds use in electricity generation. This is an environment-friendly approach with sustainability. In this process, formate dehydrogenase performs a vital part in hydrogen formation, which is needed for converting CO_2 into methane. One opening is to employ carbon dioxide-reducing methanogens for biological methane production (BMP), an intriguing group of an archaeal candidate for the effective conversion of carbon dioxide (Mayumi et al. 2013; Rittmann et al. 2013).

Bacterial methanogens are bio-inspired innate CO_2 encounters of effective sequestration. They have the power to be called bioremediator and CO_2 sequesters (Mistry et al. 2018). It uses non-photosynthetic CO_2 fixation pathways. Mainly use a 3-hydroxypropionate and 4-hydroxypropionate cycle (Mistry et al. 2018). These archaea can fix and convert around 70–80% of CO_2 to methane, while the leftover 20% is utilized for cell-mass build-up. Acetyl CoA/propionyl CoA carboxylase and carbonic anhydrase (CA) enzyme play a most critical role. Among them, CA is imperative at the industrial level. Using this kind of efficient microbial system, it may be possible to develop a sustainable process for the sequestration of CO_2. Furthermore, it contributes more to the global carbon cycle for organic matter degradation at the final step by supporting many aerobic methanotrophic communities (Zeikus 1977; Zeman and Lackner 2004).

2.5.2 Anaerobic Digestion

In various anoxic natural systems, anaerobic digestion (AD) occurs. It is an anoxic multistep, microbiological process. The complex molecules are converted into

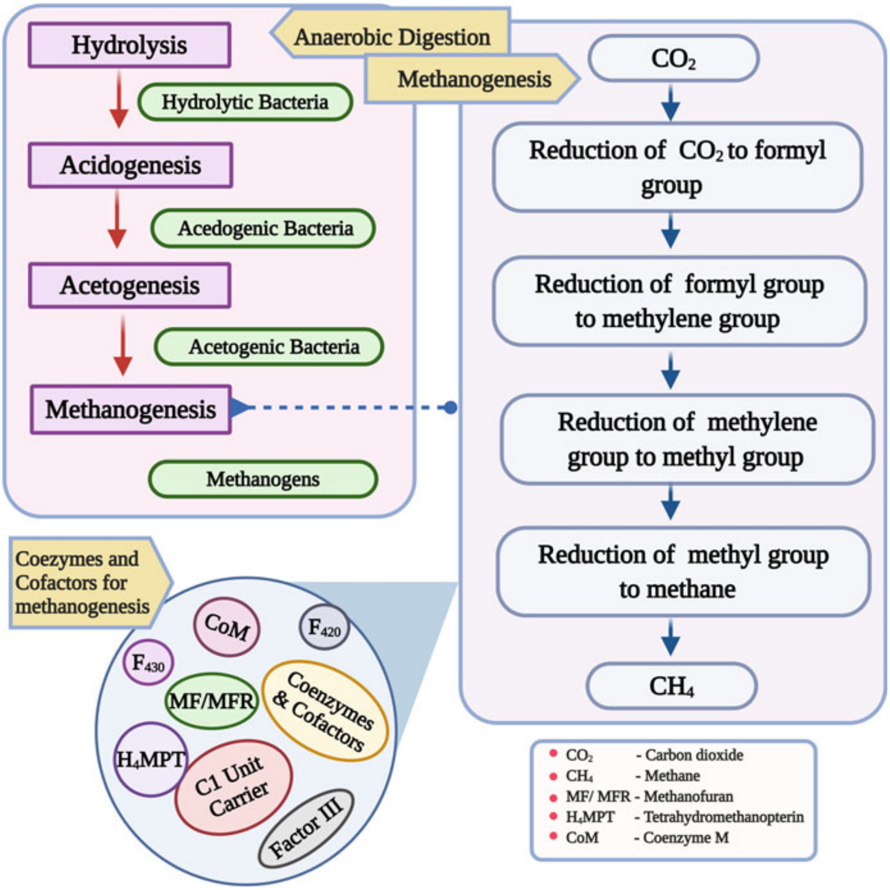

Fig. 2.3 Schematic representation of anaerobic digestion with methanogenesis

simple molecules like CH_4 and CO_2 by orderly play of following hydrolysis, acidogenesis, acetogenesis, and methanogenesis (Qiao et al. 2015). It was carried out by the various groups of microbes, viz. fermentative, syntrophic, acetogenic, and methanogenic bacteria (Shah et al. 2014). Among them, methanogens perform an energetic function in the last step of AD (Rosenzweig and Ragsdale 2011) (Fig. 2.3). Though it is the oldest method, it is now one of the latest ways to produce methane successfully. It is a reliable fourth-generation biofuel production technology involving direct sequestration of CO_2 into CH_4. Increasing energy demands and rigorous environmental and public wellness concern ensures the rising popularity of AD. Mainly, it has been exploring for waste treatment and resource recovery. It results in the production of potent biogas energy, nitrogen-rich digestate/effluent (Bolzonella et al. 2005), and wastewater treatment.

2.5.2.1 Microbial Food Chains of Anaerobic Digestion

There are five microbial candidates involved in anaerobic digestion, which decrease the biometabolic burden of individuals. The hydrolytic fermentative, obligate hydrogen-producing acetogenic, hydrogen-oxidizing acetogenic, carbon dioxide-reducing methanogenic, and methanogenic bacteria. It endures vitally within all the stages that interpolates hydrolysis (heterogeneous reaction), acidogenesis, acetogenesis, and methanogenesis (Chandrasekar 2006; Anukam et al. 2019). During biomethanation, complex polymers are converted into simple, soluble compounds using fermentative bacterial enzymes. It can metabolize the substrate to short-chain fatty acids, hydrogen, and carbon dioxide. Fatty acids, longer than acetate, are converted into acetate using obligate hydrogen-producing acetogenic bacteria. The principal outcomes after digestion of the substrate by these are hydrogen, carbon dioxide, and acetate. Hydrogen and carbon dioxide can be metabolized by hydrogen-oxidizing acetogens or carbon dioxide reducing, hydrogen oxidizing methanogens. These are produced metabolites, viz. acetate or methane, respectively. Besides, acetate is changed into methane by aceticlastic methanogens (Chandrasekar 2006; Anukam et al. 2019).

2.5.2.2 Methanogenesis

Methanogenesis, the biological production of methane by methanogenic archaea through the biological pathway, is a well-known natural, oldest bioactive anaerobic respiration mechanism and has little reducing potential than others (Gilmore et al. 2017). It occupies the terminal part in AD (Fig. 2.3). It has engrossed a high concentration of CO_2 and hence methane fuel production. It is a boon for developed and developing countries toward global warming mitigation and biofuel production through useful biotechnological applications (Zhu et al. 2020). Well-controlled methanogenesis is used for effective energy creation (Angelidaki et al. 2011). Methanogen's genomes display a paradigm shift toward energy creation and conservation (Gilmore et al. 2017). They are accountable for methane production by substrate-specific different methanogenic pathways (Singh 2009). The methanogenesis pathways include aceticlastic, hydrogenotrophic (Conrad et al. 2006), and methylotrophic pathways (Conrad 2007). Methane is mostly obtained from the aceticlastic methanogenesis pathway (Zinder 1990). However, the study of the metabolic background of methanogenesis can give the best key for energy lock.

Predominantly, methanogens possess two sets of specific unusual methanogenesis-involved coenzymes. C1 unit carrier coenzymes embrace methanofuran (MF/MFR), tetrahydromethanopterin (H_4MPT), factor III (modified B_{12}), coenzyme M (CoM), F_{430}, and the electron transferring coenzymes. It can transfer the electrons to the C1-intermediates, which includes coenzymes of F_{420}, F_{430}, and N-7-mercaptoheptanoyl-*O*-phospho-L-threonine (McBride and Wolfe 1971; Cheeseman et al. 1972; Gunsalus and Wolfe 1980; Noll et al. 1986). High concentrations of cofactor F420 act as a distinctive biochemical pointer, which is used to detect methanogens. The methanogens were observed as a bluish-green or greenish-yellow fluorescent cell under an epifluorescent microscope for UV at 440 nm. The F420 level differs in methanogenic populations based on its growth conditions (Gorris and Van der Drift 1994). The terminating part of methanogenesis is induced by MCR (methyl–coenzyme M reductase), which also induces the first step of methanotrophy. Its friendly anaerobic methane conversion depends on the coenzyme F430 (Zheng et al. 2016). The MCR alpha subunit has an *mcrA* gene, which acts as the molecular marker for methanogen detection (Lueders et al. 2001). Other than these, there are other coenzymes involved in improved methanogenesis, viz. FMN, FAD, and Coα-[α-(5-hydroxybenzimidazolyl]-cobamide. Moreover, various transition metals such as iron, nickel, cobalt, molybdenum, and tungsten are also required for active methanogenesis (Schonheit et al. 1979). In addition, methanogens also have many hydrogenases for vital methanogenesis (Fox et al. 1987).

2.5.3 Energy Pool: Biofuel

Almost all countries, including India, need alternatives to fossil fuels. The escalating energy demand worldwide, volatile and decreasing petroleum sources, and climate change concerns created a particular interest in super low carbon fuels, including biofuel, biogas, etc. Methanogens are called as the master of methane on earth (Hofer 2016). Methanogenic methane is one of the better options for future fuel due to its calorific value. The producer methanogens are the group of hydrocarbon-based methane gas producing anaerobic prokaryotes (Barker and Buswell 1956; Garcia et al. 2000; Enzmann et al. 2018). It is an energy-efficient and highly diffusible fuel. Most developing countries like India show interest and investment in biogas due to its fuel potential. It can be a suitable replacement for firewood, dung, kerosene, agricultural residue, diesel, and petroleum. Besides electricity, it can be utilized for local supply and price constraints. Also, the biogas system affords energy, fertilizer, and waste.

While it acts as a biofuel, methane production also reduces and probably meets the net accumulation of CO_2 in the environment. According to Lackner (2009), the burning of methane fuel converts into carbon dioxide, which re-backs the carbon dioxide in our environment and net gathering reduction. Hence, biogas is a technology-based environment-neutral energy resource with influential applications (Ravichandran et al. 2015). It is predicted that after 2020, the availability of fossil fuel would be depleted, and the emerging fuels replace only 30–35%. However, biofuel from methane is one of the potential alternates for economic energy demand (Haynes and Gonzalez 2014). It would accomplish 90–95% of global energy demand.

2.5.4 MEOR: A Combining Hand of Biodegradation and Biotransformation

The ever-increasing oil demand warrants an efficient oil recovery process since, even after primary and secondary treatments, most of the oil remains in the oil reservoir zone unrecovered. So, we need a potent tertiary treatment for an efficient recovery mechanism. Even though numerous studies have been conducted in this area focusing on the identification of suitable technology, several possible routes are seen, among them, microbial enhanced oil recovery (MEOR) is one of the viable energy changing equational approach (Brown 2010; Wawrik et al. 2012), which becomes the best holder of economic energy. The methanogenic archaeal community actively offers a most excellent route for MEOR mechanisms. Here, metabolizing oil through methanogenic biodegradation (methanogenesis) is carried out, producing methane by biotransformation of oil with great economic significance globally (Yernazarova et al. 2016; Nikolova and Gutierrez 2020). It is the most influential driving force for the growing energy-based economy. Methanogenic biodegradation is one of the emerging and impressing arrays for our energy sustainability. Methanogenic consortia biologically transfer the methane from the degradation of hydrocarbons under anoxic environments. In this biological treatment, no need for the replacement of an exogenous electron acceptor. So, it is a better viable bio-option compared with other anoxic treatment. It is also a critical biodegrading engineer for the hydrocarbon-contaminated sites and a potent energy creator through methanogenesis (Gray et al. 2010).

The proper hydrocarbon-consuming, non-pathogenic indigenous, or microbial methanogenic archaeal populations were injected along with nutrients at the oil reservoir area. It influences in situ microbial growth and produces microbial metabolites (methane) through its effective biodegradative methanogenic mechanism. It directly metabolizes, which influences the oil, its productivity enhancement, and reservoir life span. Commonly hydrogenotrophic methanogenic communities such as *Methanothermococcus, Methanobacterium,* and *Methanoculleus* were found in high-temperature oil recoveries (Yernazarova et al. 2016; Nikolova and Gutierrez 2020). Many field trials and best action strategies designed and developed for deep analysis of inoculums type, supporting nutrients, microbial communities, well injection methods, and microbial metabolites for enhanced oil recovery applications are conducted in developed countries.

2.5.5 Microbially Enhanced Coal Bed Methane (MECoM)

The energy and climate crisis has been paid an underlined special attention in current to-do lists. So we need to urge to develop innovative and environmentally welcoming technology. Diversified research work is going on for the identification and utilization of the new route. Coal is the key energy basis worldwide. The CO_2 emission reduction is actively made by CO_2 geological utilization and storage (CGUS) in coal beds (Talapatra 2020). Coal bed methane (COM) or biogastification, or coal bioconversion, is a growing research option landing in coal seams, which reduces the gap between energy demand and supply with a continuous sustainable future fuel cycle.

Nowadays, many potent approaches are followed to enhance the potential of Microbially Enhanced Coal Bed Methane (MECoM). It includes microbial stimulation and augmentation, physically enhancing microbial access to coal and the bioavailability improvement for coal organics. Among them, stimulation of methanogenic communities by injecting climate-changing nutrients such as carbon dioxide and inducing methanogenesis by indigenous methanogenic archaea for coal bed sequestration of carbon dioxide and effective methane recovery are environmentally preferred sound technologies. It is acknowledged as carbon dioxide enhanced coal bed methane (CO_2-EMBM) fills the sequestration and energy gap simultaneously (Budwill 2003; Gale and Freund 2008; Li and Fang 2014; Rathi et al. 2015; Ritter et al. 2015). It also expands the coal bed life and value with effective carbon dioxide sequestration. Nevertheless, it is also challenged by many qualms that make it very difficult. However, research in this area is still the initial phase and should learn about the microbiology-based technology for the future productivity of CBM. It acts as the potential storage of carbon dioxide and controls climate change.

2.5.6 Electromethanogenesis

The use of electric current directly induces microbial methane synthesis, an endowment technology for our economic growth. Potent conversion of electrical power into methane by electroactive microbes (methanogens) through the electrochemical system or microbial electrolysis is known as electromethanogenesis (Cheng et al. 2009) or microbial electrosynthesis (MES) (Enzmann et al. 2018). Cheng and his coworkers first described electromethanogenesis in the year 2009 (Cheng et al. 2009). BEP2G (bioelectrical power-to-gas) is a novel method for storing superfluous electricity through CH_4 (Geppert et al. 2016). However, several research studies are still going on electroactive microorganisms. Among them, an ecological and economical friendly worker is methanogenic archaea. It can uptake extracellular electrons from a stable donor directly (Yee et al. 2019). Methane forms by direct electromethanogenesis (direct uptake of an electron from the electrode) or mediated electromethanogenesis. It is effectively involved in treating waste, CO_2 fixation, and renewable energy storage (Cheng et al. 2009; Enzmann et al. 2018). Many studies investigated electromethanogenesis for better economic growth. Among all methanogenic communities, the thermophilic methanogens act as potent biocatalyst (Fu et al. 2015). Electromethanogenic activity would be enhanced for improved electromethanogenesis toward future fuel-making processes.

2.5.7 Corrosion Prevention

Generally, iron corrosion leads to significant economic damage. Interestingly, the recent finding figures out that the profusion of Methanobacteriales acts as the potential fighter against iron corrosion by its underlying mechanisms (Zandt et al. 2019). Recently, microbially induced corrosion (MIC) inhibition by microbiologically influenced corrosion inhibition (MICI) is an eco-friendly emerging corrosion controlling technology with socio-economic outputs. Remarkably, Kip et al. (2017) in the Netherland documented the high abundance of the

Methanobacterium population and their role in MIC and MICI technology. It paves a new path for developing novel technology of rust-fighting, which creates a better in situ screening for sustainable alternate methods. Commonly, it is used in protection against floods, dike contractions, and reinforcement of riverbank (Zandt et al. 2019). We need deep research attempts for a more beneficial perception of the ecological and biochemical alignment of methanogenesis. However, these low-cost biological complements from methanogens hold many promises.

2.5.8 Waste Management

Biomethanation is an effective waste treatment technology for a sustainable green environment (Qiao et al. 2015). An interdependent microbial community and methanogens carry out the degradation of waste. The methanogens complete the final step through methanogenesis, which is producing methane. The methanogenic consortium is commonly used to treat agricultural and toxic waste (Taherzadeh and Karimi 2007; Venkata et al. 2008). Anaerobic wastewater treatment is an established, environmentally, and economically beneficial process. The industrial and municipal solid waste (MSW) organic compound degradation produces methane, which finds use as alternative energy. Methanomicrobiales are the most abundant community in MSW treatment (Bareither et al. 2013). Methanogens are prominent members of communities within the engineered environment of anaerobic wastewater treatment. Currently, *Methanosaeta* is a strong candidate for effective wastewater treatment (Pyzik et al. 2018). It is a cost-effective treatment (Dhakephalkar et al. 2019). Besides, Tabatabaei et al. (2010) reviewed the methanogenic community's importance and characteristic features in wastewater treatment and revealed their possible applications.

2.5.9 Bio-Hydrogen Production

Some recent studies bracket that few methanogens produce potentially and economically supportable biohydrogen (Valentine et al. 2000; Goyal et al. 2016). As the condition of limited ambient hydrogen availability, some methanogenic species produce hyper, reducing equivalents and convert them into hydrogen (Valentine et al. 2000). Methane was excluded from electron sources, and organic metals were added for its active growth. It is the temperature-dependent mechanism. Lupa et al. (2008) strongly reported that the formate consuming *Methanococcus maripaludis* produced an increased amount of hydrogen in the mesophilic environment. Now, the research demand shows a much-increasing trend toward biohydrogen production through anaerobic digestion. Many exciting research studies are going on hydrogen consuming and hydrogen-producing methanogens, which open the window toward sustainability.

2.5.10 Other Active Applications

Methanogens are also dynamically involved in the biological nitrogen-fixing mechanism. The biochemical analysis of nitrogen-fixation is well studied in methanogens (Leigh 2000). Methanogens are also produced as effective biofertilizers through anaerobic digestion (Abdulkarim et al. 2019). In 2016, Goyal and his colleagues recorded that the manipulated *Methanococcus maripaludis* produced terpenoids, methanol, and other value-added products. Rother (2020) reported that metabolically modified methanogens also synthesizes valuable by-products such as isoprenoids, mainly used for medical, food, cosmetics, and other industries. In Lyu et al. (2016) produced geraniol instead of biofuel using genetically modified *Methanococcus maripaludis*. The metabolically engineered *Methanosarcina acetivorans* used methane for lactate production (McAnulty et al. 2017). Methanogenic archaea could exist in subsurface environments, and it is one of the ideal organisms for the Martian surface (Formisano et al. 2004; Koffmar 2014), which produces biogenic methane on Mars. It also acts as the model microbe for habitability assessment (Maus et al. 2020). The detailed study of the methanogenic microbiome also creates a new lightening path for microbial ecological studies with interspecies hydrogen transfer mechanism (Zhu et al. 2020). The detailed knowledge and its application process of bio-inspired candidate methanogenic archaea are not still fully understood. It is a “black box process” up to the present day. So, a fundamental understanding of methanogenic microbial populations would result in future sustainable, effective environmental solutions.

2.6 Conclusion and Future Perspectives

In conclusion, this chapter efficiently brackets that the in-depth study of mysterious methanogenic archaea offering a great scientific platform for sustainable revolution and solution for our economic and eco-friendly environment. Also, sharing knowledge about primary characters and workhorse applications of methanogens would lead to their full promised exploration. Nevertheless, this chapter also contributes to the most extensive evaluation of methanogenic activities, particularly for enhanced biofuel production and effective carbon dioxide sequestration. Additionally, it involves other activities like anaerobic digestion, waste management, nitrogen fixation, and improved bioproducts production. Eventually, it gives an outlook on the feasibility studies on Mars.

A different perspective is to develop a broader study for building up a potent electroactive methanogenic consortium for effective electromethanogenesis, which would create a robust biomethanogenic research and methanoeconomy-based bioresource management foundation system for our future. It would lay the foundation for the development of small-scale-level electromethanogenesis-based eco-factories. Genetic manipulation study is needed to develop an effective bioeconomy. So, a study on methanogenic archaea would formulate a touch of excitement in our environmental research realm. The methanogenic pathway itself has captured the curiosity of many for decades. Today methanogens have improved standards of living through energy crisis and paradigm shift. It will be a need for future enhancement.

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Chitosan Coating Biotechnology for Sustainable Environment

3

Amr Sayed Emam Ismail

Abstract

Chitosan biotechnology has received more attention in the past years owing to its potential usages in successful implementations attributed to eco-friendly modification and improvement of life. This chapter reports that chitosan (CHI) as a natural polysaccharide polymer can be integrated well with long-chain synthetic polymers such as polyester (PE), polyurethane (PU), polyvinyl acetate (PVA), and carboxymethylcellulose (CMC) throughout standard blending procedures. These polymeric resins are miscible with CHI to produce chitosan-based systems. The experimental results revealed that the integration of CHI with the polymeric blends improved its physicochemical characteristics and microbial activity. From the obtained investigations, the CHI polymeric composites can be used in several technologies such as coating, hydrogel, membrane separation, food preservation, and medical engineering.

Keywords

Chitosan · Polymers · Composites · Eco-friendly · Biotechnology

Abbreviations

BW	Butanol water
CCl_4	Carbon tetrachloride
CHI	Chitosan

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CMC	Carboxymethyl cellulose
EW	Ethanol–water
FCl	Fumaryl chloride
FTIR	Fourier transform infrared spectroscopic
IW	Isopropanol–water
MA	Maleic anhydride
MW	Methanol–water
PBS	Polybutylene succinate
PE	Polyester
PECs	Polyelectrolyte complexes
PEG	Polyethylene glycol
PEGF	Polyethylene glycol fumarate
PEGM	Polyethylene glycol maleate
PHA	Polyhydroxyalkanoate
PLA	Polylactic acid
PU	Polyurethane
PVA	Polyvinyl acetate
RT	Room temperature
SD	Swelling degree
SEM	Scanning electron microscopy
TDI	Toluene diisocyanate
UPECMs	Uniform polyelectrolyte complex membranes
XRD	X-ray diffraction

3.1 Introduction

Several valued research techniques have been operated by the scientific institutions to produce new generations of biopolymers. The technological attention raised dramatically for composites containing natural constituents as feed stocks require more evolution, to be more efficient (Agarwal 2020). The fabrication of bio-polymeric composites based on modified materials and components are reported (Fig. 3.1) (Vasconez et al. 2009; Avérous and Pollet 2012).

Green trends are employed in several implementations like seafood waste processing, polysaccharides investigation and crude amino acid production (Vasconez et al. 2009; Ismail and Farag 2020; Farag et al. 2018, 2019; Ismail et al. 2019). The accelerating attention in a clean and safe ecosystem plays a significant part in utilizing significant components. These eco-friendly composites have the ability to biodegrade throughout the bioremediation of microorganisms which in terms provides significant environmental impacts (Vasconez et al. 2009; Avérous and Pollet 2012; Agarwal 2020; Lucas et al. 2008).

Enormous requests for obtaining bio-composites based on polymeric materials are raised significantly. Based on high potential attempts, kinetic energy and

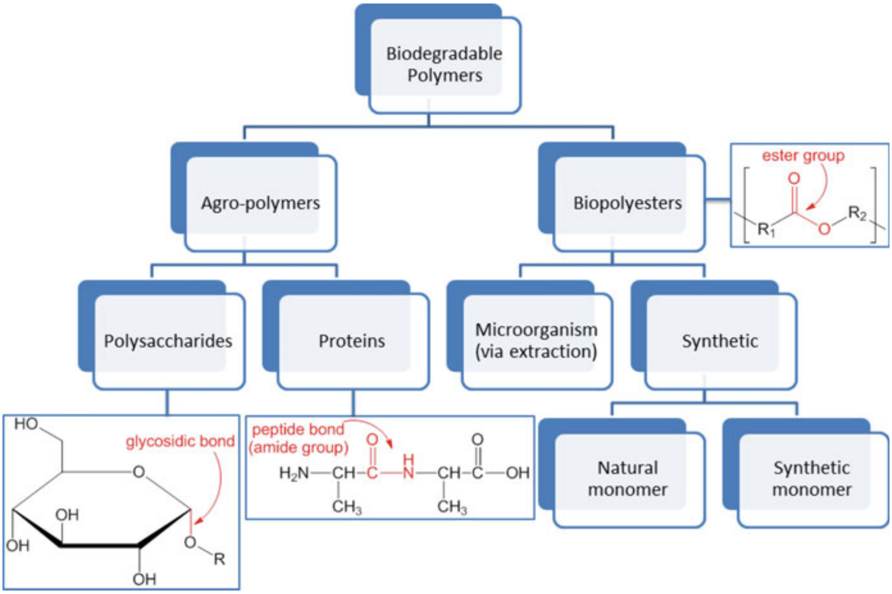

Fig. 3.1 Classification of the main biodegradable polymers

temperature control are studied (Andrew et al. 2020; Okada 2002). The present chapter discusses the characterization of chitosan as a substantial eco-meable constituent and its effective part in modern life.

3.2 Coating Technology

A broad coverage is employed under the name of coating for exterior improvement and modification. Global technologies and strategies have currently added new bio-component series according to market needs. Several utilizations for more comfortable lifestyle have been reported (Joanna 2020; Fristad 2000). The large-scale manufacturing of the coating (Fig. 3.2) reveals the modification rate.

There are several continual scientific challenges to enhance and modify the current trends. Part of these methods has been employed for many years such as physicochemical vapor deposition techniques for thin films (Mureşan et al. 2015), magnetron sputtering (Wang et al. 2016), ion beam (Hino et al. 2015), and laser deposition (Grigoriev et al. 2015) because they avoid the complexities and costs of vacuum processing. Modern researches have been published to deal with the electrodeposition of metals and alloys using multi-generations of ionic liquids (Farag et al. 2009; Ismail 2016a, b). The usage of coatings includes mechanical implementations such as hardness, wear resistance, and protective coatings with very wide industrial implementations (Tracton 2007).

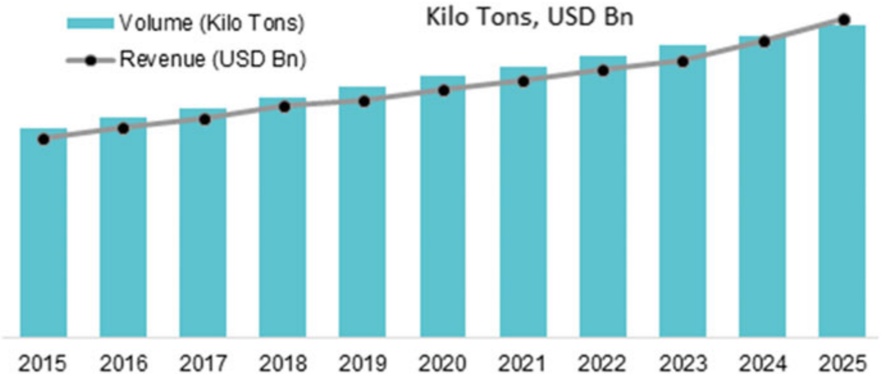

Fig. 3.2 The global coating market size

For decorative fields, the coating process is applied using several advanced components employed to modify the decoration performance (Binyamini et al. 2015). The large-scale utilization of coatings in the preservation of fresh food (Sousa et al. 2016) and catalysis (Essakhi et al. 2011) was studied. New types of composites are being developed and modified, based on green biodegradable polymeric substances, which provide significant properties and implementations. Several biopolymers have achieved great success in various implementations such as medical, chemical, and food technology. These biosystems offer the availability to get healthy life (Vasconez et al. 2009; Avérous and Pollet 2012; Agarwal 2020).

Biodegradables show a lot of properties like nontoxicity, nonpollution, biocompatibility, good performance, gas barrier, and low cost (Agarwal 2020; Lucas et al. 2008). For food preservative technology, crude protein of seafood group is more perishable than animal flesh (Venugopa 2011). The rising demand for preserving seafood using new processes and techniques is dependent on modified edible coatings (Kafrani et al. 2016). An increasing concern in the formation of antimicrobial edible chitosan-based coatings has been reported (Vásconez et al. 2009). Also, edible chitin and chitosan composites are employed nowadays in medicine as surgical sutures and artificial skin (Dutta et al. 2004).

3.3 Chitosan

Recently, chitosan as an abundant basic biopolymer has been utilized during the last period. Chitosan is prepared by chitin processing method in a basic system (Fig. 3.3). Chitin is a naturally occurring biopolymer derived from the exoskeleton of crustaceans and fungi (Farag et al. 2018; Dutta et al. 2004; Marguerite 2006; Kumar et al. 2004). CHI with an average molecular weight of 110,000 and deacetylation degree of 84.7% was prepared locally from brown shrimp (*Metapenaeus monoceros*) shell wastes (Ismail 2015; Methacanon et al. 2003; Yaghobi and Hormozi 2010; Feng et al. 2012). Chitosan has a lot of

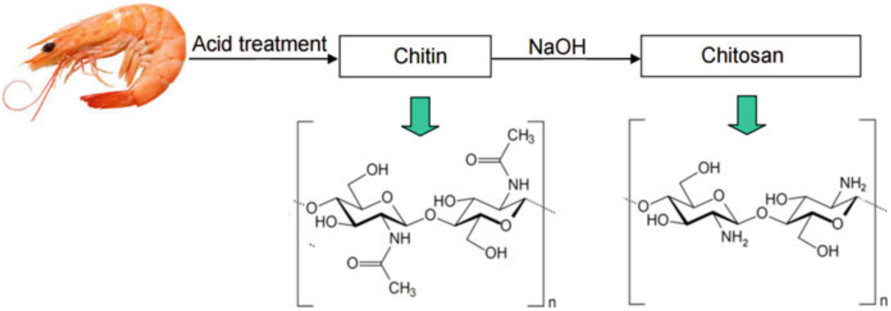

Fig. 3.3 Schematic diagram of the chitosan processing method

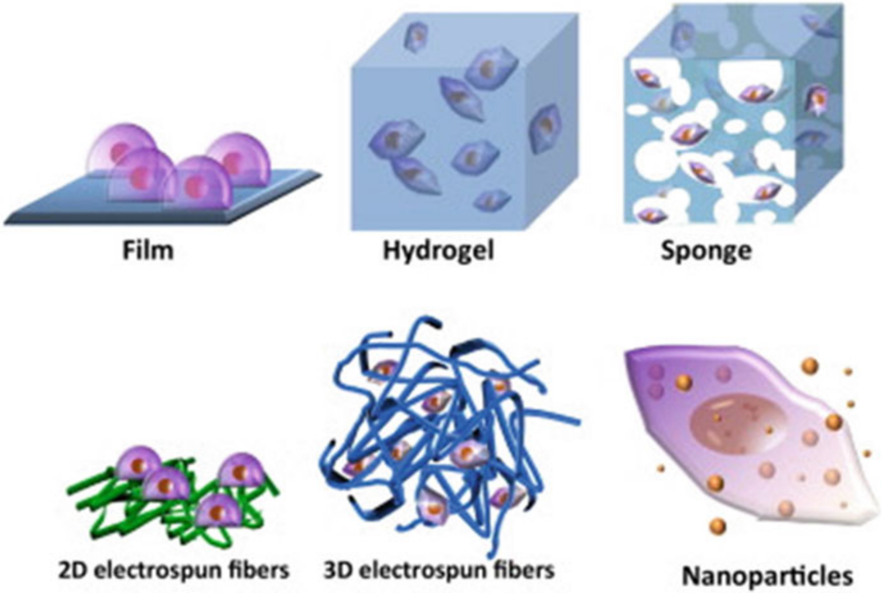

Fig. 3.4 Some biomedical applications of chitosan

implementations owing to its nontoxicity, toughness, gas permeability, and simple production method (Choi et al. 2016; Anirudhan et al. 2016; Chen et al. 2011; Baldrick 2010; Davis 2006).

Due to its polycationic nature, CHI was used in some biomedical applications (Fig. 3.4) such as anti-oxidant (Hafsa et al. 2016), anti-microbial (Hafsa et al. 2016; Chien et al. 2016), anti-tumor (Chien et al. 2016; Jiang et al. 2016), hydrogels (Jiang et al. 2016), anti-bacterial (Huang and Peng 2015), anti-fungal (Huang and Peng 2015), analgesic effects (Okamoto et al. 2002), and nanoparticles (Madureira et al. 2016).

Owing to its high hydrophilicity (Clasen et al. 2006), CHI gained special attention in gas separation technology (Liu et al. 2008a; Xiao et al. 2007), reverse osmosis (Musale et al. 1999), and pervaporative dehydration (Ismail 2015; Liu et al. 2008b; Tsai et al. 2008; Rao et al. 2007; Kittur et al. 2005). Moreover, a lot of separation methods were used for azeotropic mixtures throughout pervaporation techniques (Ulbricht 2006). Separation of alcohol from water is vital in organic synthesis.

Among the several methods, pervaporation gives high separation results and low cost (Chapman et al. 2008; Shao and Huang 2007). For this technique, the preparation of specific membranes with high hydrophilicity achieved high swelling degree and good permeation flux. For improving the CHI membranes, several methods were applied such as polymeric blends (Ismail et al. 2016; Veerapur et al. 2007; Devi et al. 2006; Zeng et al. 2014; Ariyaskul et al. 2006; Dubey et al. 2005), hybrid polymer (Lu et al. 2006; Hu et al. 2007; Liu et al. 2005), and crosslinking (Fiamingo and Filho 2016; Zhang et al. 2007; Choudhari et al. 2007).

3.4 Chitosan-Based Coatings

In the last few years, the chitosan-based polymers and their derivatives find great concerns in coating technology due to their unique characterizations. The following sections discuss the investigation of chitosan crosslinking with some selective polymers such as PE, PU, PVA, and CMC.

3.4.1 Chitosan-Based Polyester (CHI-PE)

Polyesters are commonly referred to as high molecular weight compounds containing ester links in the main structure of the molecules. These compounds incorporate acidic and alcoholic entities bonded alternately to one other. The polyester molecules may have a linear structure, from functional alcohols and acids, or a branched structure, if the constituents are greater than two. The polyester resin molecules may be terminated by other groupings besides hydroxyl and carboxyl groups, depending on the polycondensation reaction conditions and the starting compounds (Fig. 3.5). However, even for the alcohol to acid ratio of 1:1, the numbers of terminal groups of each kind may not be equal owing to side reactions that occur during synthesis (McKeen 2015).

Owing to the large spectrum of characteristics and applications of polyester compounds, thermoplastic, or thermosetting, it is mostly used in molding, films, coatings, composites, fibers, rubbers, and plasticizers (Kandelbauer et al. 2014). PEs are produced by a variety of manufacturing techniques such as direct polycondensation esterification, melt transesterification, acylation, interfacial polycondensation, and ring-opening polymerization (Rosato et al. 2004). Currently, essential bio-based polyesters have been widely used in the biological industry (Zia et al. 2016). Additionally, many thermoplastic polyesters based on renewable biomaterials are produced from bio-based diols with dioic acids like PET, PBT, and PTT (Oulame et al. 2015; Fuessl et al. 2012).

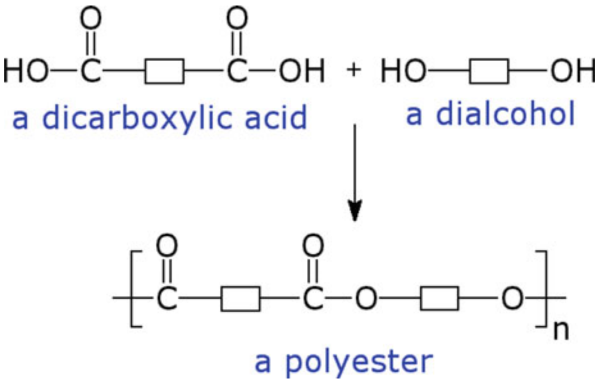

Fig. 3.5 The schematic reaction of dioic and diol for preparing polyester

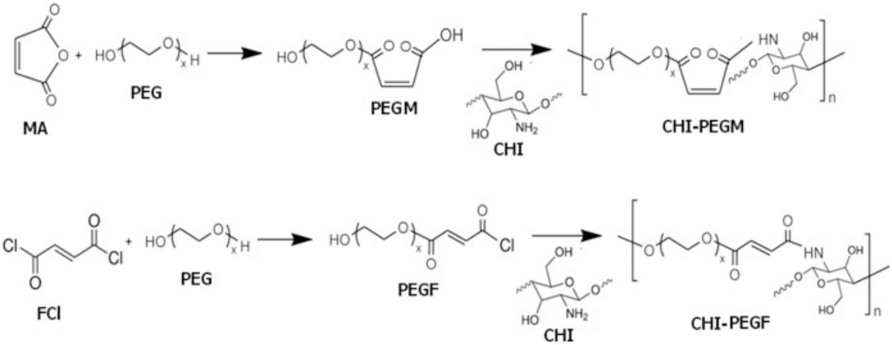

Fig. 3.6 A schematic pathway for preparing CHI-PEGM and CHI-PEGF

3.4.1.1 Synthesis

CHI-PEGM macromeres were prepared over stepwise pathway. First, the interaction between the anhydride (MA) and the glycol (PEG) with benzoyl peroxide (catalyst) to obtain the polymer resin (Fig. 3.6) which is detected by GPC analysis. The second step is the casting method by the addition of CHI (dissolved in acidic media). After several standard procedures, the CHI-based polyester resin is obtained (Doulabi et al. 2008a, b, 2013).

3.4.1.2 Fourier Transform Infrared Spectroscopy (FTIR)

The investigated FTIR analysis (Fig. 3.7) of the obtained polyester with and without CHI are illustrated based on the following sequence: 3500–3200 cm^{-1} (OH) and (NH) for both CHI and CHI-PE, 2910 cm^{-1} (CH), 1724 cm^{-1} (C=O), 1100 cm^{-1} (C-O), and 810 cm^{-1} (CH). The current performance assured that CHI and PE interacted well to form new materials (Feng et al. 2012; Fuessl et al. 2012; Doulabi et al. 2008a, b).

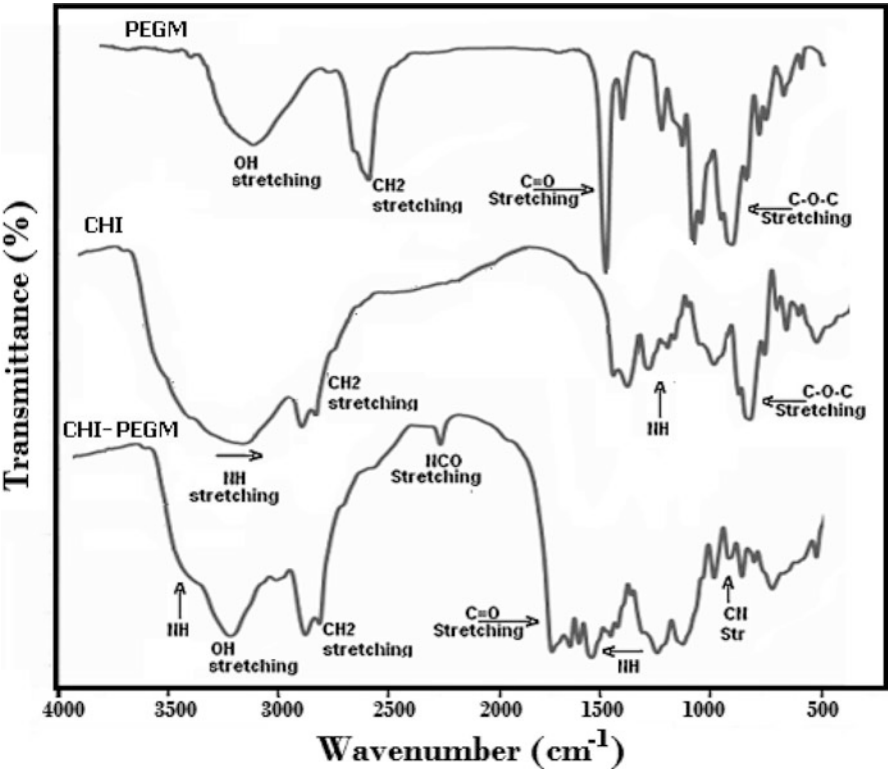

Fig. 3.7 FTIR of PEGM, CHI, and CHI-PEGM

3.4.1.3 X-Ray Diffraction (XRD)

Diffraction analysis (Fig. 3.8) exhibits patchy manner for both the systems (Lucas et al. 2008; Feng et al. 2012). Strong peak appeared at 2 h = 20° for polyester resin and two significant peaks revealed at 2 h = 12.2° for chitosan in which this value reduced after modification (Doulabi et al. 2008a, b).

3.4.1.4 Scanning Electron Microscope (SEM)

The morphological style was illustrated (Fig. 3.9). The obtained film was reduced with phase separation to some extent. The surface of CHI-PEGM was rougher than the CHI film (Baimark and Srihanam 2010).

3.4.1.5 Swelling Performance

The performance of the swelling effect of CHI-PEGF films (Fig. 3.10) was investigated based on equilibrium water uptake (EW_u %) using Eq. (3.1). The values of EW_u showed that all systems had the capability for water retention. Moreover, the blend films with 60% and 80% CHI content ratio yielded 258% and 305% of EW_u, respectively, than the others. This may be correlated to the existence of the

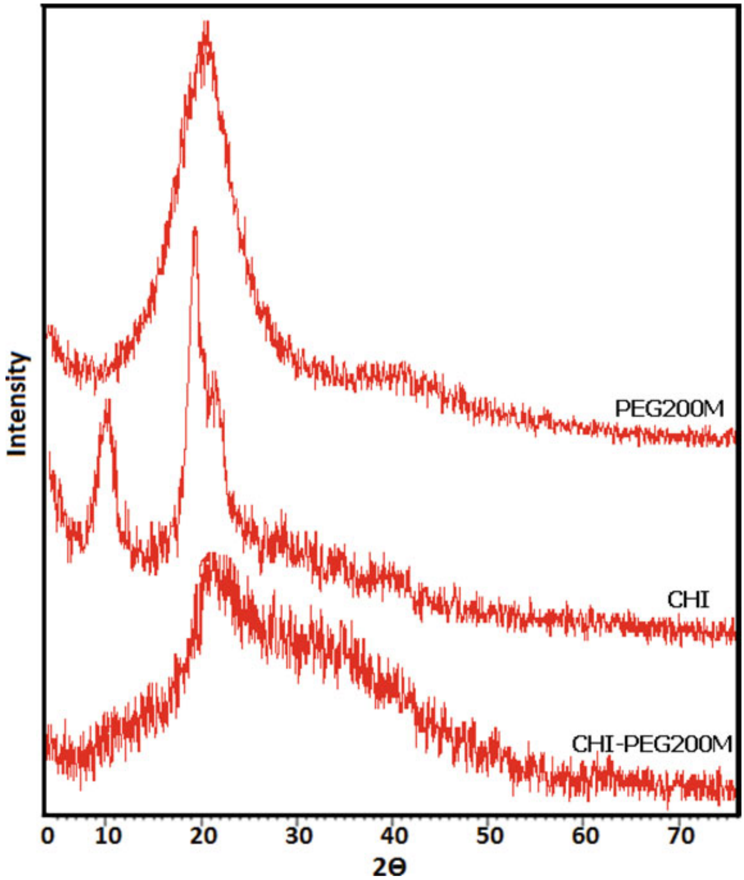

Fig. 3.8 XRD of PEGM, CHI, and CHI-PEGM

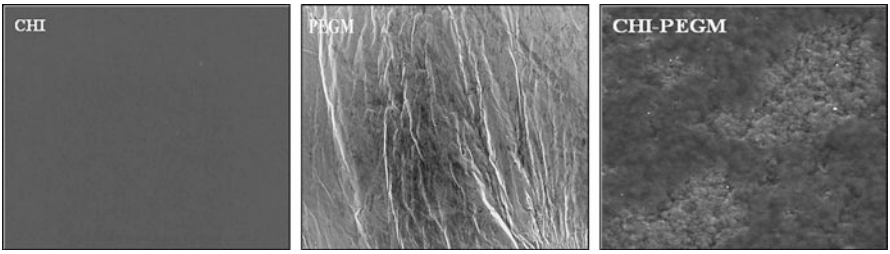

Fig. 3.9 SEM images of CHI, PEGM, and CHI-PEGM

functional OH in the films. This trend reduced in the case of 40% CHI content ratio may be due to the interactive effects between CHI and PEGF (Doulabi et al. 2013; Correlo et al. 2007).

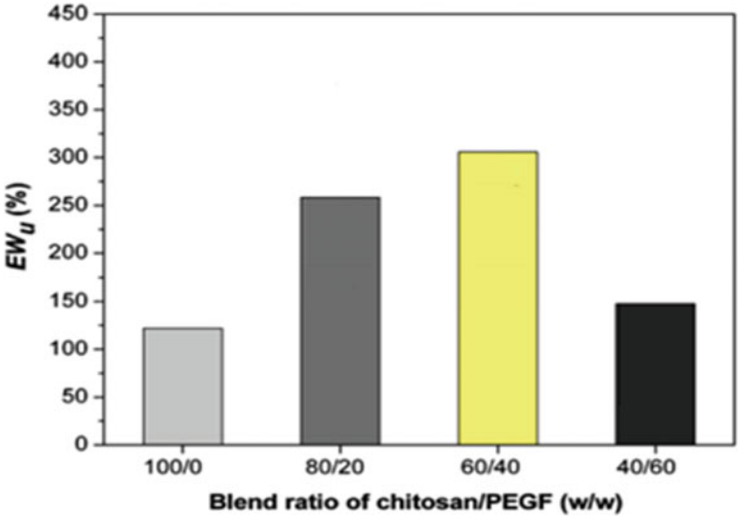

Fig. 3.10 Swelling based on water uptake (EW_u) of CHI-PEGF films

$$W_u(\%) = (W_t - W_o)/W_o \times 100 \tag{3.1}$$

3.4.1.6 Antibacterial Activity

Bio-activity of CHI and CHI-PEGF is reported in Fig. 3.11. All samples of CHI and CHI-PEGF affected on *Pseudomonas* and *Staphylococcus* bacteria. CHI composites exposed significant antibacterial resistance against *Pseudomonas aeruginosa* except for 80% CHI content films. By increasing PEGF, the interaction of CHI with bacteria may be decreased because CHI reduces the bacterial growth as well as the hydrophilicity of the film (Li et al. 2011a, b).

3.4.2 Chitosan-Based Polyurethane (CHI-PU)

PU has significant characterizations such as flexibility and abrasion resistance that finds a large scale of applications for various purposes. Currently, polyurethanes are used in various industries and biomedical implementations such as synthetic resin, coatings, vehicles, fibers, foams, paints, adhesives, elastomers, and artificial skin (Hepburn 1992; Lucas et al. 2008).

3.4.2.1 Synthesis

Polyurethane elastomers are mainly prepared from hard isocyanate portion with soft diol portion like PEG. Many research works studied the influence of chemical structures of diisocyanate compounds on crystallinity degree, surface morphology, and thermal stability of polyurethane films. Soft polyurethanes accept plasticizers

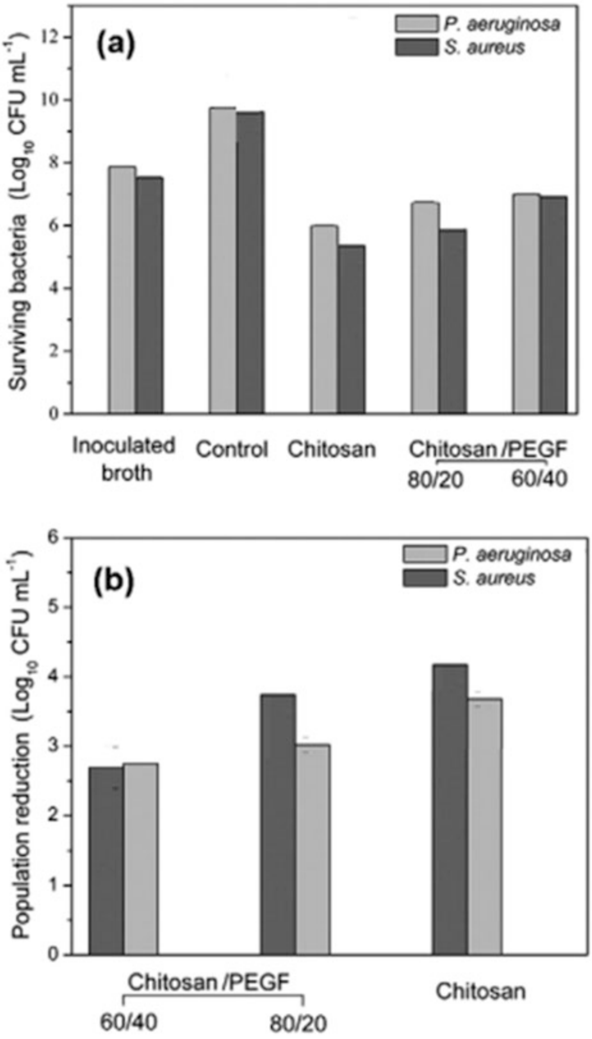

Fig. 3.11 Antibacterial activity of CHI-PEGF films for different blend ratios

with almost predictable changes in physical characterizations, meanwhile hard polyurethanes which a large increase in elasticity and decreasing of T_g would be undesirable. Small additions of plasticizers or other liquids can produce valuable foam. Polyurethane prepared from polyols and diisocyanates by a prepolymer method gives good mechanical properties (Zia et al. 2014; Alves et al. 2009). In the present section, polyurethane (PU) elastomers were prepared from TDI and PEG. First, PU with molar ratio NCO/OH = 4 was prepared by the addition of TDI to a solution PEG and CCl_4 under stirring and heating. CHI-PU was prepared by the addition of CHI (5 mg/mL chitosan in 1% ethanoic acid) giving an amorphous layer (Lucas et al. 2008).

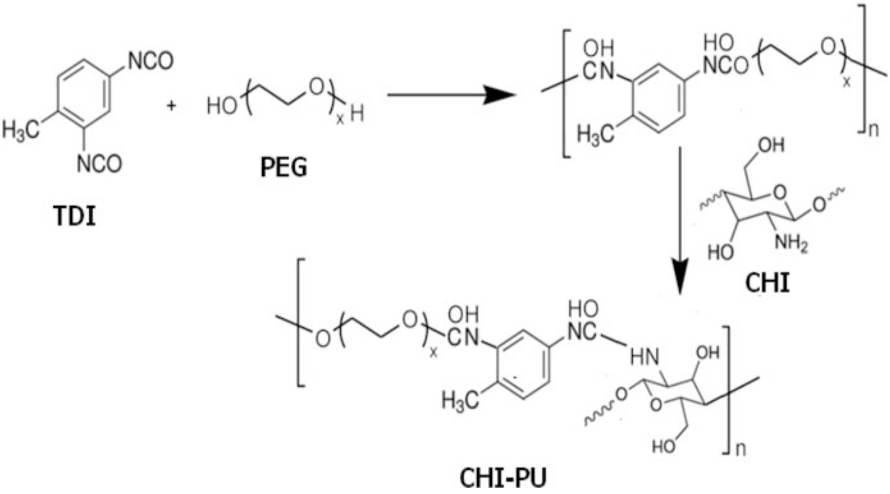

Fig. 3.12 A schematic pathway for preparing CHI-PU elastomers

The obtained products of PU and CHI-PU were characterized for comparison. Thus, chemical modification has led to opportunities for new compositions with significant properties for many end-user applications (78). The preparation pathway of CHI-PU composites is illustrated in Fig. 3.12.

3.4.2.2 Fourier Transform Infrared Spectroscopy (FTIR)

CHI-PU structure was detected by FTIR analysis (Fig. 3.13) and compared with the unmodified PU sample in the absence of CHI. CHI-PU spectra were appeared at 3300–3500 cm^{-1} (OH and NH), 2910 cm^{-1} and 2880 cm^{-1} (CH_2), 1720 cm^{-1} (C=O), 1440 cm^{-1} (CH_2), 1260 cm^{-1} (C-O), and 1173 cm^{-1} (C-O-C). The behavior of the CH_2 revealed owing to the glycol portion as shown for CHI-PU as compared with PU.

The FTIR spectra of PU exposed a spectrum at 3223 cm^{-1} due to the OH at 2277 cm^{-1} attributed to the N-C=O group attached to TDI. The spectra also show sharp peaks at the range 1500–1600 cm^{-1} due to the C-C of the aryl group. Other spectra at 1445 cm^{-1} (CH_2 bending) show that te peak intensity of the OH group is increased and the NCO group is decreased slightly (Zia et al. 2014; Murugan et al. 1998).

3.4.2.3 X-Ray Diffraction (XRD)

Figure 3.14 for CHI and PU were generally amorphous, as described in the previous report (Murugan et al. 1998; Feng et al. 2012). For CHI-PU composition, it showed strong peaks at 2 h = 19.2° and 2 h = 22.6°, meanwhile, this performance is reduced at 2 h = 14.7°, 2 h = 23.5°, and 2 h = 25.7°, as a result of the PU segments. The peaks associated with their crystalline structure were also observed. Similar behavior

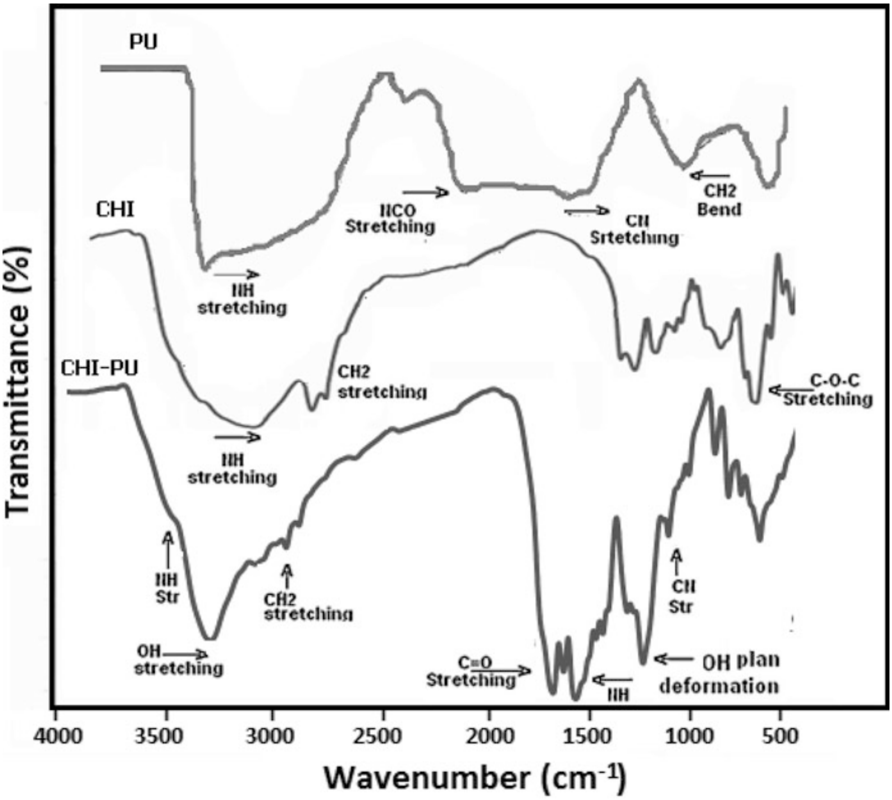

Fig. 3.13 FTIR of PU, CHI, and CHI-PU

was observed for CHI-PU but yielded residue that was difficult to handle. The segment exposure was also verified (Zia et al. 2014; Alves et al. 2009).

3.4.2.4 Scanning Electron Microscope (SEM)

The PU matrix, CHI segments, and CHI-PU composite were studied (Fig. 3.15). PU revealed a bad surface film. These formations are commonly due to the employed condition of O_2 and temperature, and under high energy beam of the electron, the macroparticles are decomposed into gases like CO or CO_2. CHI-based PU appears as small particles and micro-holes which might be physical crosslinking occurred. This appearance may be due to tri-functional CHI molecules which were impeded into the matrix to obtain a 3D structure, which assured the formation of CH-PU composites (Zia et al. 2014; Murugan et al. 1998).

3.4.2.5 Wettability

The surface hydrophilicity of CHI-PU films based on the measured water contact angles (θ) was investigated (Table 3.1). PU values are decreased by increasing polarity. It is reduced significantly for CHI-PU films. Moreover, CHI contains

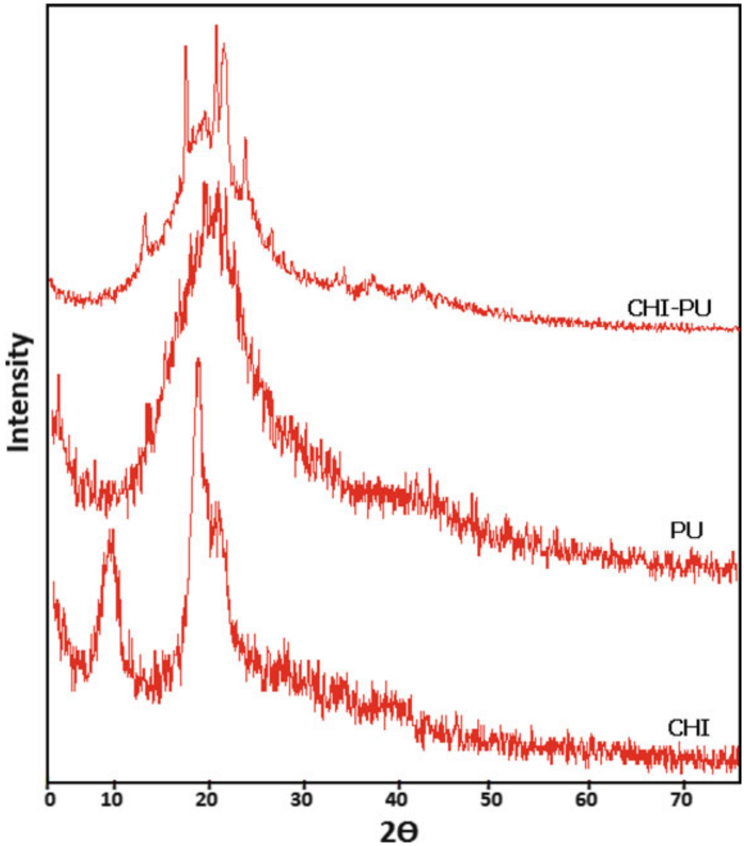

Fig. 3.14 XRD of PE, PU, and EDA-CHI-PEU

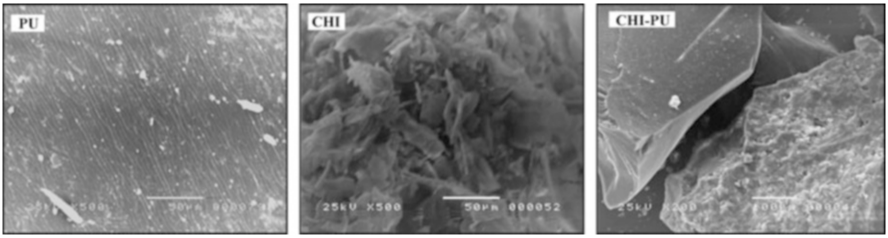

Fig. 3.15 SEM image of PU, CHI, and CHI-PU

hydrophilic OH and NH_2 groups which increased the hydrophilicity. The dropping of angle values depends on the functional groups of both CHI and PU. The results expose enhanced hydrophilicity and reduced contact angle of about 57° and 41° for PU-CH-0.5 and PU-CH-2.0, respectively (Alves et al. 2009).

Table 3.1 Wettability measurements of PU and CHI-PU

Sample	Water contact angle (°)	Total SFEγ_s (mJ m^{-2})	Dispersive component SFEγ_s^d (mJ m^{-2})	Polar component SFE γ_s^p (mJ m^{-2})	Rq (nm)
PU	91 ± 3	38.43	37.54	0.89	2.8
PU-CHI-0.5	57 ± 3	49.66	35.01	14.64	15.5
PU-CHI-2.0	41 ± 3	54.68	29.81	24.87	19.6

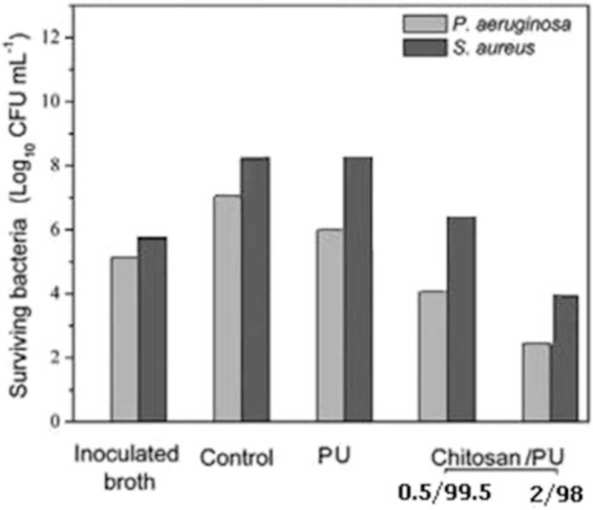

Fig. 3.16 Antibacterial activity of CHI-PEGF films for different blend ratios

3.4.2.6 Antibacterial Activity

The microbial activity of all CHI-PU films was investigated using strains of bacteria (Fig. 3.16). For the *Pseudomonas aeruginosa* control sample, bacteria contents were reduced significantly in all PU films. With *Staphylococcus aureus* bacteria, there is no change in control or PU films, but the values were reduced in the case of CHI-PU films. For both the bacteria species, CHI films have strong antibacterial efficiency parallel to the CHI content (Kara et al. 2014).

3.4.3 Chitosan-Based Polyvinyl Acetate (CHI-PVA)

PVA is a polymeric material with a rubbery style mainly utilized in several industrial coating methods and valued medical implementations. The aqueous emulsion of the PVA has been employed in the adhesion industry for many substances like paper, leather, textile, plastics, and wood (Misra et al. 2015). Although PVA polymer has many significant utilizations, it can easily degrade by the action of microorganisms

(Cappitelli and Sorlini 2008). The characterizations of PVA films can be improved and enhanced by the action of biodegradable polymers like CHI. In this way, the obtained CH-PVA copolymer film has more stability, hydrophobicity, and mechanical properties than pure CHI (Radhakumary et al. 2007).

3.4.3.1 Synthesis

CHI-PVA films were obtained by the addition of a definite ratio of CHI solution to PVA emulsion (Fig. 3.17). By increasing the content of CHI, the rate of diffusion raised, and the dispersion increased with more blending action (Ismail et al. 2016).

3.4.3.2 Fourier Transform Infrared Spectroscopy (FTIR)

From the PVA graph (Fig. 3.18), no functional OH appeared but a significant spectrum of C=O and C-O exposed at 1727 cm^{-1}, 1370 cm^{-1}, and 1220 cm^{-1}, due to the presence of $COOCH_3$. In another view, CHI graph shows the popular bands of OH and NH_2 revealed at 3460 cm^{-1} with C=O spectra. At the CHI-PVA graph, the strong spectra of NH_2 at 3450 cm^{-1} decreased strongly owing to the blending process. New COO exhibited at 1725 cm^{-1}, 1369 cm^{-1}, and 1290 cm^{-1}. Moreover, two significant spectra were revealed at 2011 cm^{-1} and 2165 cm^{-1}, attributed to the different bonding action. This result is owing to some of the NH_2

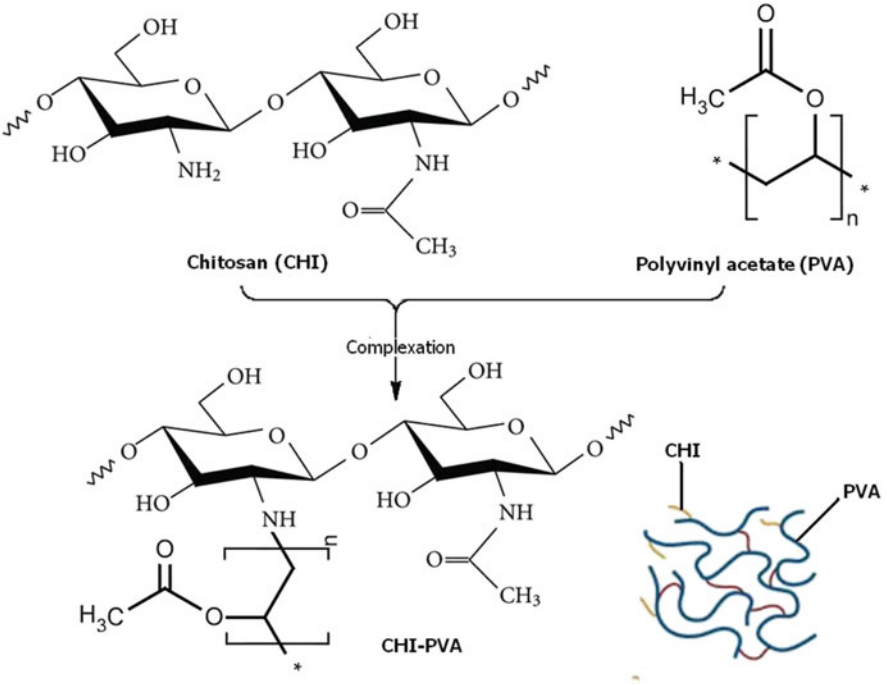

Fig. 3.17 A Schematic diagram for preparing CHI-PVA films

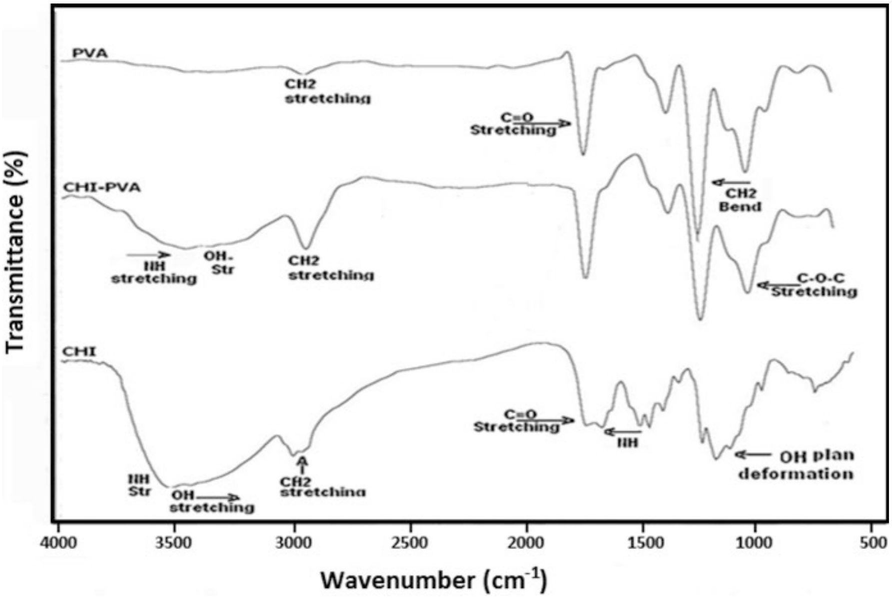

Fig. 3.18 FTIR spectra of CHI, PVA, and CHI-PVA

converted to NH during the PVA-CHI blending. The intensity of bands varied due to the CHI-PVA linking (Ismail et al. 2016; Maciel et al. 2005).

3.4.3.3 X-Ray Diffraction (XRD)

XRD (Fig. 3.19) shows the obtained peaks at diffraction angles of 10° and 20° for CHI and CHI-PVA, respectively, revealing the partial crystallinity of CHI-PVA. The crystalline structure of CHI exhibited the formation of two types of hydrogen bonding. This bonding was carried out between NH_2 and OH. The intensity of bands at diffraction angles of 20° and 40° for CHI-PVA is reduced owing to the crosslinking between CHI and PVA which breaks down H bonds and decreased crystallinity degree. Also, the limitation of chain mobility decreased the content of NH_2 groups (Ismail et al. 2016).

3.4.3.4 Morphology

The microscopic structure is reported in Fig. 3.20. No investigated fractions appeared in the case of pure PVA. At the CHI-PVA image, small CHI spots are revealed. By increasing the CHI content to the PVA matrix, branched sites appeared clearly. Dispersed porous compositions of CHI-PVA attributed to the blending process. The degree of blending, the content of CHI, and the cured period play a significant role in the molecular diffusion rate of CHI-PVA. For this purpose, several methods are employed to investigate the action of CHI content such as the box-counting method (De Souza et al. 2003).

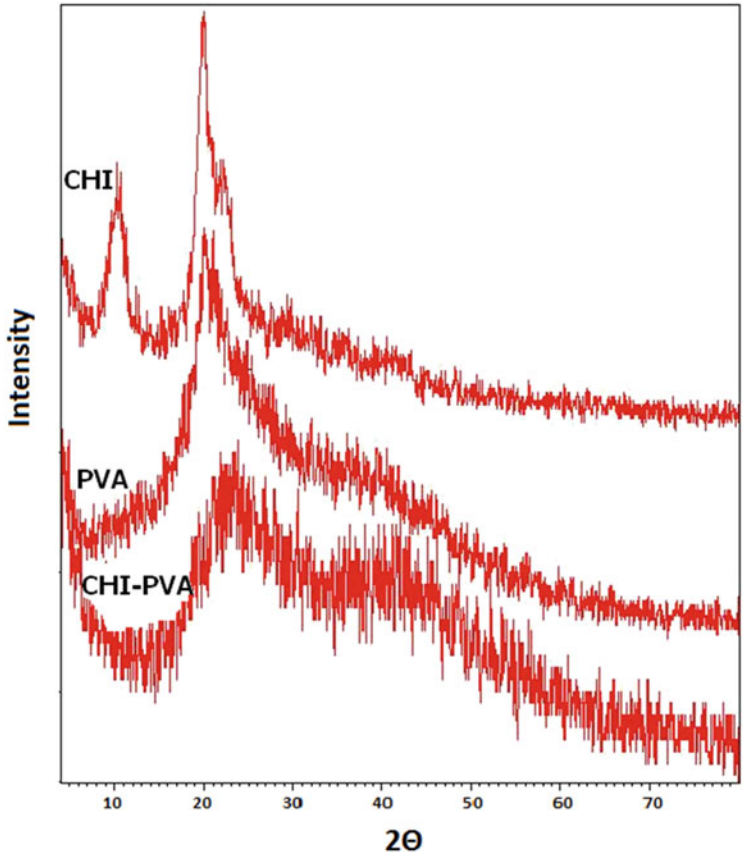

Fig. 3.19 XRD diagrams of CHI, PVA, and CHI-PVA

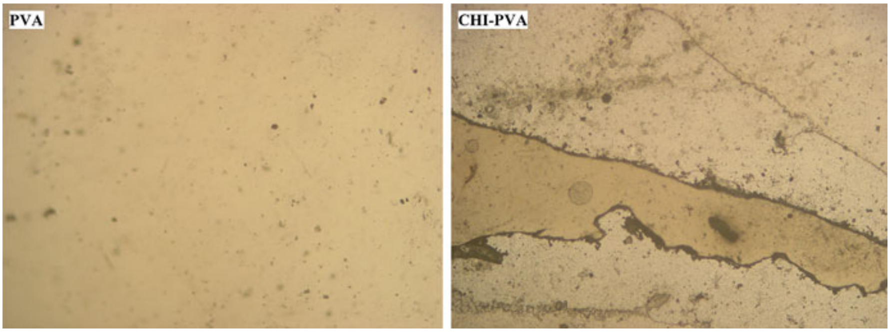

Fig. 3.20 Optical microscopic images of PVA and CHI-PVA

3.4.3.5 Swelling Performance

Swelling behavior of these blends in definite alcohol–water ratio mixtures is shown in Fig. 3.21. In pure H_2O, these films obtained higher values than in pure alcohol due to the flexibility degree of CHI (Ismail et al. 2016). The swollen systems reduce the resistivity to permeate due to the relaxation and the diffusion of the hydrophilic blends. By increasing the swelling degree with high water content, more alcohol is permeating at the same time with the water owing to the sorption coupling process (Das et al. 2008).

3.4.3.6 Conductivity

In the conductivity diagram of CHI-PVA blends (Fig. 3.22), the conductivity values increased with elevating temperature, compatible with the common polyelectrolyte performance, which attributed to the interaction of NH_2 in the CHI matrix. The high σ values of CHI-PVA are probably due to the potential interaction between CHI and PVA. The variations in conductivity are related to the change of the ionization method (Li and Tang 2016).

3.4.4 Chitosan-Based Carboxymethyl Cellulose (CHI-CMC)

Cellulose is the most abundant eco-friendly biodegradable polymer that exists in nature (Ismail 2015). Hydrophilic cellulose derivatives such as CMC can be prepared throughout the etherification reaction. Among all cellulose derivatives, CMC is an anionic polyelectrolyte that shows significant physicochemical characterizations in chemical reactions. CMC-based blend exposes high swelling in water due to the existence of Na^+ and also repulsion of its COO^- groups (Ismail 2015; Mandal and Ray 2016).

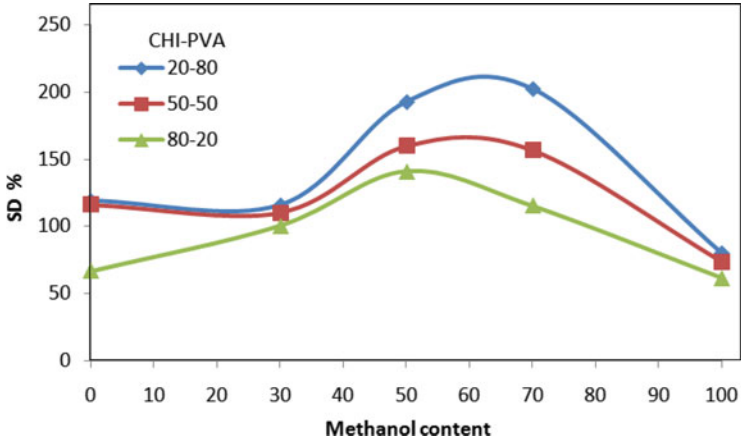

Fig. 3.21 Swelling of CHI-PVA blends in methanol–water mixtures for 48 h at RT

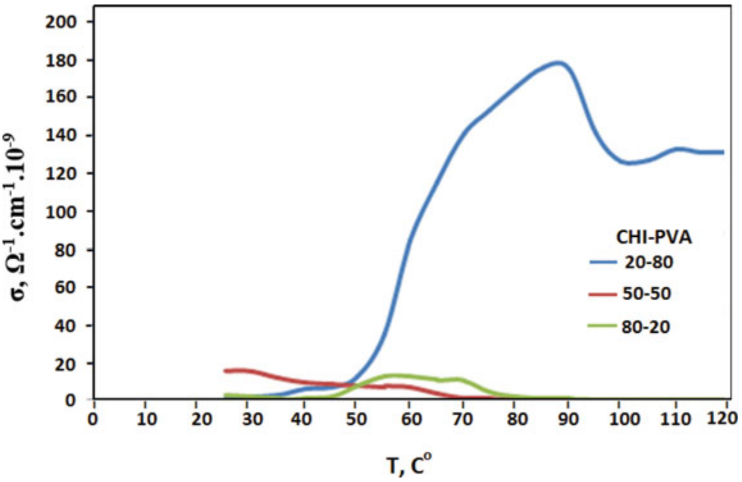

Fig. 3.22 Conductivity diagrams of different ratios of CHI-PVA copolymer

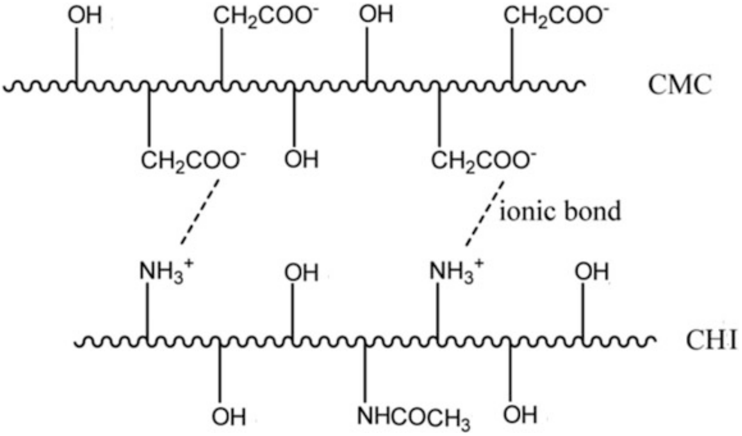

Fig. 3.23 A Schematic diagram of preparing PECs from CHI and CMC

For modification, CMC was chemically blended and grafted with other synthetic polymers. CMC-based hydrogels are used in water treatment for the removal of pollutants such as heavy metal ions (Samandari et al. 2016) and dyes (Wang and Wang 2013). Composites of CHI and CMC fibers have been modified and investigated for biomaterial applications (Kawasaki et al. 2016).

3.4.4.1 Synthesis

For preparing CHI-CMC blends, the ionic bonding was carried out between the cationic NH_2 groups of CHI and the anionic COO groups of CMC (Fig. 3.23). Complexes of CHI-CMC were prepared in an aqueous acidic medium, then the solid

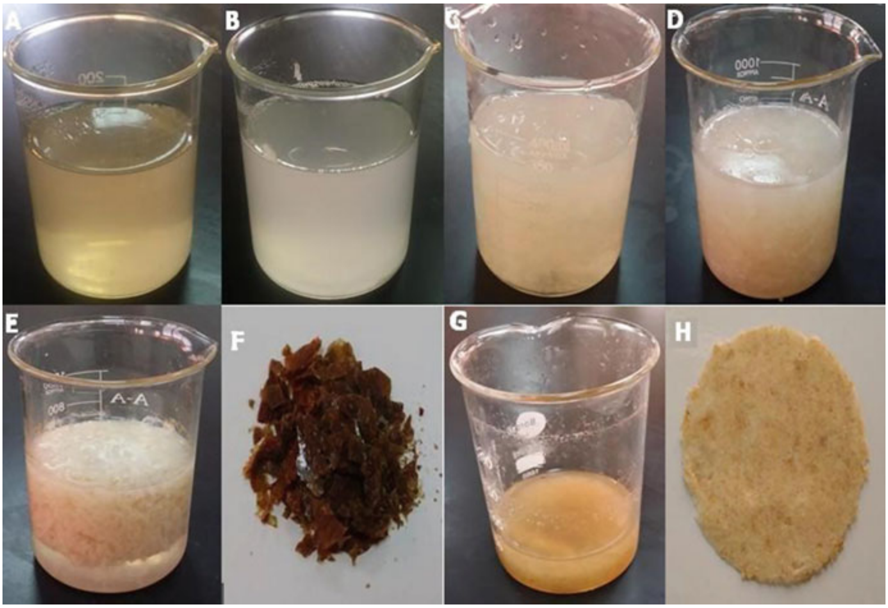

Fig. 3.24 Photographs of (**a**) CHI solution; (**b**) CMC solution; (**c**, **d**) addition of CHI solution to CMC solution; (**e**) complexation; (**f**) PECs; (**g**) casting; and (**h**) UPECMs

complex was dissolved in aqueous NaOH to prepare uniform polyelectrolyte complex membranes (UPECMs) (Ismail 2015).

UPECM films have an ionic-rich form which enhances their dehydration behaviors over a series of binary contents of MW, EW, IW, and BW mixtures. From the optical images, by adding CHI solution (Fig. 3.24a) to CMC solution (Fig. 3.24b), the turbidity is shown in CMC solution (Fig. 3.24c) with the first edition of CHI solution, correlating the production of insoluble PECs due to the ionic crosslinking between CHI and CMC. With the high CHI content, the turbidity increased (Fig. 3.24d), and two phases were obtained (Fig. 3.24e) at the crosslinking endpoint. The PECs were accumulated, separated, and dried. PECs (Fig. 3.24f) and its casting solution (Fig. 3.24g) which produces polyelectrolyte complex membranes UPECMs (Fig. 3.24h) were obtained (Ismail 2015).

3.4.4.2 Fourier Transform Infrared Spectroscopy (FTIR)

FTIR diagrams of CHI, CMC, and PECs are given in Fig. 3.25. Absorption bands of C=O groups are at 1600 cm^{-1} and 1627 cm^{-1} for CMC and PECs, respectively, attributed to the free COOH groups. Broadbands of OH groups appeared at 3500 cm^{-1}, 3422 cm^{-1}, and 3384 cm^{-1} for CHI, CMC, and PECs. For CMC, a strong band was observed at 1600 cm^{-1} for the COOH groups of CMC. In PECs, two bands appeared at 1736 cm^{-1} and 1627 cm^{-1} for both COOH and COO groups. This is due to some COO groups in CMC converted to COOH. So, the variation

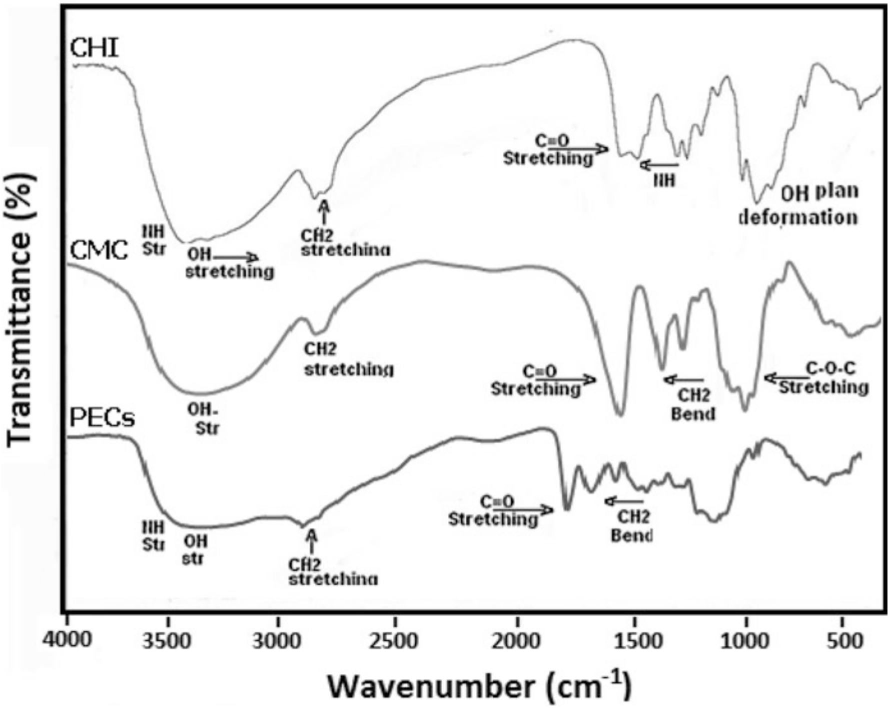

Fig. 3.25 FTIR spectra of CHI, CMC, and PECs

between the band intensities at 1736 cm^{-1} and 1627 cm^{-1} refers to the crosslinking degree of PECs (Maciel et al. 2005).

3.4.4.3 X-Ray Diffraction (XRD)

XRD of CHI, CMC, and PECs is exhibited in Fig. 3.26. Peaks at 10° (CHI) and 20° (CHI and CMC) phases are due to two types of hydrogen bonding (Ismail 2015). However, PECs are decreased, correlating the interactions between the two systems. Consequently, the ionic crosslinking reactions in turn reduce the crystallinity of PECs (Wan et al. 2006).

3.4.4.4 Swelling Performance

The swelling of prepared UPECMs in alcohol–water mixtures was studied (Fig. 3.27) based on the hydrophilic behavior of the films. SD of UPECMs in the MW mixture rose with increasing water value. The flexibility of UPECMs in water is higher than that in alcohol due to the outer particles of UPECMs (Samandari et al. 2016). In general, the high SD in all alcohol–water mixtures, except BW, was observed in the range of 30–70%, especially in the case of the IW mixture. Up to 50%, water content ratio in both MW and EW, the penetration of alcohol and water in UPECM films is the same.

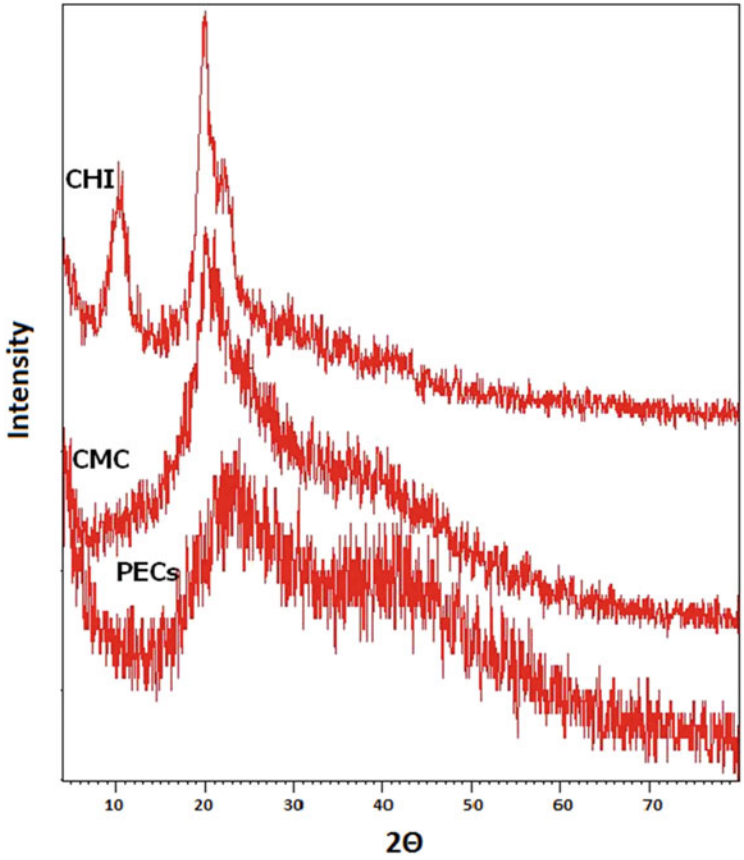

Fig. 3.26 XRD of CHI, CMC, and PECs

For IW binary feed mixture, high swelling was exposed at a range of 40–60% more than in both MW and EW systems. The high swelling of the IW mixture at 50% water content is due to the more permeation of water than alcohol. The membrane degradation begins from 50% as correlating to the high relaxation. Besides, the low polarity of isopropanol allows more water molecules to permeate through the membrane. In BW mixture, degradation of membranes was observed clearly from 10% water concentration in the feed, indicating the poor stability of membranes in butanol (Ismail 2015).

3.4.4.5 Antimicrobial Activity

An antimicrobial test is employed to check the antibacterial efficacy of films. The activities of CHI and CHI-CMC films against *Escherichia coli* and *Staphylococcus aureus* is shown in Table 3.2. The mechanism is the interaction of NH_4^+ groups with the negative bacterial cell membranes, causing membrane leakage and decompose of intracellular components, which kill the bacteria. More inhibition activity of the

Fig. 3.27 Swelling degree (SD) of UPECMs in (**a**) methanol–water, (**b**)ethanol–water, (**c**) isopropanol–water, and (**d**) butanol–water mixtures at room temperature for 48 h

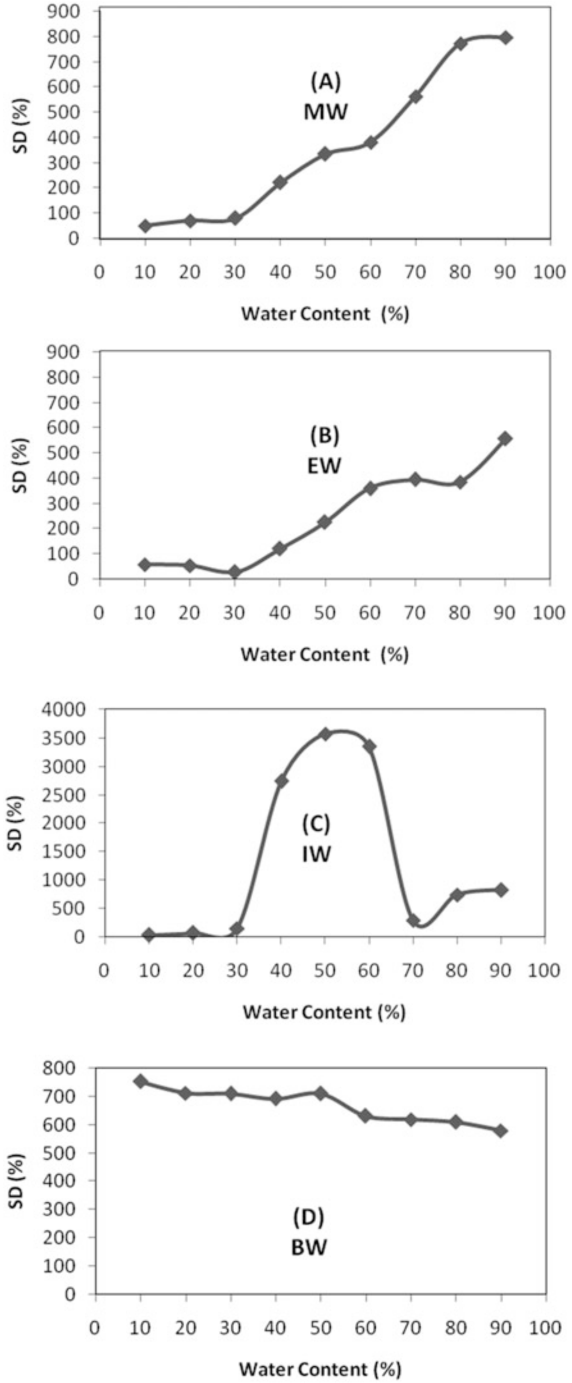

Table 3.2 Antibacterial activity of CHI and CHI-CMC films

Samples	Inhibition zone (cm) *E. coli*	Inhibition zone (cm) *S. aureus*
CHI	2.40 ± 0.12a	3.00 ± 0.20a
CHI 90/CMC 10	2.20 ± 0.10a	2.60 ± 0.10a
CHI 70/CMC 30	1.60 ± 0.20a	2.20 ± 0.15a
CHI 40/CMC 60	1.40 ± 0.12a	1.60 ± 0.20a

films is against *Staphylococcus aureus* than against *Escherichia coli*. The difference is because *Staphylococcus aureus* is more receptive to antibiotics than is *Escherichia coli* because the latter has a relatively less permeable, lipid-based outer membrane (Hu et al. 2016; Yu et al. 2013).

3.5 Summary

Bio-based polymers have received much more interest in the last decades due to their potential implementations in several fields correlated to environmental maintenance and protection of physical health. The present review reported that CHI could be incorporated into synthetic polymers such as PE, PU, PVA, and CMC by simple blending techniques. These polymeric materials are completely miscible with CHI to produce unique chitosan-based polymers. The results explained that incorporation of CHI into the polymeric blends improved its strength, wettability, resistivity, and antimicrobial activity ultimately. Based on the obtained investigations, polymeric composites containing CHI can be used in several technologies such as coating, painting, hydrogel, membrane separation, food preservation, vesicular drug delivery, and tissue engineering.

3.6 Future Perspectives

The most dynamic development of CHI-based polymer production is foreseen. Thus, the global market is expected to shift dramatically to new natural polymers derived from animal and plant origins as the following:

- Bio-based monomers: using bacterial fermentation of biomass, and PBS.
- Eco-meable polymers: directly by bacteria PHA.
- Natural bio-based polymers: proteins, nucleic acids, collagen, starch, cellulose, and chitosan.

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Bacterial Biodegradation of Bisphenol A (BPA)

4

Snehal Ingale, Kajal Patel, Hemen Sarma, and Sanket J. Joshi

Abstract

Microorganisms play a key role in saving the environment by metabolizing and removing toxic harmful chemicals and wastes from the environment. Bisphenols (BPA) are one such group of chemicals, which are widely used in the production of plastic items, meant for day-to-day activities. It was popularized due to its sturdy and clear appearance. However, over the years, it is identified as possibly one of the key pollutants, which can leach in to soil and water bodies, and could enter the food chain. Several studies reported it to be quite a harmful chemical at different concentrations, leading to disruption of neuro-endocrine systems, reproductive systems and more. Different techniques are currently utilized to degrade and transform it to less harmful intermediates or complete degradation. Biodegradation is one such environmentally friendly technique, where BPA is metabolized and biotransformed into less toxic intermediates and non-toxic end products. We isolated few bacteria strains that can degrade BPA, from Industrial effluent contaminated soil. BPA degrading bacterial strains are isolated using enrichment techniques in MSM medium containing BPA as a sole source of carbon and energy. The current chapter aims to bring together the current state of knowledge on the widespread applications, presence and occurrence in

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environment and harmful effects of BPA and to assemble updates about different microbial groups involved in biodegradation.

Keywords

Xenobiotic · Bisphenol A · Endocrine disruptor · Biodegradation · Oxidative enzymes

4.1 Introduction

Presence and recalcitrant nature of aromatic compounds are a matter of great concern due to their toxicity and persistence in the environment, and the role it plays in human health and environmental pollution (Sarma and Joshi 2020). Several of those polycyclic aromatic compounds such as phenolic are ones among the most studied chemical pollutants, since its widespread applications in synthesis of different types of pigments (used for dyes, paints), varnishes, herbicides, pharmaceutical intermediates, by-products of petroleum processing, steel industries and others (Bui et al. 2012). Over decades, several of those chemical pollutants and their degradation intermediate products are known as 'xenobiotic' and are toxic, mutagenic, carcinogen and endocrine disrupters which have been released and accumulated in the environment (Fouda 2015; Patel et al. 2021). Halocarbons, alkylbenzene sulphonates, synthetic dyes and oil mixtures are some of those recalcitrant xenobiotic compounds commonly encountered in the environment (Al Mujaini et al. 2018; Patel and Chhaya 2019; Patel et al. 2021). Halogenated hydrocarbons, also known as halocarbons, contain different numbers of halogen (e.g. Cl, Br, F, I) atoms in the place of hydrogen atoms. Some common examples are: 1-bromopropane (C_3H_7Br), methylene chloride (CH_2Cl_2), chloroform ($CHCl_3$), tetrachloroethylene (C_2Cl_4), carbon tetrachloride (CCl_4), chlorofluorocarbon (freon, also known as trichlorofluoromethane, or CFC-11), insecticides/herbicides like DDT (dichlorodiphenyltrichloroethane), chlordane (octachloro-4,7-methanohydroindane), 2,4-D (2,4-dichlorophenoxyacetic acid) and 2,4,5-T (2,4,5-trichlorophenoxyacetic acid). Polychlorinated biphenyls (PCB) have two covalently linked benzene rings, where halogens substitute hydrogen, which are widely used as in electrical equipment, hydraulic and heat exchange fluids, lubricants, plasticisers and insulator coolants in transformers. As such, it is more or less inert to biological or chemical actions, which further increases with the increase in number of chlorine atoms in the molecule (Elangovan et al. 2019). Alkylbenzene sulphonates (ABS) are chemical surfactants, containing sulphonate group and non-polar alkyl end (with branching also), which makes it recalcitrant and difficult to biodegrade. Non-branched ABSs could be biodegraded by oxidation from their alkyl ends. Crude oil is quite a complex natural product, containing different types of toxic constituents, hydrophobic nature, making it a bit difficult to biodegrade molecule (Diana Anderson et al. 2001; Al Mujaini et al. 2018; Joshi et al. 2019). The xenobiotics present several impending hazards to both flora and fauna. Many halogenated xenobiotics and PAHs are reported to be quite toxic to prokaryotes, eukaryotes and humans. Even at low concentrations, it can lead to severe skin problems and could reduce reproductive potential. It is also reported to be carcinogenic and neurotoxic. Because of its recalcitrant nature, it can persist for longer duration in the environment, leading to slow build-up, and concentration over a period, also leading to bioaccumulation or biomagnifications (Omiecinski et al. 2011).

4.2 Xenobiotic Metabolism and Biodegradation

The metabolic process of converting hydrophobic chemicals to polar intermediates or end products occurs in two distinctive stages (Anderson et al. 2001). Where, in the first stage—functionalization, an oxygen atom is incorporated into the chemical, and functional groups such as -OH and -COOH are generated, following the reduction. Such metabolites are more polar than the parent compound, which could undergo the second stage of metabolism—conjugation, where endogenous substrates such as sulphate and glucuronic acid are incorporated, leading to highly hydrophilic molecules, warranting its further removal. Generally, xenobiotic compounds containing such functional group(s) directly participates in the second stage of conjugation reactions. Stage I metabolic pathway includes aromatic hydroxylation, epoxidation, aliphatic hydroxylation, dealkylation reaction, nitrogen and sulphur oxidation, oxidative deamination, oxidative dehalogenation, nitroreduction, azoreduction, reductive dehalogenation and hydrolysis. Whereas stage II metabolic pathway includes glucuronide conjugation, sulphate conjugation, glutathione conjugation, amino acid conjugation, hydration, methylation and acetylation (Omiecinski et al. 2011). Degradation of alkenes and aromatic hydrocarbons generally occurs as: an oxygenase enzyme introduces a hydroxyl group making it reactive; the hydroxyl group is further oxidized to a carboxyl group; for the cyclic compounds the ring structure is opened making it easier to be further degraded; and the linear molecule is degraded by β-oxidation to yield acetyl-CoA and further metabolized via normal pathway (Jha et al. 2015).

4.2.1 Bisphenol A

Bisphenols (BPA: 2,2-bis(4-hydroxyphenyl) propane or 4,4′-(propane-2,2-diyl) diphenol) are a group of chemical compounds (bisphenol B (BPB), bisphenol F (BPF) and bisphenol S) that consist of two phenolic rings joined together through abridging carbon or other chemical structure (Spivacks et al. 1994). BPA is an organic synthetic compound with chemical formula $(CH_3)_2C(C_6H_4OH)_2$ belonging to the group of diphenylmethane derivatives and bisphenols, with two hydroxyphenyl groups. Physical and chemical properties of BPA (Fig. 4.1) are: 228.291 g/mol, molar weight; 155–159 °C, melting point; 360 °C, boiling point; 120–300 ppm, water solubility; 10.4, dissociation constant (Press-Kristensen 2007).

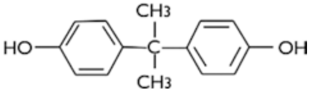

Fig. 4.1 Chemical structure of bisphenol A

4.2.1.1 Production and Uses of BPA

The projected global demand for BPA was expected to reach ~9618.7 kilo tons by 2020, with market size of ~20 billion USD (https://www.grandviewresearch.com/press-release/global-bisphenol-a-bpa-market#:~:text=The%20global%20demand%20for%20BPA,USD%207.00%20billion%20in%202013; as accessed on January 2021). This compound is synthesized by the condensation of acetone with two equivalents of phenol. The reaction is catalysed by a strong acid such as HCl. Industrially, a large excess of phenol is used to ensure full condensation; the product mixture of the cumene process (acetone and phenol) may also be used as starting material. Other applications are: BPA is employed to make certain plastics, flame-retardants, and different types of resins (polycarbonate, epoxy, unsaturated polyester). BPA-based plastic is clear and tough, and could be moulded into a variety of consumer goods, including many common and household products such as water bottles, sport equipment, computer accessories (Husain and Qayyum 2013; Noszczyńska and Piotrowska-Seget 2018).

4.2.2 Hazards of BPA

Typically food grade plastic materials consisting of polycarbonate and epoxy resins are the main route of entry for BPA, where sub ppm level BPA leaches into solid or liquid food materials (Crain et al. 2007). Extensive usage of stronger, clear and durable plastics made of polycarbonate and resins was mainly responsible for the prevalence of BPA exposure (Neri et al. 2004). Several studies showed that on an average the daily BPA intake per kilogram of body weight, among populations, was ~0.23μg/kg/day. Different concentrations of BPA have been measured in human tissues, fluids (such as urine) in many countries (Richter et al. 2007). BPA is reported to be an endocrine disruptor, and exposure to which has different effects, depending on the developmental stage of the exposed animal. Where it significantly affects during organ development stage, it may lead to irreversible changes (Richter et al. 2007). Humans exposed to BPA has been reported to suffer from different types of adverse health issues (Schug et al. 2011; Noszczyńska and Piotrowska-Seget 2018), which includes reproductive endocrine disorders (such as cancer, early puberty, infertility, diabetes/metabolic syndrome, obesity), pulmonary and cardiovascular complications during different stages of life. Most reproductive effects of such compounds are exerted through the hormonal disturbances (especially oestrogen and androgen). In laboratory studies using rodents, exposure to BPA during different developmental stages is reported causing many changes in the male reproductive system, including genitourinary abnormalities, decreased epididymal weight, decreased sperm production and increased prostate weight (Knez 2013). BPA has also been reported to inhibit thyroid-receptor-mediated transcriptional activity by binding to thyroid hormone receptor (Boas et al. 2012). BPA is a neuro-endocrine disrupting compound, which is associated with a variety of adverse health effects in the immune system, where a reduction in the amount of regulatory T lymphocytes was observed, when mice were exposed to BPA either in prenatal stages or during adulthood (Goto et al. 2007; Zielińska et al. 2019).

4.2.3 Microorganisms Involved in BPA Degradation

Various microorganisms capable of degrading BPA such as different bacterial species, fungi, algae, planktons from soils, fresh water bodies and waste water treatment plants have been reported (Kang et al. 2006), which are shown in Table 4.1. Various enzymes are responsible for BPA degradation or metabolism.

Table 4.1 Biodegradation of BPA by different types of organisms

Microorganisms	Strains	References
Bacteria	*Pseudomonas paucimobilis*; *Pseudomonas* sp.; *Pseudomonas putida*; *Streptomyces* sp.; *Sphingomonas* sp.; *Sphingomonas* sp. strain BP-7 and *Sphingomonas yanoikuyae* BP-11R; *Staphylococcus* sp.; *Bacillus* sp.; *Bacillus megaterium* strain ISO-2; *Micrococcus* sp.; *Streptococcus* sp.; *Lactococcus* sp.; *Lactobacillus reuteri*; *Bacillus pumilus*; *Enterobacter cloacae, Klebsiella* sp. *and Pantoea* sp.; *Acinetobacter* sp. K1MN	Kamaraj et al. (2014), Kang and Kondo (2002), Kang et al. (2004), Endo et al. (2007), Yamanaka et al. (2007, 2008), Telke et al. (2009), Toyama et al. (2009), Suyamud et al. (2018), Ju et al. (2019), Louati et al. (2019), Sarma et al. (2019), Eltoukhy et al. (2020), Jia et al. (2020) and Noszczyńska et al. (2020)
Fungi	*Pleurotus ostreatus* O-48; *Phanerochaete chrysosporium* ME-446; *Pleurotus eryngii*; *Schizophyllum commune*; *Trametes versicolor* IFO-7043; *Trametes villosa*; *Aspergillus fumigatus*; *Fusarium sporotrichioides* NFRI-1012; *Fusarium moniliforme* 2–2; *Aspergillus terreus* MT-13; *Emericella nidulans* MT-98; *Stereum hirsutum*; *Heterobasidion insulare*	Fukuda et al. (2001), Tsutsumi et al. (2001), Uchida et al. (2001), Shin et al. (2007), Kim and Nicell (2006), Kim et al. (2008), Mizuno et al. (2009) Subramanian and Yadav (2009) Mtibaà et al. (2018) and Hongyan et al. (2019)
Algae/ planktons	*Chlorella fusca var. vacuolata*; *Nannochloropsis* sp.; *Stephanodiscus hantzschii*; *Chlorella sorokiniana*; *Desmodesmus* sp.WR1; *Ulva prolifera*	Hirooka et al. (2003, 2005), Li et al. (2009), Eio et al. (2015), Wang et al. (2017) and Zhang et al. (2019)

Different microorganisms are responsible for the production of variety of enzymes for BPA degradation such as manganese peroxidase, laccase, peroxidase, polyphenol oxidase, cytochrome P450, UDP-glucuronosyltransferase and sulfotransferase (Hirano et al. 2000; Fukuda et al. 2001, 2004; Tsutsumi et al. 2001; Uchida et al. 2001; Kang et al. 2006; Kim et al. 2008; Subramanian and Yadav 2009; Husain and Qayyum 2013; Im and Löffler 2016; Moussavi and Haddad 2019; Jia et al. 2020)

4.2.4 BPA Degradation Pathway and Intermediates

Different types of bacteria and fungi are reported to degrade BPA, and several types of intermediates and end products were detected. Spivacks et al. (1994) reported different oxidative intermediates of BPA degradation process: 2,2-bis (4-hydroxyphenyl)-l-propanol, 1,2-bis(4-hydroxyphenyl) b2-propanol, 4,4-′-dihydroxy-a-methylstilbene, 2,2-bis(4-hydroxypheny1) propanoic acid, 2,3-bis (4-hydroxyphenyl)-l,2-propane-diol, 4-hydroxyphenacyl alcohol, 4-hydroxybenzaldehyde, 4-hydroxybenzoic acid and 4-hydroxyacetophenone. Han et al. (2015) studied and reported intermediates of BPA oxidation by ‘ferrate(VI)’ as analysed by GC/MS–MS: styrene, maleic acid, 1-(4-methyl phenyl) ethanone, p-isopropenyl phenol, hydroquinone, α-methylstyrene and 2-phenyl propenal. These metabolites are further oxidized to 2-phenyl propenal, 1,4-pentadien-3-ketone, maleic acid, and CO_2 and water. Peng et al. (2015) reported six compounds as the intermediates of BPA biodegradation by *Pseudomonas knackmussii* isolate: 2,2-bis(4-hydroxyphenyl)-l-propanol, 1,2-bis(4-hydroxyphenyl)-2-propanol, carbocationic isopropylphenol, 4-isopropenylphenol,4,4-dihydroxy-α-methylstilbene and 2,2-bis(4-hydroxyphenyl) propanoic acid. Daâssi et al. (2016) reported different types of carboxylic acid derivatives (such as pyroglutamic acid, tartaric acid, hydrocinnamic acid, 3-phenyl-3-hydroxy propanoic acid, β-hydroxybutyric acid) during BPA degradation by laccase from *C. gallica*. Ike et al. (2000) reported different bacterial isolates from activated sludge and river water samples, which showed degradation of BPA, with two major metabolites reported were 2,3-bis(4-hydroxyphenyl)-1,2-propanediol and p-hydroxyphenacyl alcohol. The exact mechanism of BPA degradation by manganese peroxidase and laccases is not yet clear, but it is proposed that BPA is metabolized via oxidation mechanism. Where BPA is initially converted to free radical, followed by random cleavage of the free radical at aromatic rings and C–C linkages, leading to production of 4-isopropenylphenol, 4-isopropylphenol and hexesterol (Chouhan et al. 2014). Different types of intermediary metabolites produced during the fungal degradation are also reported, such as 2-hydroxy-3-phenyl propanoic acid, 1-ethenyl-4-methoxybenzene and phenylacetic acid, produced via dehydroxylation, carboxylation and hydroxylation on the phenolic side chain of BPA, respectively. Some bacterial isolates also showed intermediates such as 4-hydroxybenzoic acid (HBA), 4-hydroxyacetophenone (HAP), 2,2-bis(4-hydroxyphenyl)-1-propanol and 2,3-bis(4-hydroxyphenyl)-1,2-propanediol (Chouhan et al. 2014). Laccase is reported to be a better performing enzyme, as compared to manganese peroxidase,

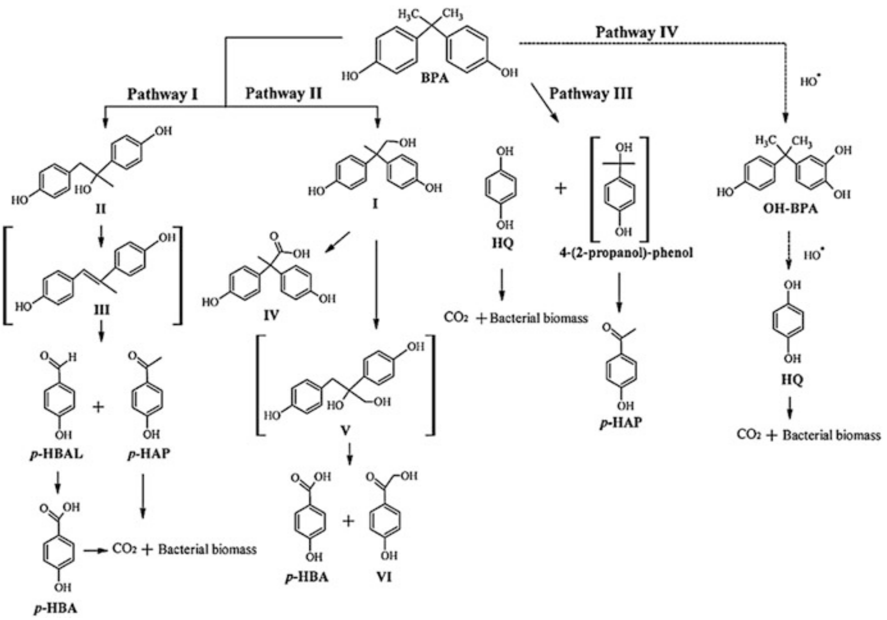

Fig. 4.2 Metabolic pathway for bacterial biodegradation of BPA (Reprinted from Eio, E. J., Kawai, M., Tsuchiya, K., Yamamoto, S., & Toda, T. (2014). Biodegradation of bisphenol A by bacterial consortia. *International Biodeterioration & Biodegradation*, *96*, 166–173. Copyright 2014, with permission from Elsevier)

and is suggested to have a key role in BPA biodegradation (Tsutsumi et al. 2001). Eio et al. (2014) proposed four BPA degradation pathways, as analysed by bacterial action (Fig. 4.2). They also reported different types of intermediate products: 2,2-bis (4-hydroxyphenyl)-1-propanol, 1,2-bis(4-hydroxyphenyl)-2-propanol, 4,4-dihydroxy-alpha-methylstilbene, 2,2-bis(4-hydroxyphenyl) propanoic acid, 2,3-bis(4-hydroxyphenyl)-1,2-propanediol, *p*-hydroxyphenacyl alcohol, *p*-hydroxybenzaldehyde, *p*-hydroxyacetophenone, *p*-hydroxybenzoic acid, *p*-hydroquinone and hydroxy-BPA.

Hongyan et al. (2019) reported that *T. versicolor* laccase could transform and degrade BPA to obtain 2-(4-hydroxyphenyl)propan-2-ylium, to isopropenylphenol free radical and further oxidized to 1-methyl-4-isopropenyl-2-cyclohexene. They also proposed that it might also be oxidized to p-xylene and toluene and transformed to ethylbenzene and cyclohexanone. Lin et al. (2020) reported different types of oxidation products during BPA degradation, such as monohydroxylated BPA, quinone of dihydroxylated BPA, 1-methylbenzene, 2-glutaric acid dimethylmethane, phenol, 2,4-bis(1,1-dimethyl ethyl), phenol, p-isopropenyl phenol and p-benzoquinone. Im and Löffler (2016) have reported a summarized view of possible pathways and intermediates of BPA degradation by different types of microorganisms and plants (Fig. 4.3). They mentioned that major intermediates are 4-isopropenylphenol, 4-hydroxybenzaldehyde, 4-hydroxybenzoate,

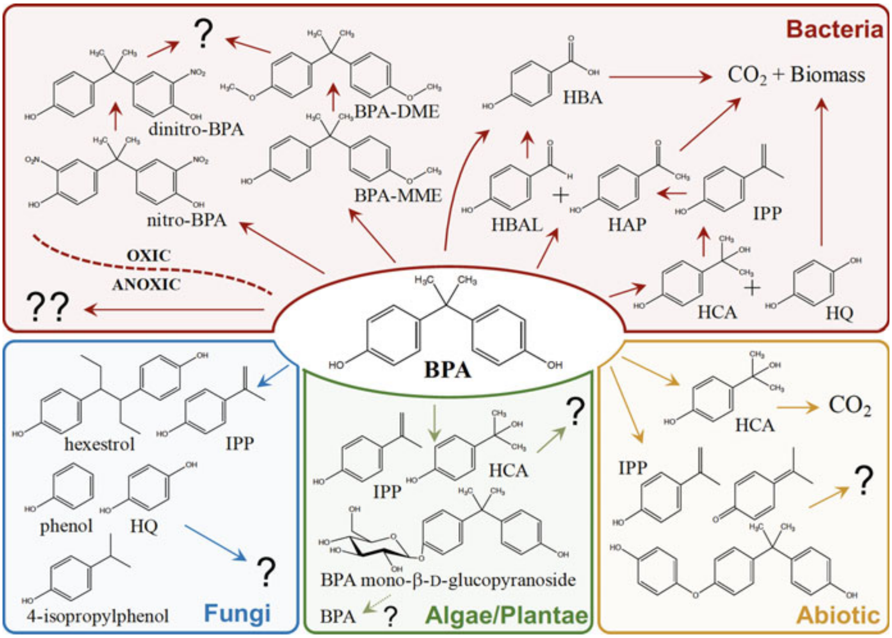

Fig. 4.3 Reported biotic and abiotic BPA degradation/transformation process pathways and intermediates. (Reprinted with permission from Im, J., & Löffler, F. E. (2016). Fate of bisphenol A in terrestrial and aquatic environments. *Environmental Science & Technology*, *50*(16), 8403–8416. Copyright 2016, American Chemical Society)

4-hydroxyacetophenone, 4-hydroxycumyl alcohol, hydroquinone and monomethyl/ dimethyl ether.

4.3 Case Study

In this study, bacterial isolates capable to degrade BPA were assessed, and the enzyme activity (laccase, MnP and LiP) was compared. Soil samples were collected in sterile bags from industrial area of Anand and Nadiad, Gujarat, India, and were transported to the laboratory. Following mineral salt medium (MSM) was used for the enrichment and isolation of microorganisms (g/L): KH_2PO_4 (0.5), K_2HPO_4 (1.5), NaCl (0.5), $MgSO_4{\cdot}7H_2O$ (0.5), NH_4NO_3 (1.0), $FeSO_4{\cdot}7H_2O$ (0.01), $CaCl_2{\cdot}2H_2O$ (0.01) and NH_4SO_4 (0.5). One gram of each soil samples was added to a 100-mL conical flask containing 50 mL of MSM medium containing 1000 ppm of BPA as a sole source of carbon. After 5 days of incubation at 30 °C, 100 rpm, the enriched soil samples were serially diluted and streaked onto MSM agar plate containing BPA as a sole source of carbon, to obtain well-isolated bacterial colonies. Morphological characterization of isolates showing BPA degradation capacity was performed using colony morphology, Gram's nature, and cell shape and size of the isolated bacteria were studied using the microscope. Various biochemical tests were performed such as carbohydrate hydrolysis test, IMViC test (indole production test, methyl red test, Vogues–Proskauer test, citrate utilization test), triple sugar iron agar test, urea test, H_2S production test, nitrate reduction test, lactose fermentation test, catalase test.

Isolated bacterial colonies were inoculated into nutrient both media, to prepare seed medium, and mineral salt media containing 1000 ppm BPA was used as a degradation medium, and incubated on a shaking incubator at 37 °C and 100 rpm. Samples were collected after every 24 h of incubation, the sample was estimated for dry cell weight, and the supernatant was used for estimation of BPA. Standard solution of BPA was prepared, and different aliquots were taken to achieve final concentration of 10–100 ppm of BPA, to it added 3 mL of 15% Na_2CO_3 and 0.5 mL of Folin–Ciocalteu Regent and incubated at 50 °C for 5 min, cooled to room temperature. The absorbance values were recorded at 765 nm using spectrophotometer. Similar steps are followed for the determination of BPA from the MSM experimental flasks. Effects of BPA concentration, temperature, pH, and inoculum size was also analysed.

4.3.1 Determination of Enzyme Activity

The activity of laccase was determined by spectrophotometric method using DMP (2,6-dimethoxy phenol) as a substrate. The method was based on oxidation of DMP at 420 nm. The reactive mixture consisted of 0.6 mL sodium acetate buffer, 0.2 mL enzyme extract and 0.2 mL DMP. The activity of manganese peroxidase was determined at 610 nm by spectrophotometric method using phenol red as a substrate. The reactive mixture consisted of 0.25 mL enzyme extract, 0.05 mL phenol red (0.1%), 0.1 mL sodium lactate (250 mM), 0.025 mL $MgSO_4$ (2 mM), 0.1 mL BSA (0.5%), 0.025 mL H_2O_2 (0.2 mM) and 0.5 mL citrate buffer (0.1 mM, pH 5). The activity of lignin peroxidase was determined at 664 nm by spectrophotometric method using methylene blue as a substrate. The reactive mixture consisted of 2.2 mL enzyme extract, 0.1 mL methylene blue (1.2 mM), 0.6 mL sodium acetate buffer (0.5 M, pH 4) and 0.1 mL H_2O_2 (2.7 mM). Production of enzymes were analysed in the presence of different BPA concentrations (500 ppm, 1000 ppm, 1500 ppm).

4.3.2 Observations

Totally 13 bacterial isolates were isolated from different soil samples and were screened for the degradation of BPA as shown in Fig. 4.4. Two sets of experiments were carried out for the isolation of bacteria, and the whole procedure was repeated for each set of experiment. Then highest BPA degradation colony was further used for optimization, enzyme production and degradation experiments. The highest BPA degradation strain (S1) was examined for colony morphology, Gram strain characters and biochemical tests. It was observed to be Gram-negative short rods,

Fig. 4.4 BPA degrading bacterial isolates obtained after enrichment of the soil samples

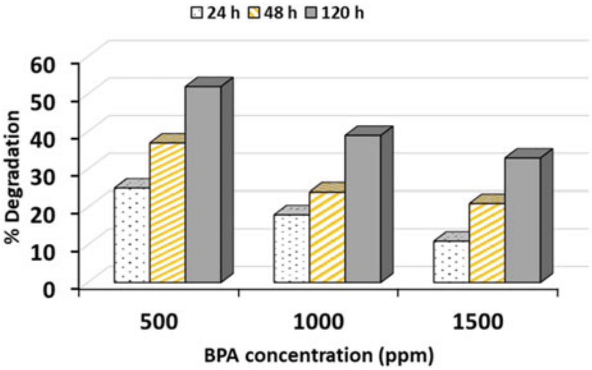

Fig. 4.5 The effect of concentration on the percentage BPA degradation

with smooth, colourless colony, and the bacteria was aerobic, with all biochemical tests positive, except urea hydrolysis and lactose fermentation tests, which were negative. From results of various tests in biochemical characterization and morphological characterization, and from the microscopic view of bacterial gram staining, it was preliminarily identified as *Pseudomonas* sp.

4.3.3 Bisphenol A Degradation

The degradation of bisphenol A was investigated by using Folin–Ciocalteu method. For that, calibration curve was prepared for quantification of BPA, and a linear standard curve was obtained. Slope value obtained from the linear graph was used to calculate the degraded bisphenol A concentrations in further experiments. The influence of substrate concentration (500–1500 ppm) on the degradation rate of BPA was investigated and percentage BPA removal and enzyme production were studied. It was observed that the conversion was constant with the increase of concentration in the selected range (Fig. 4.5). The effect of concentration on the degradation of bisphenol A and production of enzyme (laccase, MnP, LiP) is represented in Fig. 4.6. Flasks containing MSM medium with different

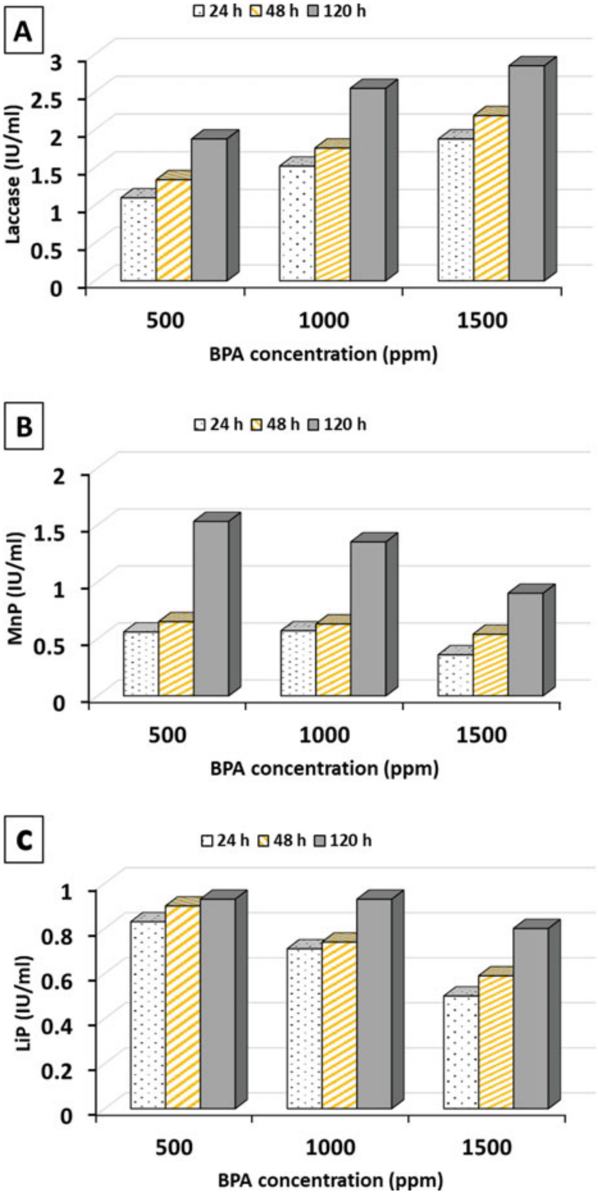

Fig. 4.6 The effect of BPA concentration on the enzyme production: (**a**) Laccase (IU/mL); (**b**) MnP (IU/mL) and (**c**) LiP (IU/mL)

concentrations of BPA were incubated at 37 °C and bisphenol A estimation was done at 24–24 h intervals. The reaction mixture containing 1000 ppm of BPA was degraded up to 39% after 120 h by *Pseudomonas* sp. This degradation rate was comparatively quite faster than the *Pseudomonas* sp. and *Pseudomonas putida* isolated from the river water in an experiment, where ~52% degradation of BPA, where concentration of BPA was 10 times lower (Kang and Kondo 2002).

The removal of BPA was carried out at 27 °C, 37 °C and ambient temperature for 120 h, and the highest BPA removal by *Pseudomonas* sp. was observed at ambient temperature. Whereas, *Klebsiella pneumoniae* showed the highest degradation of 55% between 35 and 40 °C for the initial BPA concentration of 500 ppm. The laccase, MnP and LiP production was found to be maximum at ambient temperature, whereas *Bacillus* sp. showed that the maximum production was at 35 °C (Shukur 2015). The highest MnP production was by *B. pumilus* at 25 °C and by *Paenibacillus sp.* was at 35 °C (Shukur 2015). The effect of pH on the removal of bisphenol A was investigated at a range of pH from 5 to 9. The experiment was performed at 37 °C for 120 h, where the degradation rate of BPA was 67% by *Pseudomonas* sp. which was observed highest at pH 7. Similar kind of result was reported for *Bacillus* sp. (Fouda 2015) and *Aspergillus* sp.

4.4 Conclusion and Future Prospects

Although widely criticized, BPA is still being used in several day-to-day appliances and applications. Several studies showed a wide and varied amount of damaging effects of BPA on flora and fauna. The environmental bioaccumulation and biomagnifications of BPA due to excessive use and improper disposal lead to such detrimental effects. Public awareness on such issues led to pressurizing manufacturers to look for viable alternatives to BPA. However, it is not quite a difficult task to completely ban its use, as it is widely employed in different types of plastic items, and contradictory reports are published, both in favour and against its usage. Thus, instead of immediate ban, it is a more favourable option to find suitable strategies to degrade it in environmentally friendly manner. Among different methods being suggested for the BPA removal or transformation of less toxic by-products, biodegradation has been advocated to be a promising alternative. Several types of microorganisms are reported to survive varied concentration of BPA, both on land and in water, and utilize it as a carbon source for metabolism. Both isolated strains and consortia isolated from different wastewater treatment plants or fresh water bodies showed promising results. In our preliminary study, we also successfully isolated BPA degrading bacterial isolates from soil samples near industries, which also showed that production of different types of key enzymes (laccase, MnP, LiP) reported to carry BPA oxidation/metabolism. Several studies reported the presence of different types of intermediates, when metabolized by either bacteria or fungi, and some of them are reported be toxic, but overall it was found that most of those intermediates were utilized for growth and end products such as CO_2 and water. Even though encouraging reports of microbial degradation and co-metabolism of BPA by microbes are available, we still need to search for strains with better efficiency and capable to work under harsh environmental conditions. Much research is still needed in this direction, also to utilize the wealth of knowledge in ‘omics’ and metabolic engineering.

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Microbial Degradation of Marine Plastics: Current State and Future Prospects

5

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Helge Niemann

Abstract

Millions of tons of plastics entering the sea each year are a substantial environmental problem. It is expected that ocean plastic pollution will increase when considering the rapidly rising rates in global plastic production, in contrast to the relatively slow growth in plastic recycling rates, and future projections of increasing population densities in coastal areas. However, a significant discrepancy exists between the vast quantities of plastic entering the ocean and the orders of magnitude lower amounts afloat at the sea surface, indicating a substantial sink for ocean plastics. Plastics are probably degraded in a multi-step process facilitated by abiotic and biotic factors. Abiotic factors, such as shear stress

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induced by wave action, solar ultraviolet radiation, and heat embrittle and fragment plastics. Fragmentation of macroplastics results in micro and nanoscale particles. Photooxidation causes the release of chain scission products from the polymer matrix, e.g., nanoplastics, low-molecular-weight polymer fragments, and hydrocarbon gases. Biodegradation of plastics is mediated by microbes that have enzymes capable of inducing (1) chain scission and depolymerization, and (2) assimilate and terminally oxidize the intermediate products of initial degradation. Plastic degradation products from UV radiation could be a useful carbon source for microbes, while the role of marine microbes as initial degraders is not well understood. Several terrestrial microorganisms (bacteria, fungi) are known to degrade specific plastic polymers. For example, the bacterium *Ideonella sakaiensis* hydrolyses polyethylene terephthalate (PET) with a novel cutinase (termed PETase) and utilizes the degradation products as energy and carbon source. In the marine environment, complex hydrocarbon-degrading bacteria have repetitively been found in association with plastics. These bacteria have genes encoding for monooxygenases, peroxidases, and dehydrogenases, enzymes which can, in principle, facilitate the initial breakdown of plastics. Most commonly applied methods to investigate plastic biodegradation are based on monitoring weight loss of plastic over time, determining chemical changes of the polymer, investigating colonization of plastics by microbes, and measuring CO_2 production rates. However, these evaluation methods often lack rigor in confirming initial depolymerization, assimilation, and mineralization. This chapter provides an overview of plastic biodegradation in the marine realm. Identified and potential microbial plastic degraders will be covered. Their metabolic and enzymatic capabilities will be highlighted with respect to valorization their potential in the future.

Keywords

Plastic pollution · Plastic polymers · Microbial plastic degradation

5.1 Introduction

5.1.1 Plastics: The Marvel and The Global Problem

The term ‘plastics’ is commonly used to refer to a diverse group of chemically synthesized materials, long chains of repetitive monomers that have a unique chemical structure and specific physicochemical characteristics (Cole et al. 2011; Thompson 2015; PlasticsEurope 2019). Plastics can be subdivided into thermoplastics, thermoset plastics and elastomers. Thermoplastics can be remolded upon heating, in contrast to thermoset plastics and elastomers. Thermoplastics are the most abundant plastic types and are the main subject of this chapter.

The invention of plastics dates back to the late nineteenth century when Alexander Parkes synthesized the first plastic, termed ‘Parkesine’ from cellulose (Parkes

1866). In 1907, Leo Baekeland produced one of the first fully synthetic plastic, became 'Bakelite' (Baekeland 1909), but only in the mid-twentieth century, plastics were more widely used in many industrial processes (Ryan 2015). The technological advancements and increasing demand for durable and versatile products that were effortless to manufacture at minimal costs created a niche for plastics to take over a role that was traditionally occupied by natural materials such as wood, leather, stone and glass. Nowadays, plastics are an essential part of human lives and even fulfil the needs of short-lived products that became characteristic of our 'throw-away' culture. Countless modifications of the virgin polymer structure, co-polymerizations and mixture of additives such as fillers, plasticizers, colourants, stabilizers, flame retardants and reinforcing fibres (among others) have enabled the development of a vast diversity of plastic formulations. These additives affect the properties of the polymer and serve the purpose to tailor polymer characteristics needed for the intended application (Deanin 1975). Polymers are used for packaging, construction and building, the automotive and electronic industry as well as agriculture and fishing, household, sports, warfare or medical applications, among many others (Fig. 5.1). Production of plastics has thus turned into a global multi-billion € business in less than 100 years. Currently, the most produced plastic types are polyethylene (PE), polypropylene (PP), polyvinylchloride (PVC), polyurethane (PU), polyethylene terephthalate (PET), polystyrene (PS) and polyamides (PA) (Fig. 5.2). In addition to conventional plastics of petrochemical origin, biobased plastics, i.e. plastics made from renewable sources such as cellulose, lactic acid, caprolactone, proteins, food waste and starch offer an alternative to conventional plastics. The market share of biobased plastics is rising but still comparably minor with 2.11 million tonnes produced in 2019 (Bioplastics 2019) accounting for about 1% of the global plastic production.

The invention of plastics created an unprecedented turning point in the evolution of humankind. Plastics, initially considered as the marvel of materials, controversially turned into a colossal and growing environmental hazard of the twenty-first century. Plastic production increased exponentially since mass production began in the 1950s. Solely in 2018, nearly 360 million tons were produced worldwide (PlasticsEurope 2019). Out of the world's plastics production, around 20% was manufactured in Europe and 50% in Asia, with China alone producing 30% (PlasticsEurope 2019). Unfortunately, the enormous increase in plastic production has not been accompanied by efficient waste management strategies in many countries. Since 2006, plastic waste recycling rates have only doubled, and about 25% of plastic post-consumer waste still ended up in landfills (PlasticsEurope 2019). In many tropical countries, plastic with other waste is either directly discarded to the environment or incinerated albeit in non-adequate infrastructures. While the latter prevents plastic debris from entering the environment, it dramatically contributes to other environmental problems because of the released greenhouse gases and probably more importantly, toxic fumes. These can also pose a direct hazard to human health (Wright and Kelly 2017). The prevalent popularity of plastics, in contrast to the failures of post-consumer waste management, makes plastics an indispensable material with a negative reputation—from an environmental point of view.

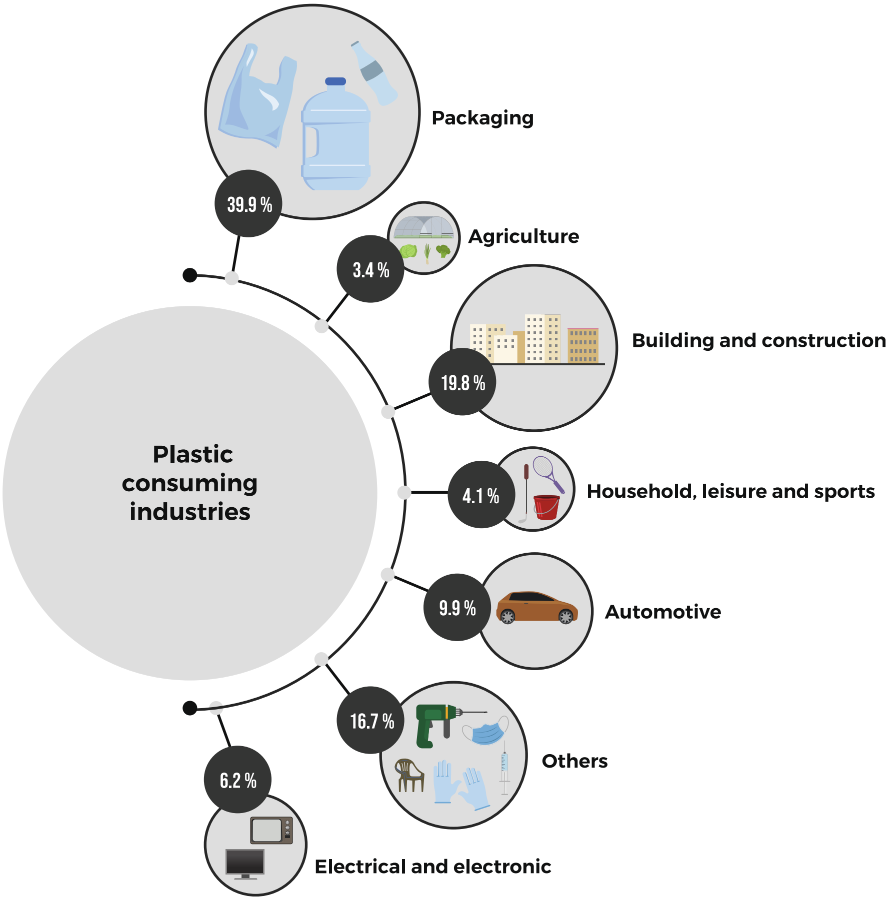


Fig. 5.1 Use of plastics by segments, based on 2018 data of 51.2 millions of tons as input for total European plastics converters demand (PlasticsEurope 2019)

5.2 The Oceans Plastic Problem

Improperly managed plastic waste often gets transported to the ocean (Jambeck et al. 2015; Geyer et al. 2017; Lebreton et al. 2017). First reports of plastic pollution in the marine environment date back to the early 1970s (Heyerdahl 1971; Carpenter et al. 1972; Carpenter and Smith 1972; Cundell 1973), and since about two decades, it has become an even more relevant and intensely investigated research topic. The quantity of plastic released into the marine realm is linked to the quantity of globally produced plastics, but even closer related to the amount of plastic waste generated in coastal areas, and the strategies enforced in those areas to manage plastic waste (Jambeck et al. 2015). For 2010, it has been estimated that the 4.8–12.7 Mt of plastic

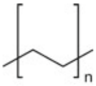
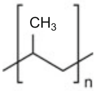
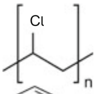
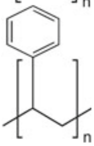
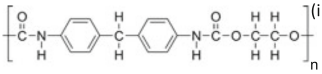
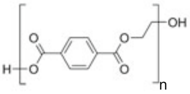
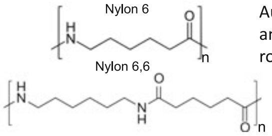
Polymer	Abbreviation	Repeating unit	Demand	Recycling code
Polyethylene	PE		Packaging, building & construction, agriculture	02 HDPE 04 LDPE
Polypropylene	PP		Packaging, automotive, household, leisure & sports	05
Polyvinyl chloride	PVC		Building & construction	03
Polystyrene	PS		Packaging, others (lab consumables like test tubes or petri dishes, soft drink lids)	06
Polyurethane	PU		Building & construction, automotive, electrical & electronic, others (insulation foams, mattresses)	07 Others
Polyethylene terephthalate	PET		Packaging	01
Polyamide	PA		Automotive, electrical & electronic and others (clothing, fishing gear, rope or thread)	PA

Fig. 5.2 Polymer molecules of plastic reoccurring in marine environments. These can be categorized based on the chemical structure into carbon-carbon backbone based (PE, PP, PS, PVC) or polymers with heteroatoms in the main chain (PET, PC, PA, PUR)

waste generated in that year ended up in the oceans (Jambeck et al. 2015). Partially, plastic waste is transported via rivers, which were found to contribute to 1.15 to 2.41 Mt of plastics to the oceans annually (Lebreton et al. 2017). Catastrophic events, such as hurricanes or floods, can also transport substantial amounts of plastics into the marine environment (Law 2017). Other pathways for plastic entering the oceans include atmospheric transport, beach littering, maritime cargo loss, and loss of commercial fishing gear. By now, no ocean habitat has remained untouched from plastic pollution: plastics were found in as remote areas as Arctic Sea ice (Peeken et al. 2018) and the deep sea (Van Cauwenberghe et al. 2013; Ramirez-Llodra et al. 2014; Peng et al. 2020). Considering the exponentially rising rates of global plastic production in contrast to the relatively slow growth of plastic recycling rates, and considering future projections of increasing population densities in coastal areas, it seems probable that the problem of oceans plastic pollution will increase.

However, in stark contrast to the expected, possibly hundreds of millions of metric tons of plastic debris that should be present in the sea (Jambeck et al. 2015), field measurements and modelling data estimate that 0.09–0.25 Mt (van Sebille et al. 2015) to 0.4–4 Mt (when considering an underestimation of macroplastics in global budgets (Lebreton et al. 2018)) of plastics are afloat in the ocean.

Several theories have been developed to explain the 'missing plastic paradox': (1) polymers, with a higher density than water, are removed by vertical transport to deeper depths/sediments. The overgrowth of floating particles with biofilms (Andrady 2011; Tu et al. 2020; Zhao et al. 2020) as well as the entanglement of plastic particles in marine snow (Porter et al. 2018) increases the density of the initially floating polymer to a point where it sinks. As a result of these biofouling-induced buoyancy changes, the amounts of plastics exported from the surface ocean to deeper water layers or sediment could be substantial. Accumulation of up to 1.9 million (microplastics) particles per m^2 sediment have been measured (Kane et al. 2020). Based on modelling and observations, this removal mechanism could be size-dependent, with smaller particles being less abundant at the surface or removed faster (Cozar et al. 2014; Kooi et al. 2017). However, the number of studies on plastic fallout as well as sedimentation is limited, and abundances of sinking/sedimented plastic detected are variable and in some cases seem even to be insubstantial (Martin et al. 2017; Willis et al. 2017; Barrett et al. 2020; Egger et al. 2020). Besides, vertical transport should not lead to disproportionation of polymer types with PE and PP dominating surface waters and polyesters and PA deeper waters and sediments (Erni-Casola et al. 2019). (2) Estimates of ocean plastic concentrations are often based on observations of floating macroplastics (i.e. plastic pieces >5 mm) or microplastics (i.e. plastic pieces 1μm to 5 mm) typically from surface trawls using nets with a mesh size of typically >300μm. However, a considerable contribution to ocean plastic pollution is attributed to smaller size classes of microplastic and possibly also nano plastics (1–1000 nm). Using nets with a mesh size of 100μm resulted in 2.5-fold and tenfold greater microplastic concentrations than with 333μm and 500μm meshes, respectively (Lindeque et al. 2020). Besides, more plastic is found in samples when improved detection methods and techniques are applied (Anger et al. 2018). (3) Finally, plastics may also be degraded by physicochemical processes, e.g. photooxidation through UV radiation, which leads to the incorporation of oxygen atoms into the polymer as well as chain scission (Gewert et al. 2018). Possibly, microbial degradation of polymers could also constitute a sink for ocean plastic debris as several microbes have been shown to degrade plastics (Yamada-Onodera et al. 2001; Gilan et al. 2004; Sheik et al. 2015; Yoshida et al. 2016; Paço et al. 2017). Nevertheless, ocean plastic degradation and the contribution of the above-mentioned factors to the 'missing plastic paradox' have not been enumerated (Fig. 5.3).

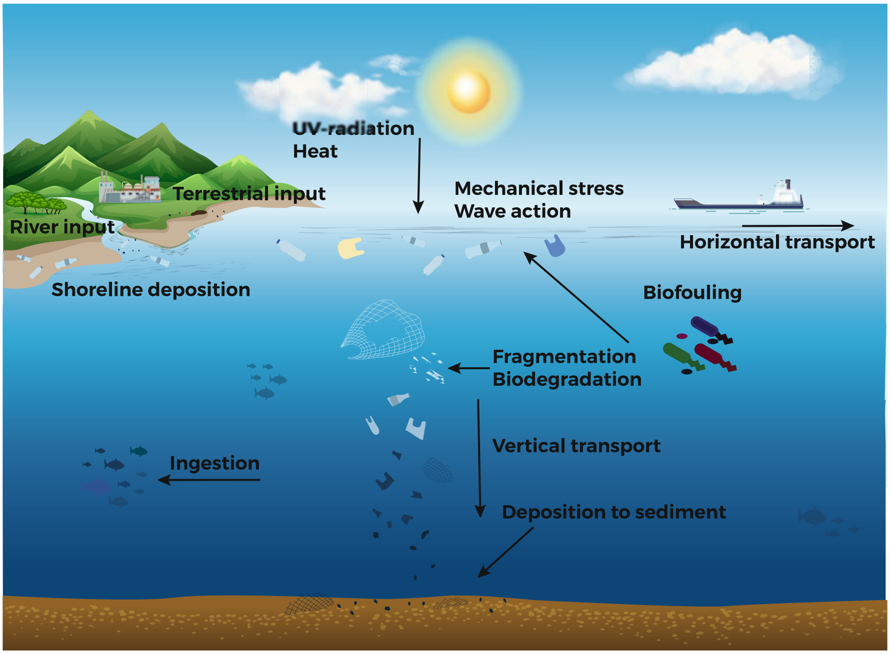


Fig. 5.3 Pathways of plastic input into the ocean and the potential further fate of plastic marine debris

5.2.1 Impacts of Plastic on Marine Life

Plastics in the oceans introduce various problems (Wayman and Niemann 2021). This includes socio-economic losses caused by the visually repulsive littering of shorelines, and physical and biochemical damage inflicted on marine mammals, fish, seabirds and other eukaryotic and possibly prokaryotic life. The effects of plastic interactions with marine life are largely dependent on the characteristics of the debris, for example size, shape, type and concentration of additives added to the basic polymer (Law 2017). First scientific records of plastics being ingested by marine fauna date back to the late 1960s, when gastrointestinal tracts of Laysan albatrosses *Phoebastria immutabilis* were inspected for plastic items in their body cavities (Kenyon and Kridler 1969). Later, regurgitation of plastics from adult specimens to chicks of Laysan albatrosses, causing intestinal obstruction and ulcerations in the gastrointestinal tract, was reported (Pettit et al. 1981). Marine animals can get entangled in ghost fishing gear or ingest plastic debris (Croxall et al. 1990; Cadée 2002; Gregory 2009; de Stephanis et al. 2013; Schuyler et al. 2014; Thiel et al. 2018). Ingestion of plastic leads to nutritional deficiencies and reduction of energy budgets in marine biota (Van Cauwenberghe et al. 2015; Watts et al. 2015). Dolphins (Hernandez-Gonzalez et al. 2018), other whales and turtles (Mascarenhas et al. 2004; Campani et al. 2013; Clukey et al. 2018), seals (Bravo

Rebolledo et al. 2013), various fish species (Boerger et al. 2010; Bucol et al. 2020), squids (Braid et al. 2012) and jellyfish (Iliff et al. 2020), among others, have ingested plastic either directly or possibly via trophic transfer. Furthermore, it has been proposed that smaller plastic particles are more detrimental to many organisms (Koelmans et al. 2015), which makes fragmentation and degradation of larger plastics into smaller micro and nanometre-sized plastics as well as the immediate release of such plastic size classes to the marine environment particularly problematic (Mattsson et al. 2017). Small microplastic particles and nanoplastics were enriched in filter feeders (von Moos et al. 2012; Van Cauwenberghe et al. 2015). Nanoplastics can be transferred in the food chain from algae through zooplankton to fish where these may cross the blood–brain barrier and cause behavioural disorders (Mattsson et al. 2017).

Plastics often contain chemical additives such as plasticizers, for enhancing polymer properties (Hahladakis et al. 2018). Some of these components act as endocrine disruptors, even at extremely low concentrations (Gallo et al. 2018; Galgani et al. 2019). Plastic additives can leach into the water, with the potential to contaminate soils, groundwater, rivers and the marine environment. Phthalates get released from PE bags and PVC cables, exposed to seawater (Paluselli et al. 2019). Polymers are lipophilic and absorb persistent organic pollutants (POP) to their surface where these compounds may become concentrated by a factor of 10^6 compared to the surrounding seawater (Mato et al. 2001). PE and PP, for example, were found to absorb higher amounts of polyaromatic hydrocarbons (PAH) and polychlorinated biphenyls (PCB) in comparison to PVC and PET (Rochman et al. 2013). As a result of microplastic ingestion in worms and mussels, bioaccumulation of PAHs occurs, and adverse effects were reported (Browne et al. 2013; Avio et al. 2015). In addition, plastics can accumulate heavy metals (Holmes et al. 2012; Rochman et al. 2014), which, if released, can cause a diversity of toxic effects. PVC and PP particles absorbed more cadmium and lead than PE, PA and particulate organic matter (Gao et al. 2019). Micro-and nanoplastics have a high surface to volume ratio and, thus, can absorb relatively high amounts of hydrophobic contaminants. These plastic size classes are readily ingested by many organisms where the contaminants may be rereleased. However, the end effect depends on microplastic particle concentration in nature, the chemical equilibrium between water and plastics, species and microniches, such as gut systems (Diepens and Koelmans 2018; Menéndez-Pedriza and Jaumot 2020). Although plastics function as a potential transport vector for harmful compounds, it remains challenging to quantify the amounts released into biota, accumulation in the marine food web and their contribution in nature. In particular, if the same toxic compounds are present in the food, surrounding water and sediment, these may be incorporated from these compartments, too (Ziccardi et al. 2016).

5.3 Plastic Degradation

Plastics are typically designed for durability, which makes them a valuable material. However, the chemical structure of the primary polymer and admixture of additives make plastics rather resistant to degradation in the environment where they persist for elongated periods of time. This poses critical questions: How do plastics degrade? What is the contribution of abiotic and biotic factors? What is the rate of this process? What is the identity of degradation products? Plastic degradation is considered a process resulting in changes of polymer properties due to chemical, physical or biological forcing factors (Singh and Sharma 2008). It is generally believed that recalcitrant plastics are not biodegradable and can persist from 10 years to hundreds or perhaps even thousands of years in nature, to the extent of being the marker of 'Anthropocene' in the geological record (Corcoran et al. 2014; Zalasiewicz et al. 2016; Geyer et al. 2017; Joly and Coulis 2018; Krause et al. 2020). However, this argument is not substantially founded because hardly any data exists on the kinetics of plastic degradation in the marine realm, neither physicochemical nor biological. Laboratory simulations revealed HDPE to be more resistant to fragmentation when exposed to seawater in comparison to samples on beach sand in 6-month experiments (Kalogerakis et al. 2017). It thus seems that ocean plastic degradation might be slower than terrestrial plastic degradation, where higher rates of solar radiation and higher temperatures facilitate enhanced breakdown of plastics (Andrady and Neal 2009).

5.3.1 Abiotic Factors Influencing the Degradation of Plastic

In the marine environment, plastic fragments into smaller particles due to mechanical shear stress imposed by e.g. wave action, causing collision and embrittlement of the polymer structure (Barnes et al. 2009). In addition, fragmentation is accelerated by the weathering process (most notably, photooxidation, see below), which breaks chemical bonds, allowing oxygen incorporation into the chemical structure (Kalogerakis et al. 2017; Gewert et al. 2015; Wayman and Niemann 2021). Fragmentation of macroplastics results in large fragments as well as particles on the milli-, micro- and nanometre scale. In the ocean, small microplastic particles dominate in abundance, while nanoplastics have only been detected recently (Ter Halle et al. 2017), and their abundance and distribution in marine systems are unknown.

Plastic degradation is facilitated by UV radiation, causing photooxidation. Photooxidation is a multi-step process, resulting in changes in the chemical structure as well as in the physical appearance of the polymer. UV radiation causes chemical bonds to break, and the formation of free radicals, which may react further with the polymer. This chain reaction proceeds until stable products have been formed and typically involves incorporating oxygen in the carbon backbone. Moreover, UV-induced photooxidation causes the release of chain scission products from the polymer matrix, e.g. low-molecular-weight polymer fragments with carboxyl groups (Gewert et al. 2018), and hydrocarbon gases such as methane, ethylene, ethane and propylene (Royer et al. 2018). Nanoparticle formation due to UV irradiation has been reported from PS (Lambert and Wagner 2016). Furthermore, UV exposure causes dissolved organic carbon to leach from the plastics (Zhu et al. 2020; Romera-Castillo et al. 2018). Extrapolation to the global scale revealed that 23,600 metric tons of DOC might leach annually to the marine environment (Romera-Castillo et al. 2018). Due to variations in chemical structure, polymers react to mechanical and oxidative stress differently, with PE having higher dissociation energy in comparison to PP (Gewert et al. 2015; Min et al. 2020). PE is characterized as more susceptible for oxidative stress, possibly due to additives it contains, as heavily oxidized patches have been observed on otherwise intact plastics (Cooper and Corcoran 2010). The number of factors affecting the fate of plastics in natural environments can be considered ‘unlimited’, as the environment and the polymer itself can vary. Besides UV exposure, variables such as temperature, mechanical forces (wave action, wind), visible light and, potentially, microbial growth (Gu 2003; Artham et al. 2009; Gewert et al. 2015; Klein et al. 2018; Pickett 2018; Min et al. 2020) may affect the further fate of plastic in the environment.

5.3.2 The Potential for Microbially Mediated Plastic Degradation

Biodegradation is a biologically mediated process, whereby organisms convert complex compounds into simpler and smaller molecules or environmentally less hazardous ones.

In the marine environment, organic matter is both aerobic and anaerobically degraded by microbes. Aerobic biodegradation occurs at the sea surface, in the water column or oxic layers of sediments, while anaerobic biodegradation proceeds in deeper sediments or other anoxic (micro)niches. Aerobic biodegradation requires oxygen as an electron acceptor and commonly results in the production of the terminal oxidation end product CO_2. Anaerobic degradation processes, on the other hand, utilize alternative electron acceptors and may oxidize, reduce or disproportionate the organic matter substrate, yielding CO_2 and/or CH_4. Polymer biodegradation involves depolymerization, yielding oligomers and/or monomers and probably further degradation of such reaction intermediates to fuel catabolic and anabolic processes. Biodegradation may be incomplete, which is often referred to as ‘biodeterioration’ (which may also occur due to the combination of biotic and abiotic processes). However, both biodegradation and biodeterioration result in the loss of structural integrity of the polymer, which supports fragmentation and eventual breakdown.

Microorganisms can degrade, transform, convert and accumulate a wide variety of organic compounds. Polymers of biological origin such as chitin, cellulose, polyhydroxybutyrate and pullulan, for example, undergo rapid and complete mineralization in nature. Microbes and microbial consortia can also degrade complex hydrocarbons, such as petroleum and polycyclic aromatic hydrocarbons (Yakimov et al. 1998; Huy et al. 1999; McKew et al. 2007; Zhao et al. 2008; Zhou et al. 2008; Joutey et al. 2013; Kim et al. 2015), which chemically resemble some polyolefin-type plastics. In principle, plastic polymers could thus be potential substrates for microorganisms (Wayman and Niemann 2021). However, plastics are synthetic and, on an evolutionary time scale, a new substrate for microorganisms. Even though plastics hold chemical energy, it is unclear to which extent microbes can make use of these. Plastic polymers are structurally large and complex molecules. Microorganisms have developed strategies to break down large molecules extracellularly, which also appears to be the case for some plastics. The organisms secrete exoenzymes, which act on the plastic surface, releasing smaller scission products. These smaller molecules may then be degraded further extracellularly or taken up into the cells (see Sect. 5.5 for details).

Several microorganisms depolymerize/degrade specific plastics, for example *Rhodococcus ruber* (Gilan et al. 2004; Sivan et al. 2006; Mor and Sivan 2008; Yang et al. 2018), *Ideonella Sakaiensis* (Tanasupawat et al. 2016; Yoshida et al. 2016), *Brevibacillus borstelensis* (Hadad et al. 2005), several strains of *Pseudomonas* sp. (Ward et al. 2006; Ronkvist et al. 2009; Kyaw et al. 2012; Pramila et al. 2012), several strains of *Bacillus* sp. (Sudhakar et al. 2008; Harshvardhan and Jha 2013; Yang et al. 2014; Syranidou et al. 2017; Ingavale and Raut 2018; Novotný et al. 2018), *Zalerion maritimum* (Paço et al. 2017), *Penicillium simplicissimum* (Sowmya et al. 2015), *Penicillium citrinum* (Liebminger et al. 2009), *Fusarium solani* and *Fusarium oxysporum* (Nimchua et al. 2007) and also several strains of *Aspergillus* sp. (Pramila and Ramesh 2011; Esmaeili et al. 2013). However, less is known about the potential plastic biodegradation in the oceans, the involved microorganisms and metabolic pathways. The following sections address the most commonly applied techniques and methods to detect plastic degradation and identify responsible microorganisms.

5.4 Methods and Techniques Applied in the Assessment of Polymer Biodegradation

5.4.1 Methods to Evaluate Biodegradation

Monitoring alterations of polymers' physicochemical properties, including changes in crystallinity, molecular weight, the topography of samples and the functional groups, during experiments, is a common way to evaluate the involvement of microbes in plastic degradation. These may be specific strains or consortia of cultured microbes as well as natural communities. In contrast, the genesis of degradation products is less frequently measured. These are some of the most frequently used physicochemical methods:

1. One of the most common methods to evaluate biodegradation is measuring the gravimetric mass loss of plastics, i.e. monitoring plastic weight-changes exposed to natural or laboratory (cultures, microbial consortia or environmental communities) conditions. Marine isolated bacteria from pelagic coastal waters of the Arabian Sea were assayed for the ability to utilize polyethylene as the sole

carbon source (Harshvardhan and Jha 2013). Three of the strains identified as *Kocuria palustris* M16, *Bacillus pumilus* M27 and *Bacillus subtilis* H1584 caused a weight loss of the tested PE film of 1%, 1.5% and 1.75% after 30 days of incubation, respectively. Marine acclimated consortia (indigenous and bio augmented consortia) reduced the weight of PS films more efficiently than non-acclimated bacteria (Syranidou et al. 2017). *Penicillium oxalicum* NS4 and *Penicillium chrysogenum* NS10 strains reduced the weight of PE films by ~60% over 90 days period (Ojha et al. 2017). Environmental studies assessing gravimetric mass loss have been carried out in the water column, sand beds and sediments (Kalogerakis et al. 2017; Syranidou et al. 2017; Welden and Cowie 2017). Gravimetric measurements do not require extensive resources, but they lack accuracy to detect small mass changes (as is typically the case during exposure experiments). Determination of the polymer weight is complicated further by the fact that post-incubation treatment of plastic samples is necessary to remove biofilms and residues of organic matter, which bears the risk of accidentally altering the mass of the plastic itself. Finally, when this method is applied in natural conditions, it is imprecise to distinguish between abiotic (weathering, ageing and/or loss) and biotic (biofragmentation, biodegradation and/or biomineralization) degradation.

2. An alternative method to gravimetric measurements in assessing microbial degradation is to monitor weigh-average-molecular-weight (Mw) and weigh-number-molecular-weight (Mn). An increase in both Mw and Mn is related to the consumption of the low Mw of the polymer (see Sect. 5.5) and might also be related to crosslinking reactions of the carbon backbone. Shifts of the spectra towards high Mw and Mn have been reported in several studies (Albertsson et al. 1995, 1998; Kawai 1995; Erlandsson et al. 1998; Hakkarainen and Albertsson 2004; Koutny et al. 2006b; Yoon et al. 2012), including marine-related strains.
3. Atomic force microscopy (AFM) (Binnig et al. 1983), measurements offer quantitative and qualitative data on the surface topography and occurring changes, with high spatial resolution. Exposure of silicone rubber, polyurethane, polyester, syntactic foam, glass fibre reinforced polymer and carbon fibre reinforced plastic for 1 year in the water column increased surface roughness (Muthukumar et al. 2011). Similarly, an increase in roughness, development of cracks and grooves were monitored on HDPE and LDPE films after exposure to *Penicillium oxalicum* NS4 and *Penicillium chrysogenum* NS10 strains for 90 days (Ojha et al. 2017). Scanning electron microscopy (SEM) has been used to visualize physical deterioration of polymer surface (pits, cracks, grooves or other abnormalities on the plastic surface) as a function of exposure to the environment and/or microbes. Simultaneously, SEM allows visualization of adhering prokaryotes and eukaryotes (Zettler et al. 2013; Eich et al. 2015; Bryant et al. 2016; Paço et al. 2017; Dussud et al. 2018b; Delacuvellerie et al. 2019) (Fig. 5.4).
4. Fourier transformation infrared spectroscopy (FTIR) that can be coupled to attenuated total reflectance (ATR) is one of the most widely used technique for polymer identification and evaluation of degradation. FTIR allows molecular and

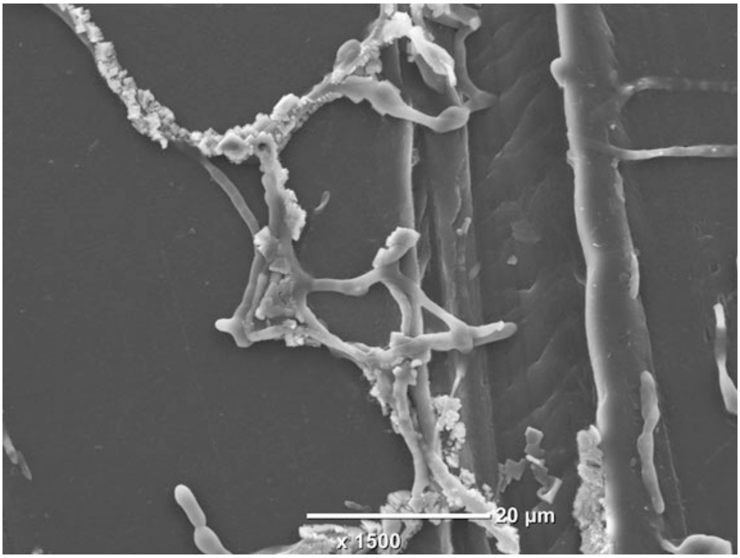


Fig. 5.4 SEM image of photo-oxidized PE, colonized by a marine fungus

structural characterization of the polymers, including plastics, copolymers and rubbers and their products (Bhargava et al. 1970; Chalmers 2006; Mecozzi et al. 2016). FTIR is mostly applied to detect oxidative damages on plastic polymers (Almond et al. 2020), by either abiotic or biotic factors. Degradation by microorganisms is monitored by the detection of specific peaks in the IR spectrum. The changes of carbonyl groups (as a result of UV oxidation or biodegradation) and the relative size of these peaks, described by the carbonyl index (peak intensity at 1850–1650 cm^{-1} in relation to the reference peak) (Almond et al. 2020), is routinely taken as a measure to determine the magnitude of degradation. It has become a standard method for identification of microplastics in sediments and the water column (Veerasingam et al. 2020). Examples of FTIR applications range from identification of polymers ingested by sea turtles (Jung et al. 2018) to the formation of carbonyl groups on PE exposed to seawater (Da Costa et al. 2018) and evaluation of the degradation of PET bottles in seawater (Ioakeimidis et al. 2016). Although FTIR can be applied to assess the biomass attachment to the polymers, revealing the presence of nucleic acids, lipids and protein content of the biomass (Paço et al. 2017), the same biomass signals may interfere with the absorption spectra of the polymer (Bonhomme et al. 2003).

Besides the above-mentioned methods, several other methods are applied to assess plastic degradation. Respirometric measurements evaluate the production of excess CO_2 and biomass variations when the polymer is the only available carbon source for microorganisms. Mechanical properties, such as tensile strength, crystallinity, hydrophobicity/hydrophilicity of the surface, are, as well, taken as a measure of degradation (Pegram and Andrady 1989). Labelled polymers (^{14}C) were used already in the 1970s, to demonstrate PE and PS degradation by fungal strains (Guillet et al. 1974; Albertsson 1978). Recently, isotopically labelled polymers were applied in the terrestrial realm to evaluate plastic-derived carbon assimilation into living cells (Zumstein et al. 2018). Similarly, labelled ^{13}C-polyethylene has been used to evaluate aquatic microbial biodegradation and eventually trace microbial-animal trophically transfer (Taipale et al. 2019). The combination of isotopically labelled polymers with molecular analyses to evaluate the mineralization (incorporation of labelled carbon into cells, nucleic acids or lipids, for example) are promising techniques to be explored.

Despite various evaluation methods to investigate plastic biodegradation, many lack rigor in fully confirming the initial depolymerization, assimilation and mineralization, and thus remain non-quantitative and inaccurate.

5.4.2 Colonization of Prokaryotes and Eukaryotes on Marine Plastic

Cultivation-independent studies often investigate microbial community composition by next-generation sequencing methodologies, mainly amplicon sequencing of the 16S rRNA (for prokaryotes) or the 18S rRNA gene (for eukaryotes). Only sparsely have eukaryotes been targeted by sequencing of the Internal Transcribed Spacer (ITS) region with specific primers (De Tender et al. 2017) or by metagenomic sequencing (Bryant et al. 2016; Pinnell and Turner 2019), to unravel which organisms adhere to the plastic surface. Applying next-generation sequencing for detecting unknown plastic degraders involves the premiss for substrate-driven selection of plastic degraders during colonization (i.e. that plastic surface will be colonized preferentially by plastic degraders). However, attachment to surfaces is a universal and fundamental trait of many microorganisms across the three domains of life, because life in a biofilm offers critical advantages (De Tender et al. 2015; Dang and Lovell 2016). In marine environments, any available surface will be colonized rapidly by microbes since forming assemblages and biofilms on a surface protects microbes from fluctuating environmental parameters such as UV radiation and predation (Eich et al. 2015). Thus, caution has to be taken when interpreting colonization data while trying to detect potential plastic degraders.

Biofouling is a multi-step process composed of priming the surface, attachment of early settlers, secretion of extracellular polymeric substances and the formation of mature biofilms (Flemming and Wingender 2010; Rummel et al. 2017). In a mature biofilm, microorganisms have proliferated, and secondary settlers are incorporated into the biofilm and may have replaced primary settlers. For plastic marine debris, the surrounding seawater provides the initial inoculum of microbial assemblages which can adhere to the polymer surface. Sequentially, the early colonizers influence the mature biofilm composition and dynamics (Dang and Lovell 2000; Dang et al. 2008). Microorganisms colonize submerged surfaces rapidly, including plastics

(Salta et al. 2013; Harrison et al. 2014). That raises the question if plastics host a specific microbial community when compared to seawater and other hard surfaces?

For plastics, it has been shown that the community of settlers and the surrounding seawater share a high degree of the same taxa; however, plastics harbour distinct and significantly different microbial assemblages (Bryant et al. 2016; De Tender et al. 2017; Kettner et al. 2017; Dussud et al. 2018b; Frère et al. 2018; Vaksmaa et al. 2021). A recent review suggests to refrain from using seawater as a control because free-living and biofilm communities differ (Wright et al. 2020a). Using hard/inert surfaces (PET, PHA and ceramics) during benthic incubations revealed that the degree of similarity between the settling communities is higher than the seawater inoculum (Pinnell and Turner 2019). Indeed, microbial communities on PS, PP and PE were different from the communities developed on glass and cellulose (Ogonowski et al. 2018). A specific bacterial community was detected on PVC, but communities were similar on glass, LDPE, HDPE and PP (Kirstein et al. 2019). The approach by Kirstein et al. was novel as it focussed on the tightly adhered community members, assuming that direct contact with plastic could indicate more relevance in the potential role in biodegrading the plastic. Specific microbial community on ‘wild plastic’ was found on PS in comparison to PE and PP (Frère et al. 2018). In contrast, no difference was observed in the microbial communities, which developed on PET bottles and glass slides exposed at different locations and during different seasons (Oberbeckmann et al. 2016). Microbial communities on plastic have been reported to be more distinct in the early stage of biofilm formation (Pinto et al. 2019). However, currently, the hypothesis that plastics harbour a distinct microbial community in comparison to other inert surfaces can be neither confirmed nor rejected (Wright et al. 2020a) particularly in mature biofilms that are highly diverse.

Another critical question is: what is the role of additives in polymers in structuring the microbial communities? The majority of studies focus on the polymer type as the determining factor for biofilm composition. However, plastics are rarely used in their pure form. Consumer plastics usually contain additives, yet only a few studies investigated the potential influence of additives on the microbial community composition. Differential microbial communities were observed on PVC compared to other tested polymers (Kirstein et al. 2019); however, the authors hypothesized that this might have been caused by PVC additives and not per se the polymer. To better understand microbial colonization dynamics on plastics, future research endeavours need to investigate early stage community succession, the role of polymer type and additive admixture, and weathering in determining microbial community composition and succession.

5.4.2.1 Prokaryotic Colonizers on Marine Plastic

The core members of microbial assemblages on plastic are seemingly the same taxa independent of geographical location and whether the sample has been in the water column or sediments. For example, *Flavobacteriaceae* and *Rhodobacteraceae* were detected on PET bottles (Oberbeckmann et al. 2016), on sheets and dolly ropes of PE (De Tender et al. 2017), PVC (Dang et al. 2008), PE and PP (Zettler et al. 2013) and on PE, PP and PS (Vaksmaa et al. 2021) based on 16S rRNA gene amplicon sequencing. Similar results for PE and PP were obtained by metagenomic sequencing (Bryant et al. 2016). Other commonly detected families are *Alteromonadaceae* (Bryant et al. 2016; Xu et al. 2019) and *Saprospiraceae* (Bryant et al. 2016; Oberbeckmann et al. 2018; Kirstein et al. 2019), *Hyphomonadaceae* (Zettler et al. 2013; Bryant et al. 2016; Dussud et al. 2018b; Oberbeckmann et al. 2018; Ogonowski et al. 2018), *Sphingomonadaceae* (Debroas et al. 2017; Oberbeckmann et al. 2018; Ogonowski et al. 2018) and *Vibrionaceae* (Zettler et al. 2013; De Tender et al. 2015; Frère et al. 2018). Nevertheless, the functioning of these commonly detected taxa (often termed the 'core community of the plastisphere') remains unresolved, and it is unclear if and in how far these organisms play a role in potential plastic degradation. Indeed, plastic can be colonized by opportunistic microbes for which life in a biofilm is advantageous. It seems that general microbial colonizers are often early colonizers, such as members of Rhodobacterales (Dang and Lovell 2000; Dang et al. 2008; Elifantz et al. 2013; Schlundt et al. 2020; Tu et al. 2020). Other commonly detected groups in biofilms such as *Saprospiraceae* and *Flavobacteriaceae* have a preference for an adhered lifestyle (DeLong et al. 1993; Fernández-Gómez et al. 2013).

Several studies have also detected hydrocarbon-degrading bacteria (HCB) in plastic-associated biofilms (Zettler et al. 2013; Oberbeckmann et al. 2016; Debroas et al. 2017; Dussud et al. 2018b; Pinto et al. 2019; Erni-Cassola et al. 2020; Vaksmaa et al. 2021). A comparison of 2229 datasets of 35 independently published investigations of biofilm communities on plastic polymers was conducted to reveal common taxa in these biofilms (Wright et al. 2020b). This overview study highlighted that often, a fraction of the plastic colonizers were hydrocarbon degraders, specifically Oceanospirillales and Alteromonadales. Obligate hydrocarbon-degrading bacteria were found on thermo-oxidatively weathered and non-weathered polyethylene after 2 days of exposure to coastal waters and constituted 5.8% and 3.7% of relative sequence abundance, which stands in stark contrast to glass controls where they constituted only 0.6% (Erni-Cassola et al. 2020). Similarly, hydrocarbon degraders (mainly *Erythrobacter*) were found to comprise 7.4% of amplicon reads of plastic-associated biofilms in comparison to 7.8% of organic particle-associated (>3μm), while a lower abundance of 4.7% was found free-living (Dussud et al. 2018b). In a seawater flow-through reactor, where, LDPE, PE with added pro-oxidant, thermally aged PE, polyester and poly (3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) were exposed for 6 weeks, HCBs constituted 34% of the biofilm community on the polymers. Especially *Alcanivorax* sp., *Alteromonas* sp., *Marinobacter* sp. and *Oleiphilus messinensis* were abundant, forming >5% of the total OTUs in one sample (Dussud et al. 2018a).

Genomes of hydrocarbon degraders encode for mono- and dioxidases, hydrolases and peroxidases (Brzeszcz and Kaszycki 2018). These enzymes can break down long chain or complex hydrocarbon molecules, which resemble some polyolefin-type plastics. Hydrocarbon degraders are thus potential candidates to break down plastic polymers (Dussud et al. 2018b; Basili et al. 2020; Erni-Cassola et al. 2020), and they may rapidly colonize plastic surfaces, because they are common in marine environments where hydrocarbon compounds (e.g. oil) are available (Kimes et al. 2013; Joye et al. 2014; Beckmann et al. 2019).

The argument that a versatile hydrocarbon degrader might indeed be able to degrade plastic is further underscored by the ability of the HCB *Rhodococcus ruber* (strain C208), an actinomycete, to degrade PE, PP and PS. *Rhodococcus ruber* possesses the ability to facilitate degradation of both linear, branched polymers and PS, which contains aromatic styrenes (Mor and Sivan 2008). Upregulated pathways during PE degradation by *Rhodococcus ruber* were those that are also active during alkane degradation and β-oxidation of fatty acids (Gravouil et al. 2017). Santo et al. demonstrated the upregulation of laccase upon UV-treated PE exposure in *Rhodococcus ruber* C208, indicating the possible role in the oxidation of the PE. An extracellular isoform of the laccase of the copper-induced *R. ruber C208* cells reduced 20% the Mw of the PE (Santo et al. 2013). Among Rhodococci, the ability to degrade aliphatic, aromatic and polyaromatic hydrocarbons has been shown. The genome of *Rhodococcus ruber* strain IEGM 231 harbours 45 dioxygenases, 73 monooxygenases, 22 cytochromes P450 oxygenases and 285 dehydrogenases (Ivshina et al. 2014). *Rhodococcus ruber* YC-YT1 strain, isolated from plastic in coastal seawater, is able to degrade di-(2-ethylhexyl) phthalate, a plasticizer of polyvinyl chloride, into phthalate (Yang et al. 2018). *Rhodococcus* sp. was isolated from terrestrial and marine environments (Sorkhoh et al. 1990; Hackbusch et al. 2020). Hydrocarbon degraders may also be fuelled indirectly with plastic derived carbon. Photooxidation by UV radiation initiates chain scission and the formation of carbonyl groups (Gewert et al. 2018). Hence, HCB might utilize the released hydrocarbons or take advantage of partially oxidized polymers. Indeed, it was recently found that DOM released during plastic photooxidation can be utilized by microbes (Romera-Castillo et al. 2018; Zhu et al. 2020).

5.4.2.2 Eukaryotes as Plastic Colonizers and Degraders

Immersed plastic surfaces are subjected to biofouling, a process that entails that a succession of microorganisms and multicellular organisms from different trophic levels attach to plastic debris. Plastic polymers in the marine environment harbour a diverse eukaryotic community as has been validated by visual observations, microscopy together with amplicon and metagenomic sequencing. Metagenomic sequencing revealed that in some cases, sequencing reads assigned to eukaryotes are more abundant than those assigned to prokaryotes (Bryant et al. 2016). Twenty-seven eukaryotic species were identified by Nanopore minION sequencing as plastic specific on PE bags exposed for 1 month to the Mediterranean Sea (Davidov et al. 2020). Microscopy-based investigations found that diatoms are early plastic surface colonizers, but they are also present in more mature biofilms (Carson et al. 2013; Zettler et al. 2013; Eich et al. 2015). Algae were also found to adhere to marine plastic surfaces (Zettler et al. 2013; Oberbeckmann et al. 2014; Bryant et al. 2016; Dussud et al. 2018b). In brackish environments, different eukaryotes were found on PE and PS: Ulva, Trebouxiophyceae, ciliates, rotifers Adinetida and Ploimida, the nematodes Diplogasterida and Rhabditida, mollusk Caenogastropoda and crustacean Podocopida (Kettner et al. 2019). Plastic debris offers for eukaryotic invasive species a path to disperse (Kiessling et al. 2015).

In the marine realm, fungi are understudied in general and in particular for fungi–plastic interactions. Until now, more than 50 studies have investigated 16S rRNA genes related to prokaryote–plastic interaction, but only less than ten have addressed the fungal community in marine and brackish environments (Wright et al. 2020b). The taxonomic composition of fungi in a seafloor PE biofilm community was investigated in the harbour of Ostend in Belgium during long-term incubation over 44 weeks (De Tender et al. 2017). Biofilm formation occurred within a week of exposure. In contrast to previous studies, this study could not detect bacteria that are known to degrade plastics. On the other hand, it found the fungal strains *Cladosporium cladosporioides*, *Fusarium redolens* and *Mortierella alpine*, which were previously identified as PE degraders (Albertsson 1978; Bonhomme et al. 2003; Koutny et al. 2006b; Restrepo-Flórez et al. 2014). The fungal community composition on PE and PS has also been investigated in the Baltic Sea, the River Warnow and a wastewater treatment plant. The results revealed that fungal communities on microplastics were different when compared to communities in the surrounding water and on wood (Kettner et al. 2017). The taxonomic composition of plastic-associated fungi in surface waters of the Antarctic Peninsula and the western South Atlantic was compared by using several molecular markers; ITS2 and the V4 and V9 regions of the 18S rRNA gene. At both locations, PE, polyamide, PUR, PP and PS were colonized by fungi. Besides identification of *Aspergillus, Cladosporium*, *Wallemia, Chytridiomycota* and *Aphelidomycota*, as well as *Zoopagomycota* and *Mucoromycota* were identified, taxa previously detected in the marine environment, but so far not on plastics (Lacerda et al. 2020). Only a few marine isolates of fungi have been evaluated for their ability to colonize and degrade plastics. A marine fungus, *Zalerion maritimum*, was isolated and able to utilize PE (250μm to 1 mm particles) in a minimal growth medium, already within 7 days (Paço et al. 2017). Concerning the generally diverse enzymatic potential of fungi, it is valid to assume that there are more fungal species able to degrade plastics in the marine environment than previously thought. This hypothesis is further supported by the already diverse set of fungal strains retrieved from lacustrine and terrestrial environments that are seemingly able to degrade plastic. From freshwater environments, ~100 fungal isolates from floating plastic debris in Lake Zurich, Switzerland were obtained and evaluated for their ability to degrade plastics. Different species, including saprotrophic and plant pathogenic fungi, were isolated. Although none of the strains isolated from plastic appeared to degrade PE, *Cladosporium cladosporioides*, *Xepiculopsis graminea*, and *Penicillium griseofulvum* (saprotrophic fungi) and the *Leptosphaeria* sp. (plant pathogen) were able to degrade PUR (Brunner et al. 2018). Furthermore, *P. simplicissimum* YK, a soil fungus, was capable of degrading previously irradiated PE and using it as a carbon source (Yamada-Onodera et al. 2001). *Aspergillus flavus*, isolated from the gut of the wax moth *Galleria mellonella*, is able to degrade HDPE. However, the HDPE was UV irradiated for sterilization prior to the experiment (Zhang et al. 2020). Fungal strains isolated from endemic plants *Aspergillus* sp., *Paecilomyces lilacinus* and *Lasiodiplodia theobromae* were able to degrade gamma-irradiated LDPE as shown by the decrease in intrinsic viscosity and average molecular weight. However, only *Lasiodiplodia theobromae* was able to degrade irradiated polypropylene as well (Sheik et al. 2015).

5.5 Enzymatic Potential of Microbes

5.5.1 General Considerations

Enzymes are large biomolecules acting as biological catalysts for the numerous (bio)chemical reactions that sustain life (Gurung et al. 2013). They are present in cells of all living organisms, including eukaryotes and prokaryotes, and are crucial to maintaining the organism's metabolism as most essential biochemical reactions proceed very slowly or may not occur spontaneously when uncatalysed (Harris and Hopkinson 1976; Berg 2002; Robinson 2015; Sheel and Pant 2018). Thus, biochemical reactions and metabolic pathways depend upon enzymes to catalyse each step by lowering the reaction's activation energy or changing the mechanism (Berg 2002; Lucas et al. 2008; Blanco and Blanco 2017). Structurally, the vast majority of enzymes are proteins, often containing or requiring other components, e.g. inorganic elements such as minerals or metal ions (Fe^{2+}, Mn^{2+}, Zn^{2+}), non-protein organic compounds (e.g. vitamins) and other cofactors (Kamerlin and Warshel 2010). Microbial enzymes accomplish numerous functions, including breaking down large molecules extracellularly. Microbes can excrete depolymerizing exoenzymes, which produce smaller sub-products that can be assimilated and further metabolized intracellularly. Microbes attach to the substrate and form biofilms of commensal and/or syntrophic consortia to enhance extracellular degradation. Although many microorganisms can break down natural complex organic polymers, the enzymatic capability to catalyse depolymerization of synthetic polymers such as plastics is rare (Albertsson 1978; Albertsson et al. 1995; Yoshida et al. 2016). Debate persists whether microbial enzymes used to degrade complex natural polymers (e.g. cutin, a waxy compound coating leaves, containing ester-bonds as in PET) have had enough time to evolve and to adjust to equivalent functions on synthetic polymers. Biodegradation of plastics is described as slow and complex process (Albertsson 1980; Hakkarainen and Albertsson 2004). Although several microbes have been shown to facilitate the breakdown of plastics, the key enzymes and metabolic pathways involved in the degradation process are not well understood, and little knowledge exists on the degradation intermediates and if these are assimilated (Ru et al. 2020). Like other complex organic compounds, plastic degrading microbes would need to attack plastics extracellularly and (partially) depolymerize the complex and large molecule to compounds that could be utilized as carbon and energy source (Gu 2003).

Research on microbial plastic degradation in terrestrial environments gained momentum over the last decades (Gilan et al. 2004; Zhao et al. 2004; Hadad et al. 2005; Sabev et al. 2006; Matsumiya et al. 2010; Latorre et al. 2012; Rajandas et al. 2012; Ali et al. 2014; Yoshida et al. 2016; Gravouil et al. 2017; Wei and Zimmermann 2017a, b; Austin et al. 2018). However, considerably fewer studies have been carried out in marine environments (Pegram and Andrady 1989; Artham et al. 2009; Balasubramanian et al. 2010; Lobelle and Cunliffe 2011; Yoon et al. 2012; Harshvardhan and Jha 2013). The next sections address extracellular and intercellular biodegradation.

5.5.2 Extracellular Biodegradation

Early studies have shown that only molecules <600 Da can pass the cellular membrane (Haines and Alexander 1974; Decad and Nikaido 1976). As prokaryotes cannot perform exo- or endocytosis, molecular weight is a critical factor in biodegradation (Albertsson et al. 1995, 1998; Kawai 1995; Erlandsson et al. 1998; Gu 2003; Hakkarainen and Albertsson 2004; Koutny et al. 2006b). High molecular weight results in a sharp decrease in solubility, rendering plastics unfavourable for the microbial attack, and a decrease in molecular weight increases the biodegradability (Gu 2003). Microorganisms apply extracellular enzymes to initiate degradation, and these bind to the plastic and catalyse bond cleavage in a single or a series of reactions (Kopeček and Rejmanová 2019), such as oxidation, reduction, hydrolysis and deesterification. In marine and aquatic environments, it is thus fundamental for the organisms to attach to the plastic surface to use it most efficiently as a substrate, although other free-living microorganisms might utilize intermediate products that are released during the initial extracellular degradation steps.

Concurrent abiotic and biotic processes have been suggested to facilitate plastic degradation (Albertsson et al. 1987; Hakkarainen and Albertsson 2004). An initial step of e.g. photooxidation, introducing carbonyl groups to the polymer backbone would quasi activate it for further enzymatic degradation (Gewert et al. 2015; Romera-Castillo et al. 2018; Wayman and Niemann 2021). By weathering, mainly the plastic surface gets initially affected; i.e. <100μm in photooxidation processes (Ter Halle et al. 2016). Therefore, studies investigating microbial degradation of pre-treated plastics need to be interpreted carefully, and additional information on the pre-treatment process needs to be taken into account for the results to be comparable and reproducible.

Plastics with functional groups, like esters, amides, carbonates and urethanes, are more prone to microbial attack because equivalent functional groups are present in other natural compounds. These heteroatoms and functional groups allow for a faster bond cleavage via enzymatic hydrolysis (Min et al. 2020). While genes and enzymes involved in the microbial degradation of such plastic types have been better characterized (Ghosh et al. 2013; Wei and Zimmermann 2017b; Ru et al. 2020), knowledge on the degradation of plastics with no hydrolysable chemical bonds in their backbone such as PE, PP, PVC is scarce (Zheng et al. 2005; Singh and Sharma 2008; Wei and Zimmermann 2017b; Ru et al. 2020). The exoenzyme group of depolymerases are able to degrade the substrate into smaller molecules. Nevertheless, the critical depolymerases involved in plastic degradation and the resulting depolymerization products remain often unknown (Ru et al. 2020). For instance, in the case of PE, past studies have identified enzymes related to peroxidases, laccase and homologous sequences related to laccases/multicopper oxidases as potential candidates involved in biodegradation. Furthermore, a manganese peroxidase (MnP) has been found to decrease the tensile strength and average molecular weight of PE (Iiyoshi et al. 1998). Similarly, the combination of soybean peroxidase (SBP) and hydrogen peroxide can oxidize and diminish the surface hydrophobicity of PE film (Zhao et al. 2004). Generation of carbonyl groups in PE films by oxidation could be mediated by a laccase exoenzyme (Santo et al. 2013). Three homologous sequences related to laccases/multicopper oxidases were identified in the transcriptome of *Rhodococcus ruber*, although the genes encoding for those sequences were neither up- nor down-regulated in an experiment with PE as a sole carbon source (Gravouil et al. 2017). In addition, recombinants from three alkane hydroxylase genes (alkB, alkB1 and alkB2) of *Pseudomonas aeruginosa* E7 strain from a contaminated beach soil were able to degrade low-molecular-weight PE (Yoon et al. 2012; Jeon and Kim 2015, 2016a). *P. aeruginosa* can use a wide range of substrates as a carbon and energy source and is a common microbial community member in soil and water (Jeon and Kim 2016a). *P. aeruginosa* strains PAO1 and RR1 contain enzymes involved in the degradation of n-alkanes: 2 alkane monooxygenases, 2 rubredoxins and 1 rubredoxin reductase (Marín et al. 2003; Jeon and Kim 2016a). In the case of PS, only a hydroquinone peroxidase was able to depolymerize PS into low-molecular-weight products in the presence of non-aqueous medium (dichloromethane) (Nakamiya et al. 1997) (Table 5.1). To the best of our knowledge, there are no reports on enzymes that degrade PP, and potential biodegradation processes remain to be clarified (Arutchelvi et al. 2008; Ru et al. 2020). Biodegradation of PP is expected to be more challenging than PE due to the stable methyl group in every monomer (Arkatkar et al. 2009; Jeon and Kim 2016b). Biodegradation studies of PP have, therefore, mostly been carried out with pre-treated substrates (UV irradiation, thermo-oxidation, γ-irradiation) under laboratory conditions (Alariqi et al. 2006; Jeyakumar et al. 2013; Sheik et al. 2015). For instance, two bacterial isolates of *Bacillus* sp. and *Rhodococcus* sp. (strain 27 and strain 36, respectively) from mangrove environments were able to grow in aqueous synthetic media containing UV-radiated PP microplastics (Auta et al. 2018). However, neither the biodegradation of the untreated plastic nor the processes and metabolic enzymes involved in the weight loss were studied. In the case of polypropylene blends (Jeyakumar et al. 2013; Jain et al. 2018) and PP with prooxidants (Fontanella et al. 2013), it is difficult to discern if the recalcitrant plastic is degraded. To date, only a few studies have shown biodegradation of untreated PP (Arkatkar et al. 2009; Jeon and Kim 2016b). However, these promising reports of PP degradation were based on weight loss or on the increase of the average molecular weight. It needs to be further tested if this is the result of degradation of the long-chain PP polymer or other shorter-chain molecules that might have been present in the plastic, too.

Regarding polyamides, few studies have shown biodegradation of different types of nylons (Negoro et al. 1992; Gold and Alic 1993; Klun et al. 2003; Tomita et al. 2003a, b), but only one study investigated PA degradation by marine bacteria and found the formation of new functional groups in the polymermatrix (i.e. NHCHO, CH_3, $CONH_2$, CHO and COOH; Sudhakar et al. 2007). Interestingly, the authors observed a greater extent of PA degradation by the marine bacteria when compared

Table 5.1 Marine microorganisms and enzymes associated with plastic biodegradation

Polymer	Relevant enzymes/ microorganism	Sample source	Test culture/environmental conditions, carbon source and time of exposure in days (d)	Weight loss (%)	Reference
PE (LDPE and HDPE film, unpretreated/thermal pre-treatment)	*Bacillus sphaericus* Alt; *Bacillus cereus* BF20	Marine water (shallow oceanic water)	MSM + PE SCS/150–365 d	Un-PE: 2–10 therm-PE: 6.52–19	Sudhakar et al. (2008)
PE (LDPE film)	*Bacillus subtilis* H1584	Marine water	30 d	1.75	Harshvardhan and Jha (2013)
PE (Pellets)	*Zalerion maritimum*	Marine environment	28 d	–	Paço et al. (2017)
PE (LDPE film)	*Alcanivorax borkumensis*	Mediterranean Sea (macroplastics afloat and in sediments)	Medium (0.05% hexadecane) + PE/80 d	3.5	Delacuvellerie et al. (2019)
PE (LMWPE: Thermal decomposition of HDPE and LDPE)	*Pseudomonas* sp. E4/*alkane* *hydroxylase gene* (alkB)	Marine environment (contaminated beach soil with crude oil)	Recombinant *E. coli* BL21 with alkB gene + compost with PE/80 d	4.9, 10.3, 14.9 and 28.6^a	Yoon et al. (2012)
PE (LMWPE: Thermal decomposition of HDPE and LDPE)	*Pseudomonas aeruginosa* E7/*alkane* *Monooxygenase, rubredoxin* and *rubredoxin reductase* genes (alkB, rubA1, rubA2 and rubB)	Marine environment (contaminated beach soil with crude oil)	Recombinant *E. coli* DH5-a with different genes + compost with PE/80 d	40.8^a	Jeon and Kim (2015)
PE (LMWPE: Thermal decomposition of HDPE and LDPE)	*Pseudomonas aeruginosa* E7/*alkane* *Monooxygenase, rubredoxin* and *rubredoxin reductase* genes (*alkB1*, *alkB2*, *rubA1*, *rubA2* and *rubB*)	Marine environment (plastic polluted coastal)	Recombinant *E. coli* DH5-a with different genes + compost with PE/50 d	$19.6–27.6^a$	Jeon and Kim (2016b)

PVC (LDPE and HDPE films (20 min UV radiation))	*Bacillus* sp. AIIW2	Marine environment (contaminated beach soil with crude oil)	BH minimal medium + PVC/ 90 d	0.96–1	Kumari et al. (2019)
PP (UV pre-treated microplastics from grated/cut PP granules (9003-07-0, sigma Aldrich) and commercial PP plastic materials)	*Bacillus* sp. strain 27; *Rhodococcus* sp. strain 36	Marine environment (mangrove sediment)	BH medium + PP/40 d	4–6.4	Auta et al. (2018)
PVC (PVC film without plasticizers (20 min UV radiation))	*Bacillus* sp. AIIW2	Marine environment (plastic-polluted coastal)	BH minimal medium + PVC/ 90 d	0.26	Kumari et al. (2019)
PA (Nylon 6; Nylon 6,6)	*Bacillus cereus, Bacillus sphaericus, Vibrio furnisii* and *Brevundimonas vesicularis*	Marine water	MSM + SCS	2–7	Sudhakar et al. (2007)
Marine plastic debris	*Alcanivorax* sp. 24/genes encoding for 2 cytochrome P450, 3 alkane Monooxygenases AlkB, and 2 monooxygenase-related AlmA (enzymes involved in the degradation of long-chain alkanes)	Marine environment (high intertidal zone)	BH medium + polyhydroxybutyrate (PHB)	–	Zadjelovic et al. (2020)

Mineral salt medium (MSM); sole carbon source (SCS); period of time in days (d); average molecular weight (Wm); Bushnell Haas (BH) medium

[a] Weight loss measured as a ratio of CO_2 consumption with and without PE assuming that all carbons in the PE are mineralized into CO_2

with soil microorganisms (Sudhakar et al. 2007). One study reported on a laccase-mediator system (LMS) and showed that a fungal laccase, a multicopper-containing enzyme, was able to degrade PA (Fujisawa et al. 2001).

In the case of PVC degradation, most studies have been performed with both PVC and plasticizers (Moriyama et al. 1993; Gumargalieva et al. 1999; Sabev et al. 2006; Latorre et al. 2012). PVC contains commonly high proportion of plasticizers (up to 50%). Two studies were carried out with PVC films in soil (Kırbaş et al. 1999; Ali et al. 2014) and one in the marine environment (Kumari et al. 2019); however, the degradation was evaluated based on weight loss. In the case of marine bacteria *Bacillus* sp. AIIW2 PVC, only a weight loss of 0.26% was observed in the pre-treated (UV radiated during 20 min) PVC films after 90 days (Kumari et al. 2019). Thus, the enzymes involved in the microbial degradation of this polymer are still unknown (Ru et al. 2020).

Several enzymes contribute to the degradation of polyurethanes, both with polyether and polyester backbones (Cregut et al. 2013; Peng et al. 2018; Magnin et al. 2020; Ru et al. 2020). PUs are presumed to be the most susceptible types of conventional plastics to biodegradation due to the presence of urethane bonds and other hydrolysable groups in their carbon backbone (≥2 functional groups per monomer; Fig. 5.2). Seven fungal strains were found to grow on the surface of solid polyester PU (Darby and Kaplan 1968). Two fungal strains, *Aspergillus niger* and *Cladosporium herbarium*, were able to grow in cultures with polyether foam as the sole nutrient source (Filip 1979). A *Staphylococcus* bacterial strain was able to grow on polyether in the absence of organic nutrients (Jansen et al. 1991). An esterase able to degrade polyester was purified from a fungal strain, *Curvularia senegalensis* (Crabbe et al. 1994), a protease was purified from *Pseudomonas fluorescens* (Howard and Blake 1998) and a lipase from *Bacillus subtilis* (Rowe and Howard 2002). Since then, other enzymes with the capacity to degrade PU have been reported (Akutsu et al. 1998; Allen et al. 1999; Matsumiya et al. 2010). Cutinases, esterases and lipases are able to attack carboxylic linkage bonds, whereas endopeptidase enzymes are able to cleavage amide bonds (Lucas et al. 2008). However, these enzymes were not found to degrade solid polyester substrates, such as PU film, foam, and elastomer (Schmidt et al. 2017; Ru et al. 2020). Although it has been claimed that some enzymes possess depolymerization activity for degrading (co)polyesters (Lucas et al. 2008), to date none have been reported to be able to degrade and cleave the urethane bonds in both polyester and polyether PU (Ru et al. 2020).

Several enzymes have been reported to degrade PET since reporting of the hydrolase (cutinase-like TfH) which could degrade up to 50% of the polymer's weight at 55 °C in 3 weeks (Müller et al. 2005). Another three cutinases from three different microorganisms were reported to degrade both low- and high-crystallinity PET (Ronkvist et al. 2009). Nevertheless, two of them should be ascribed to PET surface-modifying enzymes due to their low weight loss effect (Ru et al. 2020) and only cutinase HiC from *Humicola insolens* was found as an efficient hydrolase, causing a 97% reduction of the low-crystallinity PET film at 70 °C within 96 h. This enzyme, recently renamed as *Thermobifida fusca cutinase* (TfCut2), has been shown to reduce similar amounts of weight in PET films than previously reported, and up to 56.6% in postconsumer PET packages at 70 °C within 120 h (Wei et al. 2019). PET consists of repetitive units of polyethylene terephthalate and is mainly synthesized from terephthalic acid and ethylene glycol. To date, *Ideonella sakaiensis* 201-F6 is the most in-depth studied PET degrading organism along with *Thermobifida* sp. and *Thermomonospora* sp. (Kleeberg et al. 1998; Müller et al. 2005). *I. sakaiensis* grows on low crystalline PET as a major carbon and energy source by adhering to the surface and releasing exo-enzymes (Yoshida et al. 2016). Two novel enzymes were identified to catalyse the full reaction of degrading PET: PETase, a hydrolase belonging to the α/β-hydrolase superfamily which converts PET into mono (2-hydroxyethyl) terephthalic acid (MHET) and terephthalate (TPA). The second enzyme MHETase, a tannase, hydrolyses MHET into TPA and ethylene glycol (EG). PETase has features similar to cutinases and lipases (Austin et al. 2018). Potential PETases were investigated by metagenomic mining, which revealed that with respect to the utilized database, >500 candidates, divided over different bacterial phyla exist that could express a PETase. The candidate genes coding for PETase originating from marine environment belonged mainly to Bacteroidetes and in the terrestrial environment to Actinobacteria. The database searches identified possible PET hydrolase homologs in 31 marine metagenomes and 11 terrestrial ones. The cloning of PETase and latter enzyme activity assays revealed that both PET and polycaprolactone were hydrolysed (Danso et al. 2018). Based on the low occurrence of PETase in the analysed metagenomes, they hypothesized that PET hydrolysing enzymes evolved only recently. The bacterial strain *Ideonella sakaiensis* 201-F6 also encodes another enzyme (*Is*PETase), able to degrade lcPET films but at an ambient temperature (Yoshida et al. 2016). However, the efficiency of this IsPETase at mesophilic temperatures is markedly lower than that of TfCut2 at a thermophilic temperature (Wei and Zimmermann 2017a, b; Ru et al. 2020).

5.5.3 Intracellular Biodegradation

Once the plastic has been degraded to short-chain molecules, e.g. short oligomers, dimers and monomers, these can be assimilated, and potentially further degraded and finally mineralized to CO_2, H_2O or CH_4 (Gu 2003). Concerning enzyme specificity, polymer biodegradability probably depends on molecular physicochemical characteristics such as density, crystallinity and structural complexity and the presence of functional groups (Kawai 1995; Gu 2003; Mohan and Srivastava 2010; Alshehrei 2017). A fundamental aspect to consider in this biodegradation stage is the assimilation sizes of the polymers. However, the upper size limit for plastic molecules to undergo direct biodegradation is not well constrained. In the case of PE, it is suggested that the polymer with the average molecular weight lower than ~1000 Da could be considered as biodegradable (Kawai 1995). Later, it was reported that some microorganisms were able to degrade quite rapidly previously photooxidized molecules with higher molecular weight, raising the upper limit to ~2000 Da (Kawai et al. 1999). Nevertheless, it is unclear whether those molecules were broken down extracellularly before being assimilated or not. Concerning longer n-alkanes and saccharides, earlier studies indicated that most of the microorganisms do not seem to be able to consume aliphatic chains displaying a Mw over 600 Da (Haines and Alexander 1974). During an experiment with PE wax, a consortium of bacteria could degrade molecules that were $>$ 1000 Da (Kawai et al. 2004). Lower-molecular-weight PE (1700 Mw) was biodegraded ~6 times faster than larger PE molecules (23,700 Mw) by *Pseudomonas* sp. E4, isolated from beach soil (Yoon et al. 2012). A mesophilic polypropylene degrading strain, *Stenotrophomonas panacihumi* PA3–2, was reported to degrade two low-molecular-weight PP types containing molecules with a broad chain length spectrum and one high-molecular-weight PP (Jeon and Kim 2016b). Increase in average molecular weight was observed during incubations, and the authors concluded that the lightest molecules were mostly degraded. A few isolated microorganisms have shown the ability to utilize untreated PE as the sole carbon source (Gilan et al. 2004; Sivan et al. 2006), which points to the capacity of these organisms to utilize polymers with higher molecular weights. It is not clear how large molecules might be directly assimilated. Some authors suggest the possibility of a certain effect of biosurfactants produced by microorganisms, being then able to enter the assimilation pathway known for longer alkanes (Koutny et al. 2006a). In a gene expression experiment with *R. ruber* growing on PE as a carbon source, the authors identified 19 putative transporters upregulated in at least one of the treatments supplemented with PE. Of them, nine belonged to the major facilitator superfamily (MFS) and five belonged to the ATP binding cassette (ABC) family (Gravouil et al. 2017). They also reported a gene encoding for a protein sharing a transport function and NADH dehydrogenase activity, suggesting that this protein could be involved in both the oxidation and the transport.

The controversy of the results from different studies reveals that probably the assimilation size varies between different microorganisms and is polymer dependent. However, to date, there are not sufficient studies on different polymers and diversity of microorganisms to further constrain molecular weight cut-offs impeding biodegradation, leaving several aspects of the biodegradation unclear. For instance, regarding the biodegradation of PE, it is necessary to characterize the biochemical functions of the oxidases or oxygenases, such as the enzymes encoded by the genes *alkB*, *alkB1* or *alkB2*. Future efforts are required to characterize specific depolymerases degrading plastics and the assimilation process. In future screening experiments, it is essential to characterize the ability of microbial strains to depolymerize the long-chain molecules of the different untreated polymer resins, as well as to identify the genes encoding for membrane transporters and their regulations through gene expression experiments.

5.6 Valorization and Applications

Several bacterial and fungal strains can degrade plastic polymers, with varying efficiency. Thus, utilization and marketing of microbial plastic degraders, purified plastic-degrading enzymes, and further optimizing these for biotechnological and possibly environmental (pollution mitigation) applications is a potential future market sector. Nevertheless, with a few exceptions (Son et al. 2020), research in this area is not advanced far enough and key organisms and enzymes firstly need to be identified.

A promising candidate for future biotechnological applications is the *Ideonella sakaiensis* 201-F6, able to degrade plastics and to grow on the plastic derived carbon. The enzymes PETase and MHETase, mediating PET degradation and resulting in the release of terephthalic acid and ethylene glycol (EG), have been characterized; however, PET degradation is a very slow process (Tanasupawat et al. 2016; Yoshida et al. 2016). In order to achieve PET degradation at higher rates, protein engineering of wild-type enzyme resulted in a PETase (IsPETaseS121E / D186H/S242T/N246D) that outperformed the natural PETase by 58-fold (Son et al. 2020). In this process, it was also possible to recover the released terephthalic acid, which could then serve as a base material for the new PET production. For the first time, a biotechnological application for degrading waste of conventional plastics and contributing to its recycling was demonstrated (Tournier et al. 2020). Conventionally recycled PET (i.e. remolded) has technical disadvantages such as loss of mechanical properties, and for some applications, the quality of conventionally recycled PET is too low. However, digesting PET and 'mining' for its monomer thus offers, in principle, the possibility for a truly circular PET economy.

Furthermore, TPA and EG can serve to produce, polyhydroxyalkanoates (PHA), which are useful compounds for biotechnological applications. In nature, a variety of microorganisms ranging from general sugar fermenters to methanotrophs produce PHAs as cellular carbon storage and energy source. PHAs can be produced by *Pseudomonas* species from TPA, as well as EG (Kenny et al. 2008, 2012; Franden et al. 2018). *Pseudomonas putida* has often been suggested as a candidate to serve PHA production, not only from TPA and EG but also from the styrene degradation product phenylacetic acid (PAA) (Ward et al. 2006; Nikodinovic-Runic et al. 2011). The degradation products, TPA and EG, could serve as substrates for the bioplastic industry, where PHAs, such as polyhydroxybutyrate, are produced. However, the primary degradation of waste polymers needs to be initiated. While the discoveries for PET are promising, similar knowledge of naturally occurring enzymes and the further advancements of these enzymes has not been achieved for other polymer types. Future research thus not only needs to determine if a particular polymer type is biodegradable and which key players mediate this process, it also needs to include in-depth investigations into gene expression in order to find potentially relevant genes encoding for enzymes that mediate depolymerization which then could be used for biotechnological applications.

To date, there is no applicability of plastic degraders to counteract plastic pollution in nature. One of the reasons is our lack of understanding of naturally occurring plastic degraders and their functioning, which provides the basis for creating biotechnological applications. Furthermore, both wild-type strains and engineered enzymes are ideally associated with high degradation efficiencies to serve industrial needs and yield utilizable compounds. In a second step, these organisms/enzymes would need to be cultivated/produced in large quantities. As tempting as it is to envision plastic bioremediation by microbes in nature (a quasi-biochemical clean-up solution), in situ bioremediation might be applicable solely where plastic pollution is highly concentrated and localized, e.g. in terrestrial landfills or in local hot spots of pollution as have been found in some bays. There, it appears possible to apply microbes/enzymes at sufficient quantities on a small spatial scale. In contrast, the expanse of the ocean, both horizontally and vertically, is genuinely enormous. Although plastics tend to accumulate in the subtropical gyres and enclosed basins, it would thus require an massive quantity of microbes/enzymes to reach all plastic pieces from the ocean surface to the (deep) sea bottom. Furthermore, the marine environment is in constant motion, which further complicates such applications. Alternatively, using microbes to prevent/fight marine plastic pollution could work ex situ by retrieving plastic from the ocean, and, e.g. to extract monomers after enzymatic digestion. Though speculative, it might be plausible to design large bioreactors with plastic degrading microbes that could degrade plastics in a similar fashion as organic matter and nutrients are removed in biological wastewater treatment systems at present.

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6

Mechanism and Pretreatment Effect of Fungal Biomass on the Removal of Heavy Metals

Aparna B. Gunjal

Abstract

Heavy metals cause pollution and have adverse impacts on the humans and environment. The soil beneficial microorganisms and water bodies are polluted due to the presence of heavy metals. This is a serious issue and needs immediate solution. The physicochemical ways for the removal of toxic metals have some disadvantages. The biological method includes bioremediation, which makes the use of microorganisms for the removal of heavy metals from the aqueous solutions. The fungal biomass is available easily and in abundant. The bioremediation technology is very eco-friendly and economical. The review here describes the effect of pretreatment of fungal biomass on the removal of heavy metals. The mechanism of the removal of heavy metals by the fungal biomass is also discussed. The passive and active biosorption, fungal cell wall structure, advantages of fungi as biosorbents and factors affecting biosorption process are also highlighted in this chapter. The change in the morphology of the biomass of *Aspergillus* sp. pretreated with NaOH for the removal of heavy metals is also studied by scanning electron microscopy. The physical and chemical methods of pretreatment of fungal biomass for the removal of heavy metals are also taken into consideration. The immobilization of fungal biomass for biosorption is also discussed in this chapter. The pretreatment of fungal biomass enhances the biosorption. The bioremediation technology is very easy, and also the fungal biomass can be reused. This will be an excellent and fruitful solution to solve the problem of soil and water pollution created by heavy metals.

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Keywords

Pollution · Eco-friendly · Economical · Biosorption · Heavy metals · Fermentation

6.1 Introduction

Heavy metals are very toxic chemicals present in soil, water, etc. The heavy metals include lead (Pb), chromium (Cr), arsenic (As), zinc (Zn), cadmium (Cd), copper (Cu), mercury (Hg), and nickel (Ni) (GWRTAC 1997). These heavy metals cause soil and water pollution. In soil, there are varieties of beneficial microorganisms called as plant growth-promoting rhizobacteria which enhance the yield of the crops by direct as well as indirect ways. These heavy metals found in the ground affect the population of these beneficial microorganisms. In water, these heavy metals affect the food chain of the ecosystem and cause pollution. The water contaminated with heavy metals can be used neither for agriculture nor for drinking purposes. All of these heavy metals cause immense adverse effects on the biotic and abiotic systems. The effect of toxic metals on humans is represented in Fig. 6.1. They mainly affect the respiratory, reproductive, and central nervous systems of the humans.

There are physical, chemical, and biological ways for the elimination of heavy metals from the polluted environment. But, physical method involves incomplete removal of the heavy metals from various sources (Shishir et al. 2019). Chemical methods are chemical oxidation, precipitation, flocculation, etc. (Wu et al. 2015). The chemical methods include use of various harmful chemicals and are also costly. These cause harmful effects on the humans and environment. Both physical and chemical ways for the elimination of toxic metals require many hours.

Biological method includes bioremediation which is eco-friendly, easy and economical and requires less time (Mani et al. 2017). The biomass of microorganisms is available in large amount. Bioremediation involves the use of

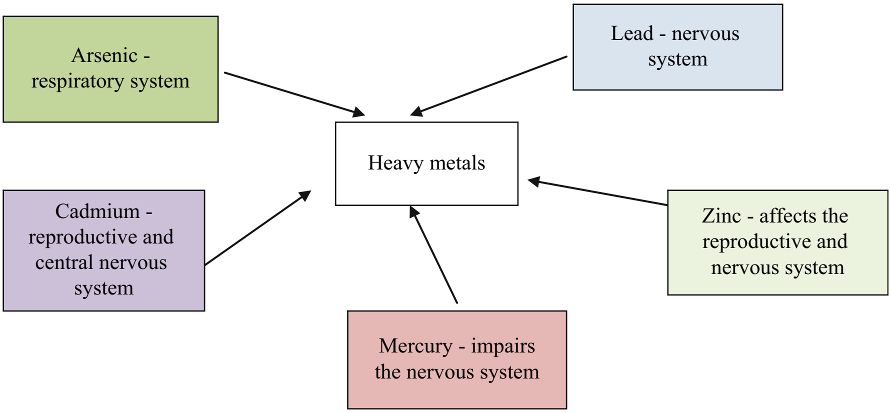


Fig. 6.1 Effect of heavy metals on humans

microorganisms, viz. bacteria, fungi, yeasts, algae, etc. for the removal of toxic metals from various contaminated environment. The microorganisms have the ability to degrade various organic compounds, heavy metals, etc. Removal of heavy metals by biosorption technology can help to improve zero-wastes economic policy in terms of reuse of biomass generated from food, pharmaceutical, and various other industries and also from wastewater treatment. This is an attractive technology for many industries to recover metals.

This chapter focuses on the mechanism and pretreatment effect of microbial biomass (i.e., fungi) on the biosorption of heavy metals.

6.2 Natural and Anthropogenic Sources of Heavy Metals

The natural sources of toxic metals are disintegration, corrosion, use of pesticides and fertilizers, and automobile batteries, while the anthropogenic sources include mining, tannery, paint, electroplating, steel industries, and smelting (Singh et al. 2018).

6.3 Passive and Active Biosorption

There are two methods of biosorption, viz. passive and active. In passive biosorption process, the dead biomass of fungi is used. The dead biomass interacts with the presence of functional groups found on the cell wall of fungi. This is an easy and rapid metabolism-independent process. In case of active biosorption, the live biomass of fungi is used and involves metabolism-independent and metabolism-dependent processes (Salam 2019). The major advantages of biosorption are very easy, economical, eco-friendly, fast, and high efficiency. Also, the biomass of fungi is easily available in large amount (Yahaya and Don 2014).

6.4 Fungal Biomass Generated from the Fermentation Industries

Fermentation industries produce excess amount of unused biomass, which are used as feed for animals, organic manure or burned. In 1 day, antibiotic fermentation industries generate around 5000 tons of fungal biomass (Paknikar et al. 1993). The fermentation industries generate about 790,000 tons of waste biomass every year, about 41,000 tons comes from citric acid production by *Aspergillus niger* (Dhankhar and Hooda 2011). The chemical and enzyme industries which produce array of enzymes and pharmaceutical industries involved in steroid transformation generate large amount of microbial biomass. The lysine fermentation industries generate huge amount of biomass of *Corynebacterium glutamicum* (Choi and Yun 2004).

6.5 Fungal Cell Wall Structure

The fungi produce spores, hyphae, and other reproductive structures. The fungal cell wall is complex and is made up of chitin, a polymer of N-acetylglucosamine, cellulose, and β(1,3) and β(1,6) glucan. In most fungi, chitin is the major component present in cell wall. The fungal cell wall also contains polysaccharides and proteins. The outer most layer of the fungal cell wall contains polysaccharide side chains made of mannose (Dhankhar and Hooda 2011).

6.5.1 Advantages of Fungi as Biosorbents

- Degrade many lignocellulosic and other complex compounds.
- Very easy to cultivate on a large-scale on different media.
- Safe.
- Economical.
- More effective than bacteria and algae.

6.5.2 Fungi as Biosorbents

The cell wall of fungi enables to bind metals. Both live and dead biomass of fungi can be used as biosorbents (Abbas et al. 2014). The fungi can uptake metals by two processes, active uptake or bioaccumulation and passive uptake or biosorption where the metal ions bind to the surface of the cell wall. The cell wall of fungi is made up of chitin, mannans, glucans, lipids, and polysaccharides. The chitin content is more in the cell wall of fungi (Mueler et al. 1992). The binding of heavy metal on chitin is represented in Fig. 6.2. Functional groups are involved in metal binding, viz.

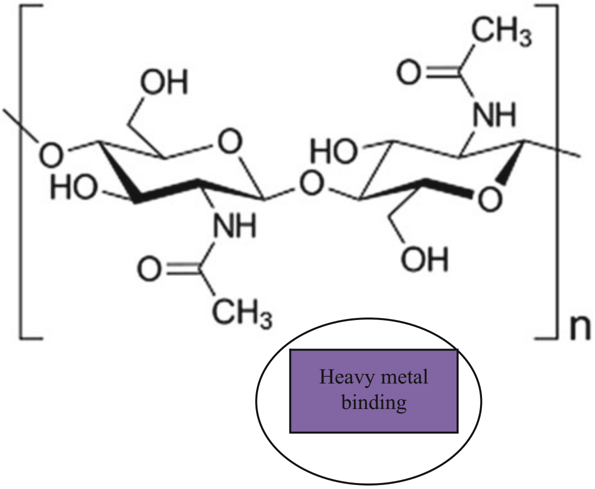


Fig. 6.2 Binding of heavy metal on chitin present in the cell wall of fungi

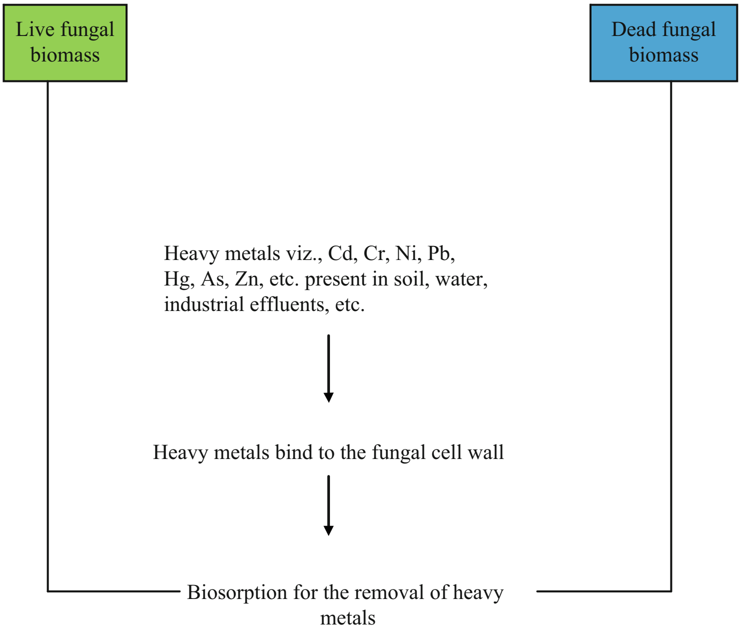


Fig. 6.3 Live and dead fungal biomass in biosorption of heavy metals

carboxyl, phosphate, proteins, nitrogen, uranic acids, etc. (Abbas et al. 2014). The live and dead fungal biomass in biosorption of heavy metals is shown in Fig. 6.3. The live and dead fungal biomass can be used for the removal of heavy metals from the waste water and industrial effluents. Studies have shown that fungi are cheaper for the removal of toxic metals due to their filamentous morphology and cell wall composition (Addour et al. 1999). Various filamentous fungi viz. *Aspergillus niger*, *A. fumigatus*, *A. clavatus*, *Penicillium simplicissimum*, *P. brevicompactum, Termitomyces clypeatus*, *Trichoderma* sp., *Rhizopus* sp., *Rhizoctonia* sp., etc. are used in sequestration of toxic heavy metals (Rana et al. 2019a, b). The fungi in the removal of heavy metals are shown in Fig. 6.4.

6.6 Factors Affecting Biosorption Process

The factors like temperature, pH, and biomass amount of the fungi affect the biosorption process. At acidic pH, the metal-binding sites are saturated by H^+ ions. Due to more H^+ ions, this minimizes the interaction between the fungal biomass and metal cations. As the pH increases, the reaction between the fungal biomass and metal cations increases and biosorption enhances. Increased temperature up to a particular point maximizes enzyme activity of fungi, thereby enhancing biosorption (Bandowe et al. 2014). Biosorption is not much affected in the temperature range 20–35 °C. If the biomass amount used is less, this increases the biosorption process.

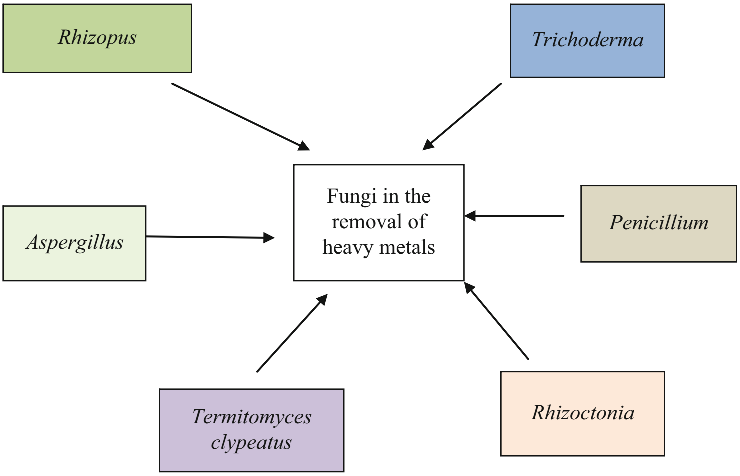


Fig. 6.4 Fungi in the removal of heavy metals

There will be more competition for the heavy metals to bind if the less biomass is used and reverse if more biomass is used (Redha 2020).

6.7 Effect of Pretreatment of Fungal Biomass on the Removal of Heavy Metals

The fungal biomass can be pretreated by physicochemical methods for the enhancement of removal of heavy metals from soil, water, industrial effluents, etc. (Yan and Viraraghavan 2000). The physical and chemical pretreatment methods of fungal biomass for the enhancement of removal of heavy metals are represented in Fig. 6.5. The physical and chemical methods cause surface modification which helps the biomass of the fungi to sequester the heavy metals from the waste water or industrial effluents. The toxic metal removal efficiency by fungal biomass through surface modification is shown in Fig. 6.6. There is a report on effect of pretreatment on biosorption of heavy metals by fungal biomass (Ilhan et al. 2004). Yan and Viraraghavan (2000) have also studied that alkaline (caustic) pretreatment has increased metal binding by fungal biomass. Biosorption of cadmium by *Aspergillus niger* has been reported (Barros et al. 2003). The physical and chemical pretreatment methods are reported to improve the biosorption efficiency by the fungi. Pretreatment of biomass of *Pleurotus florida* with NaOH has found to enhance cadmium biosorption (Das et al. 2007). Study is done on biosorption of cadmium and nickel by pretreated biomass of *Aspergillus* spp. (Gunjal et al. 2019). There is a report on where treatment with increasing concentration of NaOH from 0.002 to 5.0 g/L improved Ni (II) removal from 93.2 to 100.0%, while untreated biomass showed 64.6% Ni (II) removal. Pretreatment with higher concentrations of NaOH, 5.0–80.0 g/L showed complete removal of nickel (Rouhollahi et al. 2014). Also,

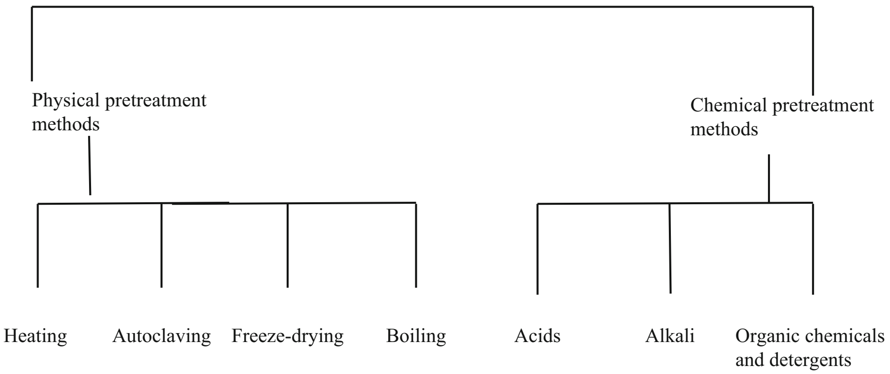


Fig. 6.5 Physical and chemical pretreatment methods of fungal biomass for the enhancement of removal of heavy metals

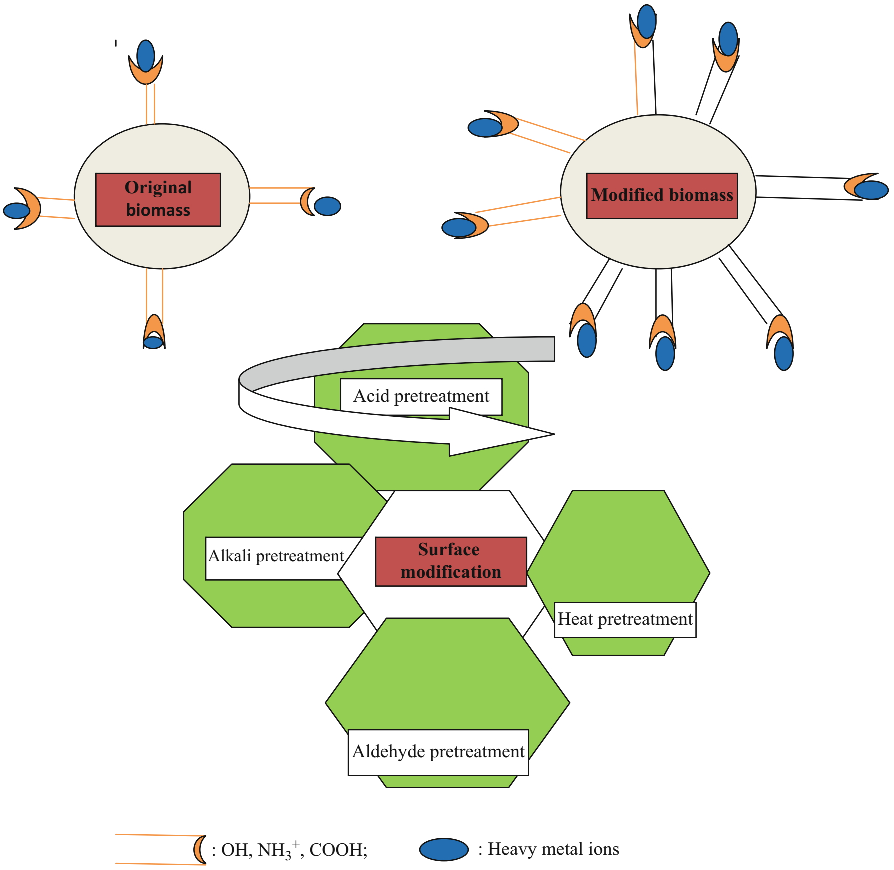


Fig. 6.6 Toxic metal removal efficiency by fungal biomass through surface modification

there is a study where biomass of *Humicola* sp. treated with NaOH showed maximum biosorption at pH 6.0 after 60 min (Netpae 2015). The pretreatment of *Aspergillus niger* with NaOH has increased the biosorption of lead and nickel from waste water and industrial effluents (Rao and Bhargavi 2013). The pretreatment of biomass of *Aspergillus nidulans* using autoclaving and formaldehyde has reported increase in the removal of cadmium compared to control (Nascimento et al. 2015). Treatment of *Mucor racemosus* biomass with 0.1 M NaOH at 120 °C for 6 h is also reported to improve biosorption (El-Morsy et al. 2013).

6.8 Physical and Chemical Methods of Pretreatment of Fungal Biomass for the Removal of Heavy Metals

6.8.1 Physical Methods

In heating method, the fungal biomass is heated for 10 min and then used for the removal of heavy metals.

In autoclaving method, the biomass in autoclave is directly kept for autoclaving at 121 °C for 20 min at 15 psi.

Freeze-drying process is also known as lyophilization. In this, the biomass is subjected at low temperature and pressure, and water molecules are removed from the biomass. The fungal biomass is kept in boiling water bath for 10 min.

6.8.2 Pretreatment Using Acids (Das et al. 2007)

In this, the fungal biomass is boiled for 15 min in 100 mL (10% v/v/) glacial acetic acid or treat with 7 mL (0.1 M) oxalic acid and keep for drying at 60 °C overnight or treat with 20 mL (10% v/v) ortho-phosphoric acid and placed on a shaker at 125 rpm for 30 min.

6.8.3 Pretreatment Using Alkali (Das et al. 2007)

In this, the fungal biomass is boiled for 15 min in 50 mL (0.5 N) sodium hydroxide or sodium bicarbonate or in 100 mL (0.5 N) sodium carbonate.

6.8.4 Pretreatment Using Organic Solvents (Das et al. 2007)

In this, the fungal biomass is given treatment with 10 mL (10% v/v) methanol or 100 mL (10% v/v) formaldehyde and placed on a shaker at 125 rpm for 3 h or boiled for 15 min with 20 mL (50% v/v) dimethyl sulfoxide (DMSO).

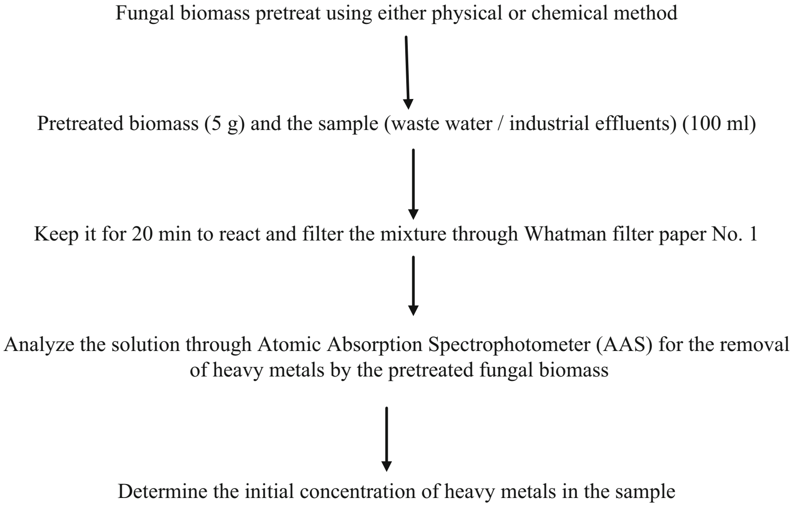


Fig. 6.7 Fungal biomass pretreated using either physical or chemical method

The fungal biomass pretreated using either physical or chemical method is represented in Fig. 6.7.

The amount of metal biosorbed per gram of the biomass is calculated as (Yan and Viraraghavan 2000):

$$Q = [(C_{\mathrm{i}} - C_{\mathrm{f}})/m] \times V \tag{6.1}$$

where

Q = metal biosorbed (mg/g)
C_{i} = initial toxic metal amount (mg/L)
C_{f} = final toxic metal amount (mg/L)
m = amount of biomass used (g)
V = amount of the reaction mixture (L)

The removal of toxic metals experiment using pretreated fungal biomass is shown in Fig. 6.8.

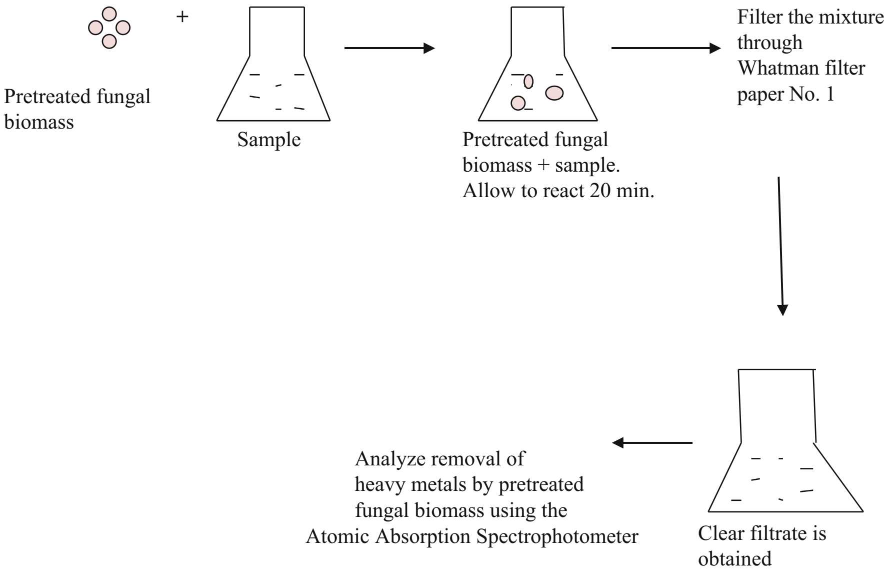


Fig. 6.8 Biosorption experiment using pretreated fungal biomass

6.9 Mechanism of the Removal of Heavy Metals by the Fungal Biomass

6.9.1 Presence of Functional Groups on the Fungal Biomass

Polysaccharides and proteins on the cell surface of the fungi are the binding sites for the heavy metals. The mechanisms for the removal of heavy metals by the fungal biomass are potential for exchange of calcium and magnesium for the metals to bind, which is reported in case of *Penicillium* sp. (Sun and Shao 2007), intracellular mechanism, and complexation mechanism and redox reaction, which is seen in case of *Aspergillus niger* (Ren et al. 2018). Another most essential factor in the removal of heavy metals by the fungal biomass is the presence of different functional groups on the cell wall of the fungi. The different functional groups present on the fungal cell wall are shown in Table 6.1. These functional groups include = C-H, C=O, C-O, -C-H, -C=N, -O-H, C-C, -N-H, and -C=C (amino, hydroxyl, carboxyl, etc. groups). These functional groups expose and make available the sites for the binding of heavy metals. The biomass of *Aspergillus* sp. without any pretreatment is shown in Fig. 6.9a–c. The change in the morphology of the biomass of *Aspergillus* sp. pretreated with NaOH for the removal of heavy metals Zn (Fig. 6.10a–c), Cd (Fig. 6.11a–c), Pb (Fig. 6.12a–c), and Ni (Fig. 6.13a–c) is studied by scanning electron microscope (SEM) (SEM; Jeol, Tokyo, Japan). The change in morphology in the pretreated biomass due to the uptake of heavy metals Zn, Cd, Pb, and Ni (Figs. 6.9, 6.10, 6.11, 6.12, and 6.13a–c, respectively) in comparison to biomass

Table 6.1 Functional groups present on the fungal cell wall

S. no.	Functional groups
1	=C-H
2	C=O
3	C-O
4	-C-H
5	-C=N
6	-O-H
7	C-C
8	-N-H
9	-C=C

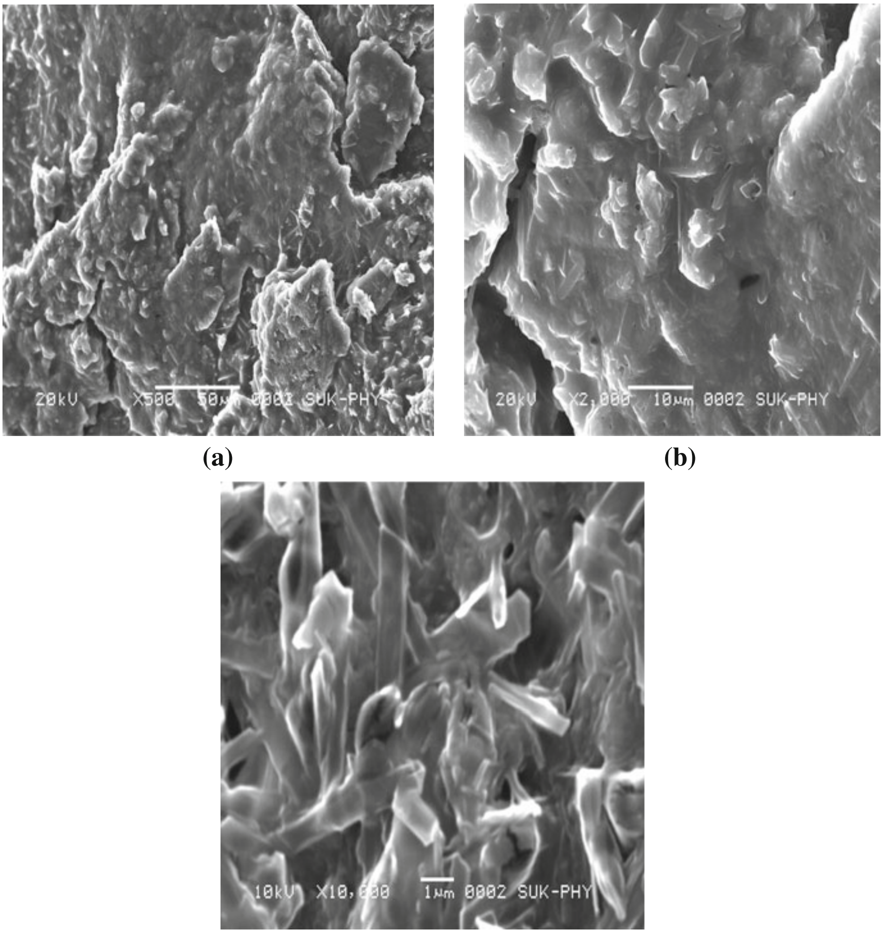
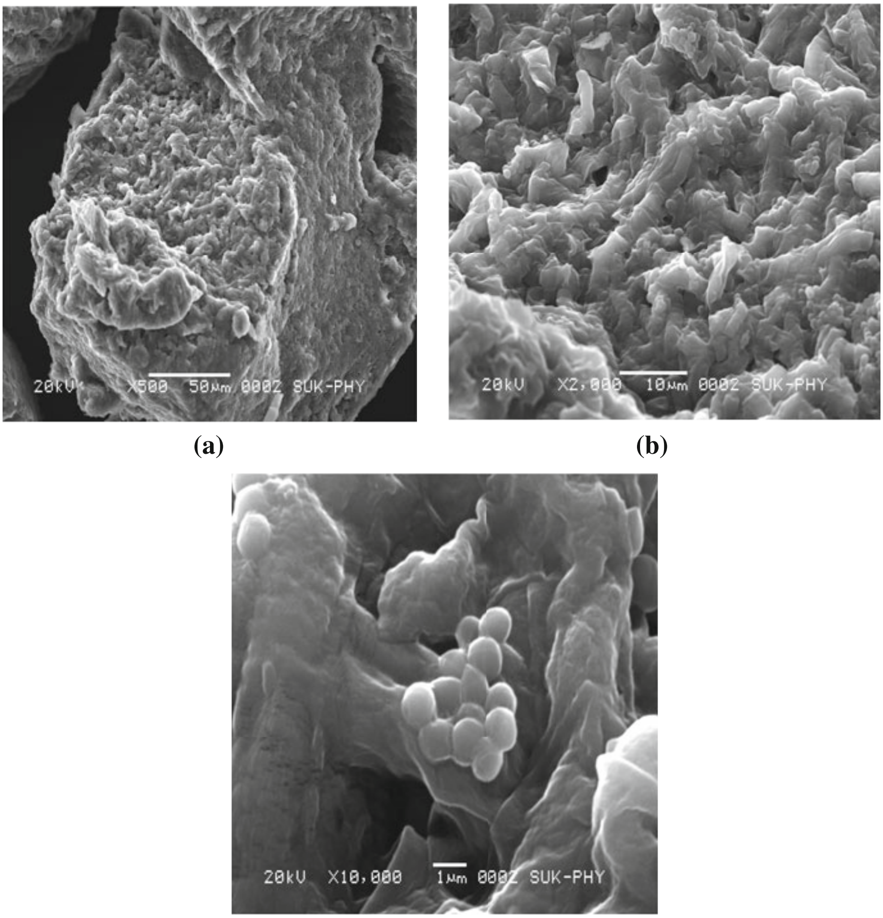


Fig. 6.9 *Aspergillus* sp. biomass without any pretreatment. (SEM image taken at different magnification, viz. ×500, ×2000, and ×10,000)



(a) (b)

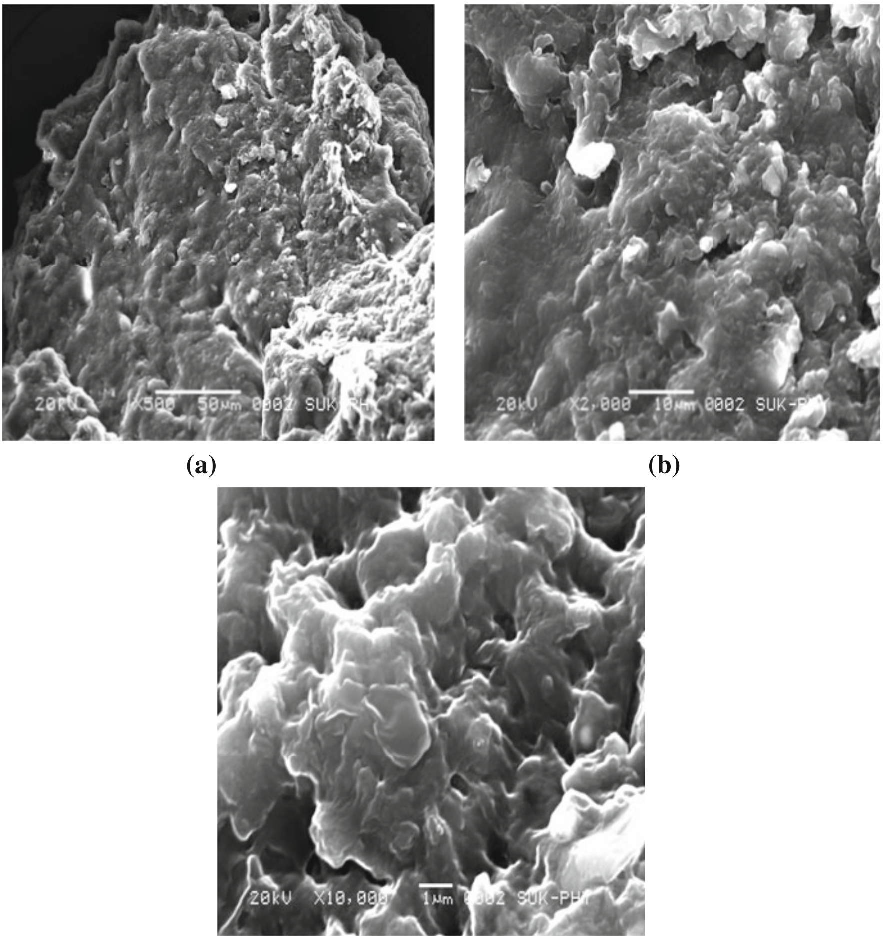
(c)

Fig. 6.10 *Aspergillus* sp. biomass pretreated with NaOH for the removal of Zn. (SEM image taken at different magnification, viz. ×500, ×2000, and ×10,000)

without any pretreatment was observed (Fig. 6.8a–c). The images are taken at different magnification, viz. ×500, ×2000, and ×10,000. The functional groups present on the fungal cell wall interact with the metals.

6.9.2 Direct Adherence on the Fungal Cell Wall

The different heavy metals directly adhere on the cell wall of the fungi, and this is one of the mechanisms for the removal of heavy metals from the waste waters, industrial effluents, and other aqueous solutions. The negatively charged cell surfaces (anions) of the fungi bind to the positively charged metal (cations). This



(a) (b)

(c)

Fig. 6.11 *Aspergillus* sp. biomass pretreated with NaOH for the removal of Cd. (SEM image taken at different magnification, viz. ×500, ×2000, and ×10,000)

is called as stoichiometric interaction, where the metal interacts with the reactive group on the fungal cell wall. There is another complex process which is known as ion exchange. There are three types of metal interaction and sorption, viz. extracellular uptake, accumulation on the inside of cells, and precipitation. Precipitation of metals can take place on the microbial surface or in the aqueous solutions.

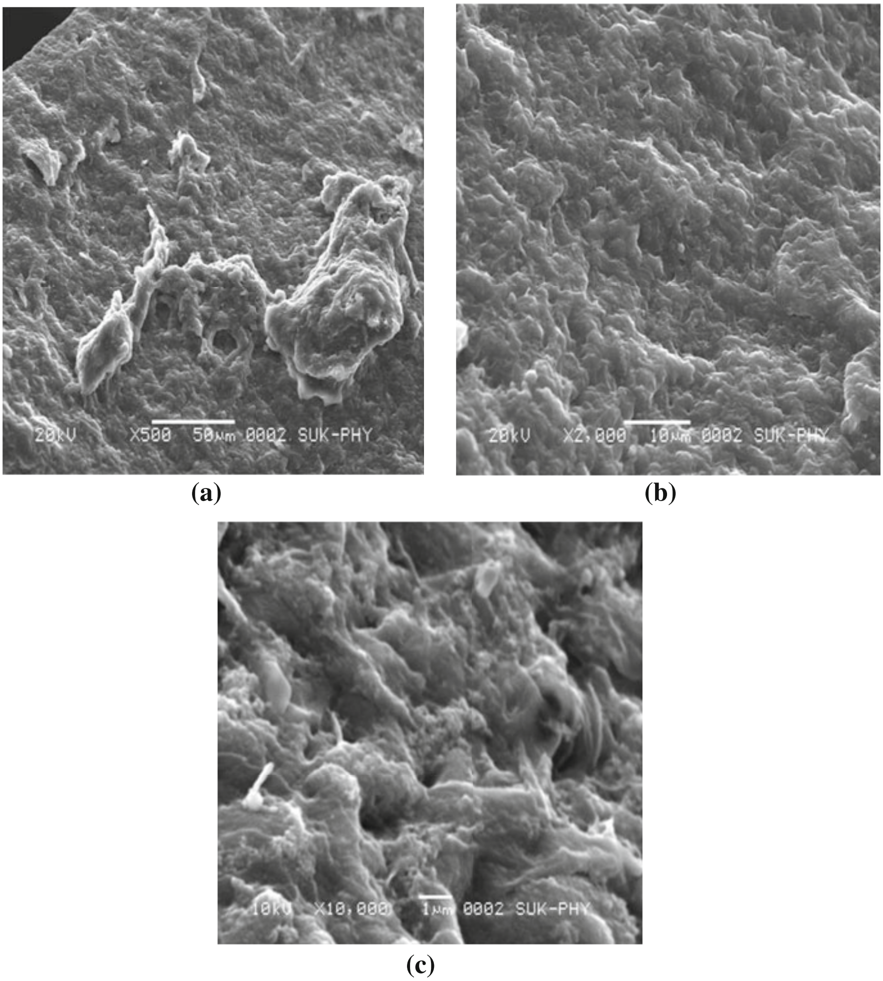
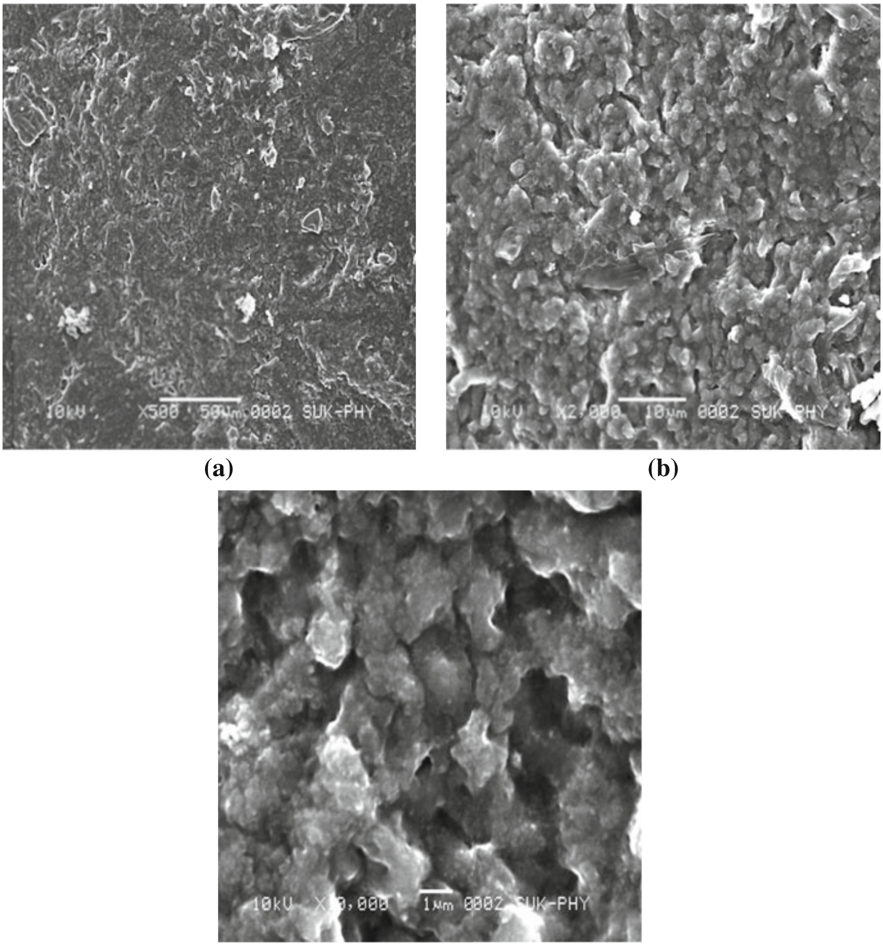


Fig. 6.12 *Aspergillus* sp. biomass pretreated with NaOH for the removal of Pb. (SEM image taken at different magnification, viz. ×500, ×2000, and ×10,000)

6.9.3 Functional Group on Chitosan

N-deacetylation process of chitin gives chitosan. This chitosan has more amine groups (Taskila et al. 2015) which is the functional group. So, this amine functional group makes available the binding site for heavy metal.



(a) (b)

(c)

Fig. 6.13 *Aspergillus* sp. biomass pretreated with NaOH for the removal of Ni. (SEM image taken at different magnification, viz. ×500, ×2000, and ×10,000)

6.10 Immobilization of Fungal Biomass for Biosorption

The microbial biosorbents exhibit low physical and chemical stability and minimum elasticity due to small size and less density. This can be resolved by immobilization of microbial biomass on a suitable carrier (Velkova et al. 2018). The immobilization methods viz. entrapment and cross linking have been studied to have applications in biosorption. Immobilization of fungal biomass within a polymeric matrix has several advantages. Various matrices used for immobilization process include sodium alginate, polysulphone, polyacrylamide, and polyurethane (Bai and Abraham

2003). The matrix used plays important role in immobilization process. The biomass will retain in internal part of the matrix, and therefore, mass transfer resistance determines the biosorption rate and efficiency. Whole-cell immobilization within a polyacrylamide gel has also been reported to have application in biosorption (Dhankhar and Hooda 2011). There is a report where *Rhizopus arrhizus* biomass immobilized on polyacrylamide gel has found application in biosorption of toxic metals, viz. copper, cobalt, and cadmium from the waste water. There is a report where *Penicillium* membrane-type biosorbent was used for Cu (II) removal from wastewater in a plate column reactor (Xiao et al. 2013). The functional groups -NH and -OH were found to play a role in biosorption of Cu (II) (Xiao et al. 2013). *Penicillium citrinum* immobilized biomass has been reported for the removal of 25 mg/g Cu (II) from the aqueous solutions with concentration of copper ions range from 20 to 90 mg/L (Verma et al. 2013).

6.11 Conclusions

The fungal biomass can be effectively used for the removal of heavy metals from contaminated soil, water, industrial effluents, etc. The pretreatment of fungal biomass with acids, alkali, organic solvents, heating, autoclaving, freeze-drying, boiling, etc. can enhance the removal of heavy metals. The immobilization of microbial biomass will also improve the efficiency of biosorption process. The biosorption technology is very easy, eco-friendly, and cheap. The pollution problem due to heavy metals will be solved due to biosorption technology. The biosorption for the removal of heavy metals from waste water and industrial effluents is 'green technology' which is definitely fruitful. As this is a biological approach, the use of physical and chemical methods will be minimized.

6.12 Future Prospects

- The pretreatment of fungal biomass for the removal of heavy metals should be studied on a large scale.
- The research on molecular mechanism involved in biosorption needs to be carried out.
- New fungi should be isolated and studied for biosorption experiments.
- The exact mechanism behind the removal of heavy metals by microorganisms needs to be studied in detail.

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Metal Bioremediation, Mechanisms, Kinetics and Role of Marine Bacteria in the Bioremediation Technology

7

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Shailesh R. Dave

Abstract

Today, the heavy metal pollution has become a major and serious concern. Heavy metals severely affect the ecosystem and also cause health hazard. Heavy metals and metalloids such as Cd, Cu, Cr, Pb, Hg, Ag, As, Zn are considered as serious systemic toxicants because they may induce toxicity even at the lower concentrations. Most of them have the tendency to bio-accumulate and undergo biomagnifications; moreover, metallic pollutants are not biodegradable. Thus, the removal and recovery of heavy metals are indispensable for the safety of environment and human health. Biosorption is a potential alternative over conventional methods for the removal of heavy metals. Biomass of microbes, agricultural waste, industrial waste, sewage sludge, etc. has been used as biosorbents. Nowadays, various marine bacteria draw a specific attention of scientists for heavy metal removal due to their unique characteristic features of adaptation to the extreme conditions. Hence, they can be better employed in bioremediation of heavy metals, as compared to the bacteria found in other natural habitats. The process of

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biosorption can be made eco-friendly and cost-effective by regenerating and reusing the biosorbents after performing desorption of metals from the biomass.

Keywords

Biosorption · Desorption · Isotherms · Kinetic · Marine bacteria

7.1 Introduction

Heavy metal pollution has emerged as one of the main issues throughout the globe in recent decades. Mostly the metals are introduced in the environment from natural sources like forest fires, volcanic emissions, deep sea vents and from anthropogenic sources such as metal manufacturing plants, textile, electroplating, plastic, leather industries, etc. (Bilal et al. 2018). Discharge of toxic metals and metalloids in the environment poses a disastrous effect and also leads to human health issues around the world. To reduce the hazards caused by heavy metal uptake and accumulation, reduction of their bioavailability or mobility is essential. Various conventional techniques that have been used for the removal of toxic metals are pyrolysis, incineration, ion-exchange, precipitation, filtration, reverse osmosis, oxidation-reduction, and membrane separation (Shirdam et al. 2006). But these methods are costly, and can also lead to the formation of some secondary toxic substances. Therefore, bioremediation methods are more suitable, as they are cost-effective, eco-friendly and more efficient for the removal of toxic metals and other pollutants. Bioremediation techniques that are used to remove heavy metals involve the use of live or dead microorganisms such as algae, fungi, bacteria or their by-products, plants, agricultural waste, and industrial waste. Bacteria have been used as biosorbents due to their ubiquitous presence, large surface area per mass, growth under controlled conditions and flexibility to the wide range of environmental conditions (Wang and Chen 2009; Srivastava et al. 2015; Ojuederie and Babalola 2017). They show rapid adaptation towards environmental changes and hence play significant role in the maintenance and sustainability of any ecosystem (Dash et al. 2013). Initially it was believed that metals only manifest toxic effects on microbes, but later on it was proved that the microorganisms can develop various metal resistance mechanisms when grown in the presence of metals. They develop some protecting strategies to defend against the penetration of heavy metal ions into the cell. Due to these metal–microbe interactions, the metals either get immobilized or transformed into non-toxic or less toxic form, which prevent their further release into the particular ecosystem. Nowadays, focus of researchers is on application of marine bacteria in the field of bioremediation. Marine bacteria as well as their enzymes, exopolysaccharides and other products can be used to remove the heavy metals. The chapter reports the different sources of toxic heavy metals, their toxic effects, mechanisms of metal removal in general and marine bacteria as biosorbents in particular as well as various factors affecting biosorption, biosorption equilibrium models, kinetics of sorption and desorption.

7.2 Heavy Metals and Their Sources

Heavy metals are present in different concentrations in all ecosystems naturally. They are defined as metallic elements owning a relatively high density compared to water. Out of 106 identified elements, about 80 of them are called metals, of which 52 are considered as industrially important (Ilyin et al. 2004; Naja and Volesky 2009). Metals can be classified as essential, toxic and very toxic based on their toxicity, solubility and chemical properties. Metals are not easily degradable, and their accumulation in living tissues can lead to some serious health hazards or death. Soil erosion of metal ions, metal corrosion, atmospheric deposition, metal evaporation from water resources, leaching of heavy metals and sediment resuspension may be responsible for environmental pollution of these metals. Natural processes like weathering and volcanic eruptions are also responsible for heavy metal pollution. The major industrial sources contribute to the metal pollution and may include plastics, electroplating, fertilizer, textiles, leather, microelectronics, wood preservation and paper processing plants as well as nuclear power stations. Some industrial procedures including metal processing in refineries, petroleum combustion, coal burning in power plants, etc. may also release the hazardous metals to the environment (Tchounwou et al. 2012; Masindi and Muedi 2018). Heavy metals persist for a long period of time in any ecosystem (terrestrial, aquatic or atmosphere). They tend to bioaccumulate and further may lead to the biomagnification. Some serious health hazards have been noted in human beings upon the consumption of metal-bearing food and water. Heavy metals can cause some deadly diseases like cardiac arrest, renal, nerve failure, etc. The sources and effect of certain toxic metal ions are listed in Table 7.1. Considering the toxicity and their harmful effect on living beings, the removal and recovery of heavy metals are essential for the protection of environment and human health. Bioremediation of metals is possible by various ways and means, so major mechanisms of the bioremediation are illustrated in the next section.

7.3 Mechanisms of Metal Bioremediation

Bio-removal of metals mainly crops up on two principles. The first one is the extraction of metals from ores or metal containing waste. The process is known as bioleaching. The second one is bioaccumulation and biosorption which includes immobilization of metals from liquid waste. The details of metal–microbe interaction for solubilization and immobilization are illustrated in Fig. 7.1.

7.3.1 Solubilization

7.3.1.1 Bioleaching

Bioleaching can be described as “the solubilization of metals from their solid sources by selected groups of microorganisms or by their activities”. Mostly, bioleaching refers to the conversion of insoluble metal (usually metal sulphides, e.g., CuS, NiS,

Table 7.1 Major sources of heavy metals and metalloids and their toxic effects

Heavy metal	Major source	Toxic effects on humans	Toxic effects on microorganisms
Antimony	Coal combustion, mining, smelting, soil erosion, volcanic eruption	Cancer, cardiovascular diseases, conjunctivitis, dermatitis, liver diseases, nasal ulceration, respiratory diseases	Inhibits enzyme activities, reduced growth rate
Arsenic	Atmospheric deposition, mining, pesticides, rock sedimentation, smelting	Brain damage, cardiovascular and respiratory disorders, conjunctivitis, dermatitis, skin cancer	Deactivation of enzymes
Beryllium	Coal and oil combustion, volcanic dust	Allergic reactions, berylliosis, cancer, heart diseases, lung diseases	Chromosomal aberration, mutation
Cadmium	Plastic, welding, pesticide, fertilizer, mining, refining	Kidney damage, bronchitis, gastrointestinal disorder, bone marrow, cancer, lung insufficiency, hypertension, Itai–Itai disease, weight loss	Damages nucleic acid, denatures protein, inhibits cell division and transcription, inhibits carbon and nitrogen mineralization
Chromium	Textile, dyeing, paints and pigments, steel fabrication	Carcinogenic, mutagenic, teratogenicity, epigastria pain nausea, vomiting, severe diarrhoea, producing lung tumours	Elongation of lag phase, growth inhibition, inhibition of oxygen uptake
Copper	Plating, copper polishing, paint, printing operations	Neurotoxicity, and acute toxicity, dizziness, diarrhoea	Disrupts cellular function, inhibits enzyme activities
Mercury	Batteries, paper industry, paint industries, mining	Damage to nervous system, protoplasm poisoning, corrosive to skin, eyes, muscles, dermatitis, kidney damage	Decreases population size, denatures protein, disrupts cell membrane, inhibits enzyme function
Nickel	Porcelain enamelling, non-ferrous metal, paint formulation, electroplating	Chronic bronchitis, reduced lung function, lung and nasal cancer, nausea	Disrupts cell membrane, inhibits enzyme activities, oxidative stress
Selenium	Coal combustion, mining	Dysfunction of the endocrine system, gastrointestinal disturbances, impairment of natural killer cell activity, liver damage	Inhibits growth rate
Silver	Battery manufacture, mining, photographic processing, smelting	Argyria and argyrosis, bronchitis, cytopathological effects in fibroblast and keratinocytes, emphysema,	Cell lysis, inhibit cell transduction and growth

(continued)

Table 7.1 (continued)

Heavy metal	Major source	Toxic effects on humans	Toxic effects on microorganisms
		knotting of cartilage, mental fatigue, nose, throat and chest irritation, rheumatism	
Thallium	Cement production, combustion of fossil fuels, metal smelting, oil refining	Alopecia, ataxia, burning feet syndrome, coma, convulsions, delirium, fatigue, gastroenteritis, hair fall, hallucinations, headache, hypotension, insomnia, nausea, tachycardia, vomiting	Damages DNA, inhibits enzyme activities and growth
Zinc	Mining, refineries, brass manufacturing, plumping	Ataxia, depression, gastrointestinal irritation, haematuria, icterus, impotence, kidney and liver failure, lethargy, macular degeneration, metal fume fever, prostate cancer, seizures, vomiting	Death, decrease in biomass, inhibits growth

Adapted and modified from Abbas et al. (2014) and Ayangbenro and Babalola (2017)

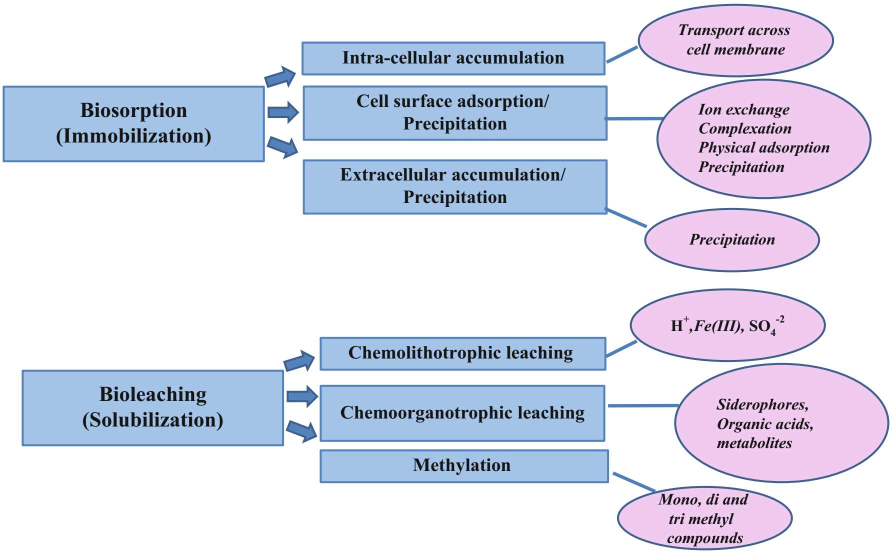

Fig. 7.1 Major mechanisms of bioremediation

ZnS) to soluble form (usually the metal sulphates, e.g., $CuSO_4$, $NiSO_4$, $ZnSO_4$) using chemolithoautotrophs or cyanogenic microorganisms. Dave et al. (2016) reported that the metal extraction can be carried out using various acidophiles like *Acidithiobacillus thiooxidans, Acidithiobacillus ferrooxidans, Leptospirillum ferriphilum, Sulfolobus thermosulfidooxidans*, etc. and a variety of other bacteria viz. *Bacillus megaterium, Chromobacterium violaceum, Pseudomonas aeruginosa, Pseudomonas fluorescens* and fungi viz. *Maramiusoreades, Clitocybe* sp., *Polyporus* sp., etc. Formation of sulphuric acid by *Acidithiobacillus thiooxidans*, Fe^{3+} ion by *Leptospirillium* spp., production of organic acids by fungi and cyanide production by cyanogenic organisms help in dissolving the metallic fraction and thus help in bioleaching of metals like Cu, Zn, Fe, Ni, Pt, Au and Ag (Dave et al. 2016). This process involves oxidation; it may also be termed as biooxidation. Biooxidation involves the microbial decomposition of mineral which enhances the metal recovery, but the recovered metal is not solubilized. An example is the recovery of gold from arsenopyarite ores where the gold remains in the mineral after biooxidation and extracted by cyanide in subsequent step (Rawlings 2011). Some other terms including 'biomining', 'bioextraction' and 'bio-recovery' are also useful to represent the mobilization processes facilitated by microorganisms or by other biological materials. Biomining mainly focuses on the economical metal recovery and the large-scale application of a microbial process in the mining industry (Mishra et al. 2005). Roy and Roy (2015) reported the bioleaching of pyrolusite ore by marine bacteria like *Bacillus, Micrococcus, Pseudomonas, Achromobacter, Enterobacter*, etc. through the enzymatic reduction.

7.3.2 Immobilization

Heavy metal ions either in particulate form or in soluble form can be immobilized on intact bacterial cells (live or dead) and their by-products. Immobilization of metals can be achieved through the biosorption or bioaccumulation process. Both the terms are frequently used as synonyms but actually they are the different processes.

7.3.2.1 Bioaccumulation

Bioaccumulation is an energy-dependent active process which is mediated by microbial metabolism. Unlike biosorption, it is a slow, irreversible and complex process (Rossi 1990; Kanamarlapudi et al. 2018).

7.3.2.2 Biosorption

Biosorption is a reversible, rapid and metabolically passive process of metal uptake. Biosorption is a physicochemical process, which includes various mechanisms (Gadd 2010; Kanamarlapudi et al. 2018; Shamim 2018). Several mechanisms of biosorption are discussed below.

7.3.3 Mechanisms of Biosorption

7.3.3.1 Cell Surface Adsorption

It is the mechanism in which the metals bind to the cell walls and external surfaces of non-living biomass. It mainly involves adsorption mechanisms like physicochemical and ionic adsorption (Joo et al. 2010). The cell wall structure of microorganisms plays an important role in the biosorption process. The cell surface of microorganisms possesses anionic functional groups, which impart the negative charge to cell surface and allow the binding of metal cations. The negatively charged groups which involves in metal adsorption are mainly alcohols, amines, carboxyl, hydroxyl, ester, sulfhydryl, phosphoryl, sulfonate, thioester, thiol, etc. (Kapahi and Sachdeva 2019). The metal uptake phenomena of microorganisms can be assessed by analysing cell wall components, which differ among the diverse microorganisms. Among the microorganisms, bacteria exhibit the highest surface-to-volume ratio and hence represent a better capacity of biosorption than any other microbes (Beveridge 1989; Shamim 2018). Functional groups located on the bacterial cell wall include amine, carboxyl, hydroxyl, phosphate and sulfhydryl, which play important role in metal biosorption. The active sites for metal binding processes in Gram-positive bacteria generally include peptidoglycan layer which contains alanine, glutamic acid, meso diaminopimelic acid, teichoic acid, polymer of glycerol, etc. and in Gram-negative bacteria glycoproteins, lipopolysaccharides, lipoproteins and phospholipids (Fomina and Gadd 2014; Lesmana et al. 2009; Gupta et al. 2015; Shamim 2018). Metals and metalloids can bind to ligands on microbial cell surfaces after which microbial cells decrease their toxicity by transforming them from one oxidation state to another (Chaturvedi et al. 2015). The cell walls of bacteria have polycations and polyanions, which interact with metal ions to maintain electro-neutrality by mechanisms of redox interactions, covalent bonding, van der Waals forces, extracellular precipitations, etc. (Gavrilescu 2004).

7.3.3.2 Extracellular Accumulation

Microorganisms also secrete various types of metal-binding metabolites such as extracellular polymeric substances. They are made up of polysaccharides, proteins, uronic acids, humic substances, lipids, capsules, slimes, sheaths and biofilms. Biofilms act as a matrix for heavy metal binding (Ayangbenro and Babalola 2017; Rehan and Alsohim 2019). Their most important component is exopolysaccharide or extracellular polymeric substances (EPS) which has an ion sequestration capability. Mainly it is composed of complex high molecular weight organic compounds such as polysaccharides and proteins. Sometimes EPS also shows the presence of other macro molecules like lipids, DNA, humic substances, etc. EPS contains abundance of hydroxyl ions due to its hygroscopic nature, which tend to interact with metal ions. The amino sugars of EPS possess the nitrogen-containing functional groups which also react with some metals. The EPS secreted by different microbial species represents varying degree of metal binding capacity (Gupta and Diwan 2017). Upadhyay et al. (2017) observed the sorption of Hg by the dead cells of EPS producing marine bacterium *Bacillus licheniformis* SR5. Igiri et al. (2018) reported the extracellular accumulation of heavy metals by the dead cells of *Pseudomonas putida, Brevibacterium* sp. and *Bacillus* sp. They also discussed the role of EPS from the biofilm produced by *Pseudomonas aeruginosa* in the accumulation of various metal ions. Jarosławiecka and Piotrowska-Seget (2014) reported the accumulation of lead on mycelia of a marine fungus *Corollospora lacera*. Priyalaxmi et al. (2014) showed the biosorption of cadmium by *Bacillus safensis (*JX126862), a marine bacterium isolated from mangrove sediments. Asksonthong et al. (2018) represented the sorption of Hg, Pb and Cd by *Halomonas elongata* and *Tetragenococcus halophilus.*

7.3.3.3 Intracellular Accumulation

The microorganisms can uptake the essential metals from the surrounding through highly specific active transport mechanisms. Detailed studies have been done on the transport system for the metals like Ca, K and Na, but less information is available about the systems regulating intracellular concentration of other metal ions (Shumate and Strandberg 1965; Kanamarlapudi et al. 2018). The intracellular uptake of metals has been observed to occur in many microbes like bacteria, yeast and fungi. It has been observed that the non-specificity of normal transport system and competitive nature of metals to bind with substrate may be responsible for the intracellular accumulation of metals (Trevors et al. 1985; Fang et al. 2016). Microbes develop metal resistance in the initial stage of the process and this may happen due to the presence of plasmid linked genes. The other mechanism accountable for metal resistance is rapid energy-dependent efflux system (Nies 2003; Ianeva 2009). Metals may be deposited in their elemental forms also. Sometimes the metals may be accumulated with the help of some specific metal binding proteins produced by the organisms. Production of low weight cystine-rich proteins and peptides called metallothioneins by certain microbes like C*yanobacterium, Synechococcus, E. coli, Pseudomonas putida* and other class of proteins called phytochelatins (short peptides rich in cysteine and glutamic acid) can accumulate the metal ions (Gupta and Diwan 2017; Kanamarlapudi et al. 2018). Jarosławiecka and Piotrowska-Seget (2014) described the intracellular accumulation of lead (1.7–5.5%) inside the cells of a marine fungus *Corollospora lacera*. They also reported the lead-resistant fungi *Penicillium* sp. Psf-2, isolated from the Pacific sediment, which showed the lead accumulation in the cytoplasm and vacuole as well as on the outer layer of the cell wall.

7.3.3.4 Precipitation

The functional groups present on the surface of the microbial cells bind with the metal ions and forms the insoluble metal precipitates. One mechanism of precipitation is the metal complexation, which plays an important role in metal–ligand interactions. Here, the metals form complex with microbial metabolites such as sulphides and phosphates. For example, the sulphate reducing microorganisms produce hydrogen sulphide, which reacts with metal ions and forms insoluble metal sulphides. Moreover, phosphatase enzyme also participates in metal precipitation (Ellwood et al. 1992; Jin et al. 2018). Igiri et al. (2018) reported that some iron reducing bacteria such as *Geobacter* spp. and sulphur reducing bacteria (SRB) like *Desulfuromonas* spp. can precipitate the metals. SRBs produce large amounts of hydrogen sulphide that causes precipitation of metal cations. The coastal marine bacterium *Vibrio harveyi* has been reported to precipitate lead as a complex lead phosphate salt (Mire et al. 2004). Jin et al. (2018) described the precipitation of Cu (II) on the spore surface of marine *Bacillus* sp. strain SG-1.

7.3.3.5 Transformation of Metals

Metals and metalloids are transformed by microorganisms using different processes like oxidation, reduction, methylation and demethylation. Generally, this phenomenon has been observed in metabolically active microbial cells (Beveridge and Murray 1976; Beveridge and Jack 1982; Timková et al. 2018). Metal reduction can be achieved by metabolically active as well as inactive cells. Partial metal reduction has been observed in many microbes by electron transport system (ETC) also, which renders the concerned metal less mobile and less toxic in the environment (Baldi et al. 1993; Ray et al. 1992; Banerjee et al. 2018). An intracellular or extracellular component of cell acts as reducing agent in passive reduction. For example, *Bacillus subtilis* is reported for the reduction of Au^{3+} to Au° on its cell wall (Beveridge and Murray 1976). Fungi like *F. oxysporum* and *R. oryzae* have been reported to transform Au^{3+} to Au°. In this case, the Au^{3+} ions get diffused through the cell membranes and finally are reduced by cytosolic redox mediators (Singla et al. 2017).

Several microbes have the plasmid-coded specific enzyme systems for methylation-demethylation reactions. Microorganisms can develop resistance towards particular metal due to these enzymes. Metals may be volatilized during methylation and may escape from the treatment site which can pollute the atmosphere. During demethylation of mercury, the methylated mercury is cleaved by the enzyme organomercurial lyase and releases inorganic Hg^{2+}, which is further acted upon by the mercury reductase reducing Hg^{2+} to Hg°. This Hg° being volatile in nature can be removed from the system (Ray et al. 1992; Igiri et al. 2018). Specific methods must be established to prevent the atmospheric contamination by volatile mercury for the large-scale application of these processes. Several bacteria like *Bacillus* spp*.*, *Escherichia* spp*.*, *Clostridium* spp., *and Pseudomonas* spp. have been reported to methylate Hg (II) to gaseous methyl mercury (Igiri et al. 2018). *Scopulariopsis brevicaulis, A. glaucus, Aspergillus virens, Mucor ramosus, Cephalothecium roseum, Sterigmatocystis ochracea, Aspergillus fischeri,* and *Aspergillus sydowii,* are reported as the 'Arsenic fungi' by Bentley and Chasteen (2002). They observed volatilization of arsenic to a garlic aroma gaseous product by these fungal species. Moreover, they also reported some gas producing fungal strains like *Fusarium, Paecilomyces,* and a brown fungus isolated from the arsenic contaminating soil but the volatile material was not identified. They also described various faecal bacteria like *B. mesentericus vulgatus*, *B. mesentericus ruber* and *B. subtilis* produced a garlic odour when grown in the presence of arsenate or dimethylarsinate and *Corynebacterium* sp., *E. coli*, *Flavobacterium* sp., *Proteus* sp., and *Pseudomonas* sp. transformed arsenate to arsenite and produced dimethylarsine. Jin et al. (2018) have reported the reduction of Hg^{2+} to Hg^0 by aerobic bacteria, Cr^{6+} to $Cr^{3+}$by *Corynebacterium* and Pb^{2+} to Pb^0 by dead cells of *Bacillus licheniformis* R08. The application of marine bacteria in metal immobilization is gradually increasing due to their great potential for environmental clean-up processes (Amidei 1997). The arsenic amount in aquatic and terrestrial species is mostly different. The arsenic amount is usually about 1 ppm (dry weight) in land animals, but for marine species, the levels vary from a few parts per million to as much as 100 ppm (Lunde 1977; Masuda et al. 2019). Many marine photosynthetic organisms take up arsenic from seawater and accumulate it in their bodies, which finally resulted in metabolic transformations of arsenic (Masuda et al. 2019). Bentley and Chasteen (2002) described the conversion of arsenic to trimethylarsine by marine bacteria like *Clostridium collagenovorans*, *D. vulgaris* and *Desulfovibrio gigas* as well as conversion of arsenate to arsenite and methylarsonate by marine bacterial strain of *Serratia marinorubra*. Furthermore, they reported the conversion of arsenate to arsenite, methylarsonate, dimethylarsinate and unidentified volatile arsines by a strain of marine yeast *Rhodotorula rubra*.

7.4 Marine Bacteria

Marine bacteria are highly abundant in nature. The overall a number of bacteria on earth may be as high as $4–6 \times 10^{30}$, with the largest proportion of bacterial cells possibly residing in the oceanic and terrestrial subsurface which correspond to 3.5×10^{30} and $0.25–2.5 \times 10^{30}$, respectively (Whitman et al. 1998). Marine bacteria can grow in unique and extreme habitats and can be differentiated according to their optimal growth conditions, including halophiles (saturated brine water/sea water), thermophiles (hot water), alkalophiles (alkaline water), psychrophiles (cold water), barophiles (pressurized conditions) and some more groups (Gontia-Mishra et al. 2017). They are distributed in natural saline areas which vary from aquatic (e.g. oceans, salt marshes and lakes) to terrestrial (e.g. marine sediments and saline lands) as well as in hypersaline environments. They are also found in artificial solar salterns, saltpans, mangroves as well as an endosymbiont (Surve et al. 2012; Gontia-Mishra et al. 2017). Totally 60 different marine bacterial species were studied more than 75 years before by ZoBell and Upham (1944). They reported that the diversity, abundance and count of marine bacteria vary at different depths of the ocean. The abundance of heterotrophic and autotrophic bacteria in the marine environment is noted by Stanley (2005). Dash et al. (2013) described that at all depths, α- and γ-*Proteobacteria* are abundant, whereas *β-Proteobacteria, Firmicutes, Actinobacteria, Chloroflexi, Planktomycetaceae, Verrucomicrobia,* and *Acidobacteria* are common at 800–440-m depth. Piskorska et al. (2007) categorized the bacterial communities of Indian Ocean into six major taxonomic groups such as α-, β- and γ-*Proteobacteria, Actinobacteria, Bacilli* and *Flavobacteria*. Patel et al. (2014) also reported β- and γ-*Proteobacteria, Actinobacteria, Firmicutes* as the most common bacterial community in the coastal water and sediment samples of Alang–Sosiya ship breaking yard, Bhavnagar, India. Different physiological groups of marine bacteria including *Firmicutes, γ-Proteobacteria, Bacteroidates,* and *Actinobacteria* isolated from the coastal region of Alang, Bhavnagar, India have been reported by Upadhyay et al. (2017). These different phyla contained different genera of bacteria such as members of *Firmicutes* represented by genera *Bacillus*, *Planococcus*, *Exiguobacterium*, *Peanibacillus* and *Jeotgalibacillus*. Representative genus of phylum *Bacteroidetes* belonged to *Pontibacter* and *Dyadobacter*. The class γ-*Proteobacteria* of *Proteobacteria* phylum is represented by members of the *Marinobacter, Pseudomonas, Enterobacter, Mesorhizobium, Sinorhizobium, Halomonas, Salinicola* and *Xanthomonas*. The *Actinobacteria* phylum is represented by members of *Micrococcus, Kocuria, Actinotalea, Cellulosimicrobium, Arthrobacter and Nocardia* (Upadhyay et al. 2016; Upadhyay 2017). In cobalt-rich crust regions of the Pacific Ocean, the abundance of γ-*Proteobacteria* was recorded by Liao et al. (2011). Matobole et al. (2017) demonstrated the presence of *Actinobacteria, Firmicutes,* γ-*Proteobacteria* and *α-Proteobacteria,* as major groups from the Marine Sponges; *Isodictya compressa* and *Higginsia bidentifera* that were collected from Algoa Bay, South Africa. The 16S rRNA gene taxonomic affiliation of these 26 sponge-associated bacterial isolates showed antibacterial activities against one or more indicator strains. Phylogenetic identification of marine bacteria isolated from deep-sea sediments of the eastern South Atlantic Ocean showed that 70 isolated bacteria were belonging to three phylogenetic groups, γ-*Proteobacteria*, *Firmicutes* (Gram-positives with low G + C percentage) and *Actinobacteria* (Gram-positives with high G + C percentage) (da Silva et al. 2013).

Marine bacteria have established defensive mechanisms for their survival under extreme conditions and also developed unusual metabolic processes which might have resulted in the ability to produce novel enzymes, bioactive compounds, exopolysaccharides (EPS), etc. than the organisms found in other habitats (Chi and Fang 2005; Dave et al. 2020).

7.5 Marine Bacteria in Biosorption of Metals

Marine bacteria exert a number of mechanisms to remove toxic heavy metals like precipitation, volatilization, physical exclusion in membranes and on EPS, intracellular sequestration, etc. (Naik et al. 2012). Many researchers have reported the heavy metal removal using marine bacteria (Canstein et al. 2002; Iyer et al. 2005; Das et al. 2007, 2009; El-Deeb 2009; Panwichian et al. 2011). Mohapatra et al. (2017) reported several marine bacteria showing resistance to chromium (IV), lead (II) and cadmium (II). They described several halophilic and halotolerant bacteria such as *Halomonas* sp. TA-04, *Vigribacillus* sp., *Planococcus maritimus* VITP21, *Bacillus subtilis, Halomonas sp*. CB5, *Bacillus subtilis* SHB13, *Exiguobacterium indicum* MW-1, showing resistance to chromium (IV), whereas *Alcaligenes* sp., *Enterobacteriaceae* sp., *Kurthia* sp., *Staphylococcus* sp., *Vibrio* sp., *Halomonas elongate, Tetragenococcus halophilus, Bacillus* sp. *Pb15, Halomonas* sp., *Micrococcus luteus* DE2008, *Alcanivorax* consortia, *Klebsiella* sp. 3S1 and *Acinetobacter* sp. THKPS16 showing resistance to lead (II). Furthermore, they discussed the role of *Pseudoalteromonas* sp. SCSE709–6, *Vibrio harveyi* 5S-2, *Pseudoalteromonas* sp. CD15, *Alteromonas macleodii* ASC1, *Bacillus* sp. NT-1, *Enterobacter* sp. NT-5, *Aeromonas* sp. NT-10, *Pseudomonas* sp. TT-10, *Pseudomonas stutzeri* N-1, *Pseudomonas mendocina* C-1, *Alcaligenes faecalis* C-8, *Acinetobacter baumannii* C-10, *Bacillus licheniformis* C-12 and *Lysinibacillus fusiformis* C-14 in resistance of cadmium (II).

Metals in soluble form or in particulate form can be accumulated by live or dead cells of bacteria and by their products such as EPS (Gupta and Diwan 2017). EPS helps the organisms to grow and survive under adverse environmental conditions by helping them in uptake of nutrients, by providing adherence capacity for the attachment to various surfaces, in biofilms formation, in aggregation, etc. (Dave et al. 2016; Poli et al. 2010; Shukla and Dave 2018). Various ionisable functional groups and non-carbohydrate substituents such as carboxyl, hydroxyl, amine, phosphate, sulfhydryl and sulphate groups of EPS impart the negative charge to the polymer due to which various heavy metals can bind to EPS (Gupta and Diwan 2017). Moreover, the presence of uronic acids in some marine bacterial EPS also play important role in metal removal. The role of EPS-producing heavy metal-resistant isolates from deep-sea hydrothermal vents and their purified EPS for metals and toxic substances binding ability has been described by Loaec et al. (1998) and Wuertz et al. (2000). Bhaskar and Bhosle (2006) have discussed the sorption of lead and copper by EPS of *Marinobacter* sp. Gutierrez et al. (2012) have demonstrated the chelation of calcium, aluminium, iron and magnesium by *Halomonas* sp. associated with marine micro-alga. The sorption of cadmium (65%), copper (20%) and hexavalent chromium (75%) was reported by the EPS of marine *Enterobacter cloacae* (Iyer et al. 2004, 2005). Qin et al. (2007) have recorded the sorption of Cu^{2+} (52.77%), Fe^{2+} (85.00%), Zn^{2+} (58.15%), Mg^{2+} (30.69%), Co^{2+} (48.88%), Cr^{6+} (5.15%) and Mn^{2+} (25.67%) by EPS of *Pseudoalteromonas* sp. SM9913. Details about the EPS-producing marine bacteria and their metal removal efficiency are enlisted in Table 7.2.

7.6 Use of Genetically Modified Microorganisms in Biosorption

Genetic engineering is mainly used to develop more potent strains having necessary selectivity and affinity for metal ions. Using recombinant DNA technology, the interested genes of another organism of the same or different species have been incorporated into the host genome. The genetically modified microbial strains have enhanced capability to degrade organic and inorganic pollutants. Dixit et al. (2015) and Dash et al. (2013) have described the genetic manipulation of marine bacteria by transforming merA gene responsible for the conversion of toxic form of mercury to nontoxic form. Furthermore, they also mentioned several genetically altered marine bacteria like *Vibrio harveyi, Synechococcus* sp., *Staphylococcus aureus, Thalassospira lucentensis, Nocardia* sp., *E. coli, E. coli* FM5/pKY287 and

Table 7.2 Use of marine bacteria and their EPS for metal remediation

Marine bacteria	Metal ions	Sorption capacity (mg/g)	Reference
Idiomarina fontislapidosi F23^T	Cu^{2+}	16.30	Mata et al. (2008)
	Pb^{2+}	40	
	Co^{2+}	8	
Idiomarina ramblicola R22 ^{T}	Cu^{2+}	26.25	
	Pb^{2+}	44.65	
	Co^{2+}	10	
Pseudoalteromonas sp. strain TG12	Na^+	154.5	Gutierrez et al. (2008)
	Mg^{2+}	31.0	
	K^+	10.6	
	Sr^{2+}	2.7	
	$Fe^{2+/3+}$	0.14	
Salipiger mucosus A3^T	Cu^{2+}	15.7	Llamas et al. (2010)
	Pb^{2+}	43.5	
	Co^{2+}	8.7	
Desulfovibrio desulfuricans	Cu^{2+}	98.2	Kim et al. (2015)
	Ni^{2+}	90.1	
	Cr^{6+}	99.8	
EPS M1	Cu^{2+}	400	Deschatre et al. (2015)
	Ag^+	256	
Alteromonas sp. JL2810	Cu^{2+}	140.8 ± 8.2	Zhang et al. (2017)
	Ni^{2+}	226.3 ± 3.3	
	Cr^{6+}	251.2 ± 5.1	
Bacillus licheniformis SR5	Hg^{2+}	200	Upadhyay et al. (2017)
Bacillus xiamenensis PbRPSD202 (live cells)	Pb^{2+}	216.75	Mohapatra et al. (2019)
Bacillus xiamenensis PbRPSD202 (dead cells)	Pb^{2+}	207.4	

sulphate-reducing bacteria (SRB) for the enhanced bioremediation of heavy metals, hydrocarbons, poly chlorinated biphenyls (PCB), trichloroethylene (TCE), toluene, benzene, etc. In their review, Ojuederie and Babalola (2017) mentioned the use of several genetically modified bacteria such as *Deinococcus geothermalis*, *Cupriavidus metallidurans* strain MSR33 and *Pseudomonas* strains for the bioremoval of Hg. They demonstrated the insertion of mer operon which codes for Hg^{2+} reduction into *Deinococcus geothermalis* from *Escherichia coli*. The modified organism showed enhanced ability to reduce mercury contamination at high temperatures. Likewise, the pTP6 plasmid having merB and merG genes that regulate mercury degradation and also have the ability to synthesize organomercurial lyase protein (merB) and mercuric reductase (merA) enzymes was inserted into the *Cupriavidus metallidurans* strain MSR33 and the modified strain showed Hg degradation. Several strains of *Pseudomonas* having resistance to Hg were developed by inserting the pMR68 plasmid into them.

Moreover, several metal-binding proteins such as metallothioneins (MTs) and phytochelatins have been used to develop the microbial strains that can tolerate the stressful environmental conditions as well as exhibit higher accumulation of metal ions (Kanamarlapudi et al. 2018). Valls and De Lorenzo (2002) discussed that the expression of MTs was found to be higher in the outer membrane compartments (periplasmic space) as compared to the cytoplasm. Furthermore, they reported 15- to 20-fold increase in Cd^{2+} binding by the strain of *E. coli* expressing MT fused to the Lam B (an outer membrane maltose protein) as compared to its wild-type counterpart. Li et al. (2016) isolated a cDNA clone, encoding 74-a.a. metallothionein type 1 protein (ZjMT), from the cDNA library of *Ziziphus jujube* and incorporated it in *Escherichia coli* cells. The cells of *Escherichia coli* expressing ZjMT showed enhanced metal tolerance and higher accumulation of metal ions as compared to control cells. In recent times, new transgenic and adapted *S. cerevisiae* strains are reported to have an increased sorption capacity for Cu (II) (Farcasanu and Ruta 2017). Different strains of *E. coli* are modified to express phytochelatin 20, Hg^{2+} transporter, metalloregulatory protein and nixA genes. Such modified strains showed the improved biosorption ability for the metals like mercury (Hg), arsenic (As) and nickel (Ni) (Kanamarlapudi et al. 2018). Deng and Jia (2011) have constructed a recombinant strain of a photosynthetic bacterium, *Rhodopseudomonas palustris*, expressing the genes of (pSUTP+pGPMT) mercury transport system and metallothionein for Hg^{2+} removal from heavy metal wastewater. Some improvements have been done for the biological heavy metal uptake in several microorganisms like *Escherichia coli*, *Corynebacterium diptheriae, PseudomonasK-62, Pseudomonas K-12, Serratia marcescens, Streptomyces coelicolor, Saccharomyces cerevisiae* with the use of recombinantly expressed inner membrane importers from three major transporter classes such as channels, secondary carriers and primary active transporters (Diep et al. 2018).

Although the genetically altered microbes are beneficial for environmental clean-up processes, some preventive measures should be taken to minimize environment risk before introducing them into the environment. One such potential risk of this is the horizontal gene transfer (HGT), which occurs between the modified and indigenous microbes. It might result into the spread of the resistant strains in the environment and also cause ecological imbalance (Phillips 2008; Keese 2008). HGT can be prevented by using suicidal gene systems and also with the use of anti-sense technology which involves the insertion of antisense RNA-regulated plasmids and protein plasmids into the microbe (Azad et al. 2014; Ojuederie and Babalola 2017). Considering all these points, the genetically altered microbes should be used with carefulness and in agreement with bio-safety regulations.

7.7 Factors Affecting Biosorption

Different microbial biomasses have different biosorption abilities, which also vary considerably within each group. The biosorption ability of every single biosorbent depends on several factors such as pH, temperature, initial metal ion concentration, temperature, biosorbent concentration, biosorbent size, and contact time (Igiri et al. 2018; Timková et al. 2018). In biosorption process, pH seems to be the most important parameter. It affects the metal ion solubility and total charge of biosorbent (Friis and Myers-Keith 1986; Galun et al. 1987; Oyewole et al. 2018). The lower pH favours the competition of hydrogen ions with metal ions for binding to active sites of sorbent surface. In highly acidic system, the removal of metal ions is almost negligible, and it increases with increase in solution pH up to a certain limit while less amount of H^+ and greater number of ligands with negative charges results in greater metal ions biosorption at higher pH values (Feng et al. 2011; Bilal et al. 2018). The initial metal ion concentration also plays a key role in biosorption. Sometimes the biosorption capacity increases with the increasing initial metal ion concentration but it is also observed that at higher metal concentrations, more ions are left un-adsorbed in the solution due to the saturation of the binding sites. The low concentration of metal ions facilitates more sorption because more binding sites are available and sometimes shows less sorption because the metal ions diffuse slowly to the biomass surface due to their inadequate concentrations. This depends on the type of the metal and biomass (Naiya et al. 2009; Oyewole et al. 2018; Shamim 2018). The biomass concentration determines its potential to remove metal ions at a given metal concentration. The increased adsorbent dose provides greater surface area and availability of more active sites, and hence leads to the enhancement of metal ion uptake for a fixed metal concentration. But the adsorption capacity is reduced when the biomass dosage increases beyond some limits as a result of lower adsorbate to binding site ratio where the ions are distributed onto more empty binding sites (Kumar and Gaur 2011; Kanamarlapudi et al. 2018). Another important factor which is studied in most of the batch biosorption experiments is the optimum contact time required to attain the maximum metal removal. Due to a larger surface area of the adsorbent, the rate of metal removal is higher in the beginning, and it gets slow down afterwards as there would be a competition for binding to available active sites by the remaining metal ions (Abdel-Ghani et al. 2007; Bilal et al. 2018). Temperature may exert positive or negative effect on the process of biosorption. Biosorption capacity of the biosorbent may get changed due to increase or decrease in the temperature. Mostly the chemical reaction rates increase at higher temperatures, but it can be fatal for living microbial cells as it damages the cell membranes (Timková et al. 2018). The optimum temperature for the sorption of heavy metals may vary from microbes to microbes. Moreover, the same organism may also show the sorption of various metals at different temperature (Goyal et al. 2003; Hu et al. 2010; Jin et al. 2018). Shamim (2018) demonstrated that the biosorption mostly takes place between 20 and 35 °C, and at higher temperature (above 45 °C), the process may get affected due to the protein damage.

7.8 Biosorption Isotherm Models

Sorption isotherms can be best explained by studying the equilibrium relationships between adsorbent and adsorbate. The biosorption isotherms describe the adsorption capacity of sorbent for the removal of sorbet at constant condition. Biosorption isotherms are characterized by definite parameters, which express the surface properties and affinity of the biosorbent. The equilibrium parameters provide important information for designing batch biosorption systems (Witek-Krowiak et al. 2011). To study the nature of adsorption process, several equilibrium isotherm models are employed. These comprise one parameter isotherm like Henry's isotherm, two parameter isotherms such as the Langmuir, Freundlich, Hill–Deboer Model, Flory–Huggins, Dubinin–Radushkevich, Jovanovic and Temkin isotherms, three parameter isotherms such as Toth, Sips, Koble–Carrigan and Redlich–Peterson, etc. (Abdel-Ghani and El-Chaghaby 2014; Ayawei et al. 2017). The solid–liquid absorption system can be studied by investigating the equilibrium batch sorption tests and dynamic continuous flow sorption studies. The Langmuir and Fruendlich equations are widely used to study the linearized equilibrium adsorption isotherm models for single-solute system. The Langmuir isotherm model accounts for the surface coverage with homogeneous binding sites by balancing the equivalent sorption energies (Gupta et al. 2010; Dave et al. 2012). According to Langmuir model, no further sorption can take place at the site which is occupied once. The surface will ultimately reach a saturation point where the maximum adsorption will be achieved (Areco et al. 2013). The Langmuir equation is represented by: $q_e = q_{\max} bC_e/(1 + bC_e)$, where q is milligrams of metal accumulated per gram of the biosorbent material; C_e is the residual metal concentration in solution; $q_{\max}$ is the maximum specific uptake corresponding to the site saturation and b is the ratio of adsorption and desorption rates (Langmuir 1918). This theoretical model is used to represent a monolayer adsorption. To study the multilayer adsorption process, Freundlich model is used. The Freundlich isotherm model is applicable to those adsorption processes which occur on heterogeneous surfaces. The Freundlich equation is given as: $q_e = K_F.C_e^{(1/n)}$, where K_F and n are Freundlich constants (Freundlich 1907). The n value specifies the degree of nonlinearity between solution concentration and adsorption as follows: if $n = 1$, then adsorption is linear; if $n > 1$, then adsorption is a physical process and if $n < 1$, then adsorption is a chemical process. These models can be applied at a constant pH, and used for modelling of biosorption equilibrium in the presence of one metal (Abdel-Ghani and El-Chaghaby 2014; Upadhyay et al. 2017).

7.9 Biosorption Kinetics

The rate at which the metal ions or contaminants are removed from the aqueous system can be determined by the kinetic study. The sorption kinetics describes the mechanism through which the biosorption process takes place (Gupta et al. 2010). The optimum operating conditions for batch biosorption processes can be determined by kinetic study (Tuzen and Sarı 2010). To study the biosorption data of different metal ions sorbed by various biosorbents, several kinetic models such as pseudo-first order, pseudo-second order, Elovich, intraparticle diffusion, etc. have been applied (Jin et al. 2018). According to the pseudo-first-order kinetic model, metal ion binds only to one sorption site on the surface of sorbent, and the rate of occupied sites is proportional to the number of unoccupied sites (Ghaedi et al. 2013). The model is represented by: $dq_t/dt = k_1(q_e - q_t)$, where q_e is uptake of metal per unit weight of biosorbent (mg/g), q_t is the amount of metal sorbed at any time (mg/g), and k_1 is the rate constant (min^{-1}). The values of k_1 and the calculated q_e can be obtained respectively from the slope and intercept of the linear plot of ln $(q_e - q_t)$ versus (t) (Dave et al. 2012; Upadhyay et al. 2017). The second-order kinetic model is based on the assumption that the reaction rate is dependent on the amount of solute present on the adsorbent surface. Here, the rate limiting step involves chemisorption, due to which metal ions bind to the surface by strong covalent bonding. The pseudo-second-order equation based on equilibrium adsorption is expressed as: $dq_t/dt = k_2 (q_e - q_t)^2$, where k_2 is the equilibrium rate constant (mg/g/min). Plots of (t/qt) versus (t) give the values of q_e as the slope and k_2 as the intercept. Elovich's equation is used to understand the chemisorption process of adsorption. In this model, interactions or desorption between the adsorbed species could not affect the adsorption kinetics at low surface coverage. Furthermore, as the amount of adsorbed solute increases, the rate of adsorption of solute decreases exponentially (Gupta and Bhattacharyya 2011). Elovich's equation is given as: $q = \alpha \ln(\beta\alpha) + \alpha \ln(t)$; $q = \alpha \ln(\beta\alpha) + \alpha \ln(t)$; where a (initial sorption rate mg/g min) and b (loading capacity g/mg) are related to the extent of surface coverage and activation energy for chemisorption (Dave et al. 2012). The suitability of the model to fit the experiment data is mainly determined by the correlation coefficient (R^2) and the calculated q_e value. The model gives the best fit to the experimental data, when the model's calculated q_e is equal to the experimental q_e and R^2 approaches unity. Detailed insights of biosorption mechanism by the application of intra-particle diffusion model are studied by many researchers. The Weber and Morris or intra-particle diffusion model determines the adsorption rate in most of the liquid systems (Witek-Krowiak et al. 2011). This model assumes that the adsorption can occur through three successive steps including film diffusion, intra-particle diffusion and sorption. Moreover, it represents the proportionality between the adsorption capacity and the square root of the time (Boparai et al. 2011; Abdel-Ghani and El-Chaghaby 2014). The model's equation is expressed by: $q = Kt^{1/2}$, where intra-particle diffusion rate constant, K, is deduced from a graph of qt vs. $t^{1/2}$ (Dave et al. 2012). Biosorption mechanism can be very well understood by studying the plots of qt against $t^{0.5}$. These plots are represented by single or multiple lines. When the Weber–Morris plot of qt vs. $t^{0.5}$ gives a straight line, the adsorption process is believed to be controlled by intra-particle diffusion only. If the regression of qt vs. $t^{0.5}$ is linear and passes through the origin, then intra-particle diffusion is the sole rate-limiting step. When the data show multilinear plots, then it indicates two or more steps are influencing the sorption processes. The intercept of these plots reflects also the boundary layer effect (Abdel-Ghani and El-Chaghaby 2014; Upadhyay 2017).

7.10 Analytical Techniques to Analyse Biosorption Process

The active sites of biomass involved in the binding of metals can be analysed through a number of sophisticated analytical techniques (Mohapatra et al. 2017), and some of them are enlisted in Table 7.3.

7.11 Living and Non-living Systems for Metal Sorption

Metabolically active as well as inactive cells and dead biomass can be used as biosorbents for heavy metal removal as they all can accumulate metals. The main advantages of live biomass are:

- The cellular ion transportation systems of the living cells transport metal ions directly into cells.
- It can be self-renewing, resulting in an increase of cell mass, enabling biosorption of more heavy metal ions.
- Some excreted metabolic products such as H_2S, $H_2PO_4^{2-}$, metallothioneins can contribute to the heavy metal removal.

Table 7.3 Techniques used to analyse biosorption process

No.	Analytical techniques	Remarks
1	Atomic absorption spectroscopy (AAS)	Determines metal concentration in aqueous phase
2	Inductively coupled plasma (ICP)	Determines metal concentration in aqueous phase
3	UV-vis spectrophotometer	Determines metal or dye concentration in aqueous phase by measuring its colour intensity
4	Scanning electron microscope (SEM)	Visual confirmation of surface morphology of the biosorbent
5	Transmission electron microscope (TEM)	Visual confirmation of inner morphology of the biosorbent
6	Energy dispersive X-ray spectroscopy (EDS)	Elemental analysis and chemical characterization of metal bound on the biosorbent
7	X-ray diffraction (XRD) analysis	Crystallographic structure and chemical composition of metal bound on the biosorbent
8	Fourier transform infrared spectroscopy (FT-IR)	Determines active sites of the biosorbents
9	Nuclear magnetic resonance (NMR)	Determines active sites of the biosorbents
10	Thermogravimetric analysis (TGA)	Characterizes thermal stability of the biosorbent
11	Differential scanning calorimetry (DSC)	Characterizes thermal stability of the biosorbent

All these may lead to a higher level of metal ion uptake. Though the live cell systems provide higher metal uptake, there are some disadvantages using them at large scale. Maintaining the living systems is difficult because the waste streams are mostly toxic, lacking nutrients and also have extremely varying conditions. Thus, most of the biological metal removal systems make use of non-living or non-metabolizing systems.

There are a number of advantages in using non-living cells as biosorbent for heavy metal removal, and some of them are enlisted below:

- The non-living systems are growth-independent and do not require nutrients for the cell growth.
- The sorption process is not limited by metal toxicity as there is no metabolic or physiological activity.
- The non-living biomass represents more efficient metal uptake and higher metal loading capacity.
- The operating conditions including pH, temperature, time and metal concentrations can be controlled easily as compared to live systems, and no sterile conditions are required.
- Non-living biomass can be stored for a longer period of time.
- Metal desorption from the used biomass is relatively easy allowing reuse of biomass.
- The disposal problems do not arise, if the amount of metal recovered is insignificant. In such case, the biomass could be incinerated.
- Various industries may provide a cheap source for such biomass.
- Low operational cost as compared to live systems.

Disadvantages of Non-living Systems

- As the cells are not metabolizing, the biological process improvement potential becomes limited.
- The biomass can get saturated at early stage of the process.
- There is no possibility for biological altering of the metal valency state.
- If the biomass contains some recovery chemicals, then it can affect the metal sorption performance adversely (Junlian et al. 2010; Hassan et al. 2010; Upadhyay 2017).

7.12 Desorption and Metal Recovery

Desorption of biosorbents is significantly important in biosorption process as economical point of view. The selection of eluent used for metal recovery depends upon the chemistry of metal and mechanism of its sorption. If the process of biosorption of metal occurs through adsorption/cell surface accumulation which is metabolically independent, then it could be considered similar to an ion exchange process. In that case, metal could be recovered easily by mild and non-destructive methods. If the sorption occurs by intracellular accumulation such as sequestration or binding with induced proteins and other metabolically depended mechanisms, then it requires drastic methods for recovery. In this case, the biosorbent may be damaged to such level that it cannot be reused. The metal recovery is achieved through incineration or dissolution of biosorbents in acids, alkali and complexing agents. Dilute mineral acids (HCl, HNO_3 and H_2SO_4), organic acids (citric, acetic, lactic acid), alkali (NaCl, KCl, NaOH, sodium bicarbonate) and complexing agents (EDTA, thiosulphate, etc.) can be used for metal elution without affecting the biosorbent (Faison et al. 1990; Dixit et al. 2015; Shamim 2018). Furthermore, the metals showing pH-dependent sorption can be recovered by changing pH of the solution. The metal sorption is generally maximum between pH 5 and 7, which can be eluted using eluent at pH 2, whereas metals showing better sorption at low pH (i.e. pH ≤ 2) could be removed by increasing the pH. Metals showing relatively pH-independent behaviour for biosorption process are difficult to remove that by changing the pH of solution (Upadhyay 2017).

For effective desorption, the selected eluent should have the following characteristics:

- The eluent should yield the metals in a concentrated form.
- It should not cause any physical changes or damages to the biomass and restore the biosorbent close to the original state for effective reuse with maximum metal uptake.
- It should not alter or block the sites for metal uptake.

Different metals from loaded biomass can be eluted out using appropriate eluents. Dixit et al. (2015) reported desorption of Cd (II), Cr (VI), Pb (II) and Zn (II) from dry biomass of *E. crassipes* and *C. indica*. They reported that the maximum sorption of these metals is achieved in the range of 80–100% even after the four repeating cycles of desorption by desorbing agents including HNO_3 and KCl. Desorption of Au (I) sorbed on eggshell membrane was successfully achieved using NaOH and NaCl; the sorption capacity for Au (I) did not decrease after five continuous sorption–desorption cycles (Niazi et al. 2016).

7.13 Future Work

As compared to the conventional techniques, biosorption is a more favourable technique. Biosorption has been applied at laboratory, pilot and industrial scales to treat industrial effluents using various biomass (Vijayaraghavan et al. 2009; Vinodhini and Das 2010; Javaid et al. 2011; Rao and Ikram 2011; Singh et al. 2012). Actually, there is a dearth of instances of biosorption processes at highly organized levels such as in pollution treatment centres, municipalities, large industries, etc. This may be due to the presence of a variety of chemical and biological contaminants at different concentrations in waste. For different types of the effluents, some promising biosorption processes need to be established. Another developing approach in the field of bioremediation is the use of genetically modified organisms and metal binding proteins with the use of genetic engineering. Only few reports are available for molecular approach, and the technique is not yet implemented for the wide range of microbial strains. Therefore, more detailed research in this field is needed to enhance the metal remediation using microbes.

7.14 Conclusion

The microbial biosorption process is very efficient and economically viable. It offers several advantages, including cost-effectiveness, high efficiency, minimization of chemical/biological sludge and regeneration of biosorbent with possibility of metal recovery. The selectivity of biosorbent for a particular metal may create difficulties when applied to multimetal effluents. Moreover, the residual metal concentrations after sorption process should be within the permissible limits. These hurdles could be overcome by selecting appropriate biosorbent and/or appropriate recovery methods. There is an urgent need for the development of an economical and eco-friendly technology in countries having rapid industrial development. Bioremediation technology provides exciting prospects for metal recovery and environmental protection, but more work is required in several areas in order to understand the complete potential of biological systems.

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Biofilm-Associated Metal Bioremediation

8

Amrita Jasu, Dibyajit Lahiri, Moupriya Nag, and Rina Rani Ray

Abstract

One of the biggest challenges to the developing societies is metal pollution, especially in the regions of mining and plating that is affecting people worldwide. The use of conventional strategies in removing the waste is expensive and generates a large amount of toxic wastes, thereby affecting the environment adversely. This has resulted in the drift from the normal strategies to the use of eco-friendly strategies for the removal of metallic wastes being present within the soil. This technique of remediation uses the microbial organisms or microbial biomass that helps in detoxifying the soil from the toxic effects of inorganic metallic salts and heavy metals. Microbial biomass mainly comprises of extracellular polymeric substances (EPS) which increases the efficiency of metal sequestering for the purpose of field bioremediation. The EPS is released by the microbial cells for the purpose of self-defense which mainly occurs during various environmental stresses such as starvation, temperature, pH, and other physiological or rheological stress conditions. It contains a large amount of anionic charge hence it causes large sequestering of metallic ions. Thus this chapter will focus on the biofilm-associated bioremediation of heavy metals, and the mechanism which is helping the process to occur.

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Keywords

Heavy metal · Biofilm · Remediation · Mechanisms

8.1 Introduction

In order to maintain the standards of the environmental regulatory bodies, significant strategies are to be undertaken to reduce the organic and inorganic pollutants that contaminate ecosystems (Trapani et al. 2010). Heavy metals are natural high density elements that are required in various biological processes in trace amounts. Recalcitrant and toxic compounds containing heavy metals are a result of numerous urban and enhanced industrial activities. Accounting to its ease in solubility, products from pharmaceuticals, pesticides, plastics, rubbers, tanneries, and organic chemical industries lead to the accumulation of metals like Pb^{2+}, Cr^{2+}/ Cr^{3+}, Cd^{2+}, Ni^{2+}/ Ni^{4+}, Zn^{2+}, Cu^{2+}, Hg^{2+}, etc. that further triggers bio-magnifications (Lakherwal 2014). Metals tend to be toxic on accumulation, as they are nondegradable and can only be altered to different valance states and transformed by sorption, methylation etc. So this nonbiodegradability and the rising accumulation of free forms of heavy metals can lead to irreversible and indigenous health issues that affect bones, kidney, heart, and even brain. They can pose mutagenic, cytotoxic, or even carcinogenic effects (Ray and Ray 2009). The common physicochemical practices to reduce the load of these heavy metal ions include coagulation, reverse osmosis, chemical precipitation, nanofiltration, ultrafiltration, etc. But these procedures are not very favorable due to high reagent demand, costing, operational effectiveness, high energy demand, etc. Thus, for sustainable environmental detoxification, to reduce heavy metal ions to a safer level of concentration, there is a need to undertake environment friendly methods and strategies (Prasad and Prasad 2012). Here, microbial communities of bacteria, microalgae, fungi, and yeast play a vital role in degradation, neutralization, and mineralization as processes of remediation in order to remove organic and inorganic terrestrial and aquatic pollution of heavy metal ions. Efficient utilization of microbial metabolism in place of physicochemical procedures is a safe and efficient approach (Pandey and Jain 2002). Sorption of metal ions is an electrostatic or covalent interaction of the metal with peripherally available functional group on the sorbent. When such sorbents are of biological origin of eukaryotic or prokaryotic microbial biomass, such phenomenon is called biosorption (Comte et al. 2008; Lau et al. 2005). An environment-friendly approach is thus biofilm-mediated bioremediation. In order to thrive in stringent environmental conditions like high temperature, pH, salinity, and heavy metal-contaminated site, the microbial community produces extracellular polymeric substances (EPS) (de Carvalho 2018). EPS are biosynthetic polymers made up of mainly polysaccharides, proteins, and uronic acid that are produced in light of self-defense against environmental stress. The EPS composition hinders the metal ion penetration into the cellular surface and thereby helps in sequestration of the metals. Biofilms are formed by the association of single or various species of bacteria, algae, or fungi, and its subsequent structure and composition of EPS produced can alter on the basis of environmental conditions (Yin et al. 2019).

As biofilms are capable to absorb, sequester, and immobilize various environmental pollutants (namely heavy metal ions), hence biofilm-mediated bioremediation is an effective approach. Thus, the attempt of this chapter is to focus on survival strategy of the microorganisms in the impacted (heavy metal contaminated) environment, the protective and beneficial approach of biofilms for the producing community, the biosynthesis of the polymer, i.e., the biofilm's life cycle and thereby metal ion to the biofilm interaction. Finally, in order to understand the mechanisms involved in metal ion uptake by the EPS, the interaction mechanism is also studied. Lastly, the scope and miscellaneous uses of biofilm and possible adverse environmental impacts have also been discussed.

8.2 Heavy Metals and Their Toxicity

As an important part of the global ecobiological system and vital component of metabolic pathways, heavy metals are sufficient even in trace amount. Such are the metals with atomic numbers more than 20 and of metallic density more than 5 g/cm^3 with distinct chemical characteristics. Majority are present in all ecosystems in varying concentrations along with trace quantities of essential metals like Mn, Ni, Zn, Mg, Cu, etc. They tend to have prolonged half-lives and resist biodegradation, thus are potent toxic to the ecosystem (Hussain et al. 2013). Environmental accumulation of these metals as a result of overloaded byproducts and toxic sludges from industries over long period of time is a serious matter of concern now.

When human body gets exposed to elevated amounts of these metals, they bind to various biomolecules and exert toxicity via different mechanisms like disrupting enzymatic functions, hinder in process of DNA formation and production of reactive oxygen species (ROS) (Yu 2001; Gauthier et al. 2014). Metals like lead, cadmium, and mercury are able to interact with cell membrane ligands, disrupt channels, and confer metabolic disturbances and cytotoxicity (Tavares and Carvalho 1992). Methylation or harmful thiol formation by chromium and arsenic, binding of cadmium to certain protein to render it dysfunctional, saturation and lipid peroxidation due to iron, lead causing free radical imbalance are few of the toxicological manifestations of heavy metal ions (Jaishankar et al. 2014).

Various plants and animal diseases have been reported due to metal toxicity. A prominent and burning example is the Minamata Bay (Japan) tragedy, where due to the Minamata disease, several people lost lives on consumption of shellfish that were heavily mercury contaminated. Zinc plays a vital role in plant growth and its defense mechanism. Upon accumulation, it reacts with oxygen and acids to produce potential toxic compounds harming the plants (Fosmire 1990). According to WHO recommended guidelines, arsenic concentration above 10μg/L might cause bronchitis, liver cirrhosis, or even cancer. Dietary intake of cobalt above 6μg may cause

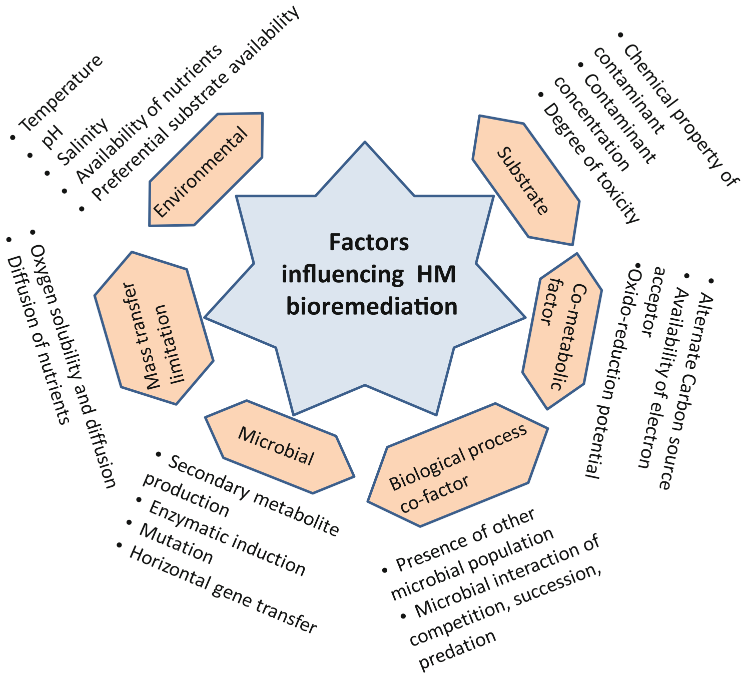

Fig. 8.1 Factors influencing bioremediation of heavy metals (HM)

chronic asthma or pneumonia. Renal and hepatic systems, and even the immune system are affected on overexposure to lead or copper ions (Flora et al. 2012).

Taking into consideration the cost, effectiveness, and end products produced after the physical treatments like flocculation, ion exchange, electrodialysis, etc. to reduce these metal ion concentrations to safer limits in the environment, it urges for imperative need to find environment-friendly and economic strategies (Alluri et al. 2007). Many microbes can thrive in metal contaminated sites and potentially accumulate soluble or particulate metal ions. Microbial biomasses of fungi, yeasts, and bacteria act as bioabsorbents of these heavy metal ions (Das et al. 2008). To survive in stringent environment, these eukaryotic and prokaryotic biomasses produce biosynthetic polymers that act as a shield and help in sequestering the metal ions via bio-immobilization or biotransformation (Fig. 8.1).

8.3 Biofilm: Composition and Structure

8.3.1 Composition

Biofilm is a cluster of microorganisms of same or different species that organize to attach themselves on living or nonliving surfaces while shielding themselves with a protective EPS layer from antibiotic agents, predators, or environmental stress.

Mostly composed of water, the EPS is made of mostly homo or hetero polysaccharides, proteins, nucleic acids, humic acid, lipids, as well as low molecular weight non-polymeric substances. FTIR spectroscopic analysis shows predominantly polysaccharides and proteins as the components of biofilm (Mosharaf et al. 2018). This microbial mucoid polysaccharide helps in cell aggregation, maturation of microcolonies, attachment to surface, and water-holding capacity and protects the cell cluster from stringent environment (Czaczyk and Myszka 2007). They are best differentiated not on the basis of origin but on the basis of separation, as soluble or bound EPS (Pal and Paul 2008). Ionic bridges that interlink the polysaccharide chains are due to uronic acid and other divalent metallic cations, which also entrusts the acidic character of EPS (Majumdar et al. 2013; Dogan et al. 2015) .The exopolysaccharide composition differs with diverse microbial aggregates, phase of growth, and its extraction method, like production of capsular EPS during the log phase of growth, whereas slime is formed during stationary phase (Wingender et al. 1999). EPS with polysaccharide backbone of repetitive units of same or different monosaccharides are differentiated on the basis of monomer units and linkages. The heteropolysaccharides are composed of units of N-acetylglucosamine or N-acetylgalactoseamine, D-glucose, L-rhamnose with intermittent presence of glycerol, phosphate, or acetyl groups, whereas homopolysaccharides are classified as Fructan, polygalactan, α-D-glucan, and β-D-glucan (Sharon 1966). Variety of EPS are produced upon alteration of the polysaccharide backbone by change in polymeric length due to variations in side chains and functional groups (Whitfield 1988). Those EPS with abundant anionic functional groups are reported to be better heavy metal remediator and a suitable biosorber. Moreover, as a nonliving adsorbent, EPS is considered more useful than living microorganisms to avoid pathogenecity concerns (Gavrilescu 2004). Some of the anionic bacterial EPS reported are xanthan (*Xanthomonas campestris*), galactopol (*Pseudomonas oleovorans*), hyaluronan (*Pseudomonas aeruginosa*), gellan (*Sphingomonas paucimobilis*), alginate (*Azotobacter vinelandii*) (Freitas et al. 2009, 2011). PsI polysaccharide produced by *Pseudomonas aeruginosa* is reported to maintain biofilm architecture and is responsible for intracellular and cell surface adhesion (Ma et al. 2012).

eDNA or extracellular DNA is a constituent of EPS that are formed from lysed cells within the biofilm. Recent studies show that these eDNA are important constituents of the EPS as they maintain the structural integrity of the biofilm, enhance cell–cell communication, and act as nanofilaments for electron transfer (Martins et al. 2010). These eDNA ranges in length of 1000–10,000 bp (Romero et al. 2018).

8.3.2 EPS Synthesis

The EPS is synthesized intra- or extracellularly depending upon the type of polysaccharide it produces. Precisely considering bacterial EPS biosynthesis, it is mostly seen homopolysaccharides are produced extracellularly when responsible precursors are transferred from substrate to the growing end of polysaccharide chain by suitable

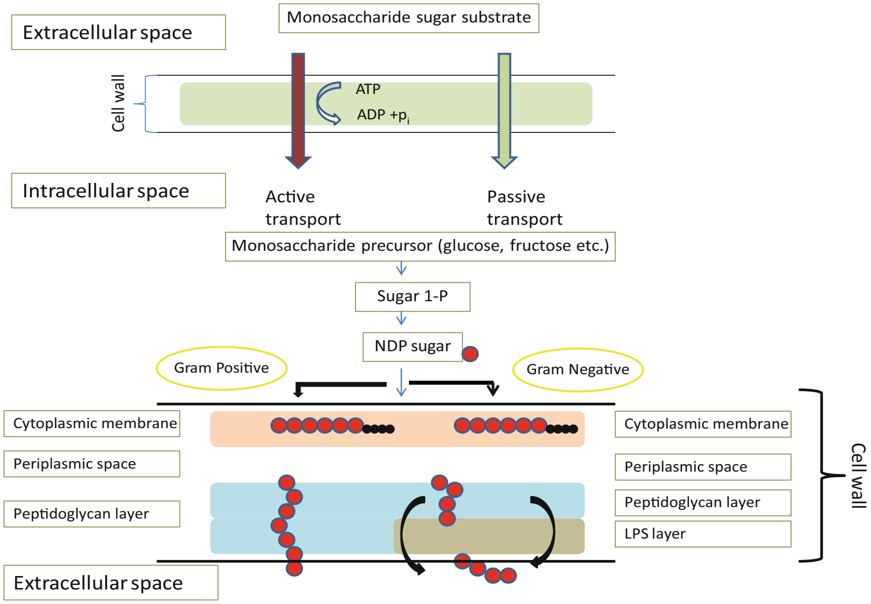

Fig. 8.2 Biosynthesis of EPS in Gram-positive and Gram-negative bacteria. Initiating with substrate diffusion, conversion within the cytoplasm, and thereby transport via polysaccharide assembly for the growing chain in the cytoplasmic membrane, elongation of the assembled polysaccharide in the peptidoglycan, maturation and transportation by ABC-dependent or Wzx–Wzy pathway to the extracellular environment

enzymes. The polysaccharide then matures and assembles itself with varying branches (Boels et al. 2001).

Comparatively a complex sequence occurs during the intracellular synthesis and transport of the polysaccharides. Regulatory molecules of interconnected metabolic pathways and various enzymes are involved during such synthesis process. It involves active or passive passage of substrate sugar into the cell which then gets catabolized by phosphorylation or periplasmic oxidation (Freitas et al. 2011). Intracellular synthesis involves the assembly of the high molecular weight, hydrophilic polymer in the cytoplasm and its transport via the cell membrane. The Gram-negative bacteria is studied to biosynthesize via the following pathways (Fig. 8.2).

(a) ABC transporter-dependent pathway: polymerization occurs at the inner cell membrane, in the cytoplasm side (Cuthbertson et al. 2009) (i) the Wzx–Wzy-dependent pathway, wherein the polymer monomeric units are accumulated at the inner face of the cytoplasmic membrane and polymerized at the periplasm.
(b) Wzx–Wzy-dependent pathway: The monomer units are assembled in the inner face of the cell membrane, whereas polymerization takes place in the cytoplasm.

(c) Synthase-dependent pathway: Here the stand is secreted across the cell wall membrane, where the homopolymers are translocated and polymerization takes place with a single precursor of sugar and single synthase protein (Table 8.1).

8.3.3 Biofilm Structure and Its Formation

Unlike the planktonic growth, the development of biofilm has distinct and significant mechanism. The biofilm formation and growth are ubiquitous to bacterial cells and occur sometimes under diverse stringency. The biofilm formation, its attachment, and maturation mostly requires a moist environment. Attachment surface may be biotic or abiotic, and their interaction interface can be of any form of solid-liquid, liquid-liquid, or solid-air. Different stages of microbial life cycle, is reported to produce various forms of biofilm.

The overall process of formation involves four distinct steps initiating with the attachment of the planktonic forms of microbes to a moist surface by Van der Waals or electrostatic forces of attraction. Following it is the procedure of microcolony formation, maturation, and development of biofilm and finally detachment from the surfaces (Jamal et al. 2018).

- *Initial surface attachment*: Intricate study on biofilm formation reports the initial attachment of the microbes on a moist surface. Their arrival instigates a formation of conditioning layer with the organic matters within a short span of time (Qi and Christopher 2019). The microbial population attaches itself to the surface with its pili, fimbriae, by van der Waals force or via electrostatic forces of attraction. The adhesion to the surface brings about cohesive forces among the cells to expand the film formation.
- *Colony formation*: The intercellular signaling calls for rapid cell multiplication and thus microcolony formation. The spatial arrangement of the clustered microcolonies determine the architecture of the biofilm. The side chains of the formed EPS by these colonies further strengthens the biofilm's anchoring to the surface. This confirms the further maturation at that surface (Randrianjatovo-Gbalou et al. 2017).
- *Architectural maturation*: Intracellular signaling, replication, and nutrient transfer increase the microbial population to the desired density (Vasudevan 2014). Maturation-specific genes are expressed during this stage of development that is facilitated by the phenomenon of Quorum Sensing. This three-dimensional biofilm development can produce as thick as few inches of film by some bacteria.
- *Microbial detachment*: Biofilm detachment may be caused by physical forces of shear strain like natural abrasion or hydrodynamic forces which erodes off biofilm layers (Webb 2009). This passive dispersion of sloughing might occur due to aged biofilm, deprivation of nutrients, natural erosion, etc. But regardless of the detachment process, the conditioning layer tends to remain attached to the substratum, chopping off the outer layer with saccharolytic enzymes produced by the microorganisms (Fig. 8.3).

Table 8.1 Microbial EPS: homopolysaccharide and heteropolysaccharide

EPS	Class	Monomers	Linkages	Example of EPS-producing microorganisms	Reference
Levan	Homopolysaccharide	Fructose	β-2,6	*Halomonas smyrnensis, Streptococcus salivarius, Streptococcus mutans*	Sima et al. (2011) and Czaczyk and Myszka (2007)
Curdlan	Homopolysaccharide	Glucose	β-1,3 & β-1,2	*Bradyrhizobium japonicum, Streptococcus pneumoniae*	McIntosh et al. (2005) and Czaczyk and Myszka (2007)
Pullulan	Homopolysaccharide	Glucose	α-1,4	*Aureobasidium pullulans*	Özcan et al. (2014), Cheng et al. (2011) and Elshafie et al. (2017)
Reuteran	Homopolysaccharide	Glucose	α-1,4	*Lactobacillus reuteri 121*	Kralj et al. (2002)
Cellulose	Homopolysaccharide	Glucose	β-1,4	*Gluconacetobacter hansenii*	Sun et al. (2010) and Shah and Brown (2005)
Alternan	Homopolysaccharide	Glucose	α-1,3 & α-1,6	*Streptococcus salivarius*	Simpson et al. (1995)
Xanthan	Heteropolysaccharide	Glucose as backbone and glucuronic acid as side chain	(1–4)-β-D-glucan	*Xanthomonas campestris*	Schatschneider et al. (2013)
Alginate	Heteropolysaccharide	β-D-Mannurosyl & α-L-guluronosyl	1–4 bonds	*Pseudomonas aeruginosa, Azotobacter vinelandii*	Czaczyk and Myszka (2007)
LAB EPS	Heteropolysaccharide	Fructose, rhamnose, glucose, and galactose	1–6; 1–4; 1–2; 1–3 bonds	*Lactobacillus* sp.	Nishimura (2014)
Sphingans (gellan, rhamsan, welan)	Heteropolysaccharide	Glucose, rhamnose and glucoronic acid	1–3; 1–4 bonds	*Sphingomonas elodea, Sphingomonas* sp. *ATCC53159*	Pollock (1993) and Coleman et al. (2008)
Schizophyllan	Homopolysaccharide	Glucose	β-1,3 with β-1,6 branching	*Schizophyllum commune ATCC38548*	Joshi et al. (2016)

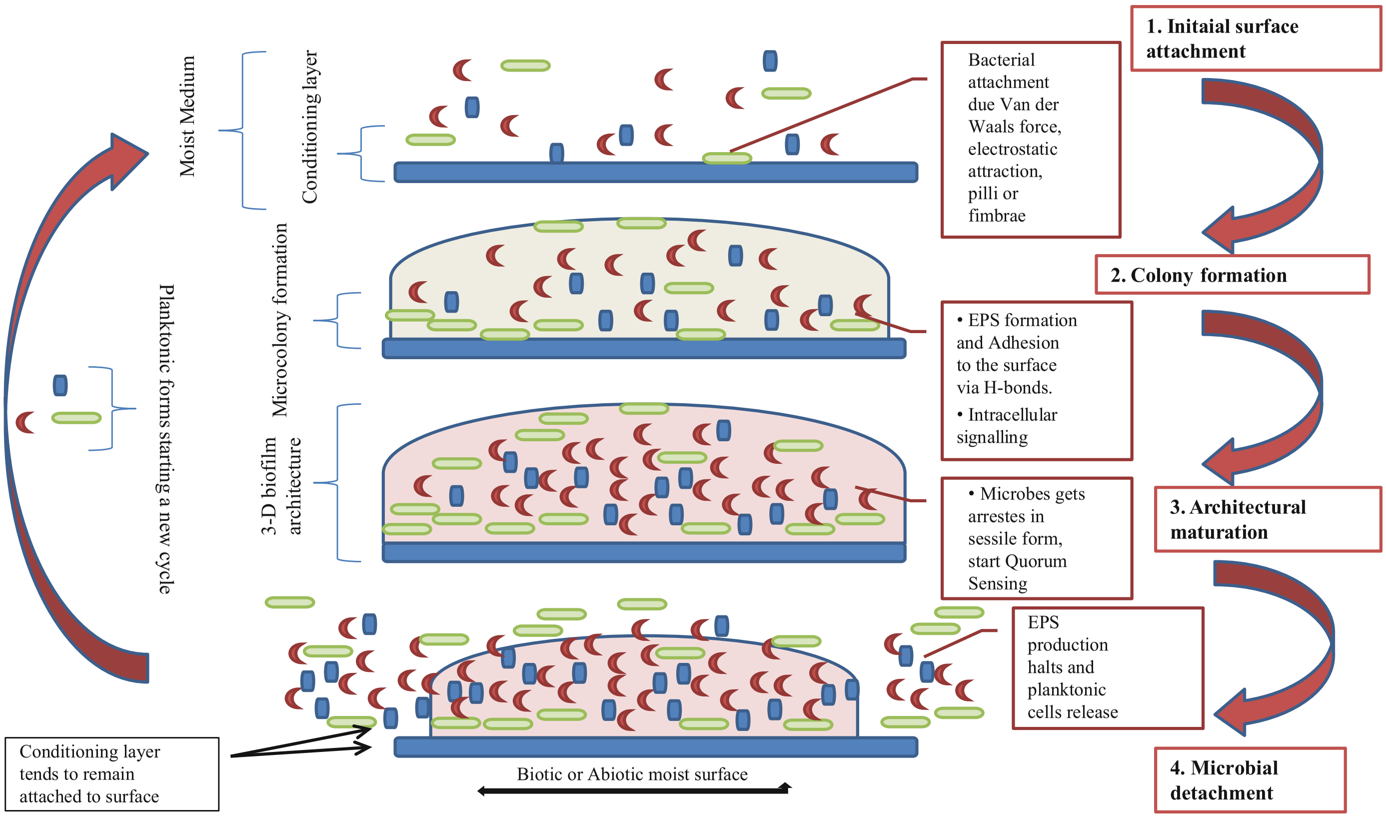

Fig. 8.3 Stepwise events of microbial biofilm formation

8.4 Biofilm-Producing Microbiota

Biofilm producing microbial biomass have been widely reported for heavy metal bioremediation. The microbial population predominantly includes bacteria along with fungi, microalgae, and yeast. Mostly the metal sequestration takes place by adsorption or bioaccumulation. These metal-resistant strains are efficient producers of biosynthetic polymers (EPS), and their immobilized forms are better-off with metal interaction and its reaction kinetics as reports suggest that production of EPS enhances due to attachment to a substrate (Vandevivere and Kirchman 1993).

8.4.1 Bacteria in Bioremediation of Heavy Metals

The lab-scale efficiency of microbial biomass varies with the onsite physicochemical and other environmental factors (Ayangbenro and Babalola 2017). Almost all bacterial cells, due to varying size, cellular composition like teichoic acid, N-acetylglucosamine, or N-acetyl muramic acid, their robust adaptivity, and metabolic secretion are able to produce biofilms and thrive in harsh environments. This helps the bacterial biomass to accomplish efficient removal of the nonbiodegradable heavy metals (Hassan et al. 2010; Özer and Özer 2003). Numerous species have been reported as important biosorbents like *Listeria monocytogenes* (Colagiorgi et al. 2017), *Pseudomonas fluorescens* (Lopez et al. 2000), *Pseudomonas putida, Sphingomonas* sp. (Douterelo et al. 2018), *Micrococcus luteus* (Puyen et al. 2012), *Staphylococcus xylosus* (Aryal et al. 2010), and metabolically superior consortium of *Acinetobacter* sp. and *Arthrobacter* sp. (De et al. 2008), *Acinetobacter* sp. IrC1 and *Cupriavidus* sp. IrC4 (Irawaiti et al. 2018). The teichoic acid on the bacterial cell wall is the potential site for bacterial chemisorptions (Mosa et al. 2016). Upon bacterial exposure to contaminants, enzymatic induction calls for remediation by metal chelation or electrostatic interaction. Reports suggests that zeolite-immobilized *Desulfovibrio desulfuricans* used in a batch reactor show metal removal efficiency of 90.1% 98.2%, and 99.8%, of Ni, Cu, and Cr^{6+}, respectively (Kim et al. 2015). Also efficient Pb reduction by *Bacillus megaterium* (2.13–0.03 mg/L), Cr reduction (1.38–0.08 mg/L) by *Aspergillus niger*, and Cd reduction (0.4–0.03 mg/L) by *Bacillus subtilis* has been reported by Abioye et al. (2018).

8.4.2 Fungi in Bioremediation of Heavy Metals: Mycoremediation

Fungi are the decomposers that are involved in vital nutrient cycling in the nature and adapt to harsh conditions similar to that of bacteria. But the bacterial and fungal glycan and other biosynthetic enzymes differ in sequence homology (Sheppard and Howell 2016). Yet these organisms are capable of biofilm formation by clustering and attaching to a surface or entrapping itself inside the EPS to shield from environmental stringency. Reports suggest that in the course of biofilm formation, the fungal ability of surface attachment is due to the presence of hydrophobin protein. This protein signals the adhesion of fungal spore on hydrophobic surfaces (Pérez-Mendoza et al. 2011). Various members those are able to form biofilm are species of *Neocosmopora* and *Acremonium* (Douterelo et al. 2018), *Candida albicans* (Rajendran et al. 2016), and those with prominent metal removal efficacy are *Coprinopsis atramentaria* reported to bioaccumulate 94.7% of 800 mg/L of Pb^{2+} (Luna et al. 2016), *Candida sphaerica* has removal efficiency of Pb, Zn, Fe of 79%, 90%, and 95%, respectively. Reduction of Cr (VI) to Cr (III) by yeast strains of *Rhodotorula mucilage, S. cerevisiae, Pichia guilliermondii*, and *Yarrowia lipolytica* has also been reported (Chatterjee et al. 2012). Fungi are studied to biosorp and accumulate heavy metals by means of ion-exchange or complexation mainly in their fruit bodies (Ogbo and Okhuoya 2011). Fructification, age of mycelia, and metal exposure time-span determine the degree of removal efficacy (Floudas et al. 2012).

8.4.3 Algae in Bioremediation of Heavy Metals: Phycoremediation

Algae are the organisms that produce a large quantity of biomass, able to survive in toxic environments with low nutrient availability. The most prominent members with effective heavy metal bioremediative ability are those of microalgae. Their various cell surface chemical moieties are the metal binding sites like with amide, carboxyl, hydroxyl groups (Abbas et al. 2014). Algae like bacteria and fungi, bioremediate heavy metals by adsorption, cellular integration of the toxicants or by degradation (Chabukdhara et al. 2017). Among the various algal groups, heavy metal phycoremediation by biosorption is best reported by Phaeophyta (brown algae) (Brinza et al. 2007; Oyedepo 2011). Table 8.1 enlists the heavy metal remediating bacteria, fungi, and algae (Table 8.2).

8.5 Metal–Microbe Interaction and EPS-Mediated Strategies for Remediation

The physical methods involved to clean up the environment from metal accumulation involve high-cost techniques and production of toxic byproducts. This calls for cost-effective and environment-friendly biological methods. Exploiting the microbial metabolism is a gentle approach to combat heavy metal toxicity. But not all the time these metals pose to be toxic to the microbes. The concentration of metal and microbes, mode of interaction with the metals as active or passive uptake and subsequently develop metal tolerance or resistance. (Alluri et al. 2007). This microbial metal tolerance can be specific or nonspecific. Mercury methylation is an example of specific tolerance that produces volatile toxic methyl mercury, whereas nonspecific tolerance may be constitutive or inducible. Production of cysteine-rich metallothionine peptides by *Pseudomonas putida* or *E. coli* is an example of nonspecific inducible resistance (Rajendran et al. 2003). On the other hand,

Table 8.2 List of the heavy metal remediating bacteria, fungi, and algae

Metal	Bacteria	Fungi	Algae
Fe	*Rhodobacter capsulatus*	*Aspergillus flavus* (Bano et al. 2018) *Pleurotus ostreatus* (Arbanah et al. 2012)	*Chlorella vulgaris* (Kwarciak-Kozlowska et al. 2014)
Pb	*Methylobacterium organophylum* (Bharagava and Mishra 2018) *Pseudomonas putida* (Chen et al. 2005)	*Agaricus bisporus* (Frutos et al. 2016) *Pleurotus florida* (Prasad et al. 2013)	*Nostoc* sp. (Kumaran et al. 2011) *Corallina mediterranea* (Ibrahim 2011)
Zn	*Pseudomonas putida* (Pardo et al. 2003) *Bacillus firmus* (Salehizadeh and Shojaosadati 2003)	*Schizophyllum commune* (Javaid and Bajwa 2008) *Pleurotus ostreatus* (Arbanah et al. 2012)	*Sargassum* sp., *Ulva* sp., *Padina* sp., *Gracilaria* sp. (Sheng et al. 2004) *Scenedesmus quadricauda* (Bayramoglu and Arica 2009)
Cr (VI)	*Bacillus laterosporous* (Zouboulis et al. 2004) *Bacillus cereus* (Nayak et al. 2018) *Bacillus circulans* MN1 (Chaturvedi 2011)	*Rhizopus arrhizus* (Shoaib et al. 2013) *Pleurotus ostreatus* (Arbanah et al. 2012)	*Isochrysis galbana* (Kadimpati et al. 2013) *Sargassum polycystum* (Senthilkumar et al. 2010)
Cd	*Bacillus laterosporous* (Zouboulis et al. 2004) *Pseudomonas aeruginosa*, *Klebsiella pneumonia* and *Bacillus cereus* (Kafilzadeh et al. 2013)	*Alternaria alternata* (Bahobil et al. 2017) *Aspergillus flavus* (Cardenas-Gonzalez et al. 2017)	*Ceramium virgatum* (Sari and Tuzen 2008) *Caulerpa fastigiata* (Sarada et al. 2014)
Cu	*Desulfovibrio desulfuricans* (Kim et al. 2015) *Micrococcus luteus* (Puyen et al. 2012)	*Agaricus bisporus* (Frutos et al. 2016) *Termitomyces clypeatus* (Ramrakhiani et al. 2011)	*Chlorella kessleri* (Horvathova et al. 2009) *Caulerpa lentillifera* (Apiratikul and Pavasant 2008)
Ni	*Pantoea agglomerans* and *Enterobacter asburiae* (Bhagat et al. 2016) *Desulfovibrio desulfuricans* KCTC5768 (Congeevaram et al. 2007)	*Aspergillus versicolor* (Taştan et al. 2010) *Schizophyllum commune* (Javaid and Bajwa 2008)	*Chlorella vulgaris* (Klimmek et al. 2001) *Spirogyra hyaline* (Kumar and Oommen 2012)

exopolysaccharide production is a nonspecific tolerance. Adsorption, bioleaching, bioaccumulation, and redox transformations are the modes of interaction among the microorganisms to uptake metal ions.

8.5.1 EPS-Mediated Metal Biosorption: Mechanism, Advantages, and Disadvantages

Among various biotransformation procedures, bioadsorption has been seen to be more feasible due to its inherent advantages and applicability. Unlike bioaccumulation or redox transformations, biosorption is a metabolism-independent procedure which can be achieved by both live and dead biomass via different physicochemical procedures (Vijayaraghavan and Yun 2008). While it is a challenge for the live cells beyond lethal concentration of metals or in restricted nutrient availability, to survive metabolically actively, dead cells on the other hand are independent of such constrain to sequester metal by adsorption and its storage (Aksu 2005). Moreover, since the dead biomass only accumulate the metals within, without involving it in metabolic processes, so they can be profitably desorbed and the metal ions can be utilized, acting as reversible ion exchanger. Even though these microbial biomass (live or dead) are efficient and economical solution for metal removal, but its efficiency of application on industrial scale is still a constraint which needs to be worked upon further (Wang and Chen 2009).

8.5.2 Strategies of Heavy-Metal and EPS Interaction and Its Remediation

The exopolysaccharides are made up of repetitive sugar units that are responsible for metal chelation by interacting with their functional groups via ion exchange, physical sorption, or precipitation mechanisms. The charged metal ions interact with anionic EPS for the presence of ionizable carboxylate, phosphate, and amine groups on the EPS backbone (Liu and Fang 2002). The hydroxyl, carboxyl, and amino functional groups aid in metal binding and stabilize the metal–polymer interaction by forming co-ordination bonds (Cozzi et al. 1969).

8.5.3 Types of EPS and Its Remediation Strategies

8.5.3.1 Dead Biomass EPS

EPS of a dead biomass and favorable environmental condition gives maximum metal uptake capacity as a biosorbent. An acidic pH is mostly favorable for metal uptake as an overall positive charge is created due to lowered pH that protonates the functional group which interacts well with the negatively charged EPS (Sultan et al. 2012). However, the adsorption capacity of immobilized EPS is head over dead biomass EPS. Freundlich–Langmuir adsorption model in a research report suggested that in acidic pH of 2, copper ions were maximally chelated by dead biomass EPS with a concentration of 26 mg/g of EPS where the metal load was 91.66 ppm (Ozdemir et al. 2005).

8.5.3.2 Homogeneous EPS

Many single species microbiota are reported to thrive in multi-metal contaminated sites, bind to or sequestrate metal specifically or nonspecifically, by either tolerance or resistance. Cells along with their produced polysaccharide together are seen to be more efficient in adsorption of heavy metals. Various microbes and specially bacteria have been widely reported for such bioremediating activity like *Methylobacterium organophilum* (copper and lead removal) (Kim et al. 1996), *Rhizobium radiobacter* (biosorption of zinc and lead) (Wang et al. 2013), microalgae *Halomonas* sp. (calcium, iron, magnesium, aluminum) (Gutierrez et al. 2012), cyanobacteria *Anabaena spiroids* (manganese) (Freire-Nordi et al. 2005), etc.

8.5.3.3 Immobilized EPS

Studies on specificity and reaction kinetics have shown the excellency of the immobilization techniques. Attachment of microbial cells on a surface enhances not only EPS production but also metal binding ability without elevating the growth rate (Vandevivere and Kirchman 1993). The EPS secreted by *Paenibacillus polymyxa* immobilized on agar beads (Hassiba et al. 2014) and *Chryseomonas luteola* immobilized on alginate beads (Ozdemir et al. 2005) are able to efficiently adsorb various metals like cadmium, cobalt, nickel cobalt, etc.

The test microorganism and the metal concentration are more of a concern than other factors of colony size, procedure of metal–microbe interaction, etc. (Cruz-Vega et al. 2008). Hence for isolating a powerful bioremediating microbe, such criteria must be essentially considered.

8.6 Challenges with Biofilm and Future Prospects

The aggregation of microbial population called biofilms, able to attach to various surfaces, are explicitly studied for environmental pollution remediation. An economical as well as sensitive application of the ubiquitous exopolysaccharide (EPS) produced by the microbial biomass is an efficient approach to metal ion bioremediation. Along with the various environmental factors, the EPS matrix composed of various polysaccharides, nucleic acids, humic acid, and proteins helps in formation and stabilization of the biofilm, defining its 3D structure. These biofilm-forming microbial population adapt to thrive in harsh conditions; hence, several dynamics governs the EPS efficacy of metal removal. As discussed before, the charge composition of EPS, types, quantity, and charge distribution of the metal pollutants, environmental factors of temperature, pH, and salinity are all, in combination, determining factors of the onsite sorption efficacy of the biopolymer. Moreover, the lab-based efficacy must also be replicated at the actual site of pollution which needs to be monitored and checked for proper desorption of the metals from the polymer for sustainable remediation. Desired modification of the polymer for immobilization and improved selectivity for metals can be made of EPS hybrids with ceramic, porous silica, etc. (Kariminiaae-Hamedaani et al. 2003; Shi et al. 2011). Selection of anionic EPS producers and mixed culture EPS gives better results in bioremediation, but scaling up the technology, out from laboratory as field trials, needs more research for successful application (Kiliç et al. 2015).

Microbial biofilms are also utilized in several promising ways as in production of microbial fuel cell (MFC) that oxidizes organic matter to produce electricity, as bioemulsifier and biocapsule, as biofilm traps and microbial canaries (Moreno-García et al. 2018). As an emerging scope, Biofilm-Integrated Nanofiber Display (BIND) is a nano-biotechnological approach to develop programmable biomaterials like adhesive biofilm that are able to bind various surfaces (Nguyen et al. 2014).

Uncontrolled growth of biofilms on suitable surfaces are nowadays a potent matter of concern in the health sector. The growth of biofilm on medical instruments, surface of catheters, and medical implants may cause nosocomial infections, on teeth (plaque), it is also implicated in the otitis media (an acute ear infection). Even avid growth of these biofilms are seen over household items like cutting board, toilet sink, wash basin, etc., which need to be checked and sanitized. Biofilm formation is a major concern in the food industry as they cause havoc product spoilage with growth of pathogenic bacteria, mechanical blockage, and post processing contamination. Hence, to combat with such biofilm problems, effective and systematic antibiofilm strategies are to be undertaken for large-scale application.

The great versatility of the microflora helps in with proficient method of metal ion sequestration and restoration of contaminated ecobiological systems. Thus, in order to effectively use EPS for bioremediation, it must be studied properly from its formation, maturation, EPS–metal binding, and therefore sustainably remove heavy metals from the environment.

Conflict of Interest A mutual declaration by all the authors states that there is no conflict of interest.

Moreover, this chapter does not contain any study conducted upon human or animals, by any authors.

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Phytoremediation of Mine Waste Disposal Sites: Current State of Knowledge and Examples of Good Practice

9

Ksenija Jakovljević, Dragana Ranđelović, and Tomica Mišljenović

Abstract

Mining activities are considered to be one of the main sources of environmental pollution, and lead to the production of vast amounts of waste materials. Mining leads to soil degradation, loss of biodiversity, and pollution of soil, water, and air. The disposal of mine waste is a global problem, and its composition depends on ore characteristics. In addition to high concentrations of metal/metalloids, mine tailings and spoils of abandoned mines are characterized by unfavorable physicochemical characteristics, such as extremely low or high pH, low nutrient status, and water retention capacity. Various remediation technologies could be applied to improve the environmental characteristics of mine waste disposal sites, reduce the amount of pollutants, and prevent their mobilization to the surrounding soil and waterbodies. One of the most cost-effective, and environmentally friendly technologies is phytoremediation, with phytostabilization, phytovolatization, phytofiltration, and phytoextraction as main techniques. An overview of the past experiences is discussed together with future trends in phytoremediation.

Keywords

Phytoextraction · Phytostabilization · Hyperaccumulation · Plant species · Tailings

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9.1 Introduction

Mining activities are one of the bases for the economic development of many regions worldwide. The mining industry generally extracts and processes three basic types of minerals: energy minerals (such as coal, oil, gas, oil shale), metallic minerals (e.g., iron, aluminum, copper, lead, zinc, nickel, gold ores), and non-metallic minerals (gravel, limestone, clay, salt, phosphate rock, etc.). Areas with mining activities are usually with altered landscape, hydrological and ecological processes, hosting anthropogenic constructions that follow the extraction and processing of minerals. In addition, during mine operations, large areas of land become occupied with mine waste deposits. It is known that mining activities have a deteriorating effect on the surrounding environment, such as soil, surface or ground water, and air (Rocha-Nicoleite et al. 2017). Furthermore, mining is considered one of human activities with the most far-reaching environmental and social impacts, and one of the main causes of the land degradation worldwide (Barkemeyer et al. 2015). For this reason, mining companies in the countries in which they operate are obliged by different regulations to reclaim the mined area once the mining process is complete. The reclamation of the mine site is an integral part of the mine life cycle, especially its closure phase.

As mining activities vary according to their nature, scale, and exploitation techniques applied, they leave different ecological footprints in their surroundings (Fig. 9.1). Compared by mining techniques, surface mining severely damages the aboveground landscape while compared to underground mining, which has more indirect and limited influence on the surface environment, it takes up large areas of land resources. The negative impacts of surface mining include a considerable change in soil structure, altered hydrology of the area, and the long-term leaching of contaminants from excavated grounds and waste deposits (Lima et al. 2016). In addition, surface exploitation of mineral resources leads to the production of larger volumes of waste rocks, as these have to be removed to reach the ore. As newly discovered mineral deposits usually have a lower ore grade and are found at greater depths (International Council of Mining and Minerals 2014), it is expected that their exploitation will result in significant amounts of additional mining waste (Wieszczycka 2018).

Generated waste is characterized by different physicochemical properties depending on the extracted mineral, ore composition, and deposit type (Wieszczycka 2018). Two main types of solid waste produced by mining activities are waste rock and mine tailings. Waste rock consists of various geological materials that have been excavated to reach the ore. This type of waste is usually deposited in the form of heaps or piles. The surface of the rock piles is exposed to external temperature conditions, oxygen and water infiltration, which leads to weathering of the rock material. This weathering can produce metal-contaminated drainage water, the control and mitigation of which is a long-term challenge to the surrounding environment. Long-term monitoring by Vriens et al. (2018) showed that the primary weathering rate of waste rock varies with its composition and particle size, while

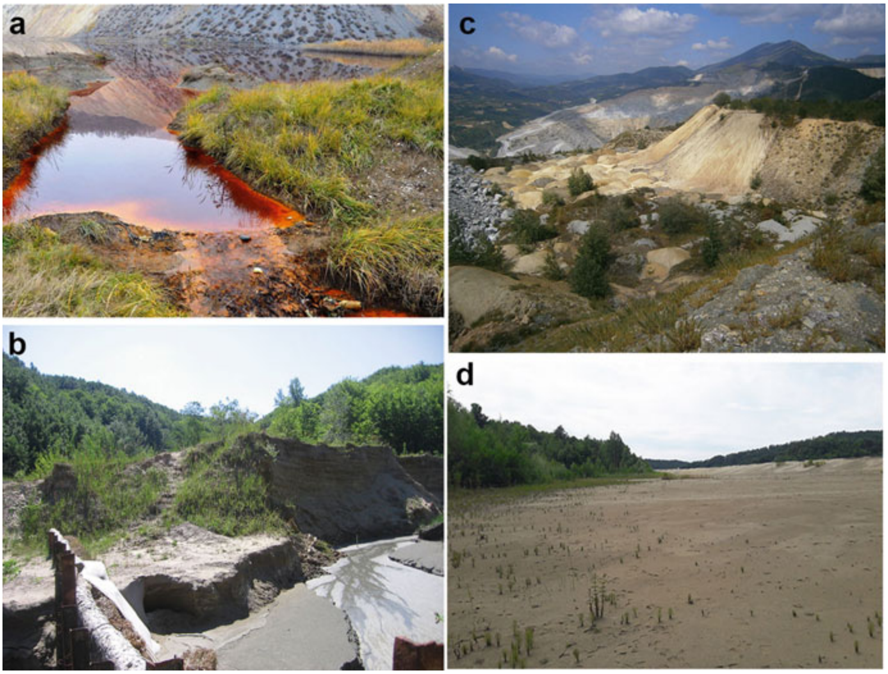

Fig. 9.1 Various types of generated mine wastes and possible environmental consequences: (**a**) sulfidic rock waste and generated acid mine drainage at Bor copper mine site; (**b**) Stolice antimony flotation tailing dam spill; (**c**) Veliki Krivelj copper mine waste rocks; (**d**) Flotation tailings of Rudnik polymetallic mine

the secondary mineral formation process and the presence of reactive rocks influence the drainage chemistry.

Certain geological environments, such as metallic ore deposits, coal seams, oil shale, or mineral sands, may contain abundant content of sulfide minerals (Lotermosser 2010), which can easily be weathered when exposed to oxygen, producing acidic drainage water enriched with sulfate and metals that can be easily released into the environment. This process, known as acid mine drainage (AMD), is one of the major environmental problems facing the mining industry today (Dold and Fontboté 2001). One of the most common sulfide minerals is pyrite (FeS_2), which is commonly found in metal ore deposits and coal seams. Air movement and associated oxygen transport through waste rock dumps have the potential to significantly enhance the oxidation rate of pyrite-bearing material. High concentrations of dissolved metalloids and metals, low pH, and high sulfate in the acid mine drainage pose a serious threat to surface and underground water, soil, and biota. Although extensive research has been conducted in recent decades, there is still no reliable method for dealing with acid mine drainage process and its effects (Dold 2014; Skousen et al. 2017). The colonization of such environments by vegetation or other biota is usually quite inhibited due to the harsh conditions they contain, and it can occur within a considerable period of time after the waste is deposited (Lotermosser 2010). In cases where waste rock contains sulfide minerals and the minerals that can consume released ions through buffering reactions (such as carbonates, silicates, or hydroxides), the formation of acid mine drainage can be inhibited or controlled (Dold and Fontboté 2001).

Mine tailings are another type of mine waste produced during ore processing in order to separate valuable minerals. They consist of ground rock and process effluents, and their grain size is relatively homogeneous as they undergo a grinding process before the selective separation of the useful components. The composition of mine tailings differs considerably from the mined waste rock in terms of texture, mineralogy, and chemistry (Lotermosser 2010; Ranđelović et al. 2014). In addition, the tailing characteristics can vary as they are conditioned by ore mineralogy and physicochemical processes used during ore extraction.

Most of the major ore minerals are geochemically associated with certain metals and metalloids. In addition, the different forms of metals or metalloids found in mine wastes vary greatly in terms of solubility and mobility, which also determines their potential environmental impact. Important factors influencing the release of these elements into the surrounding environmental media are the geology of the mining area, climate, topography, and the type of mining and processing activities applied (Dybowska et al. 2006). Understanding metal speciation in such complex environments is crucial to gain insights into the mobility, bioavailability, and toxicity of metals. Various abiotic and biotic processes influence element speciation and distribution, including adsorption and desorption of mineral surfaces, precipitation, release through mineral dissolution, and interactions with plants and microbes (Brown et al. 1999). When assessing the transport mechanisms and fate of elements originating from mining sites, it is necessary to consider mineralogical and geochemical conditions at the site (Ranđelović et al. 2019a).

The proportion of the total content of a chemical element or compound in an earth material that can be released into the surface or near-surface environment by various mechanical, chemical, or biological processes is known as geo-availability (Plumlee 1994), which refers to the susceptibility of the resident mineral to weathering reactions (Rivera et al. 2015). Although elements originating from mineralized resources may be present in large quantities in mine wastes or in the surrounding environment, they may also be highly chemically bound and therefore not readily available for release and mobilization. When elements are loosely bound in soluble, exchangeable, or different adsorbed phases, they tend to be easily moved and dispersed when environmental conditions change. The transition of an element from the unavailable to the available form is regulated by various physicochemical or biological conditions such as pH, redox potential, clay, and organic matter content, competition from other ions (Smith 2007; Leita et al. 2009).

However, living organisms react to the specific part of the current concentration of the element that is considered biologically available or bioavailable. Therefore, many of the regulatory frameworks for the remediation of contaminated sites include the bioavailability of the contaminant in the risk assessment processes (Kim et al. 2015). However, to date there is no generally accepted definition of bioavailability, and the method of assessing it is not clearly defined. One of the widely accepted definitions was created by the National Research Council Comitee (2003) and refers to bioavailability processes, defined as the individual physical, chemical, and biological interactions that determine the exposure of plants and animals to chemicals associated with soils and sediments. Bioavailability can be used as a tool for the selection of appropriate remediation strategies for contaminated sites (Leita et al. 2013), whereby the effects of speciation on the bioavailability of elements must be assessed and quantified. The bioavailability of metals includes metal species that are biologically available and are absorbed or adsorbed by an organism that has the potential for further distribution, metabolism, elimination, and bioaccumulation (Drexler et al. 2003). Certain plants or animals living in or around mining areas are often exposed to the accumulation of various elements present. Similarly, living organisms that are able to cope with increased internal or external concentrations of metals or metalloids may be used in remediation programs.

Some challenges for modern mining remained from past experiences include remediation of legacy impacts, active protection of the environment and human health, and the reduction of the negative impacts and ecological footprint of mining operations (Carvalho 2017). To meet these challenges, there is a constant need for research and application of clean technologies, sound environmental legislation, and waste management.

9.2 Phytoremediation

Remediation of mine waste disposal sites is one of the main priorities of environmental protection, particularly with regard to industrial development and its impact on the environment. There are different schemes for mine waste sites remediation, such as excavation, chemical stabilization, capping, electrokinetic treatments, thermal procedures (Mendez and Maier 2008; Bech et al. 2014), with different implementation effectiveness and variable costs. In addition to these conventional approaches, the use of plants in the treatment of these surfaces, i.e., the process of phytoremediation, is increasingly emphasized. Four phytoremediation techniques have been singled out, depending on the remediation mechanism of the contaminated soil or water: (1) phytostabilization, or retention of pollutants in plant roots, limiting translocation into the aboveground tissues and diffusion in the soil; (2) phytoextraction or absorption of metals or metalloids in the roots with intensive translocation into the shoot, where they are being accumulated; (3) phytovolatization or absorption of organic pollutants by plants and their removal in the atmosphere by volatilization; and (4) phytofiltration or removal of pollutants from aqueous systems by their absorption and concentration in plant roots or their submerged organs (Ali et al. 2013; Favas et al. 2014). However, phytoextraction and phytostabilization can primarily be used in the treatment of mine sites.

9.2.1 Phytoextraction in Mine Waste Sites

Phytoextraction (phytoaccumulation) is a process in which plants remove metals and metalloids from the substrate through their accumulation/hyperaccumulation in the aboveground plant tissues. After reaching full growth, the plants are mowed and safely removed. The harvested material can either be disposed of as hazardous waste, reused in metal extraction or used as biofuel (Pidlisnyuk et al. 2014; Ozyigit et al. 2020). In this way the amount of metal in the soil is reduced. Two main strategies for phytoextraction are: continuous and assisted (induced) phytoextraction (Salt et al. 1998).

9.2.1.1 Continuous Phytoextraction in Mine Waste Sites

Continuous or long-term phytoextraction is based on hyperaccumulating plants that are able to absorb, translocate and accumulate exceptional concentrations of metal/metalloids in their aboveground tissues, without expressing toxicity symptoms (van der Ent et al. 2013). There are different criteria regarding the accumulation capacity. According to Baker et al. (1994), species with a shoot-to-root ratio > 1 are suitable, while Krämer (2010) proposed exceeding the hyperaccumulation threshold as a necessary criterion. In addition, the selected plants should be fast-growing with high biomass, easy to cultivate, resistant to pathogens, and well adapted to the local climate (Baker et al. 1991; Wójcik et al. 2017). There are several steps within the process of hyperaccumulation of metals or metalloids in plants: uptake into the root cells through the plasma membrane where specific compounds such as histidine in the uptake of Ni prevent their sequestration in the roots and enhance their movements (Deng et al. 2018). They are then transported through the xylem to the aboveground parts of the plant, mainly into the leaves as the main storage organ, and sequestered in the vacuoles in order to avoid toxicity (Tangahu et al. 2011). Compartmentalization in the vacuoles decreases the surplus of metals or metalloids ions, thus reducing the possibility of their interaction with metabolic processes in plants (Assunção et al. 2003; Ali et al. 2013). Although a significant number of hyperaccumulating plant species are known to date (about 700 species; Reeves et al. 2018), due to the low biomass only a small number of them can be used in the phytoextraction process without the additional use of the inducing agents.

Sedum alfredii Hace, found in the Pb/Zn mine area in Zhejiang Province in China, is one of the Zn hyperaccumulating plant species that also accumulates significant amounts of Cd, as a geochemically related element. Particularly high amounts of Cd were accumulated in the leaf stems with a shoot-to-root ratio of Cd concentration > 2 (Yang et al. 2004). In view of the high Zn and Cd levels in tailings, which occur in Pb/Zn mines, *S. alfredii* could be a very good candidate for use in phytoremediation.

Pteris vittata L. is a known arsenic hyperaccumulator with exceptionally high amounts of As accumulated predominantly in the aboveground tissues (with 22,630 mg kg^{-1} of As in the fronds as 93% of fully accumulated arsenic; Ma et al. 2001). Similar concentrations of As have also been found in another species of the same genus (*Pteris longifolia* L., *P. cretica* L. and *P. umbrosa* R.Br.; Zhao et al. 2002), confirming the view that closely related hyperaccumulator species are prone to accumulate the same or similar metals (Xu et al. 2020). The distribution analysis showed that higher quantities of As were detected in mature, even in senescing fronds, compared to younger ones, predominantly in the lamina of the pinnae, more than in the pinnae midrib and in the spores, and, at the cellular level, in the upper and lower epidermal cells of the pinnae, most likely in their vacuoles (Lombi et al. 2002; Han et al. 2020). In addition to the strong tolerance and the extraordinary accumulation of As, this species proved to be an excellent candidate for the remediation of As-rich tailings due to its fast growth and large biomass. Co-accumulation of Tl and As was also recorded in *P. vittata* near an As tailing site in Yunnan, SW China (Wei et al. 2020). Cadmium-tolerant ecotypes of *P. vittata* could be used for simultaneous phytoextraction of As and Cd from the sites co-contaminated with these elements (Xiyuan et al. 2008), whereas co-planting of *P. vittata* with *Morus alba* L. or *Broussonetia papyrifera* (L.) L'Hér. ex Vent. could achieve a more comprehensive phytoextraction of As, Zn, and Cd than these species individually (Zeng et al. 2019).

The great potential for uranium extraction in vetiver grass (*Vetiveria zizanioides* L. Nash) has been experimentally confirmed (Pentyala and Eapen 2020). Although at low uranium concentrations (200 mg kg^{-1}), most of the U is accumulated in the roots, a strong translocation into the shoots was observed in plants exposed to significantly higher concentrations (1000 mg kg^{-1}). In addition, a high accumulation rate was observed (17% of biomass) when exposed to 11,900 mg kg^{-1} of U. These results indicate the strong ability of vetiver grass to survive in uranium-rich tailings and to successfully remediate them.

Although related to perennial plants, effective phytoextraction could also be achieved with woody species. It has been shown that *Salix viminalis* L. thrives in soils heavily contaminated with single or multiple-metals/metalloids, especially As (Mleczek et al. 2018). Moreover, most of the accumulated elements are translocated into the leaves, which can be removed mechanically at the end of the season, thereby removing a portion of pollutants from the contaminated substrate each year. Although samples from mining sludge cannot achieve full growth compared to those from non-contaminated substrate, significant amounts of pollutants can still be removed according to Mleczek et al. (2018).

9.2.1.2 Assisted Phytoextraction in Mine Waste Sites

While hyperaccumulating plants continuously absorb considerable amounts of metals or metalloids, in the induced or assisted phytoextractions, a significant amount of pollutants is absorbed within a short time after the addition of some agents. Recently, numerous studies have been conducted on mine waste sites and sites contaminated by mining activities, in order to find effective ways to apply assisted phytoremediation (Table 9.1). There are several ways to boost extractions, such as the application of chelating agents (chelants), organic matter, fertilizers, biosolids, biochar, biotechnological modification of plants, plant growth-promoting bacteria, arbuscular mycorrhizal fungi, etc. (Fig. 9.2). These amendments improve the extraction of pollutants by increasing their availability and thus the uptake by plants and translocation into the aboveground tissues (Grčman et al. 2001). In this

Table 9.1 Examples of assisted phytoextraction of mine wastes or sites contaminated by mine activities

Mine waste/ site contaminated by mining activities	Contamination type	Amendment	Plant species	Reference
Serpentine mine tailing	Cr, Ni	EDTA, DTPA, LMWOAs	*Brassica juncea*	Hsiao et al. (2007)
Former Pb/Zn smelter	Cd, Pb, and Zn	EDTA	*Sinapis alba*	Kos et al. (2003)
Open-pit Mn mine	Mn	EDTA	*Polygonum pubescens*	Yu et al. (2019)
Cu contaminated soil	Cu	EDTA, Cu-resistant PGPB	*Cucurbita pepo*	Abbaszadeh-Dahaji et al. (2019)
Pb/Zn mine tailing	Pb, Cu, Zn	EDTA, rhamnolipid	*Atriplex nummularia*, *Zea mays*	Jordan et al. (2002)
Soil contaminated with Pb/Zn mining wastewater	Cd, Zn	Organic matter	*Sedum alfredii*	Wu et al. (2006)
Tailing pond of Pb/Zn mine	Pb, Zn, Cu, Cd	Organic (pig slurry) and inorganic (marble waste) amendments	*Atriplex halimus*	Acosta et al. (2018)
Iron mine tailing	Cu, Zn, Pb, Cd, Mn	Organic fertilizer, rice husk, biochar, ceramsite, and micromycete (*Mucor circinelloides*)	*Glycine max*	Li et al. (2019a)
Soil contaminated with Pb/Zn mining wastewater	Cd, Zn	Fertilizer	*Sedum plumbizincicola*	Wu et al. (2006)
Polluted site near Au mine	As, Sb	Fertilizer	*Brassica juncea*	Wang et al. (2018)
Cu mine tailing	Cu	Biochar	*Lolium perenne*	Santibáñez et al. (2008)
Ag-Pb extraction site	Pb	Biochar	*Salix alba*	Lebrun et al. (2017)
Au mine tailing	Au, Hg	Biosolids	*Brassica juncea*, *Daucus carota*	Alcantara et al. (2015)
Contaminated soil near Pb/Zn mine	Cd	PGPB (cd-resistant rhizobacteria)	*Brassica napus*	Li et al. (2019b)

(continued)

Table 9.1 (continued)

Mine waste/ site contaminated by mining activities	Contamination type	Amendment	Plant species	Reference
W mine tailing	Cd, Zn	PGPB (*Chryseobacterium humi*)	*Helianthus annuus*	Marques et al. (2013)
Pb/Zn mine tailing	As, Cd, Cu, Pb, Zn	PGPB (*Bacillus thuringiensis*)	*Alnus firma*	Babu et al. (2013)
Bauxite mine ore waste	Cd, Mn, Pb, Zn	PGPB (*Bacillus cereus*)	*Jatropha curcas*	Narayanan et al. (2020)
Pb-contaminated site	Pb	Biotechnological modification (*Agrobacterium tumefaciens*)	*Nicotiana glauca*	Gisbert et al. (2003)
As mine site	As	Arbuscular mycorrhizal fungi	*Holcus lanatus*	Gonzalez-Chavez et al. (2002)
Pb/Zn mine area	Cd, Cu, Pb, Zn	Arbuscular mycorrhizal fungi	*Ailanthus altissima*, *Cotinus coggygria*, *Populus simonii*, *P. purdomii*, *Robinia pseudoacacia*	Yang et al. (2015)

PGPB plant growth promoting bacteria

way, low accumulating species with fast growth rate and high biomass can be successfully used, overcoming some of the disadvantages of continuous phytoextraction, i.e., small biomass of hyperaccumulating plants with limited depth that can be assessed by the plant roots, a limited (often small) number of elements that can be absorbed this way, long time required for purification, etc. (Bech et al. 2014; Pinto et al. 2015). Various types of chelating agents are currently used in chelant-assisted phytoextractions, but ethylene diamine tetraacetic acid (known as EDTA) proved to be the most effective so far (Grčman et al. 2001). Besides the EDTA, some of the available synthetic chelants are: diethylenetriaminepentaacetic acid (DTPA), hydroxylethylenediaminetetraacetic acid (HEDTA), ethylene glycol-bis(β-aminoethyl ether)N, N, N′, N′-tetraacetic acid (EGTA), etc. There are also natural or biodegradable chelating agents, such as S–ethylenediaminedisuccinic acid (EDDS), nitrilotriacetic acid (NTA), methylglycinediacetic acid (MGDA), etc. (Evangelou et al. 2007). These chelating agents, which are also readily degradable and therefore more acceptable for use, have proven to be less effective than the more commonly used synthetic chelating agents (Luo et al. 2006). A certain chelating efficiency was also observed with low-molecular-weight organic acids or LMWOAs (oxalic, citric, malic, tartaric acid,

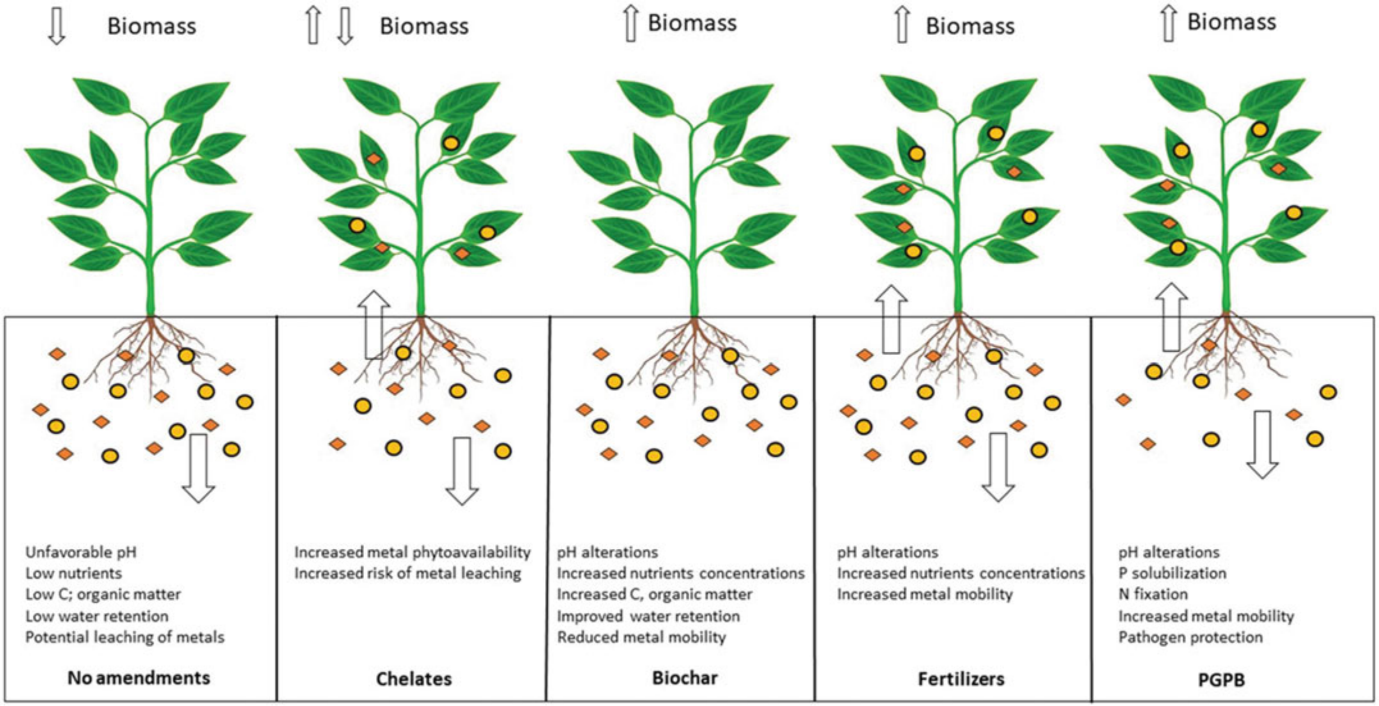

Fig. 9.2 Generalized mechanisms of assisted phytoextraction of sites contaminated by mine activities

etc.), but this was lower compared to the synthetic chelants (Liu et al. 2008). When added to the soil, the chelating agent forms a complex with the metal, which is usually absorbed by the plants via the apoplastic pathway and then translocated within the plant (Komárek et al. 2010).

One of the examples was an experimental study conducted on serpentine mine tailings, which indicated that the application of chelants could significantly increase the uptake of Cr and Ni by *Brassica juncea* (L.) Czern., one of the main model systems for various remediation techniques (Hsiao et al. 2007). Two groups of chelants were used in the study, synthetic chelants such as EDTA and DTPA, and natural low-molecular-weight organic acids (LMWOAs), such as citric and oxalic acid. The results showed that synthetic chelating agents EDPA and DTPA, although they increase the Cr and Ni concentrations in the soil solution more efficiently compared to LMWOAs, simultaneously caused a more significant reduction in the biomass of *B. juncea*. Therefore, although LMWOAs are less successful in the metal extraction process, they are a more acceptable alternative from an environmental point of view. Different *Brassica* species can also accumulate significant amounts of Cd (Angelova et al. 2008; Bauddh and Singh 2012; Cojocaru et al. 2016). Although this process depends on many factors, such as concentration and solubility of Cd and physicochemical characteristics of soil, as well as on the selection of *Brassica* species, it has been shown that Cd is absorbed in significant quantities and transported to aboveground organs, even much easier than Zn and Pb (Angelova et al. 2008; Rizwan et al. 2018). In addition to choosing the most suitable *Brassica* cultivars, Cd accumulation can be enhanced by genetic modification, plant growth regulators, soil organisms, organic acids, organic and inorganic amendments (Nehnevajova et al. 2007; Meng et al. 2009; Yang et al. 2009; Feng et al. 2013; Bauddh and Singh 2014; Zong et al. 2017; Kaur et al. 2018).

However, the use of chelating agents is not always sufficient for extraction enhancement. Wu et al. (2006) showed that addition of a mixture of organic additives not only improved the solubilization and extraction of Cd and Zn by *Sedum alfredii* from contaminated soils in S China, but also reduced the leaching of metals into the underground water, which only proved to be significant when EDTA was used. Furthermore, it was observed that irrigation and application of fertilizers particularly favors the extraction of Cd and Zn by *Sedum plumbizincicola* X.H.Guo & S.B.Zhou ex L.H.Wu in Pb/Zn mine tailing by lowering the pH of the soil and increasing metal availability (Jiang et al. 2010). Today, rare earth elements (REEs) attract special attention because of the enormous possibilities of their application, especially as essential components of electric vehicles, nuclear technologies, sensors, batteries, etc. (Negrea et al. 2018). However, surviving on soil rich in REEs is quite a challenge for plant species, and a small number of species thrive on REE mine tailings. According to the results obtained so far (Grosjean et al. 2019), *Phytolacca americana* L., a fast-growing and widely distributed perennial plant with huge biomass, has been shown to be able to hyperaccumulate up to 0.1% REEs in the aboveground tissues, which is why it can be successfully used to extract REEs and remediate their mine tailings. However, it has been found that the efficiency of phytoextraction can be improved by adding organic amendments, such as organic material and biochar, mainly through the improved physicochemical characteristic of the soil. Although both amendments are beneficial, it has been shown that treatment with biochar has a greater potential to improve RRE extraction by *Ph. americana* compared to organic material (Liu et al. 2020). Biochar is a material highly efficient in heavy metal sorption, especially from contaminated water (Shakoor et al. 2020). Due to its environmental-friendly characteristics and easy application, it is one of the most favored biomaterials (Shakoor et al. 2020). Additionally, biochar was found to be a useful amendment for immobilization of contaminants thus contributing to assisted phytoremediation of various mine wastes. Addition of biochar to the Pb/Zn mine tailings resulted in decreased mobilization of elements such as Pb, Cd, and Cr in tailings, as well as the increased level of macronutrients (K and P) followed by improved water retention, probably due to promoted formation of microaggregates (Fellet et al. 2011). Similar was found by investigations of Rodríguez-Vila et al. (2017) who stated improved soil conditions (pH, carbon content, total nitrogen content) and reduced mobility of contaminants (namely Al, Co, Cu, Fe, and Ni), coupled with increased germination of *Brassica juncea* plants used for phytoremediation experiments on copper mine soils in Spain. Generally, biochar-assisted phytoremediation is supported by a number of successful small-scale examples (see Ghosh and Maiti 2020, for more details), but it is necessary to take into account the biomass feedstock characteristics, pyrolysis temperature, and type of present pollution for successful application of biochar for such purposes.

According to Santibáñez et al. (2008), positive effects were observed in phytoremediation of Cu-contaminated mine tailings when *Lolium perenne* L., widely distributed perennial grass, was used. The results of this study show that this species, when grown on biosolid-treated tailings, is able to produce high biomass as well as to limit erosion and potential leaching of toxic elements into environment. The biosolids mixed with or applied to the surface of the tailings increased the chlorophyll and nutrient concentrations in the shoots of the *L. perenne*, thereby directly inducing plant growth. In addition, accumulated elements are mostly retained in the roots, and only a small amount is transported to the aboveground tissues.

Special attention should be paid to mine tailings in arid and semi-arid environments, due to the high risk of aeolian erosion and potential spread of contaminants. The best solution for stabilizing these areas is revegetation, but this process usually requires the addition of organic material. As the source of organic matter can be challenging to find or costly, especially for the large mine deposition sites, the growth of plants can be enhanced with plant growth-promoting bacteria (Grandlic et al. 2008). However, it is important to find the plant growth-promoting bacteria tolerant to the main characteristics of tailings, such as increased amounts of heavy metals and unfavorable pH values, so many studies involved species isolated from the rhizosphere of plants present on the mining sites. Research of Li et al. (2019b) on application of Cd-resistant plant growth-promoting bacteria isolates from Pb/Zn mine on development of *Brassica napus* L. in pot experiments showed improved Cd uptake in plant roots and shoots (48.09–79.73% in roots and

7.38–11.98% in shoots, compared to the control), thus promoting phytoremediation processes. Contrary to that, two metal-resistant plant growth-promoting bacteria strains were found to reduce the losses in biomass of *Helianthus annuus* L. and decreased concentration of metals in plant (Marques et al. 2013). Strain of *Chryseobacterium humi* was more effective in that sense, decreasing content of Zn by 67% and 64% in roots and shoots, respectively, and content of Cd by 27% in plant roots, thereby increasing phytostabilization potential of *H. annuus*. So far, efficacy of plant growth-promoting bacteria in microbe-assisted phytoremediation is recognized predominantly in terms of promoted plant growth, biomass production, and increased tolerance to metals, aiming at supporting the phytostabilization processes on mine sites (Novo et al. 2018). The positive effects of plant growth-promoting bacteria were also observed in biodegradation of polycyclic aromatic hydrocarbons (PAHs) generated in crude oil refining (Sarma and Prasad 2016). PAHs are difficult to degrade, and are therefore considered as one of the leading pollutants. However, plant-microbe consortia proved to be highly efficient in their removal, and even more effective in combination than when applying plants or microbes separately (Sarma and Prasad 2015). In addition, their output can be improved with biochars, through their positive impact on nutrient availability, microbial biomass, systems of plants and microbes, etc. (Sarma et al. 2019). Although application of these consortia is beneficial for PAH removal and soil quality, certain limitations were observed. One of the most pronounced is poor nutrient status, which can be overcome with simultaneous implementation of plant–microbe consortia and nutrients (Sarma and Prasad 2016).

Another promising method of phytoremediation is the biotechnological modification of plants to improve their metal tolerance and accumulation capacity. One of the successful applications of this method has been the introduction and overexpression of wheat genes that encode phytochelatin in *Nicotiana glauca* Graham, a fast-growing, widely distributed plant species that is repellent for herbivores (Gisbert et al. 2003). After modification, the roots of *N. glauca* elongated to 160% of the length of the wild relative, while the amount of Pb absorbed doubled (up to 1572 mg kg^{-1}). According to these results, *N. glauca* can not only be cultivated as an ornamental plant, but can also be successfully used for decontamination sites polluted with of heavy metals. Besides the various *Nicotiana* species (Misra and Gedamu 1989; Korenkov et al. 2007), *Arabidopsis* Heynh. was also widely used in various transgenic modifications. In the study by Hsieh et al. (2009), the binding protein for mercuric ion (MerP) from *Bacillus megaterium* de Bary 1884 was expressed in transgenic *Arabidopsis*. Considering that MerP is responsible for a good accumulation of several metals/metalloids, the transgenic *Arabidopsis* has the great ability to accumulate heavy metals and can be successfully used in the process of remediation of contaminated soils.

Positive effects on metal extraction were observed in plant associations with arbuscular mycorrhizal (AM) fungi. They participate in the additional supply of nutrients and water to the plants and thus increase plant growth. Additionally, they reduce the toxicity of pollutants by complexation or precipitation (Gonzalez-Chavez et al. 2002). However, these effects were much more pronounced in non-hyperaccumulator plants then in true hyperaccumulator plants (Gaur and Adholeya 2004). Due to the mycotoxic effect of copper, AM fungi cannot be used for the remediation of surfaces contaminated with this metal (Ernst 2005).

Researches with combination of different amendments for enhanced phytoremediation are recently emerging, trying to combine advantages of single amendments. For instance, Radziemska et al. (2020) investigated influence of immobilizing amendments mixtures on assisted phytoremediation with *Festuca rubra* L. in pot experiments. Use of halloysite and compost mixture showed increase in aboveground biomass of *F. rubra*, followed with decrease in concentration of total and mobile Pb in the soil compared to the control. Moreover, mean content of Pb in shoots of *F. rubra* decreased by 28%, while bioconcentration factor reached the value of 2.78 (in comparison to <1 of the control). Similarly, Abbaszadeh-Dahaji et al. (2019) showed that combined application of chelate EDTA and Cu-resistant plant growth-promoting bacteria increased uptake of Cu in shoots of *Cucurbita pepo* L. four times compared to the control, while additionally increasing its mobility in soil by reducing the Cu concentration bound to Fe and Mn oxides and increasing the soluble and exchangeable Cu concentration.

9.2.2 Phytostabilization in Mine Waste Sites

In addition to often enormous quantities of metals/metalloids, mine waste deposits are rather inhospitable habitats for most plants due to their other hostile characteristics, such as unfavorable pH values (often extremely low or high), nutrient deficiency, low water retention capacity, and susceptibility to erosion (Conesa et al. 2007; Shi et al. 2011). Of particular importance is the risk of erosion due to the effects on the stability of tailings and eventually their collapse, as well as the potential leaching of toxic material (e.g., acid mine drainage) into the environment (Salomons 1995; Conesa et al. 2007). In contrast to phytoextraction, the main objective of phytostabilization is the immobilization of contaminants in soil by reducing their availability and the possibility of leaching into the surrounding soil or water (Lee 2013). Bioavailability is reduced by binding to the soil matrix through root exudates or by accumulation in or adsorption by the roots (Tangahu et al. 2011). While hyperaccumulating plant species are recommended for the phytoextraction, metal-tolerant grasses are the best choice for phytostabilization (Prasad 2006). Numerous previous studies indicated that grasses could be successfully used to stabilize Pb/Zn/Cd-contaminated sites (Shu et al. 2002; Ranđelović et al. 2018).

Shu et al. (2002) investigated the potential of several grasses in revegetation and stabilization of Pb/Zn tailings in Guangdong Province, S China. They conducted a field experiment to compare the growth rate of *Cynodon dactylon* (L.) Pers., *Imperata cylindrica* var. *major* (Nees) C.E.Hubb., *Paspalum notatum* Flüggé, and *Vetiveria zizanioides* on the tailings rich in heavy metals, such as Pb, Zn, Cu, and Cd, and at the same time extremely poor in organic matter and nutrients (N, P, K). Their results showed that *Vetiveria zizanioides* reached the highest growth rate and height among the species studied, with a 100% coverage. Thanks to its favorable morphological characteristics, this species is already traditionally used in erosion control. At the same time, its physiological specificity in terms of tolerance to high concentrations of heavy metals, but also to a wide pH range (2.7–9.5), allows it to be used in the stabilization of various mining overburden, as has been demonstrated in coal and gold mines in Australia (Truong 1999). Furthermore, this species was widely used in Pb/Zn mines in China due to its exceptional revegetation potential (Prasad 2006). The phytostabilization potential of this species was also confirmed by Meeinkuirt et al. (2013) in the experimental study conducted on Pb-tailing soil in W Thailand, when it was found that *Thysanolaena maxima* (Roxb.) Kuntze also showed a significant potential for stabilization of Pb-tailings, with BCF > 1 and a shoot-to-root ratio (TF) < 1 in all experiments. Both species have favorable features for phytostabilization, such as metal tolerance, rapid growth, high biomass, massive root system, autochthonous character (Meeinkuirt et al. 2013).

Similar characteristics were observed in *Calamagrostis epigejos* (L.) Roth, which successfully inhabits various degraded habitats, thus significantly reducing the risk of erosion by extensive rhizomes and building of a dense vegetation cover (Lehmann and Rebele 2004; Ranđelović et al. 2020). Furthermore, *C. epigejos* shows tolerance to elevated heavy metal concentrations, especially Pb, Zn, Cd, and Cu, which it stores in significant amounts in the roots (Mitrović et al. 2008; Ranđelović et al. 2018). At the same time, only a small fraction of heavy metals is translocated into the aboveground tissues, suggesting exclusion as a strategy of this species (Lehmann and Rebele 2004). Although the accumulated amounts of heavy metals in the roots of *C. epigejos* were not as pronounced, its revegetation potential should not be neglected, especially in view of the wide range of anthropogenically devastated sites where it can successfully thrive (Ranđelović et al. 2018).

Due to its high biomass production and known metal tolerance, *Miscanthus* spp. could be used simultaneously in phytostabilization and energy crop production, with its growth additionally improving the substrate properties (Pidlisnyuk et al. 2014). A 2-year field trial was carried out on the flotation tailings contaminated with Pb, Cu, and Zn at Rudnik Mine (Serbia), where *Miscanthus* × *giganteus* acted as an excluder of Cu, Zn, and especially Pb, and it was shown that it could be cultivated on the abandoned flotation tailings. Fertilization with NPK fertilizer improved metal uptake by the plant roots, but did not affect their translocation into the leaves. Fertilization also had a significant positive effect on biomass yield, chlorophyll content, and the potential efficiency of the photosystem II photochemistry (Andrejić et al. 2019).

A positive effect on the phytostabilization process has been observed during application of the sewage sludge, which in combination with plant growth can stabilize metals in acidic mine tailings and thus prevent the mobilization of metals in food webs (Kacprzak et al. 2014). The reduction of heavy metal uptake was achieved by a reduced availability of metals and their stabilization in the soil after the sludge was applied, due to the effects on pH and Eh of the soil. Although there were obviously positive effects on metal stabilization, there is, however, a number of obstacles that need to be removed to make this process successful over a long period of time (disposal and exploitation of wastewater used for the production of sewage sludge, instability of the pH of the soil solution, etc.) (Kacprzak et al. 2014).

One of the consequences of mining activities with significant environmental impact is the production of acid mine drainage. Certain remediation measures could reduce acidity and improve the chemical properties of the polluted soil. According to RoyChowdhury et al. (2019), satisfactory results have been achieved by using drinking water treatment residues and perennial grass *Chrysopogon zizanioides* L. The role of drinking water treatment residues (WTRs), formed during the treatment of drinking water, is to bind metals and neutralize the acidity of mine drainage, while *Ch. zizanioides*, fast-growing grass with high biomass and extensive root system, is intended to prevent substrate erosion. Both laboratory and field studies confirmed that WTRs effectively increase pH and reduce metal bioavailability and leachability by adsorbing them on its surface. Additionally, *Ch. zizanioides* reduces the risk of erosion in great extent.

It is reported that *Brassica juncea*, Indian mustard, hyperaccumulates Pb, which is a rare phenomenon in nature. It has been shown that when this species grows in soil with 500 mg kg^{-1} Pb, it accumulates 2675 mg kg^{-1} of this element (Begonia et al. 1998). Due to its low mobility, a large part of the accumulated Pb is retained in the root, which makes this species a good candidate for the phytostabilization process. However, this process has some shortcomings. Namely, Pb availability increases under restricted phosphate conditions, such as in mine tailings, but at the same time plant yield is significantly reduced, which significantly decreases the efficiency of the process (Chaney and Baklanov 2017).

Das and Maiti (2007) investigated the accumulation potential of the semi-aquatic plant species *Ammannia baccifera* L., which grows in a marshy area of the tailings pond rich in untreated waste left behind after the closure of a copper mine in India. The observed average amounts of Cu and Ni in tailings from the rhizospheric zone of *A. baccifera* were 1779 and 564 mg kg^{-1}, respectively. Analysis of the metal concentration in the tissues of *A. baccifera* tissues showed that this species effectively accumulates Cu in concentrations up to 1000 mg kg^{-1}, exceeding the hyperaccumulation threshold of 300 mg kg^{-1} (van der Ent et al. 2013). However, considering that a large part of the accumulated Cu retained in the roots, *A. baccifera* cannot be considered a true hyperaccumulator ($TF < 1$; van der Ent et al. 2013), but can be successfully used for metal stabilization of copper tailings.

A significant potential for stabilization of mine waste through revegetation has been observed for certain woody species. Namely, it was found that shrub species such as *Sesbania cannabina* (Retz.) Pers. and *Amorpha fruticosa* L. are able to absorb significant amounts of heavy metals in Pb/Zn and Cu tailings. At the same time, the translocation and bioconcentration factors were < 1, indicating an exclusion strategy in heavy metal tolerance and thus a potential in the tailing revegetation (Shi et al. 2011). The results of this study showed that *A. fruticosa* is highly tolerant to heavy metal excess in both tailings and maintains normal growth, while *S. cannabina*, although with significant biomass, showed stress symptoms in the form of reduced growth.

In the study carried out by King et al. (2008), the phytostabilization potential of eucalyptus species was assessed taking into account their high ecological plasticity and wide distribution. Four eucalyptus species were grown in Australia in gold mine tailings particularly rich in As. The results showed that *Eucalyptus cladocalyx* F. Muell. reached a much higher height compared to the other species, which is not correlated with the As concentration in substrate. At the same time, low concentrations of As were found in the stems, with no As detected in young leaves, which is especially important to prevent As entering the food chain. Although additional studies are needed to improve the remediation characteristics of this species, *E. cladocalyx* is a good choice for the long-term phytostabilization of As-rich tailings.

9.3 Limitations of Phytoremediation

In addition to the numerous advantages of using phytoremediation techniques in the treatment of mine waste disposal sites, there are a number of shortcomings that may limit their use in practice. When considering the time required to successfully carry out the phytoextraction process, the main disadvantage is that the application of phytoremediation techniques usually takes longer than more conventional approaches (Mendez and Maier 2008; Sarma 2011; Antoniadis et al. 2017), which is particularly true for continuous phytoextraction. Furthermore, according to Wójcik et al. (2017), phytoextraction should not be the first choice for the remediation of areas hardly affected by mining activities, as it is only effective in moderately polluted soils, which is why it is necessary to start with soil stabilization and reduce the contamination rate.

An additional problem is the choice of the appropriate species, especially in the process of phytoextraction. Of the approximately 700 hyperaccumulator plant species known to date (Reeves et al. 2018), most of them are able to hyperaccumulate only one, rarely two elements, while mine waste disposal sites are mostly contaminated with a whole group of geochemically related metals or metalloids (Sarma 2011). In addition, hyperaccumulator plant species are mostly characterized by a low or very low biomass, which makes the phytoextraction process inefficient at the beginning. The low biomass is the reason why *Noccaea caerulescens* (J.Presl & C.Presl) F.K.Mey. is not considered a suitable species for the phytoextraction process, despite the enormous concentrations of Zn and Cd it can adopt (Purakayastha and Chhonkar 2010). Similarly, despite high or even extremely high concentrations of Ni, Zn, and Cd found in certain accessions of *Noccaea praecox* (Wulfen) F.K.Mey. and *N. kovatsii* (Heuff.) F.K.Mey, these species could not be considered suitable candidates for the phytoextraction of these elements due to their low biomass (Mišljenović et al. 2018, 2020). The same applies to *Potentilla griffithii* Hook.f., a newly discovered Zn hyperaccumulator which grows in the Pb/Zn mine at Yunan Province in SW China with as much as 193,000 mg kg^{-1} of Zn, without showing any serious toxicity symptoms (Qiu et al. 2006). Although a high accumulation rate of several toxic elements (Pb, Zn, Cu, Cd, As, Sb) by active absorption has also been found in samples of *Tussilago farfara* L. from several mining sites in Serbia, the species would not be a suitable candidate for phytoextraction of these elements. On the other hand, as a pioneer species, with high ecological plasticity, it could play an important role in the early stages of revegetation of heavily contaminated sites, which would reduce the risk of spreading trace elements to adjacent habitats (Jakovljević et al. 2020). However, biomass problems can be related also to the plants with high yields, considering that it varies every year, sometimes to a very large extent (Antoniadis et al. 2017).

Though their effects on the extraction of metals/metalloids from contaminated media are significant and very rapid, the use of chelants in the assisted phytoextraction process could promote the leaching of both potentially toxic elements and chelants into the surrounding soil (Tangahu et al. 2011). This way, the substrate is additionally loaded with pollutants, and the extraction process itself becomes inefficient and counterproductive. Moreover, one of the major shortcomings of chelant-assisted phytoextraction is the fact that high amounts of chelants are needed to make the process effective, which makes this activity very expensive (Chaney and Baklanov 2017). There is also a risk of increasing the availability of other metals that a particular species cannot absorb, and leaching of chelants is also very likely, raising concerns about this process (Nowack et al. 2006). Although it is possible to use biodegradable chelants, such as EDDS, the usefulness of using chelants in assisted phytoextraction remains questionable, and perhaps this method can be considered insufficiently profitable in the remediation of mine waste sites.

Large-scale trials and applications of phytoremediation technology are still rarely represented in practice, as they encounter certain obstacles. Although various phytoremediation models in the plant–soil–atmosphere system are used for the preliminary evaluation of phytoremediation results (Baltrėnaitė et al. 2017), many complex natural interactions and stress factors that can limit the success of phytoremediation are not obvious in laboratory or pot experiments.

Even though phytoremediation projects on a field-scale emerged since the 1980s and a number of companies offering commercial phytoremediation services have been established in the United States, Canada, and Europe (Glass 1999; Baltrėnaitė et al. 2017), only limited information is available on their results, cost, and duration. While the projects carried out in Canada and the United States were mainly focused on the remediation of petroleum hydrocarbons and metals, the projects in Europe were mainly focused on the remediation of metal and radionuclide pollution (Glass 1999). In a long-term field-scale phytoremediation project that has been conducted at the Anaconda smelter site (USA) since 1995, over 36 species of grasses, forbs, and shrubs were tested for their efficiency in phytostabilization of As, Cd, Cu, and Zn content in mining and smelting site (EPA 2005). Some field scale studies were carried out to explore the phytoremediation potential of triticale, *Helianthus annuus*, *Brassica juncea*, and *Sorghum bicolor* (L.) Moench for immobilization of heavy metals and radionuclides in an area of the former uranium mine in Germany. The results showed an immobilization of metals and radionuclides in the rhizosphere by phytostabilization, followed by a significant reduction of the seepage water rate, showing that phytoremediation was a suitable method for the remediation of large-scale sites with low to medium heavy metal and radionuclide contamination (Willscher et al. 2013).

Though the market for phytoremediation was growing at the beginning of the twenty-first century, recent decades have shown that its potential has not yet been fully exploited (Conesa et al. 2012). Some of the main obstacles to the development of commercially applicable phytoextraction projects are: the time-frame required to remediate the soil up to valid regulation limits and the following utilization of contaminated biomass. In the case of marginal land (such as mine wastes and other former mining sites) with no demand for immediate land use and short-term economic value, it is not the time constraints that are crucial, but the problems arising from the treatment and handling of contaminated biomass. According to Wang et al. (2020), to increase sustainability of phytoremediation, future research should focus on creating the ways of limiting secondary pollution emerging from biomass treatment, and to incorporate social and economic impacts in life cycle assessment of phytoremediation projects.

9.4 Conclusion and Future Investigations

Although phytoremediation is considered a cost-effective and environmentally friendly technology, there are currently no large areas affected by this type of soil pollution reduction. It is therefore necessary to make much more effort to develop new, and to improve existing phytoremediation procedures. In this respect, several steps should be taken to make this process feasible and more successful: understanding the mechanisms of uptake and accumulation of metals/metalloids by plants, carrying out pilot and laboratory-scale trials followed by full-scale and field trials to meet the most optimal criteria for the application of appropriate techniques, and seeking new techniques that would be more successful in solving contamination problems while minimizing environmental impact (Bini et al. 2017).

There are many challenges that must be addressed during the phytoremediation process. In the process of phytoextraction, it is necessary to properly treat or dispose the plant material removed from contaminated surfaces and to ensure that the accumulated metals/metalloids do not spread or leach out into the environment. There are, however, several possibilities for the use of this material. In addition to a significant amount of metals and metalloids extracted from the soil, plant material also contains lignin, cellulose, hemicellulose, and many other compounds with significant calorific value, all of which have significant potential for further use (Jiang et al. 2015). Coupling phytoremediation with bioenergy production is one of synergistic opportunities that offer potential economical valorization of biomass in the form of renewable energy. However, the sustainability of bioenergy production from polluted lands still needs to be proved by demonstrating feasibility by large-scale trials, as well as by selection of adequate methods for processing contaminated feedstocks to biofuels (Gomes 2012; Tripathi et al. 2016). Another way of utilizing biomass after the phytoremediation process is developed through phytomining, process in which metals/metalloids are extracted from the low-grade ores or metal-enriched soils by using hyperaccumulator plant species that concentrate metals in their aboveground parts. After moving, their biomass is processed via combustion and metals/metalloids are recovered from ash in the form of elements or their salts that could be sold on market, while energy released during the combustion can also be exploited for adequate purposes (Zhang et al. 2014; Simonnot et al. 2018).

Recently, pre-orientation from phytoremediation strategies to so-called phytomanagement approaches is taking place, focusing on gradual elimination of environmental risk while simultaneously restoring ecosystem services of mining lands (Gerhardt et al. 2017). Current environmental regulations and practices, directed to the overall concentration of environmentally harmful elements, require a shift toward the recognition of risk-based approaches taking into account the bioavailable concentration of elements that should be removed from soils during the phytoremediation process. Mean factors of contaminant transfer into bioavailable fractions within the mine soil matrices should be identified and made accessible in this sense. Furthermore, bioavailability is crucial to assess the success of the phytoremediation technology, as many researchers today consider bioavailable fractions when calculating the bioconcentration factors of plant species (Petruzzelli et al. 2015; Ranđelović et al. 2019b). Similarly, it is recognized that the overall efficiency of the phytoextraction process can be increased by manipulating the bioavailability through the application of various single or combined amendments (Khan et al. 2018). Biomass valorization for use as an energy resource, biochar, timber, or for purpose of metal recovery could provide additional economic benefits that could justify the cost and timeframe for site remediation, as showed by recent studies (Jiang et al. 2015; Gerhardt et al. 2017). Additionally, integration of phytoremediation with other remediation methods or their successive use for remediation of contaminated sites could aid to the wider application and more successful implementation of phytoremediation technologies. Similarly, emergence of new pollutants and their wider dispersion into the environment could also open opportunities for wider application of phytoremediation technologies. Generally, wider acceptance of phytoremediation as proven, effective and sustainable technology requires development of more innovative and efficient approaches that would aid to overall feasibility of the process, including the economical valorization of gained biomass and mitigation of environmental risks.

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10

Metallicolous Plants Associated to Amendments and Selected Bacterial Consortia, to Stabilize Highly Polymetallic Contaminated Mine Deposits

Manhattan Lebrun, Romain Nandillon, Florie Miard, Domenico Morabito, and Sylvain Bourgerie

Abstract

Metal(loid) contaminated soils are a big issue worldwide due to their negative effects on the environment and human health. Phytoremediation, using plants and their associated microorganisms, can reduce the toxic impacts of these soils on the environment and human health. The success of phytoremediation will depend on the plant species. Metallicolous ecotypes are good candidates due to their tolerance towards the pollutants present in the soil. Moreover, microorganisms, and especially bacteria, can influence phytoremediation by affecting plant growth and pollutant mobility and bioavailability. Finally, due to the extreme soil conditions, amendments are often required to improve soil fertility (compost, manure) and reduce pollutant mobility (biochar, red mud). This chapter will show the potential of various metallicolous plant species found on metal(loid) polluted sites, which were associated with amendments and/or bacterial consortia, isolated from the soil and selected based on their tolerance towards particular metal(loid)s, for the stabilization of mine deposits.

Keywords

Metallicolous ecotype · Bacterial inoculation · Mine deposit · Metal(loid)s · Phytoremediation

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10.1 Introduction

Soil pollution by metals and metalloids is an important issue worldwide. Indeed, more than 60% of the polluted soils have excess concentrations of metal(loid)s and total petroleum hydrocarbons (Carré et al. 2017). One of the main sources of the soil metal(loid) pollution is the mining activities, past and present, which have strongly affected the operating sites (Vareda et al. 2019). Moreover, polluted area releases a mixture of pollutants to the surrounding environment. Such metal(loid) pollutions pose a problem for the environment and are known to be toxic to humans, inducing chronic diseases and cancers (Ashraf et al. 2019). They also decrease soil fertility and thus the biodiversity (Ashraf et al. 2019). Therefore, after the cessation of the extraction activities, the mining technosols need to be remediated. To this end, many physical and chemical techniques have been used; however, such methods are expensive, destructive for the soil, can induce a secondary pollution and render the soil improper for vegetation. The biological remediation, using plants and/or microorganisms, is a more environmental friendly and cost-effective method. It is divided in two techniques: the phytoremediation, which uses plants to reduce the toxic risk of contaminated soils (Awa and Hadibarata 2020), and the bioremediation, in which microorganisms can affect metal(loid) behavior, making them less toxic and less mobile (Wang et al. 2017a). Thus, plants and microorganisms can be used together, bringing mutual benefits and leading to a more efficient soil remediation. The success and efficiency of a biological remediation process will depend on the plants and the microorganisms selected. They need to tolerate the soil conditions, especially the high pollution level and the poor soil fertility. Therefore, the use of plants that are native of the site or found in the vicinity of the polluted soil can have better results than the implementation of foreign species, since they are already tolerant to the climatic conditions and the pollution encountered on the site. For instance, Deng et al. (2007) collected *Sedum alfredii* seeds on two mines as well as a clean soil and performed a hydroponic experiment to evaluate their tolerance towards Cd and Zn. For each treatment, the populations from the contaminated sites grew better and produced a higher biomass than the population collected on unpolluted area and accumulated higher amounts of Zn and Cd in their leaves and stems, demonstrating the higher tolerance of the endemic populations. Similarly, Wu et al. (2009) grew non-metallicolous and metallicolous populations of *Pteris vittata* on an As contaminated soil and observed that the metallicolous ecotype performed better in terms of biomass production and restriction of As uptake than the non-metallicolous population. Finally, *Agrostis*, a facultative metallophyte of the *Poaceae* family, has been found on many contaminated sites and showed good potential for the remediation of those sites (Nandillon et al. 2019b; Dahmani-Muller et al. 2000; Doubková and Sudová 2016; Sudová et al. 2008). In the bioremediation strategy, microorganisms are inoculated to render metal(loid)s less toxic, as they cannot be degraded. For this, they use mechanisms such as extracellular complexation, intracellular accumulation, oxidation-reduction, and precipitation (Ashraf et al. 2019). Moreover, some microorganisms possess some plant growth promoting properties, which can ameliorate plant growth (Rahman and Singh 2020). Similarly to the plants, which have to be able to develop and grew in the soil, the inoculated microorganisms need to be tolerant to the soil conditions including the pollution present. That is why, endemic species selected from the soil to be remediated can lead to better outcomes than non-endemic strains.

Metal(loid) polluted mining technosols often have low nutrient and organic matter contents, extreme pH and high pollution levels, which hinder plant and microorganism growth and activity and thus reduce the remediation efficiency. Therefore, to increase remediation success, it is necessary to ameliorate the soil conditions, which can be obtained by applying amendments to the soil. These organic or inorganic amendments will have potentially two interests: they will ameliorate the fertility of the soil, by providing nutrients and organic matter, and they will also reduce metal(loid) stress towards plants and microorganisms, by reducing their mobility and/or availability (Lebrun et al. 2017, 2018; Nandillon et al. 2019a). Diverse amendments can be used, such as biochar, compost/manure, iron sulfate, and red mud, which demonstrated, in many studies, efficiency towards metal(loid) immobilization, fertility improvement, and plant growth amelioration (Lomaglio et al. 2018; Nandillon et al. 2019a, b; Lebrun et al. 2020).

In the present chapter, divided in four parts, we will focus firstly on soil pollution with a special emphasis on the remediation techniques. Secondly, a brief overview will be done on the potential of metallicolous plant species for the remediation of metal(loid) contaminated soils. In a third part, the selection and inoculation of single bacterial strains or microbial consortia will be described in order to explain their beneficial roles. Finally, the association of plant, amendment, and microbial inoculation will be outlined, focusing on the advantages of such combination for the alleviation of metal pollution in mining environments.

10.2 The Soil Pollution and the Remediation Techniques

Around the world, the number of contaminated sites and potentially contaminated sites has been estimated at 10–20 million (Carré et al. 2017). The sources of such pollution are natural or anthropogenic (Ahmad et al. 2015). The natural sources are the weathering of the parent material as well as the volcanic activities; these activities usually cause small concentrations of available metal(loid)s. However, the human activities are responsible for high available levels of pollution. The anthropogenic sources are the industrial and domestic waters, the use of fertilizers and pesticides in agriculture, the mining, smelting and industrial processing activities as well as the transport (Vareda et al. 2019; Ashraf et al. 2019). Many pollutants, organic and inorganic, are found in soils, such as nitrogen and phosphorus, hydrocarbons, radionuclides, and metal(loid)s. Among them, metal(loid)s are the most important ones, found in more than 50% of the contaminated soils worldwide (Rodríguez-Eugenio et al. 2018; Khalid et al. 2016). Metal(loid)s pose environmental and health issues. Indeed, contrary to organic pollutants, they cannot be degraded and thus accumulate in the soils for centuries. They are also toxic to plants, altering their metabolism and reducing their growth (Ashraf et al. 2019). Moreover, metal(loid) polluted soils are subjected to wind erosion and water leaching, which transport the contamination to the surrounding non-contaminated environment. In addition, metal(loid)s are classified as carcinogenic and induce other health problems such as chronic weakness, nervous system disorders, and biochemical imbalance (Bissen and Frimmel 2003; Jaishankar et al. 2014; Rodríguez-Eugenio et al. 2018; Ashraf et al. 2019). Consequently, there is an important need to remediate those metal(loid) polluted areas, to avoid the adverse impacts of metal(loid)s and thus reduce the environmental risk (Awa and Hadibarata 2020).

Remediation techniques are divided in three types of processes: physical, chemical, and biological. The physical and chemical remediation techniques have been used for a long time because they are faster than the biological remediation techniques.

Physical remediation is divided in four main techniques: soil replacement, surface capping, encapsulation, and thermal desorption. The removal of the contaminated soil and its partial or complete replacement by a clean soil is called soil replacement. Such technique dilutes the metal(loid) content and improves soil fertility (Khalid et al. 2016; Gong et al. 2018). In the surface capping, the polluted soil is covered by a geotextile layer covered with garden soil. This capping will support vegetation and reduce water leaching (Liu et al. 2018). For soil encapsulation, physical barriers are placed all around the polluted soil to limit leaching and horizontal metal(loid) migration (Khalid et al. 2016). Finally, in the thermal desorption, the soil is heated, using steam, microwave, or infrared radiations. This technique induces the volatilization of the pollutants that need to be collected (Gong et al. 2018).

The chemical remediation is also subdivided into different strategies, such as soil washing, solidification/stabilization, vitrification, electrokinetic and oxidation/neutralization/reduction. For soil washing, the soil is removed from the site and mixed with chemical reagents or extractants that remove the metal(loid)s. The cleaned soil is then returned to the original site (Gong et al. 2018). Similarly, the soil needs to be excavated for solidification and stabilization processes. In solidification, binding agents are applied, leading to the formation of water proof solids that encapsulate the pollutants, whereas in stabilization, chemical reagents are added to the soil to reduce the mobility and toxicity of the metal(loid)s (Liu et al. 2018; Nejad et al. 2018). When vitrification is performed, the soil is excavated and heated at high temperatures, between 1600 and 2000 °C, which incinerates the organic and mineral matters. The soil is thus transformed into glasslike solids having a smaller volume (Liu et al. 2018; Nejad et al. 2018). The electrokinetic method consists to add to the soil a buffer solution and to apply a low intensity current between a cathode and an anode placed inside the soil. Under this current, and according to their charge, the metal(loid)s migrate, to the anode or to the cathode. Metal(loid)s are then recovered and treated (Gong et al. 2018; Liu et al. 2018). Finally, the oxidation/neutralization/reduction processes use solutions to detoxify, precipitate, or solubilize metal(loid)s (Hamby 1996; Mulligan et al. 2001).

The different physical and chemical remediation techniques are summarized in Table 10.1. These techniques have been highly used in the past because they are usually fast. However, they render the soil improper for vegetation and can even

Table 10.1 Definitions of the physical and chemical methods to remediate a contaminated soil

Remediation type	Method	Definition	References
Physical	Soil replacement	Excavation of the soil and replacement by a clean soil	Khalid et al. (2016), Gong et al. (2018)
Physical	Surface capping	Geotextile cover installation on the surface, which supports vegetation and reduces water leaching	Liu et al. (2018)
Physical	Soil encapsulation	Physical barriers set up on the soil surface and underground to prevent horizontal and vertical transfer	Khalid et al. (2016)
Physical	Thermal desorption	Heating of the soil and collection of the volatilized metal(loid)s	Gong et al. (2018)
Chemical	Soil washing	Excavation of the soil, mixing with reagents or extractants and return of the clean soil to the original site	Gong et al. (2018)
Chemical	Solidification	Encapsulation of the soil by mixing with binding agents	Liu et al. (2018), Nejad et al. (2018)
Chemical	Stabilization	Application of chemical reagents to reduce metal(loid) toxicity and mobility	Liu et al. (2018), Nejad et al. (2018)
Chemical	Vitrification	Heating of the soil at high temperatures and transformation into glass like solid of a smaller volume	Liu et al. (2018), Nejad et al. (2018)
Chemical	Electrokinetic	Application of a current between two electrodes and recovery of the metal (loid)s	Gong et al. (2018), Liu et al. (2018)
Chemical	Oxidation/ neutralization/ reduction	Application of solutions to detoxify, precipitate, or solubilize metal(loid)s	Hamby (1996), Mulligan et al. (2001)

induce a secondary pollution, especially with the use of chemical solutions, and have a high cost, especially if applied on large areas. That is why, clean-up techniques are now turning towards a more sustainable and cost-effective remediation method, which advocates the implementation of biological approaches for the remediation of these contaminates sites.

The term “biological remediation” englobes the use of plants and microorganisms in order to reduce the toxic effects of polluted soils. It is divided in two main processes: the phytoremediation and the bioremediation. The phytoremediation uses plants to remove metal(loid)s or stabilize them into a harmless form (Liu et al. 2018). Different phytoremediation techniques exist, depending on the fate of the pollutant in the plants: phytovolatilization, phytodegradation, phytoextraction, and phytostabilization. They are illustrated in Fig. 10.1. Phytovolatilization is mainly applicable to organic pollutants and Hg, Se and As, which are uptaken by the plants, transformed into a less toxic and volatile form and then released into the

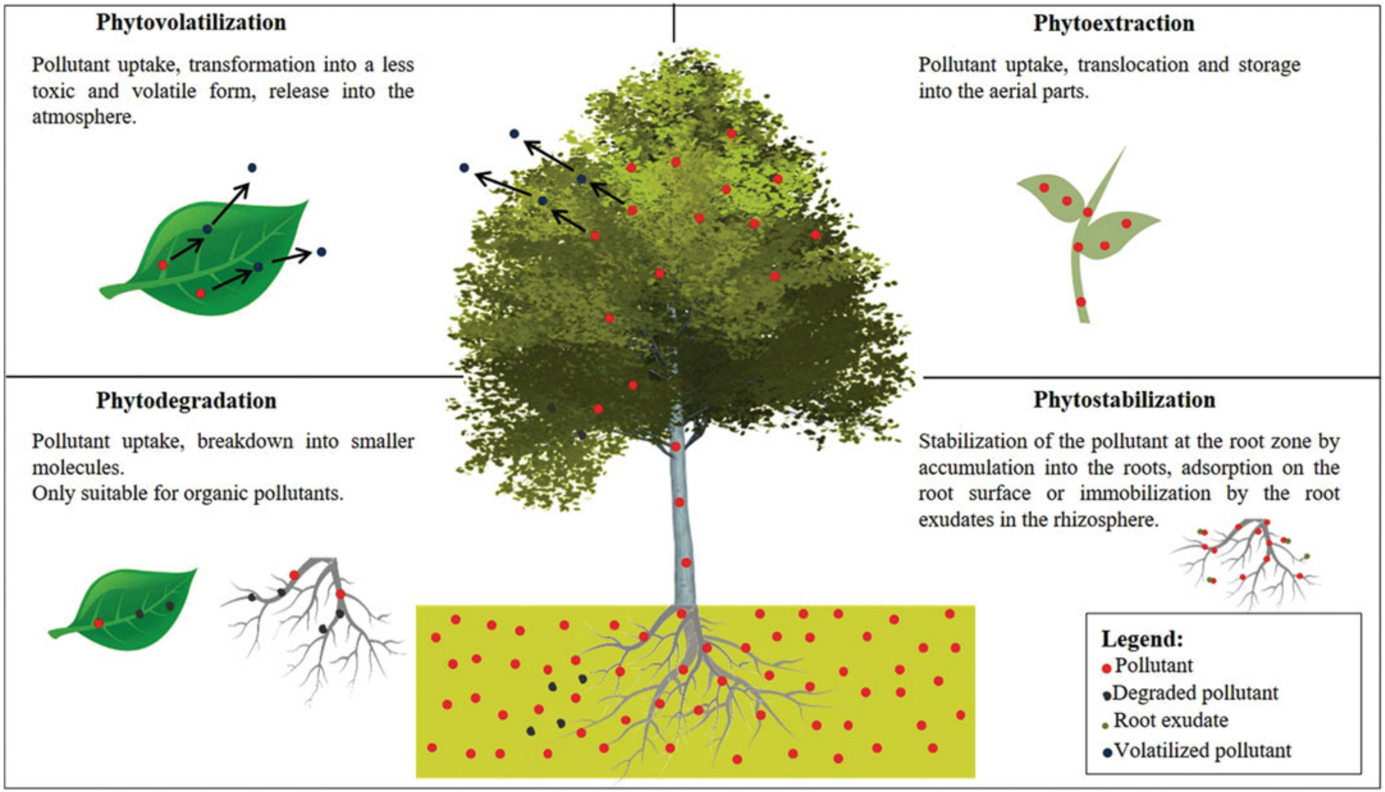

Fig. 10.1 Illustration of the main phytoremédiation techniques

atmosphere (Haq et al. 2020; Awa and Hadibarata 2020). In the phytodegradation, pollutants are taken up by the plants and break down at the root or aerial level into smaller molecules (Mirck et al. 2005; Wenzel 2009); however, this technique is only suitable for organic pollutants and cannot be used for metal(loid)s, as they are not degradable. Phytoextraction is one of the two main phytoremediation techniques. In this process, plant roots uptake the pollutants from the soil, or water, which are then translocated and accumulated in the aerial tissue. This aerial biomass is then harvested and incinerated (Haq et al. 2020; Awa and Hadibarata 2020). The other main phytoremediation technique is phytostabilization, which uses plants to stabilize the pollutants in the soil, and especially in the rhizosphere, but does not remove the pollution (Awa and Hadibarata 2020; Liu et al. 2018). The phytoremediation has many advantages over the physical and chemical remediation techniques: it has a low cost, it is environment-friendly, it requires little manpower, it is aesthetically pleasing, and it is a passive approach, relying on solar energy (Haq et al. 2020; Awa and Hadibarata 2020; Ashraf et al. 2019; Nejad et al. 2018). Additionally, the establishment of a plant cover will reduce water leaching and wind erosion, protecting the surrounding environment. It will also restore the functionality and biodiversity of the ecosystem. Finally, the biomass produced on the sites can be valorized for energy and biofuel production, adding an economical benefit to the remediation process. Such valorization of marginal sites by manipulating the soil-plant system to produce biomass of economic value is called phytomanagement (Kidd et al. 2015; Evangelou et al. 2015). However, phytoremediation also has some limitations, the most important one being time, as several plant growing periods are required to clean a soil. In addition, only the subsurface layer of the soil, prospected by the roots, is cleaned (Haq et al. 2020; Ashraf et al. 2019). Therefore, the choice to use phytoremediation, and which technique, will depend on the societal pressure exerted on the site as well as the pollution level. When a soil contains high concentrations of metal(loid)s at an important depth, it is not feasible to use phytoextraction, as it will take centuries and will require the harvest and proper disposal of the contaminated biomass, which add an extra cost. Therefore, phytostabilization is more suitable in such cases in order to stabilize the soil pollutant and prevent contamination spreading.

Other than plants, soil contains microorganisms such as bacteria, fungi, actinomycetes, protozoa, and algae (Thavamani et al. 2017). Among them, bacteria are the most important ones, with 10^8 to 10^9 cells per gram of soil on average (Yu et al. 2019). Although metal(loid)s cannot be degraded, microorganisms can affect their behavior and mobility, through different mechanisms, illustrated in Fig. 10.2. For instance, microorganisms can reduce metal(loid) mobility through biosorption, a passive process in which metal(loid)s are bound to the cellular structure of the bacteria. The attachment can be physical or chemical, with biofunctional groups (Rahman and Singh 2020). Moreover, sorption can be done through extracellular sequestration by means of biosurfactants (Etesami 2018; Yin et al. 2019). Biosurfactants are compounds secreted by microorganisms having complexing capacity towards metal(loid)s (Rahman and Singh 2020). In addition to being sorbed on the cell surface, metal(loid)s can be actively uptaken inside the

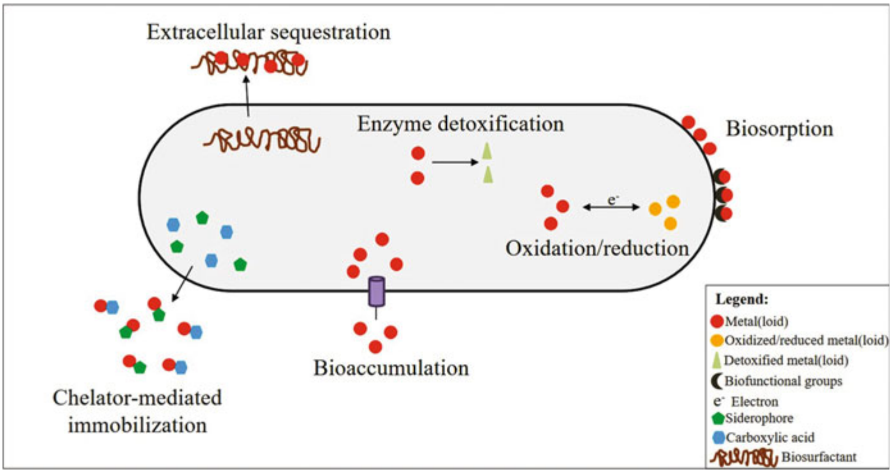

Fig. 10.2 Illustration of the main tolerance mechanisms towards metal(loid)s used by bacteria. (Modified from Yin et al. 2019; Nong et al. 2019)

cell and trapped into the cytoplasm; such process is called bioaccumulation (Etesami 2018; Rahman and Singh 2020). Inside the cell, metal(loid)s can be detoxified into a less harmful form through enzyme detoxification process (Etesami 2018; Yin et al. 2019) and can also be subjected to oxidation/reduction reactions, involving the transfer of an electron from one element to another, which changes the oxidation state of the element. Oxidation-reduction reaction can be mainly performed for As, Cr, and Hg (Rahman and Singh 2020). Finally, microorganisms can act on metal (loid)s by secreting chelators, such as carboxylic acids and siderophores (Sessitsch et al. 2013). Carboxylic acids, such as oxalate, malate, and citrate, are produced by bacteria, as well as plant roots, and are able to chelate metal(loid)s (Sessitsch et al. 2013). Siderophores are secondary metabolites released under iron-limiting conditions and qualified as iron carriers (Sessitsch et al. 2013). In addition to chelate iron, they can complex other metal(loid)s (Sessitsch et al. 2013). These resistance mechanisms make microorganisms suitable to use in the remediation of metal(loid) polluted soils. This process is called bioremediation.

In addition, microorganisms can ameliorate plant growth, as some of them possess plant growth promoting properties. For instance, siderophores produced by microorganisms can support the uptake by plants of iron, and other micronutrients such as magnesium and phosphorus, enhance chlorophyll content and plant growth, in addition to chelating toxic metal(loid)s (Sessitsch et al. 2013; Wang et al. 2017a; Rahman and Singh 2020). Microorganisms can also produce phytohormones, such as indole acetic acid (IAA). This IAA can be produced using various pathways, most of them using tryptophan as precursor but having different intermediates (Spaepen et al. 2007). The secretion of IAA by bacteria can modify the osmotic contents of the plant root cells and increase their permeability to water, stimulating cell elongation and thus enhancing plant growth (Mohite 2013; Rahman and Singh 2020; Babu et al. 2013). Finally, microorganisms can have aminocyclopropane-1-carboxylate deaminase activity, which reduces the formation of ethylene in response to stress and thus reduce the induction of senescence, chlorosis, and abscission (Santoyo et al. 2019). Therefore, microorganisms and plants can be used together for the remediation of metal(loid) polluted soils, bringing mutual benefits: microorganisms will enhance plant growth and benefit from root exudates for their activity in return.

10.3 The Use of Metallicolous Plants in Phytoremediation

For an efficient phytoremediation, plants need to have (1) an important growth, which will permit a large soil cover, reducing wind erosion, water leaching, and allowing biomass valorization; (2) a deep and wide root system; and (3) be tolerant to the pollution levels (Alkorta et al. 2004; Ernst 2005). In this goal, endemic metallicolous species that are found on polluted sites showed good results. For instance, Fahr et al. (2015) tested metallicolous and non-metallicolous populations of *Hirschfeldia incana* towards Pb in a hydroponic experiment and observed that the metallicolous population were more able to tolerate the presence of Pb and accumulated less Pb in its aerial parts. Becerra-Castro et al. (2012) sampled plants on an abandoned Pb and Zn mine and found that the species *Betula celtiberica*, *Cytisus scoparius,* and *Festuca rubra* excluded Cd, Zn, and Pb from their shoot tissues and had a good potential in phytostabilization. *Agrostis* species has been found on many contaminated area or in their surroundings. For instance, Dahmani-Muller et al. (2000) collected plants on a non-ferrous metallurgical factory and found four herbaceous species: *Armeria maritima*, *Cardaminopsis halleri*, *Arrhenatherum elatius,* and *Agrostis tenuis*. The metal(loid) concentrations they measured in *A. tenuis* suggested that the ecotype of this site was metal tolerant. Similarly, Rodríguez-Seijo et al. (2016) collected *Agrostis capillaris* plants on an old trap shooting range and found higher Pb concentrations in the roots compared to the shoots, showing its potential as a Pb phytostabilizer. Several studies compared *Agrostis* metallophyte ecotypes collected on polluted sites or non-metallicolous ecotypes collected on unpolluted soils with commercial ecotypes to evaluate their tolerance towards metal(loid)s. For instance, Doubková and Sudová (2016) found that the metallicolous ecotype of *Agrostis capillaris* was able to maintain higher chlorophyll contents under severe metal(loid) stress compared to the non-metallicolous ecotype and had lower Pb and Zn concentrations. Sudová et al. (2008) compared metallicolous and non-metallicolous ecotypes of *Agrostis* for their tolerance to Pb, Zn, and Cu. The authors found that the non-metallicolous ecotype, when exposed to metal(loid)s, had a lower root length and tiller percentage than the metallicolous ecotype sampled in the vicinity of a Pb smelter. They attributed this observation to a long-term selection pressure that allowed a tolerance towards the metal(loid)s encountered on this site; this tolerance resulted from an avoidance strategy, i.e., a restriction of the metal(loid) uptake. Finally, Nandillon et al. (2019b) compared the growing and metal(loid) accumulation capacity of *Agrostis* plants of commercial origin with the ones coming from the former mine technosol of Pontgibaud highly contaminated with As (539 mg kg^{-1}) and Pb (11,453 mg kg^{-1}). They observed that the metallicolous ecotype had a higher dry weight production and higher Pb concentrations in the roots. They concluded that the metallicolous ecotype of *Agrostis* was a good option for the amendment-assisted phytoremediation of such mine technosol.

The better growth of adapted metallicolous ecotypes is important in terms of soil vegetation cover because they produce more biomass that will cover more surface than non-adapted plants and thus reduce wind erosion more importantly. In addition, such better development is important at the root zone (rhizosphere effect). Indeed, plants can affect the rhizosphere through their root activity. For instance, the growth of *Deschampsia* and *Agrostis* plants slightly decreased the availability of Cd and Mn and increased their residual and non-available fractions; however, *Agrostis* growth had a negative impact on Cu and Pb, leading to their mobilization (Langella et al. 2014). Similarly, Abbas et al. (2016) observed that growing acacia ameliorated the chemical properties of the soil, by increasing pH and electrical conductivity, as well as its physical properties by decreasing bulk density and allowing a better water infiltration rate and availability. Plants can control metal(loid) behavior via different mechanisms such as uptake, root exudation, soil pH modulation, and dissolution of mineral phases (Becerra-Castro et al. 2012; Wenzel 2009).

Finally, plants can also affect the microbial community of the soil. As an example, Becerra-Castro et al. (2012) measured a higher microbial density in the rhizosphere of plants grown on a mine soil compared to the bulk compartment. In the rhizosphere of *Rumex acetosa*, enzyme activities, Biolog™ diversity and activity indices as well as microbial biomass C were higher than in the bulk soil, making this species the best choice for a biologically active and healthy soil (Epelde et al. 2010). Finally, Borymski et al. (2018) sampled rhizosphere soils of *Arabidopsis arenosa*, *Arabidopsis halleri*, *Deschampsia caespitosa,* and *Silene vulgaris* as well as bulk soils at three polluted locations. They measured a higher oligotrophic fraction of cultivable bacteria in the rhizosphere than in the bulk soils. The total catabolic activity was higher in the rhizosphere of *A. halleri* and *S. vulgaris*. They concluded that the microbial community was positively affected by the presence of a plant, arising from the release of plant-derived metabolites by the roots, which served as a carbon source for the microorganisms.

10.4 The Selection of Microorganisms and Their Uses as Inoculant

Generally, the presence in a soil of metal(loid)s in high concentrations reduce the microbial activity (Marques et al. 2013). However, the presence of metal(loid)s in soils exert a selection pressure to microorganisms, which favors the development of microorganisms tolerant to metal(loid)s. For instance, Nicoară et al. (2014) isolated 25 bacterial strains from the rhizosphere of *Agrostis capillaris* plants growing on a mine tailing; 21 of those had a metal(loid) resistance. Similarly, Abdelkrim et al.

(2018) isolated from the rhizosphere of *Lathyrus sativus* 12 bacterial strains, which are Pb accumulators. Finally, Thouin et al. (2019) identified microorganisms capable of As(III) oxidization and As(V) reduction in a As and Pb contaminated technosol.

Moreover, those endogenous resistant microorganisms are also characterized by plant growth promoting (PGP) properties. Langella et al. (2014) isolated microbial strains from two polluted sites, one from Germany, polluted by Ni and Mn, and one from Sweden, polluted by Cd, Cu, Mn, Pb, Sr, and Zn. All the strains isolated from the Ni-Mn polluted soil showed siderophore production, whereas only three of the ten strains were able to produce phytohormones. The reverse was observed for the strains of the soil polluted by Cd, Cu, Mn, Pb, Sr, and Zr, all were producing phytohormones, whereas only three produced siderophores. Similarly, among the 74 metal tolerant rhizobacterial strains isolated from an abandoned Pb/Zn mine, 38% had at least 1 PGP trait and/or were able to produce biosurfactants, 15% were capable of solubilizing inorganic phosphate, and 6 strains could produce siderophores and 2 IAA (Becerra-Castro et al. 2012). Therefore, strains isolated from polluted soils and selected based on their tolerance towards metal(loid)s and their PGP traits could be used for the remediation of polluted soils, either as a single strain inoculation or as a consortium of several strains having complementary features.

In addition, microorganisms can affect the mobility and availability of metal (loid)s. For instance, Nicoară et al. (2014) stated that the siderophore produced and released by the inoculated bacteria could be involved in the metal(loid) immobilization they observed. Indeed, siderophores are chelating agents that can bind several metal(loid)s such as Fe, Mg, Mn, Cd, Zn, Pb, and As (DalCorso et al. 2019; Ashraf et al. 2019). In addition, microorganisms can also increase metal(loid) solubility and modify their speciation (Wenzel 2009; Sessitsch et al. 2013) through diverse mechanisms. For instance, the release of organic ligands via the decomposition of soil organic matter and exudation of metabolites can complex metals or desorb anionic elements from soil sorption sites (Wenzel 2009). Immobilization mechanisms include uptake and accumulation in the biomass, adsorption onto the cell wall, and complexation with exopolymers released by microorganisms (Wenzel 2009; Marques et al. 2013).

In addition, due to their PGP properties, microorganisms can ameliorate plant growth and thus the phytoremediation process. This has been demonstrated previously. For example, Becerra-Castro et al. (2012) selected 14 strains among the 74 they isolated from an abandoned Pb/Zn mine for a re-inoculation experiment. Inoculating the soil with these strains improved *Festuca pratensis* plant growth without affecting leaf macronutrient levels; however interestingly, the same strains had a negative effect on *Salix viminalis* growth. The beneficial effect on *F. pratensis* was attributed to the production of the phytohormone IAA. Similarly, Marques et al. (2013) isolated two bacterial strains from a metal contaminated site, *Ralstonia eutropha* and *Chryseobacterium humi*. They inoculated these strains to an agricultural soil supplemented with Cd or Zn and grew *Helianthus annuus* plants. They observed an amelioration of plant growth in the inoculated conditions. Langella et al. (2014) observed that the shoot length of *Festuca rubra* and *Agrostis capillaris* was slightly higher in inoculated pots compared to the non-inoculated soils, collected on former mining sites. Similarly, the total biomass of *Agrostis capillaris* native of a mine was ameliorated by the inoculation of a consortium made of ten strains capable of producing siderophore and phytohormones and able to fix nitrogen and solubilize P (Nicoară et al. 2014). Furthermore, polluted soils often have low nutrient levels. Microorganisms can improve the nutritive status of soil by increasing N and P available, through: (1) the transformation of unavailable N and P present in the soil into phytoavailable forms and (2) the fixation of atmospheric N and transformation into plant assimilable N (Sessitsch et al. 2013; Epelde et al. 2010).

Finally, microbial soil inoculation can also affect the endogenous microflora of the soil. The consortium of ten strains tolerant to metal(loid)s and harboring PGP properties lead to an increase in the soil microbial respiration of a mine tailing (Nicoară et al. 2014). For instance, Marques et al. (2013) observed that adding Cd or Zn to an agricultural soil reduced its microbial activity. However, the inoculation of a polluted soil with two bacterial strains isolated from a metal contaminated soil lowered this negative effect, indicating that these inoculated strains prevented the loss of variability of the microbial community (Marques et al. 2013).

In conclusion, microbial inoculation has a double benefit to contaminated soils: it can reduce metal(loid) mobility and ameliorate plant growth, thus increasing pollutant successful soil stabilization. In return, microorganisms benefit from the metabolites exuded by plant roots (Marques et al. 2013). However, the sustainability of the inoculum over time needs to be evaluated and can be improved by adding amendments that will ameliorate soil conditions and thus the survival rate of the inoculum.

10.5 The Combination of Plants, Amendments, and Microorganism Inoculation

Many studies, as reported in previous sections, demonstrated that plants and microorganisms are efficient in reducing metal(loid) mobility and ameliorating soil conditions. However, metal(loid) contaminated technosols generally have poor agronomic properties, i.e., extreme pH, low content in organic matter, reduced level and availability of nutrients, which together with the elevated metal(loid) concentrations hinder plant establishment as well as microorganism growth and activities. Therefore, it can be necessary to apply amendments in order to ameliorate the soil properties. Amendments, which can be organic or inorganic, will serve several purposes: supply of nutrients for plants and microorganisms, improvement of soil physicochemical properties such as pH, organic matter content, and metal(loid) immobilization. Some amendments also contain microorganisms, which will be added to the soil and potentially be efficient to allow a better plant growth if adapted to encountered pollutants. Many amendments can be used but three attracted attention over the last decades, biochar, compost/manure, and iron-based amendments. Compost, an organic amendment used in agriculture, is the product of the microbial degradation of organic wastes (Huang et al. 2016). It is rich in humic substances, microorganisms, and nutrients (Fischer and Glaser 2012). Biochar is a carbon rich and porous material obtained from the pyrolysis of biomass (Paz-Ferreiro et al. 2014). Biochar is generally characterized by an alkaline pH, a high surface area, a high cation exchange capacity, and elevated contents in carbon, hydrogen, oxygen and nitrogen (Paz-Ferreiro et al. 2014; Kwak et al. 2019). Finally, iron-based amendments, such as iron grit and red mud, are especially used to overcome anion pollution like arsenic (Mak et al. 2009; Bhatnagar et al. 2011).

Previous studies demonstrated the positive effects of amendments on the soil, the plants as well as the microorganisms. For instance, Lebrun et al. (2017) demonstrated that applying a pinewood biochar to a mining technosol highly contaminated with As and Pb increased soil pore water pH and electrical conductivity (EC) while decreasing drastically Pb mobility and ameliorating the dry weight production of *Salix* plants. Similarly, the application of four hardwood biochars with various particle sizes to the same soil increased soil pore water pH and EC, decreased SPW Pb concentrations, and increased *Salix viminalis* dry weight (Lebrun et al. 2018). Furthermore, biochar, compost, and iron grit were applied alone or combined to the former mine soil and all treatments ameliorated soil properties (water holding capacity, organic matter content, pH and EC increases, Pb immobilization) and increased *Salix viminalis* growth, except iron grit alone or combined with biochar (Lebrun et al. 2019). Nandillon et al. (2019b) sown *Agrostis* seeds on the former mine, amended or not with biochar, compost, and/or iron sulfate. The authors did not observe a growth on non-amended soil; however, both the metallicolous and non-metallicolous ecotype had a higher growth when the contaminated soil was amended, due to the amelioration of the growing conditions. The best treatment was the combination of the three amendments, biochar, compost, and iron sulfate. Similarly, the metallicolous ecotype of *Agrostis* was sown on the same soil filled with different amendment combinations, using biochar, ochre, and cow manure. All the treatments containing cow manure improved plant growth compared to the non-amended condition, due to the nutritive properties of the cow manure (Fig. 10.3) (Lebrun et al. 2020).

Regarding the microorganisms, biochar was shown to increase the microbial population of a contaminated agricultural soil and to modify the bacterial community, increasing the abundance of *Actinobacteria* and decreasing the ones of *Acidobacteria* and *Chloroflexi* (Ahmad et al. 2016). Meier et al. (2017) demonstrated that biochar could stimulate the microbial activity of a Cu smelter by reducing Cu availability and providing C sources and nutrients to the microorganisms. Similarly, in the study of Farrell and Jones (2010), the application of compost to an old mine spoil caused important shifts in the structure of the microbial community due to the addition of microorganisms present in the compost. Compost can also stimulate microorganism activity through the release of nutrients (Mackie et al. 2015).

Both amendments and microorganism inoculation can be combined with plant growth for an efficient phytoremediation. For instance, Andrey et al. (2019) combined the application of a pinewood biochar with the inoculation of bacterial strains, isolated from highly polluted sites, in order to stimulate the growth of *Hordeum vulgare*. They observed that the high surface area of the biochar could enhance the

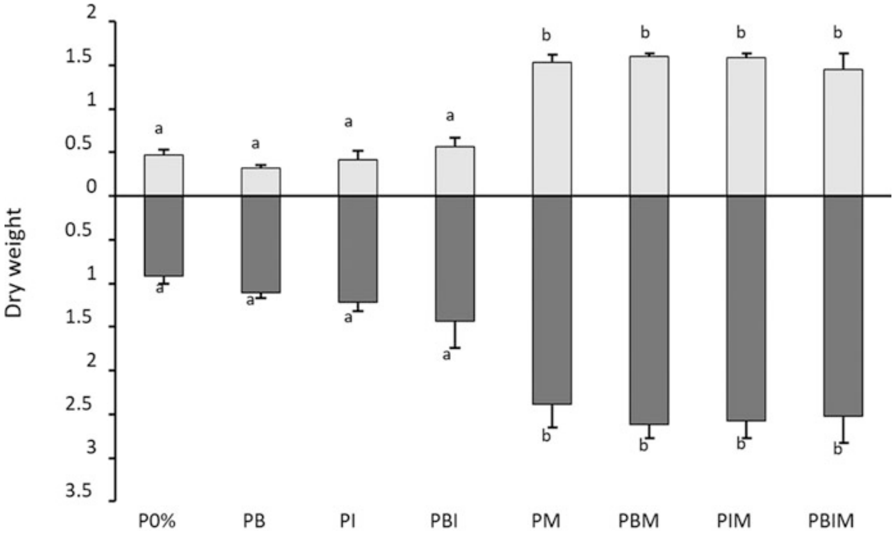

Fig. 10.3 Dry weight (g pot^{-1}) of the aerial (light grey) and root (dark grey) parts of *Agrostis capillaris* plants grown for 26 days under greenhouse conditions on a mining technosol, highly contaminated with As and Pb, unamended (P0%) or amended with 1% biochar (B), 1% ochre (I) and 1% manure (M), alone or combined. Letters indicate significant difference between treatments ($p < 0.05$) ($n = 5 \pm$ SE). (Extracted from Lebrun et al. 2020)

activity of the endogenous and added microorganisms and that the joint application of biochar and bacteria was very effective in reducing metal(loid) stress towards plant and thus induced an amelioration of *H. vulgare* growth. Similarly, Arshad et al. (2017) inoculated two bacterial strains and applied a wood chip biochar to a Cr-spiked soil. They found that amendment and inoculation improved wheat growth due to the positive effect of the biochar on the soil physicochemical properties and the reduction of Cr and supply of nutrients by the inoculated bacterial strains. Wang et al. (2017b) applied a pig manure biochar in combination with *Bacillus subtilis*, which lead to a synergistic effect on the soil fertility improvement, plant growth promotion, and soil metal(loid) immobilization. The association of rice husk biochar with the inoculation of *Pseudomonas putida* and *Bacillus megaterium* increased maize dry weight and reduced Cr uptake.

Finally, biochar, due to its porous structure, can serve as habitat for microorganisms, protecting them from harsh conditions and predators (Gul et al. 2015). Therefore, biochar can be used as a carrier for the inoculation of the microorganisms, i.e., microorganisms are fixed on the biochar surface and the biochar is then applied to the soil (Zhang et al. 2017). For instance, in their studies of 2014 and 2015, Hale et al. showed that the bacterial population of the soil was ten times higher when biochar was used as a carrier compared to the liquid inoculation. Similarly, Chuaphasuk and Prapagdee (2019) used cassava stem biochar as a carrier for *Arthrobacter* sp. and *Micrococcus* sp., two Cd resistant bacteria. The bacterial cells that were immobilized on the biochar were able to proliferate and survive in the

Cd contaminated soil, due to the protection of the bacterial cells from toxic or stressful environments. Both bacterial inoculations increased Cd bioavailability and thus Cd accumulation in *Chlorophytum laxum*. In their study published in Liu et al. (2020), Liu et al. inoculated *Delfia* sp. B9 bacteria either alone or fixed on a corn stalk biochar. They observed that rice growth was improved, Cd rice accumulation decreased, and the residual fraction of Cd in soil increased. These benefits were higher when bacteria were fixed on the biochar compared to the pure cell inoculation. Moreover, the microscopic analysis of the bacteria-loaded biochar showed that bacterial cells were attached on the surface of the biochar but also in its pore. Finally, Tu et al. (2020) amended a Cd and Cu spiked soil with maize straw biochar, loaded or not with *Pseudomonas frederiksbergensis*. They observed that the bacteria adhered well on the biochar and that biochar amendment decreased Cd and Cu availability and increased soil enzyme activity; the effects were better with the bacteria-loaded biochar compared to the biochar alone.

10.6 Conclusion

Soil pollution by metal(loid)s is an important issue worldwide, threatening both the environment and the human health. Therefore, these polluted areas need to be remediated. Compared to the conventional physical and chemical remediation techniques that are fast but expensive and often leave the soil improper for vegetation, the biological remediation, using plants and/or microorganisms, is less expensive, aesthetically pleasing and does not disrupt the soil. Among the plants that can be used in phytoremediation, endemic plant species found in the vicinity of polluted soils and already tolerant to metal(loid) stress can perform better than non-native plants and are also more acceptable ecologically as no non-endemic plants are introduced into the environment. Similarly, microorganisms found in polluted soils can be isolated and selected for their tolerance towards metal(loid)s and PGP traits and inoculated to the soil. They will immobilize metal(loid)s and provide nutrients to the plants, improving plant growth and thus remediation efficiency. Finally, amendments can be applied to further ameliorate soil conditions, improve plant and microorganism growth and activity and thus increase remediation success.

More research needs to be made to understand the mechanisms involved in the higher tolerance of metallicolous ecotypes as well as their response to amendment and microbial inoculation. Furthermore, the becoming of inoculated microorganisms needs to be studied over a longer period of time, to testify of their non-toxicity over time and potentially allow their application in the field, method that is for now forbidden in many countries, except for a handful of products having a homologation and called biofertilizers.

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11 Bioindication of Heavy Metals Contamination by Mushrooms and Mosses in Highly Industrialized Environment

Marek Pająk and Marcin Pietrzykowski

Abstract

The importance of plants in bioindicative assessment of environmental pollution is well known. Mosses and edible mushrooms, especially in the context of human health risk assessment, can be useful, as well. Contaminants generated by human activity and industrial pressure, including heavy metals, entering to the ecosystem by wet and dry deposition and are dangerous for the food chain. Upper Silesia in Poland is one of the most industrialized and populated regions in Europe, where simultaneously forest ecosystem services, including forest floor fruits and mushrooms acquisition, are important and have a long tradition. In the chapter, we present the biomonitoring of heavy metals pollution by mushrooms and mosses contamination level on the evidence from forest ecosystems around the "Miasteczko Śląskie" zinc smelter.

Keywords

Mosses · Mushrooms · Environmental pollution · Heavy metals · Biomonitoring

11.1 Introduction: Mosses and Edible Mushrooms as Bioindicators of Heavy Metals Contamination

Heavy metals are a natural and inseparable element of the natural environment. Circulation and migration of heavy metals in the natural environment are mainly related to such processes as rock weathering, volcanic eruption, evaporation of oceans, forest fires and soil-forming processes (Kabata-Pendias and Pendias 2001).

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However, various human activities have released excessive amounts of metallic elements into the environment. The most important anthropogenic sources of environmental pollution include mining and non-ferrous metals metallurgy, metallurgical and chemical industry, waste storage, application of mineral fertilizers and plant protection products at high doses, sludge fertilization, and surface runoff from roads with heavy traffic (Kabata-Pendias and Pendias 2001; Ociepa et al. 2008; Serengil et al. 2011). Heavy metals arriving from these sources are dispersed in the natural environment, polluting the soil, water and air. Once introduced into the environment, they circulate in it constantly, changing their form at best. Metallic elements are characterized by a very long period of decomposition, which can last up to several hundred years in soils (Grzebisz et al. 2002; Cabała 2009; Moosavi and Zarasvandi 2009). The risk from metallic elements results directly from their movement in the tropic chain, from soil > plant > animal > human. The movement of metals from soil to animals and humans occurs primarily through plants, which are the most important link in the food chain (Mulligan 2005).

According to the degree of risk to living organisms, trace metal elements can be divided into:

- Very high potential hazard, e.g. Cd, Hg, Pb, Cu, Zn.
- High potential hazard, e.g. Mo, Mn, Fe.
- Medium potential hazard, e.g. Ni, Co.
- Low potential hazard, e.g. Sr, Zr (Ociepa et al. 2008).

The toxicity of trace metals depends mostly on the role in the physiological processes of the living organisms and their concentration in environment. However, elements such as cadmium, lead and zinc introduced in large quantities into ecosystems pose a risk of their balance disturbance and even threaten the life of some organisms and the functioning of whole ecosystems (Cabała et al. 2008; Dokmeci et al. 2009).

The environmental pollution from heavy metals is still a major problem for many parts of the world, so it is very important to understand the relations observed in forest ecosystems in the areas heavily polluted with metallic elements. Forest ecosystems play a very important protective role against further penetration of pollutants for surrounding areas. Forests can exclude from circulation, sometimes even for a very long period of time, heavy metals that accumulate in its components. Excessive concentration of metallic elements, however, causes destabilization of this system, which, apart from economic losses, is much more serious in terms of the losses to the broadly understood non-productive functions of forests. The precipitation of pollutants under the canopy of the stand is clearly higher than in open areas (Zwoliński 1995), and the scale of the problem may be seen in the accumulation of heavy metals in individual components of forest ecosystem layers.

Bioindication is the oldest method of environmental assessment by an indicator species i.e. the species whose function, population, or status can reveal the qualitative status of the environment, for example, the stress of excessive pollution. For this reason, bioindicator species can be used to detect the presence of pollutants in a specific location and provide information on changes in air quality (Gorovtsov et al. 2017). The organism to be used as a bioindicator must have the following characteristics: be easily identifiable and retrievable; be widespread in the studied area; have a long life cycle and be present throughout the year (Stankovic and Stankovic 2013). Bioindication is an excellent alternative to the monitoring of pollutant methods using technical equipment (which can be expensive) for biomonitoring (Wolterbeek 2002).

We distinguish between two groups of bioindicators: sensitive indicators and accumulation indicators. The first group is made up of organisms that react to changes in the environment in a visual way (e.g. morphological changes), while the second group is made up of organisms that can accumulate toxic compounds whose concentration can be determined by analytical methods. Chemical analyses of higher plant tissue as leaves has been long time used in the biomonitoring (Stankovic and Stankovic 2013).

Heavy metals emitted into the atmosphere may bind to other atmospheric particles. The particles can be a mixture of various compounds in the atmosphere, many of which are harmful to plants, animals and humans. Due to their small size, particulates are considered a serious health risk, as they can be inhaled and circulated. Leafy tree crowns can be used to filter out emissions of heavy metals from the atmosphere, as the nanoparticles settle on plant leaves. As a result, chemical analyses of heavy metals concentration levels in plant leaves are often used in environmental risk assessment studies and as indicators of air pollution (Dmuchowski and Bytnerowicz 1995; Celik et al. 2005; Samecka-Cymerman et al. 2006; Gratani et al. 2008; Shahid et al. 2017). They became an effective alternative to more common monitoring methods, including the methods using mosses and lichen. In particular, Scots pine tissue has been used to assess the environmental pollution and anthropogenic pressure by SO_2 and heavy metals (Lamppu and Huttunen 2002; Rossini-Oliva and Mingorance 2004; Parzych and Jonczak 2013). Numerous authors (Brown and Wilkins 1985; Eltrop et al. 1991; Marguí et al. 2007; Pająk et al. 2017) used that the silver birch leaves, as well, as bioindicator for heavy metal contamination of the environment.

The distribution of heavy metals in individual organs of woody plants is uneven. The highest concentrations are recorded in the bark, which is related to the time of exposure to pollutants and the rough structure facilitating the deposition of particles from the air. Plant roots also show high levels of toxic substances. After the bark, the next organs with the ability to accumulate heavy metals are roots, leaves and fruits (Filipović-Trajković et al. 2012).

Nowadays, however, lichens and mosses are most often used as bioindication plants to assess air pollution by heavy metals. This is largely based on a lack of roots compared to most higher plants, which means that they are both considered to obtain almost all of their mineral resources from air sources rather than from the ground (Wolterbeek 2002). Jiang et al. (2018) compared the ability of moss and tree leaves to accumulate heavy metals. They showed that the mosses accumulated the elements better and that the accumulated elements came mainly from atmospheric deposition and not from the soil. The author's studies have also shown that moss tissue accumulates more heavy metals than tree leaves.

In particular, mosses are often used to identify forest pollution, as they are very accurate and sensitive bioindicators (Rühling et al. 1987; Türkan et al. 1995; Grodzińska et al. 1999; Szarek-Łukaszewska et al. 2002; Szczepaniak and Biziuk 2003; Dmuchowski and Bytnerowicz 2009). In Central Europe, the most popular mosses used for biomonitoring purposes of contamination with metallic elements are *Pleurozium schreberi* and *Hylocomium splendens* (Berg and Steinnes 1997; Harmens et al. 2008; Migaszewski et al. 2009). *P. schreberi* is a frequently used species in the bioindication of the natural environment, both in places with potentially high contamination and in the vicinity of metal mines (Pöykiö et al. 2001) and in places where there is no significant air pollution, such as in national parks.

As noted above, significant contamination of the forest soil ecosystems with metallic elements negatively affects the nutrient cycle, which reduces the growth, quality and vitality of trees and forest floor vegetation. High accumulation of heavy metals in forest soils limits the species structure and the number of soil microorganisms as well as their metabolic activity (Bååth 1989; Kandeler et al. 1996; Kuperman and Carreiro 1997; Wyszkowska and Wyszkowski 2003; Šmejkalová et al. 2003; Stefanowicz et al. 2008; Sardar et al. 2007). As indicated by Shen et al. (2006), soil enzyme activity, for example, urease as well as dehydrogenase (Brookes 1995), can be used to assess soil contamination with trace elements.

Wild mushrooms, i.e. macroscopic fungal fruiting bodies, particularly easily assimilate heavy metals from the forest environment. Many authors point to their high bioindicative capacity (Kalač et al. 1996; Kalač and Svoboda 2000; Falandysz et al. 2001, 2002, 2007; Falandysz and Borovička 2013; Kokkoris et al. 2019). What is more, according to numerous studies, mushrooms are able to absorb many heavy metals from the substrate. Heavy metals in mushroom fruiting bodies can be accumulated in concentrations much higher than the amounts present in the substrate (Gast et al. 1988; Wang et al. 2014; Türkmen and Budur 2018). As stated by Kalač and Svoboda (2000), depending on the species, much higher concentrations of heavy metals can be found in mushrooms than in plant tissues. These include mostly mercury, cadmium, lead, or copper.

It is the factors related to genetics and the environment that are responsible for mushrooms absorbing heavy metals as well as macro- and microelements from the soil into their fruiting bodies. According to Kalač and Svoboda (2000) and Kalač (2013), among the former factors are the individual development stage and the enzyme activity levels while the latter include the soil's organic matter content, texture and pH. Other studies (Falandysz et al. 2011; Liu et al. 2015) point to the high hazard to human health resulting from increased levels of lead, mercury, cadmium and arsenic in edible wild mushrooms. Former ore mining and processing sites are particularly dangerous when it comes to mushroom collection and consumption as they are heavily contaminated with heavy metals. What follows, edible mushrooms as well as mosses make excellent bioindicators of environmental pollution, which ought to be constantly monitored in industrialized areas.

11.2 Heavy Metals Accumulation in Mosses and Mushrooms: Evidence from Upper Silesia

Among those regions of central Europe where heavy metal contamination is one of the highest is Upper Silesia (southern Poland), in which zinc-lead ore mining and processing has lasted for centuries (Krzaklewski and Pietrzykowski 2002; Krzaklewski et al. 2004; Pająk and Jasik 2012; Pietrzykowski et al. 2014; Pająk et al. 2018). The heavy metals pollution is still one of the most important effect of industrial activity in Upper Silesia region and their accumulation in soil is the great problem for environment and biogeochemical cycles in the ecosystems (Ullrich et al. 1999; Pająk and Jasik 2011, 2012; Chrastný et al. 2012; Gruszecka and Wdowin 2013; Ciarkowska et al. 2014; Pająk et al. 2015, 2016).

In order to investigate the state of heavy metal contamination of forest areas located in the highly industrialized environment in the immediate vicinity of the zinc plant Miasteczko Śląskie, 31 permanent monitoring areas were established (Fig. 11.1, Photos 11.1 and 11.2). The surfaces are located at the grid intersection points of 1500 × 1500 m.

The research areas were situated to the east and north-east of the "Miasteczko Śląskie" zinc smelter, with the coordinatesl 50°29′01″–50°32′25″N, 18°57′10″–19°08′43″E. They were located downwind with regard to the dominant direction of winds, carrying with them the highest amounts of pollutants from the smelter. The areas were characterized by two forest types: wet mixed coniferous forest and fresh

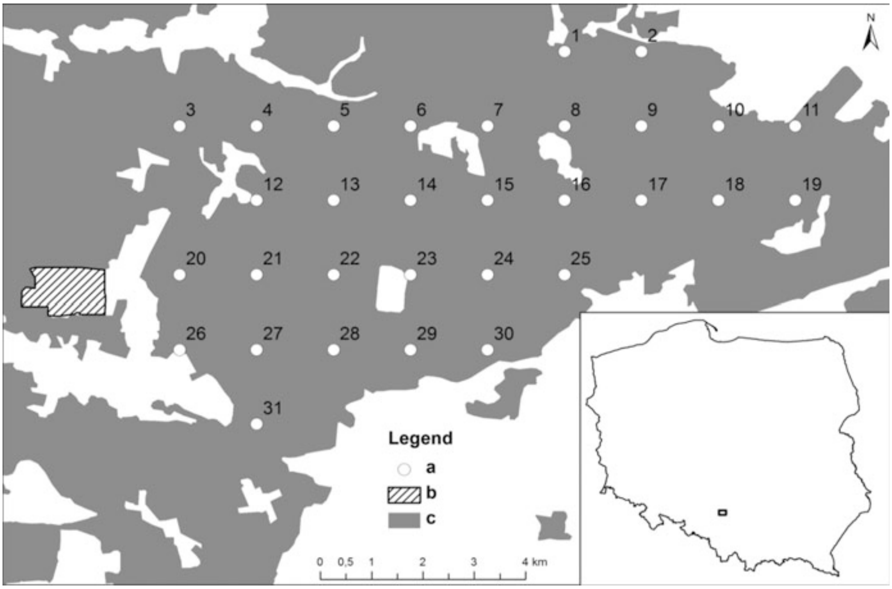

Fig. 11.1 Location of monitoring areas (*a*—monitoring areas, *b*—zinc plant Miasteczko Śląskie, *c*—forests) (Pająk et al. 2020)

Photo 11.1 The area after a die-back of forest stands, which probably are caused by interaction of critical heavy metals load in soil and the drought phenomena in the last 3 years, which is an additional stress factor in these heavily polluted sites (in the background the view of the chimney of the zinc smelter "Miasteczko Śląskie")

mixed coniferous forest, the soil types were Haplic Podzols as well as Gleyic Podzols (IUSS Working Group WRB 2007). According to the Forest Management Plan for the years 2003–2012, the research plots were situated in stands aged 40–80. Each plot had its coordinates (x, y) and its location was identified with the use of a GPS receiver.

As part of the conducted research, moss *Pleurozium schreberi* (Pająk and Jasik 2011) and wild mushrooms (Pająk et al. 2020) were collected on permanent monitoring surfaces.

Sampling of *Pleurozium schreberi* moss was done on all monitoring areas where it was found (27 plots). The samples were then transported to the laboratory where the green and brown parts of the unwashed tissue were dried at 60 °C and grounded. The concentration of Zn, Pb and Cd in plant tissues was measured by the AAS method on AA Varian 20 apparatus (Pająk and Jasik 2011).

Sampling of edible wild mushrooms was done by collecting their fully developed, ripe fruiting bodies, and included ten individuals of each species. The collected fruiting bodies were instantly cleaned by removing all impurities, such as sand, using a plastic knife. The next stage was drying at room temperature, in a dry, well-ventilated, clean space for several days. After their transport to the laboratory, the mushrooms underwent drying at 60 °C in the laboratory dryer until reaching a constant mass, which was followed by crushing in the laboratory mill and

Photo 11.2 Tree stands with the symptoms of weakened and industrial pollution pressure in the area of influence of the zinc smelter “Miasteczko Śląskie”

mineralization with the use of the “wet procedure” in a mixture of concentrated nitric and perchloric acids (ratio 3:1). This enabled the determination of the content of: Ca, Cd, Cr, Cu, Fe, K, Mg, Mn, Na, Ni, P, Pb and Zn by means of the ICP-AES plasma emission spectrometry, using the Thermo Scientific iCAP 6000 Series ICP-OES (Pająk et al. 2020).

More important results of the research (Pająk and Jasik 2011; Pająk et al. 2020) conducted on permanent monitoring surfaces are discussed below.

The concentration of the investigated heavy metals in moss tissues is presented in Table 11.1. The highest concentrations of Zn, Pb and Cd were found on the monitoring plots located closest to the metallurgical complex (Fig. 11.1; Table 11.1). The study also showed very high correlation coefficients of Zn ($R = 0.93$), Cd ($R = 0.93$) and Pb ($R = 0.90$) concentrations in moss tissue along with a change in distance from the metallurgical complex “Miasteczko Śląskie” (Pająk and Jasik 2011).

Comparing the results of our research with those of Grodzińska and co-authors’ research (1999), which were carried out throughout Poland, the Brynica district should be classified as one of the most polluted area by cadmium in Poland. According to the Grodzińska et al. (1999), only 0.8% of the samples in Poland had a Cd content of more than 2 $mg{\cdot}kg^{-1}$, whereas in the analysed moss samples from the forest areas of the Świerklaniec Forest District, higher Cd concentrations were recorded in all moss samples. Similarly, also in the case of lead, based on the content of this element in moss tissues, the whole studied area should be classified as

Table 11.1 Content of zinc, cadmium and lead in the moss *Pleurozium schreberi* [mg·kg^{-1}] (Pająk and Jasik 2011)

Number of the monitoring area	Distance from zinc smelter [km]	Moss		
		Zn	Cd	Pb
20	1.8	*	*	*
26	2.2	*	*	*
21	3.3	969	38	811
27	3.5	700	28	856
3	3.7	468	25	657
12	3.7	641	31	683
31	4.3	335	17	434
4	4.6	524	27	746
22	4.8	464	21	422
28	4.9	*	*	*
13	5.1	372	15	385
5	5.8	368	17	653
23	6.3	289	14	323
29	6.4	368	22	518
14	6.5	345	9	192
6	7.0	279	9	191
24	7.8	75	15	116
30	7.9	262	12	266
15	7.9	197	8	192
7	8.4	236	8	199
25	9.3	199	8	179
16	9.4	171	7	121
8	9.8	191	5	148
1	10.4	156	7	168
17	10.9	162	6	129
9	11.2	188	5	124
2	11.8	192	7	165
18	12.4	162	6	135
10	12.7	150	4	98
19	13.9	241	4	118
11	14.1	*	*	*

Where: * the moss did not grow

one of the most polluted areas of Poland (Grodzińska et al. 1999). Also in the case of zinc, as much as 90% of the monitoring surfaces were characterized by very high levels of contamination. For example, the concentrations of the heavy metals in question found in the *Pleurozium schreberi* moss in the forest ecosystems of the Świerklaniec Forest District were much higher than those determined in the Pieniny Mountains by Panek and Szczepańska (2005). In case of Zn, these concentrations were 20 times higher, and in case of Pb and Cd, as much as over 80 times higher. Zwoliński (1995) pointed out that it is very important to know the total concentration of all heavy metals in the forests, which are the cause of the negative impact on forest health. As a critical value he indicated a total content of about 500 mg·kg^{-1}of heavy metals in the organic layer of forest soil. In the studied forests of the Świerklaniec Forest District, as much as 80% of the area was characterized by concentrations of Zn, Cd and Pb higher than 500 mg·kg^{-1} in the organic layer of forest soil.

As shown in Table 11.2 below, the biomonitoring performed on the research plots in the years 2013 and 2014 resulted in gathering 88 samples of edible wild mushrooms. Among them, it is four species: *Imleria badia*, *Lactarius helvus*, *Russula sardonia* and *R. aeruginea* that turned out to be the most frequent.

As presented in Table 11.3 below, in most of the mushroom species sampled in the research area, heavy metal concentrations, ranging from the highest to the lowest, concerned the elements: Zn > Cu > Cd > Pb > Cr > Ni. Among the metals with the highest mean content, the values for zinc (the highest one) amounted to 79.2 mg·kg^{-1} in the species *Suillus bovinus* to 451.1 mg·kg^{-1} in *Russula aeruginea*. In the case of Cd, the highest value of 3.6 mg·kg^{-1} was found in *Lactarius deliciosus* and the lowest, 27.0 mg·kg^{-1}, was in *Imleria badia*. The highest concentration of Pb: 12.0 mg·kg^{-1}, was noted in *Imleria badia*. It is worth noting that a high mean Cu content: 36.5 mg·kg^{-1}, was also found in *Imleria badia*.

The studies carried out showed that fungi belonging to the families Russulaceae and Boletaceae were characterized by a higher accumulation of heavy metals in comparison with the family Suillaceae (Fig. 11.2); moreover, the most frequent species were similar in their content of three metals, namely Zn, Cd and Pb.

Conducting a statistical analysis revealed a number of significant relationships related to edible wild mushrooms under scrutiny. Firstly, there was an evident relationship between the distance from the source of heavy metal emissions and heavy metal concentration in the study material: $R^2 = 0.46$, $R = 0.66$, which was particularly significant for three elements: Zn, Cd and Pb. Secondly, in most of the analysed mushrooms, the highest bioconcentration was noted in the case of Cd, which turned out to be particularly well pronounced for *Imleria badia*. In that species, the BCF between soil and mushrooms amounted to 9.17 (Pająk et al. 2020).

The majority of higher fungi are able to accumulate in their fruiting bodies not only heavy metals but also other metals and metalloids (Sarikurkcu et al. 2011). To numerous living organisms, the fact that heavy metals get into the food chain and stay there constitutes a serious threat (Wang et al. 2017). Therefore, apart from the degree of environmental contamination, the toxicity of heavy metals also depends on the large degree of their absorption and excretion as well as their biochemical role in disrupting metabolism (Szynkowska et al. 2008). Due to the fact that, apart from nutrients (minerals), water and soil are also sources of toxic metals to be absorbed by mushrooms, the latter can deliver those metals to human and animal bodies in the form of food and fodder (Chen et al. 2009).

As reported by various studies, the degree of heavy metal concentration may vary in different parts of the fruiting bodies of individual species (Kojta et al., 2011). Thus, the highest average Cd content was noted in the cap (3.23 mg·kg^{-1}) and stalk (2.24 mg·kg^{-1}) of *Agaricus campestris* (Širić et al. 2016). In the case of Pb, it is *Macrolepiota procera* that revealed the highest average content of that metal in its

Table 11.2 List of monitoring areas (plots) where collected of edible mushrooms in 2013 and 2014 (Pająk et al. 2020)

Number of the monitoring area	*Imleria badia* (Fr.) Fr.	*Suillus luteus (L.) Roussel*	*Suillus grevillei* (Klotzsch) Singer	*Suillus bovinus* (L.) Roussel	*Lactarius deliciosus* (L.) Pers.	*Lactarius helvus* (Fr.) Fr.	*Russula paludosa* Britzelm.	*Russula claroflava* Grove	*Russula aeruginea* Lindblad ex Fr.
1									
2									
3	*				* ×		* ×		
4	* ×								
5	* ×				* ×				
6	×	* ×							
7									
8	* ×	* ×	* ×						
9	* ×	* ×							
10			* ×	* ×					
11	* ×		* ×					* ×	
12									
13	×	* ×		* ×					
14	* ×								
15	×			* ×		* ×	* ×		
16									
17						* ×		* ×	
18									
19			* ×						* ×
20	×								
21	×								
22		* ×							
23	*								

24									
25	* ×		* ×		* ×				
26				* ×					
27									
28	×	* ×				* ×			* ×
29	* ×	* ×	* ×						
30									
31									

Where: * plots in which the species of mushrooms was collected in 2013
× plots in which the species of mushrooms was collected in 2014

Table 11.3 Content of Cd, Cr, Cu, Ni, Pb and Zn [mg·kg^{-1}] in the edible mushrooms of the study area (Pająk et al. 2020)

Species of mushroom	Cd	Cr	Cu	Ni	Pb	Zn
	[mg·kg^{-1}]					
Imleria badia	27.0 (7.8–84.3)	7.0 (1.3–57.0)	36.5 (2.8–90.6)	2.2 (0.5–6.5)	12.0 (1.3–102.0)	187.4 (100.4–404.0)
Suillus luteus	12.3 (6.5–20.8)	5.0 (3.9–6.1)	20.4 (0.9–39.7)	2.1 (1.1–2.9)	4.9 (3.8–5.6)	146.9 (96.6–175.6)
Suillus grevillei	4.1 (2.7–5.5)	3.1 (2.3–3.8)	2.3 (0.9–4.7)	0.7 (0.5–0.9)	4.5 (3.5–5.6)	94.1 (87.3–100.8)
Suillus bovinus	4.3 (3.8–4.9)	2.7 (2.6–2.8)	0.2 (0.1–0.3)	1.5 (1.2–1.8)	4.2 (2.7–5.7)	79.2 (73.6–84.8)
Lactarius deliciosus	3.6 (1.2–5.9)	2.6 (2.3–2.8)	17.7 (0.2–35.1)	1.0 (0.5–1.4)	6.4 (4.6–8.3)	124.2 (97.1–151.8)
Lactarius helvus	11.5 (1.4–40.9)	4.4 (2.6–9.9)	24.8 (0.2–76.3)	1.6 (0.8–2.9)	6.7 (3.0–13.8)	147.4 (81.3–219.1)
Russula paludosa	14.4 (12.7–17.5)	3.5 (1.9–4.8)	48.1 (40.3–53.8)	1.7 (0.9–3.1)	14.0 (12.8–15.6)	129.5 (110.1–158.8)
Russula claroflava	11.1 (4.8–17.4)	10.2 (5.8–14.7)	36.8 (30.3–43.4)	2.2 (2.0–2.4)	10.5 (7.4–13.7)	302.7 (212.2–393.3)
Russula aeruginea	9.3 (7.0–12.7)	3.5 (2.8–4.3)	10.8 (0.3–33.5)	1.9 (1.0–2.8)	9.7 (6.4–13.8)	451.1 (251.3–599.6)

Where: 7.0 mean, (1.3–57.03) minimum and maximum value

cap (1.91 mg·kg^{-1}) and stalk (1.60 mg·kg^{-1}). Finally, the largest concentrations of Mn, i.e. 2.54 mg·kg^{-1}, and Zn, namely 60.6 mg·kg^{-1}, were noted for *Imleria badia* sampled in areas with no traces of environmental pollution (Kuziemska et al. 2018).

The results obtained in our study show that, taking into consideration the most common mushrooms in the research area, it was *Russula aeruginea* that revealed especially large Pb, Cd and Zn contents while *Imleria badia*, another mushroom that is frequently occurring and often picked for human consumption, can also serve the purpose of biomonitoring in areas with high heavy metal contamination. As found in our study, the mean Pb accumulation in *Imleria badia* amounted to 12 mg·kg^{-1}. However, in the most contaminated areas, its values exceeded 100 mg·kg^{-1} (Table 11.2).

Since human health may be seriously threatened by the presence of heavy metals, limits have been established for their permissible intake by people. As indicated by two organizations acting within the United Nations, namely the WHO (World Health

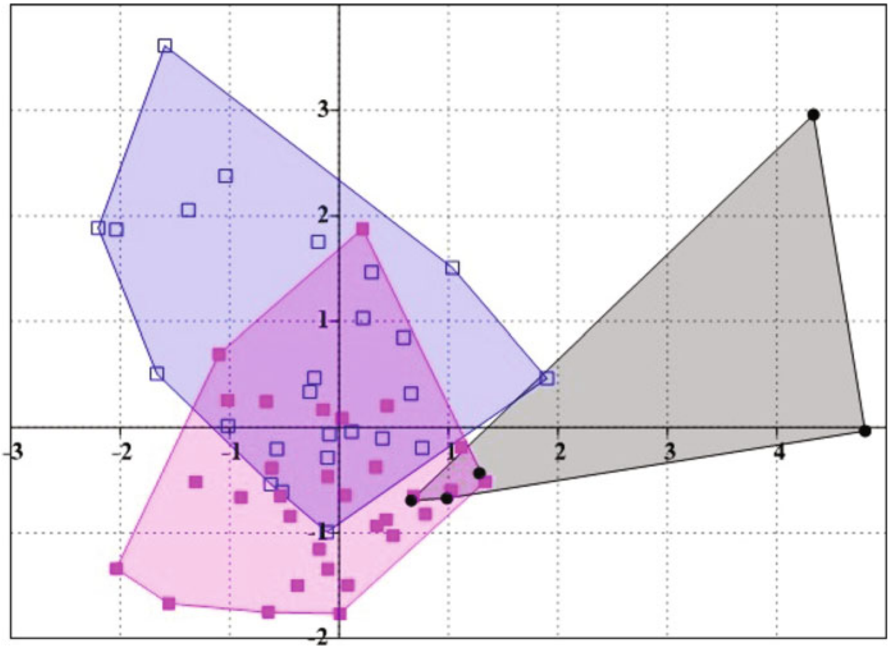

Fig. 11.2 CVA ordinance diagram of differences in heavy metal concentrations between the analysed mushroom families. Open squares stand for the *Boletaceae* family, colored squares for *Russulaceae* and black circles for *Suillaceae* (Pająk et al. 2020)

Organization) together with the FAO (Food and Agriculture Organization) (JECFA 2010), the weekly permissible content of Cd and Pb in food amounts to 7 and 15µg/kg of body weight, respectively (Fang et al. 2014; Liu et al. 2015). For example, a person that weighs 60 kg, which is the mean weight of an adult, can consume these respective metals in weekly amounts of up to 0.42 and 0.9 mg. What this means in the case of our research material is that, for instance, 1 kg of the dry matter of *Imleria badia* found in our study area exhausts the Cd consumption limit for cadmium for as long as 64 weeks. Against the background of the EU Food Guidelines (EU 2008), the Pb and Cd contamination found in *Imleria badia* exceeds the limit values (0.3 mg kg^{-1} for Pb and 1.0 mg kg^{-1} for Cd, considering fresh weight). The edible mushrooms sampled in the most contaminated forests revealed the Pb content exceeding the limit value by approx. 30 times while the Cd content was exceeded by approx. 10 times.

11.3 Conclusion

In our study the bioindicative role of mosses and edible mushrooms, especially in the context of human health risk assessment, was confirmed. Contaminants generated by human activity and industrial pressure, including heavy metals, entering to the ecosystem and are dangerous for the food chain in the studied ecosystems. Upper Silesia in Poland is one of the most industrialized and populated regions in Europe, where the metallurgic complex “Miasteczko Śląskie” was confirmed as a significant source of heavy metal pollution, which may affect ecosystem vitality. As revealed by our research, in areas with high heavy metal contamination, the *Boletaceae* and *Russulaceae* mushroom families accumulate more heavy metals than the *Suillaceae* family. The BCF values taken into consideration allow for a conclusion that it is Cd that had the highest bioaccumulation level in most of the wild edible mushrooms under scrutiny. Furthermore, our study unambiguously points to the spatial dependence that exists between heavy metal concentrations in the analysed mushrooms and the distance of the forest area from the chief source of heavy metal emission. The conducted research showed, as well, the high usefulness of the moss *Pleurozium schreberi* in conducting biomonitoring of contamination in a highly industrialized environment.

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Polycyclic Aromatic Hydrocarbons: Toxicity and Bioremediation Approaches

12

Djaber Tazdaït and Rym Salah-Tazdaït

Abstract

PAHs (Polycyclic Aromatic Hydrocarbons) constitute a particular class of organic compounds, which has been widely studied because of their genotoxic, carcinogenic, and teratogenic properties. Given their stability in the environment (Persistent Organic Pollutants (POPs)) and their toxicity, 16 of them have been declared as priority pollutants. The structure of PAHs makes them very stable in the environment. PAHs in the atmosphere are formed during incomplete combustion or pyrosynthesis of organic matter. Thus, they are present in soot and smoke of all origins (exhaust gases of internal combustion engines, in cigarette smoke, etc.). Fortunately, microbial populations and plants naturally adapted to the biodegradation of PAHs exist. Many studies have, in fact, demonstrated that in the presence of various plants and microorganisms, the degradation of PAHs is accelerated. This chapter mainly summarizes and analyzes the state-of-the-art within the PAHs toxicity at the molecular level (DNA) and the biochemical aspects of their bioremediation by microorganisms and plants.

Keywords

Biodegradation · Microorganisms · PAHs · Phytoremediation · Toxicity

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12.1 Introduction

Pollution is everywhere nowadays, whether in everyday life or at work. Pollution of outdoor air was also classified in 2013 in the group of certain carcinogens to humans by the International Agency for Research on Cancer of the World Health Organization and are considered as "the most extensive environmental carcinogens" (Kim et al. 2018). The link between a pollutant and a health effect is difficult to express with certainty given the numerous risk factors, generally chronic exposures, and the sometimes very long latency time between exposure and disease (Johannson et al. 2015). Besides, the concentration levels of pollutants are higher in the professional environment.

Ubiquitous in the environment, PAHs form a family of several hundred persistent organic compounds made up of at least two fused benzene rings (Okamoto 2019). In the atmosphere, they are always present in the form of complex mixtures comprising a particulate phase for the heaviest (four cycles and more, potentially carcinogenic) and a gas phase for the lighter ones (two to four cycles, less toxic) (Kumari and Lakhani 2018). They are very stable molecules with multiple sources of emission. They are emitted during the processes of incomplete combustion of organic matter but also the distillation of coal (pyrogenic source) and petroleum (petrogenic source), or the use of products derived from these processes or thermal inversion phenomena (Ifegwu and Anyakora 2015; Abdel-Shafy and Mansour 2016; Largeron and Staquet 2016; Zhang et al. 2016). The concentrations and the relative proportions of the different PAHs in the mixtures are very variable. In urban areas, concentrations depend on the season and are higher in winter than in other seasons due to higher emissions linked in particular to domestic heating and emissions from transport vehicles (Ifegwu and Anyakora 2015). The concentrations also depend on the sources of emission which depend in particular on the type of industrial activity (Petit et al. 2019; Alhamdow et al. 2020; Yakovleva et al. 2020).

PAHs have lipophilic properties and low aqueous solubility allowing them to readily penetrate living organisms. They also tend to bioaccumulate in soft tissues. The main routes of penetration of these molecules into the body are inhalation, ingestion (food, drinks, pharmaceuticals), and skin contact (skin, mucous membranes, and eyes) (Hamidi et al. 2016; Zhang et al. 2016). Regarding non-occupational contamination, the main routes of absorption are inhalation of smoke in smokers, and ingestion of grilled food in non-smokers, while at the professional level they are inhalation and direct and indirect skin contact with the products (Abdel-Shafy and Mansour 2016; Cattaneo et al. 2016). Once inside the body, after absorption by the lungs, skin, and intestine, PAHs are distributed rapidly to all compartments of the body and can be stored in the kidneys, liver, and fat tissue. They are then generally bio-transformed into metabolites to be more easily excreted. But during this metabolization, certain compounds are bioactivated, making them toxic (Flesher and Lehner 2016). Elimination occurs primarily through feces and to a lesser extent through urine. The heaviest compounds (four cycles and more) are mainly excreted in the feces while the lightest (two to four cycles) are mainly excreted in the urine (Ifegwu and Anyakora 2015). PAHs represent one of the most toxic families of organic compounds known to date. Short-term exposures can cause lung problems in asthmatics as well as thrombosis in people with heart disease. In the workplace, high exposure to PAHs can cause symptoms such as eye irritation, nausea, vomiting, or even diarrhea (Kim et al. 2013). PAHs are best known for their chronic effects. They are believed to be responsible for immunity disorders, for triggering or increasing asthma symptoms, especially during childhood, and would increase the risk of cardiopulmonary mortality (Zaccaria and McClure 2013; Karimi et al. 2015; Alshaarawy et al. 2016; Liu et al. 2016). They are suspected of causing reproductive disorders, of being teratogenic (premature deliveries, delays in child development as well as low birth weight), and endocrine disruptors (Zhang et al. 2016; Bolden et al. 2017; Li et al. 2018). However, the major health problems linked to exposure to PAHs concern their carcinogenicity, which is mainly due to their ability to bind to DNA, which can generate a series of disruptive effects often at the origin of the initiation of tumors (Zaccaria and McClure 2013; Hamidi et al. 2016; Kim et al. 2016; Misaki et al. 2016; White et al. 2016).

To remedy this scourge, different approaches are being explored, including bioremediation. It consists of using microorganisms (microbial remediation) or plants (phytoremediation) to reduce mobility or transform pollutants into non-toxic compounds (Mougin 2002). Microbial remediation uses the potential of microorganisms (fungi, bacteria, and algae) to break down certain types of pollutants. This biodegradation can take place under aerobic or anaerobic conditions (Liao et al. 2019). In the case of PAHs, bioremediation techniques consist of increasing their biodegradation or biotransformation, by inoculating specific microorganisms (bioaugmentation) or by stimulating the activity of indigenous microbial populations (biostimulation) by providing nutrients and adjusting the environmental conditions (oxidation-reduction potential, humidity) (Haleyur et al. 2019; Villaverde et al. 2019; Sarma et al. 2019a, b).

This chapter is a synthesis of current knowledge on the toxicity of PAHs and the capacity of microbial and plant species to capture and degrade them in air, water, and soil.

12.2 Polycyclic Aromatic Hydrocarbons (PAHs): General Considerations

Polycyclic aromatic hydrocarbons consist of a large family of organic compounds of carbon and hydrogen that contain from two to up to seven fused aromatic rings arranged in three different configurations (linear, cluster, or angular). PAHs can be found in soil, air, food, water, and sediments deposited from water, wind, and even ice (Sarma et al. 2016).

12.2.1 Sources of PAHs

PAHs are formed during incomplete combustion of organic matters with lack of oxygen. Under such conditions, C-H free radicals occur, which polymerize to form ultimately different PAHs compounds. It has been estimated that the global worldwide air emissions of the 16 priority PAHs of the US Environmental Protection Agency in 2004 were 520 giga grams per year. The Asian countries were the biggest emitters with 290 giga grams per year (Rengarajan et al. 2015). PAHs are released from several natural and man-made sources. In the natural environment, PAHs occur naturally in fossil fuels such as crude oil and coal (in which dibenzothiophenes are typically the dominant component), and are also produced during volcanic eruptions and during bush and forest fires (Alawi and Azeez 2016). PAHs can also be produced naturally (biogenic origin) by living organisms such as plants, bacteria, and fungi (Wakeham and Canuel 2016). However, human activity remains the dominant source of PAHs in the environment (Wilcke 2000). The anthropogenic sources of PAHs are categorized into petrogenic and pyrolytic. Petrogenic PAHs are released during the production of different petroleum products including coal tar creosote (wood preservative), coke and asphalt (Adeniji et al. 2018; Offiong et al. 2019). Pyrolytic sources refer to combustion processes which include biofuels and fossil fuels combustion, wood burning (Naphthalenes, Benzo[b,j,k]fluorene, Benzopyrenes, Fluorene, etc.) industrial processes, and automobile and aircraft exhaust systems (Dimethylphenanthrenes, Methylbenzanthracenes, Chrysene, Benzofluoranthenes, Benzopyrenes, Benzochrysene, etc.) (Faboya et al. 2020; Remizovschi et al. 2020). PAHs are also likely to be generated from food processes such as drying, smoking, roasting, and grilling, which are considered as significant sources of food contamination (Codex Alimentarius Commission 2004). Some PAHs compounds have been used commercially as intermediates in the manufacturing of different chemicals including dyes and pigments, pharmaceuticals, thermoset plastic, resins, and agrochemicals such as pesticides (Abdel-Shafy and Mansour 2016).

12.2.2 Physical and Chemical Characteristics of PAHs

Although PAHs are made up of only carbon and hydrogen atoms, they can contain other elements such as oxygen, sulfur, and nitrogen within the benzene ring (Fig. 12.1). These substituted derivatives are designated by a more general term, which is heterocyclic aromatic compounds (HACs), that include azaarenes (containing nitrogen) such as nitropyrene, nitroanthracene, 5-nitroacenaphthene, 6-nitrochrysene, etc.; thiophenes (containing sulfur) such as dibenzothiophene, 1,2-benzodiphenylene sulfide, thianthrene, etc.; furans (containing oxygen) such as naphthalenone, 9,10-phenanthra quinone, 1,4-naphtho quinone, and hydroxy-derivatives such as 1,5-dihydroxynaphthalene, 1-naphthol, etc. (Menichini and Bocca 2003; Goldfarb 2013; Hayakawa 2018; Balmer et al. 2019). The molecular weight of PAHs varies from 128 g/mol (Naphthalene, $C_{10}H_8$) to up to 300 g/mol

(Coronene, $C_{24}H_{12}$). PAHs are characterized by their neutral charge, semi-volatility, and low solubility in water. Some molecules with six aromatic rings such as Benzo [ghi]perylene and Dibenzo[a,e]pyrene are even practically insoluble in aqueous solutions (Table 12.1). The aqueous solubility and vapor pressure of PAHs decrease with increasing molecular mass (Albers 2002); their solubility also decreases with increasing degree of substitution and ring structure (Adeniji et al. 2018). They have high melting and boiling points, which increase with the augment of their molecular mass (Nikolaou et al. 2009). Besides, because they are nonpolar molecules, PAHs exhibit a high solubility in most organic solvents including toluene and benzene and show strong binding on particular matters and clays. This adsorption phenomenon is almost exclusively related to the octanol to water partition coefficient (K_{ow}) of PAHs, which augments with increasing molecular weight (Smith et al. 1999). It is important to bear in mind that the transformation (biotic or abiotic) and fate of PAHs in soil and sediment materials are typically influenced by the physical-chemical characteristics of both the chemicals and the sediment matrices.

12.2.3 Toxicity of PAHs

12.2.3.1 DNA Damage by PAHs

The hazardous effects of PAHs on human health or the environment are nowadays well established and admitted by all experts in toxicology and ecotoxicology from administration and academia. The deleterious effects of PAHs compounds result from their metabolic activation, which occurs intracellularly and results in the formation of electrophiles responsible for their mutagenic, cancerogenic, and teratogenic properties. This bioactivation which has been well studied involves predominantly three enzymatic pathways (Fig. 12.2): (1) the bay-region dihydrodiol epoxide pathway involving the cytochrome P450 enzymes; (2) the radical cation pathway mediated by P450 peroxidase; and (3) the *o*-quinones pathway mediated by dihydrodiol dehydrogenases also known as aldo-keto reductases (Xue and Warshawsky 2005). The most negative effect of PAHs is precisely related to their genotoxic properties (DNA deterioration) acquired after their metabolic activation, against normal cells by killing them through different processes such as necrosis (pathological cell death) (Bai et al. 2017), senescence (cellular aging) (Yu et al. 2019), or apoptosis (programmed cellular self-destruction) (Solhaug et al. 2004; Das et al. 2017). The molecular DNA damages induced by exposure to genotoxic PAH compounds include (1) non-enzymatic DNA-protein crosslinks, where there are nonspecific covalent linkages between proteins and DNA-DNA crosslinks (Yu et al. 2006); (2) single-strand breaks induced by photoirradiated PAHs, which sever the phosphate-deoxyribose backbone of one DNA strand (Yu et al. 2006); (3) double-strand breaks, in which the two strands of the DNA double helix are severed; this results in intracellular reactive oxygen species accumulation, which is mediated by PAHs (Wilk et al. 2013); and (4) PAH-DNA adducts in which covalent bounds are formed between segments of DNA and some reactive PAHs such as benzo(a)pyrene and 7,12-dimethylbenz(a)anthracene (Galván et al. 2005). The

Table 12.1 Some physical-chemical properties of PAHs of high concern

PAHs	Number of rings (aromatic + nonaromatic)	Chemical formula	Molecular weight (g/mol)	Melting point (°C)	Solubility in water at 25 °C (µg/L)	Log *n*-octanol to water partition coefficient (Log K_{ow})	Log organic carbon to water partition coefficient (Log K_{oc})	Vapor pressure (Pa) at 25 °C
Benz[a] anthracene	4(4)[a]	$C_{18}H_{12}$	228.3[b]	167[c]	9–14[c]	5.6[d]	5.3[d]	2.8×10^{-5}[e]
Benzo[b] fluoranthene	5(4 + 1)[a]	$C_{20}H_{12}$	252[f]	168.3[c]	1.51[g]	6.04[d]	5.74[d]	6.6×10^{-5}[h]
Benzo[j] fluoranthene	5(4 + 1)[f]	$C_{20}H_{12}$	252.3[b]	165.4[c]	2.49[g]	–	–	–
Benzo[k] fluoranthene	5(4 + 1)[a]	$C_{20}H_{12}$	252.3[b]	215.7[c]	0.75[g]	6.04[d]	5.74[d]	5.2×10^{-8}[e]
Benzo[ghi] perylene	6(6)[a]	$C_{22}H_{12}$	276.3[b]	278.3[c]	0.3[f]	6.5[d]	6.2[d]	1.3×10^{-8}[h]
Benzo[a]pyrene	5(5)[a]	$C_{20}H_{12}$	252.3[b]	178.1[c]	0.5–6[c]	6.06[d]	6.74[d]	7.0×10^{-7}[e]
Chrysene	4(4)[a]	$C_{18}H_{12}$	228.3[b]	255–256[c]	1.5–2.2[c]	5.16[d]	5.3[d]	5.7×10^{-7}[e]
Cyclopenta[c,d] pyrene	5(4 + 1)[f]	$C_{18}H_{10}$	226[f]	170[c]	33.52[f]	–	–	–
Dibenz[a,h] anthracene	5(5)[a]	$C_{22}H_{14}$	278.4[b]	266.6[c]	0.5[c]	6.84[d]	6.52[d]	3.7×10^{-10}[e]
Dibenzo[a,e] pyrene	6(6)[f]	$C_{24}H_{14}$	302[f]	233[c]	0.24[f]	–	–	–
Dibenzo[a,h] pyrene	6(6)[f]	$C_{24}H_{14}$	302[f]	317[c]	–	–	–	–
Dibenzo[a,i] pyrene	6(6)[f]	$C_{24}H_{14}$	302[f]	281.5–282.5[c]	0.5[f]	–	–	–

Dibenzo[a,l] pyrene	6(6)[f]	$C_{24}H_{14}$	302[f]	162.4[c]	0.24[f]	–	–	–
Indeno[1,2,3-cd]pyrene	6(5 + 1)[a]	$C_{22}H_{12}$	276[f]	163.6[c]	62[i]	6.58[d]	6.2[d]	0.166[h]
5-Methylchrysene	4(4)[f]	$C_{19}H_{14}$	242[f]	118–119[c]	65[f]	–	–	–

[a] Ifegwu and Anyakora (2015)
[b] Menichini and Bocca (2003)
[c] IARC (1983)
[d] Feng et al. (2007)
[e] Hayakawa (2018)
[f] Purcaro et al. (2016)
[g] Pearlman et al. (1984)
[h] Ghosal et al. (2016)
[i] Nikolaou et al. (2009)

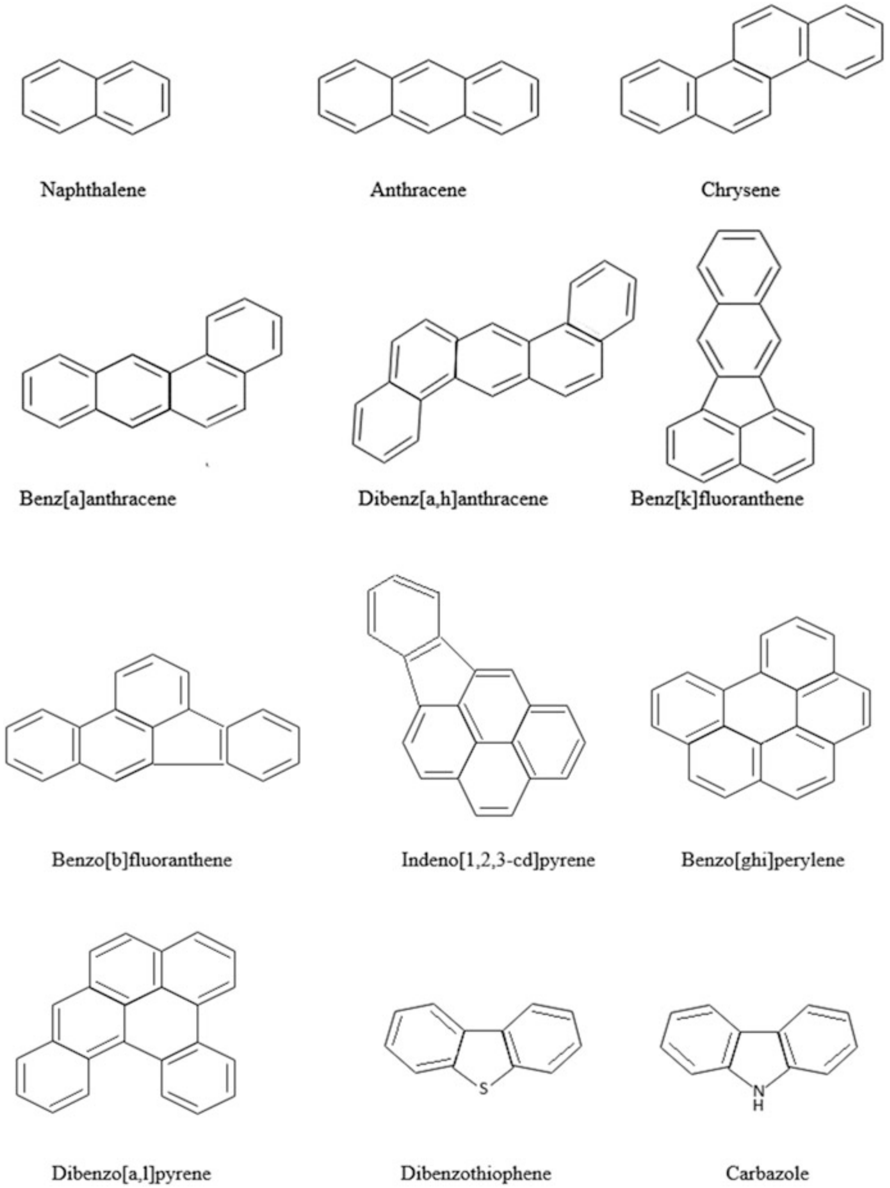

Fig. 12.1 Chemical structure of some PAHs and HACs

human carcinogen metabolite of benzo[a]pyrene (benzo[a]pyrene diol epoxide), for instance, forms a stable covalent binding with the exocyclic amino groups of guanine and/or of adenine (Ewa and Danuta 2017). The adducts formed by PAHs can induce several point mutations, including base substitution resulting in the

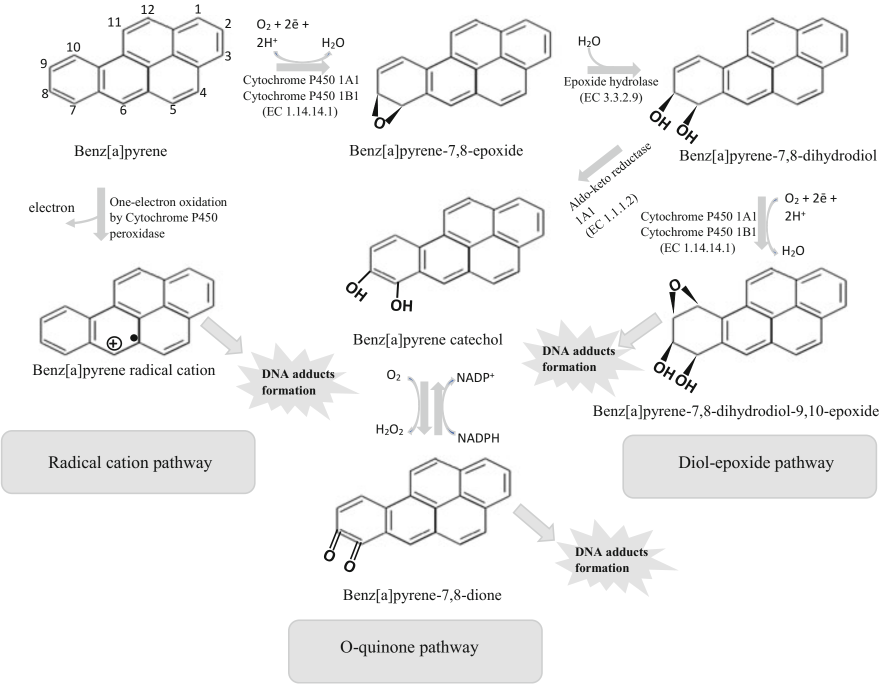

Fig. 12.2 Metabolic activation mechanism of benzo[a]pyrene through diol-epoxide, *o*-quinone, and radical cation pathways

conversion of G to T (Smith et al. 2000), frameshifts (Mordukhovich et al. 2010), small deletions, and chromosome- and chromatid-type aberrations (Wei et al. 1996; Abdel-Shafy and Mansour 2016).

The main health concern in the long term due to PAHs exposure is development of cancer (Sarma et al. 2017). There are three categories of genes whose mutation usually causes cancer formation in the case of unsuccessful DNA repair or faulty cell replication: (1) the tumor suppressor genes (anti-oncogenes) are known to act by inhibiting tumor development, examples include *APC*, *DPC4*, *RB1*, *NF2*; (2) the mutator genes such as *BRCA1* or *BRCA2* constitute a class of genes that is involved in DNA repair and replication. Their mutated versions cause several cellular damages, including carcinogenesis (Clark and Pazdernik 2016). In both cases of mutator genes and anti-oncogenes, the two mutant alleles of these genes can lead to malignant transformations (Caldas and Venkitaraman 2013); and (3) the proto-oncogenes are a group of genes that are normally responsible for regulating cell division. In this case, a single mutant allele may be sufficient to induce uncontrolled cell division. Several mammalian genes are targeted by PAHs, *ras* proto-oncogenes (family of genes encoding a GTPase), for example, whose mutation by PAHs compounds including benzo[j]anthracene, benzo(b)fluoranthene, benzo[a]pyrene, dibenzo[al]pyrene, etc. leads to cancer formation (Ross and Nesnow 1999). In another study, it was suggested that PAH *o*-quinones can inactivate the tumor suppressor gene *p53* leading to lung cancer (Shen et al. 2006).

12.2.3.2 Toxicity of PAHs on Human System

The toxicity of PAHs on human health has been extensively studied and documented for decades. Among the several hundred of PAHs that may be formed, 15 PAHs, namely benz[a]anthracene (BaA), benzo[b]fluoranthene (BbFA), benzo[j]fluoranthene (BjFA), benzo[k]fluoranthene (BkFA), benzo[ghi]perylene (BghiP), benzo[a]pyrene (BaP), chrysene (CHR), cyclopenta[cd]pyrene (CPP), dibenz[a,h]anthracene (DBahA), dibenzo[a,e]pyrene (DBaeP), dibenzo[a,h]pyrene (DBahP), dibenzo[a,i]pyrene (DBaiP), dibenzo[a,l]pyrene (DBalP), indeno[1,2,3-cd]pyrene (IP), and 5-methylchrysene (5-MCH) are known for their mutagenic/genotoxic effects in experimental animals (EFSA 2008). Out of these 15 compounds, 7, namely BaA, BbFA, BkFA, BaP, CHR, DBahA, and IP are categorized as carcinogenic (IARC 2010). The sources of PAHs human exposure include air (inhalation), contact through the skin, and diet (ingestion). With respect to the exposure through ingestion of food, the EFSA (2008) estimated that the average intakes in Europe of eight PAHs (PAH8) (BaP, BaA, BbFA, BkFA, BghiP, CHR, DBahA, and IP), which are carcinogenic indicators in food, were from 28.8 to 51.3 ng/kg by weight per day. The manifestations of PAHs toxicity on the physiological, cellular, and molecular levels are many and varied; they include acute symptoms such as nausea, vomiting, and eye irritation, or chronic symptoms such as fatal or non-fatal cancers, immune and hematological function disorders, organ (kidney, liver) damage, cataracts, etc. (Rengarajan et al. 2015). Table 12.2 summarizes some adverse effects of PAHs on human health.

12.3 Biochemical Mechanisms for the Microbial Degradation of PAHs

The popularity of bioremediation which combines fundamental biochemical mechanisms and engineering techniques is increasing because of the advantages it offers in terms of low-energy and resources consumption, making it more sustainable than conventional physicochemical treatment methods such as incineration, coagulation, and adsorption. Biological processes possess even the capability of converting the pollutants into their inorganic constituents (H_2O, CO_2, SO_4^{2-}, etc.). However, biological techniques are often slow and incomplete, so it is advisable to combine them with other remedial treatments. These methods are, however, the most respectful of soil quality (Azubuike et al. 2016).

Once released in the environment, especially in soils, PAHs are subject to different processes including adsorption, photolysis, volatilization, and chemical/biological oxidation (Ghosal et al. 2016). The biodegradation of PAHs can occur both aerobically and anaerobically in different environmental matrices, such as water, soil, and sediments. The adsorption of PAHs on microorganisms followed by their biotransformation is by far the major process determining their fate. Notable

Table 12.2 Some human health disorders associated with PAHs organism

Adverse effects	PAHs	Effects	References
Cytotoxicity	A mixture of benzo[a]pyrene, benzo[b]fluoranthene, acenaphthylene, Phenanthrene, benz[a]anthracene, benzo[ghi]perylene, dibenz[a,h]anthracene, and fluoranthene	Significant reduction of the viability and the cell cycle S-phase of the MDA-MB-231 human breast cancer cell line.	Smith et al. (2019)
	1-methyplyrene and perylene	Significant decrease of the viability and proliferation of keratinocytes, and increasing of their cell apoptosis.	Bahri et al. (2010)
Neurodegeneration	Pyrene, naphthalene, phenanthrene, and fluorene	Decrease in caudate and pallidum volumes, verbal learning and memory function.	Cho et al. (2020)
Cancer	PAHs of tobacco smoke	Early age leukemia.	Ferreira et al. (2012)
	PAHs in coal combustion products	Lung cancer	Straif et al. (2005)
Teratogenicity	PAHs of tobacco smoke	Craniosynostosis (birth deformity in which the bones in an infant's skull join together prematurely).	O'Brien et al. (2016)
		Congenital heart defects.	Patel et al. (2020)
Immunotoxicity	Benzo[a]pyrene	Suppression of the expression of *DGAT2* (a gene responsible for immune response).	Iwano et al. (2010)
		Apoptosis induction on B lymphocytes.	Salas and Burchiel (1998)

progress has been accomplished toward understanding the microbiological transformation of PAHs, which is performed by a metabolically and phylogenetically versatile group of microorganisms that spans from halophilic archaea (*Halobacterium*, *Haloferax*, etc.) to proteobacteria (Alphaproteobacteria (*Agrobacterium*, *Beijerinckia*, etc.), Betaproteobacteria (*Achromobacter*, *Acidovorax*, Alcaligenes, etc.), Gammaproteobacteria (*Escherichia*, *Enterobacter*, *Klebsiella*, *Pseudomonas*, *Proteus*, etc.) and fungi (*Neurospora*, *Mucor*, *Saccharomyces*, *Scedosporium*, *Penicillium*, Sordaria, *Candida*, *Cladosporium*, *Helicostylum*, etc.) (Prince and Drake 1999; Ghosal et al. 2016). Besides, several microalgae species have been reported to be capable of degrading different PAHs.

Examples include *Chlorella kessleri* (benzo[a]pyrene) (Takáčová et al. 2014), *Oscillatoria* sp. and *Chlorella* sp. (pyrene) (Aldaby and Mawad 2019), and *Selenastrum capricornutum* and *Scenedesmus acutus* (benzo[a]anthracene and benzo[a]pyrene) (García de Llasera et al. 2018). The microbial involvement in PAHs biodegradation has been well established through studies over the past half century. Low-molecular-weight PAHs are known to be highly reduced; they can therefore easily serve as catabolic electron/proton donors in aerobic biodegradation processes mediated by various microorganisms. The microorganisms that are capable of biodegrading PAHs compounds under aerobic conditions (with oxygen) are usually indigenous of contaminated environments. Besides, several in vitro and in situ studies have reported that biodegradation of PAHs compounds can also take place anaerobically (without oxygen) involving different terminal electron-accepting compounds including ferric ions, manganese (IV) oxide, nitrate, carbon dioxide, and sulfate (Meckenstock et al. 2016). With respect to the extent of PAHs transformation, two situations can exist. The first situation refers to biotransformation process that slightly affects the chemical structure of the contaminant, particularly in pure cultures, and can be injurious to cells by diverting energy from growth and metabolite production. In the second situation, there is a complete biodegradation (mineralization) of the contaminant yielding harmless inorganic compounds. During contaminant biodegradation processes, energy production and cellular growth do not systematically occur. This depends on the extent of the biodegradation process itself (biotransformation or mineralization). Thus, complete biodegradation of the contaminant is often associated with energy and/or biomass production through primary metabolic processes, while biotransformation which occurs co-metabolically in the presence of growth substrate (glucose, ethanol, citrate, etc.) cannot support cellular growth (Tazdaït et al. 2013, 2015; Salah-Tazdaït et al. 2018). On the other hand, release of metabolites that are similarly or more toxic than the parent contaminant is usually observed during biotransformation, which may pose health risks when released in the environment. Thus, particular care should be taken in performing biodegradation reactions by identifying final by-products to ensure that bioremediation objectives are being reached. Besides, it should be mentioned that the rate of biodegradation of PAHs is inversely related to their molecular mass, which means that compounds with high molecular weight are less rapidly degraded than those with low molecular weight (Prince and Drake 1999). On the other hand, the biodegradation rates of PAHs depend not only on microbial genetic capabilities and the culture conditions, but also on the factors that influence their bioavailability. Perhaps the most important factor that impedes the biodegradation rate of PAHs is their sorption to solid materials, resulting in limitations in interphase mass-transfer.

In this part of the chapter, a description of some recent studies dealing with the biotreatment of PAHs through aerobic and anaerobic biodegradative approaches will be presented.

12.3.1 Aerobic Biodegradation of PAHs

The highly reduced nature of PAHs, especially those with low molecular weight, makes them likely to serve as energetic substrates (electron donors) in aerobic degradation reactions; however, the very low water solubility of high-molecular-weight PAHs strongly limits their biodegradation. In the presence of oxygen, the biodegradation of PAHs by microorganisms is commonly initiated through one of the three general strategies in which molecular oxygen (O_2) is inserted into aromatic rings: (1) oxidation performed by dioxygenases, (2) oxidation by methane monooxygenase, and (3) oxidation by cytochrome P450 monooxygenases.

12.3.1.1 Oxidation by Dioxygenases

The microbial dioxygenases act by inserting two atoms of oxygen into different aromatic substrates, including phenanthrene, anthracene, naphthalene, chrysene, fluorene, etc. (Muratova et al. 2014; Jiang et al. 2018; Vaidya et al. 2018; Rabodonirina et al. 2019). Under oxic conditions, biodegradation of these compounds occurs through analogous pathways in bacteria (Prince and Drake 1999). For instance, in the case of anthracene biodegradation, the dioxygenase, which requires NADH, H^+ as a co-reactant, adds two hydroxyl groups to yield *trans*-1,2 dihydroxy-1,2-dihydroan-thracene, which is then opened between the hydroxyl groups through ortho cleavage reaction yielding 2-hydroxynaphthalene-2-carboxylic acid, whose further degradation leads to the formation of central metabolic intermediates (pyruvate, acetaldehyde, and formate) for cell growth and proliferation (Fig. 12.3). Several microbial strains were reported to initiate PAHs biodegradation with the usage of dioxygenases. A rhizobacterial strain (*Ensifer meliloti* P221) isolated from the roots of *Phragmites australis* (common reed) cultivated in soil contaminated by PAHs was capable of phenanthrene degradation through two pathways. The first pathway involves the cleavage of the terminal aromatic ring and yields benzocoumarin and 1-hydroxy-2-naphthoic acid, and the second pathway yields 2,2′-diphenic acid through central aromatic ring oxidation. The biodegradation of phenanthrene was observed in inverse dose-dependent manner with a maximum biodegradation rate of 60% at initial phenanthrene concentration of 0.1 g/L. The ability to utilize phenanthrene as an efficient carbon and energy source was also noted (Muratova et al. 2014). Another study, testing the potential of 15 halophilic Archaea strains to degrade three PAHs (naphthalene, phenanthrene, and pyrene), found that nine isolates, namely, *Haloarcula hispanica*, *Haloferax* sp. (two isolates), *Halobacterium piscisalsi*, *Haloarcula* sp., *Halobacterium salinarum*, *Halorubrum ezzemoulense* (two isolates), and *Halorubrum* sp. were able to use the three PAHs for their growth as a source of carbon and energy through the expression of catechol 1,2 dioxygenase and/or protocatechuate 3,4 dioxygenase, which acted via *ortho* cleavage pathway (Erdoğmuş et al. 2013). Besides, the authors noted that no growth was observed in the presence of 200 ppm pyrene and that no *meta* cleavage pathway intermediate was detected. In another study, *Cycloclasticus* sp. strain P1 (MCCC 1A01040) isolated from deep-sea sediments (Pacific Ocean) was shown to be capable of degrading 500 ppm naphthalene, phenanthrene, and

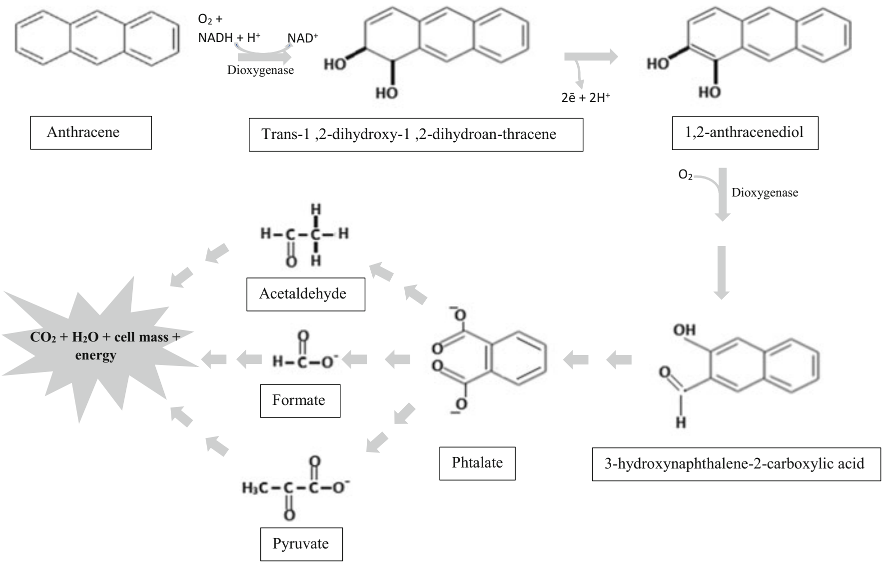

Fig. 12.3 Biodegradation of anthracene via initial dioxygenase-catalyzed reactions that lead to pyruvate acetaldehyde and formate (central metabolic pathway intermediates). Note that successive arrows designate multiple reactions

pyrene, used as the sole carbon source, after 5 days incubation with removal percent values of 98%, 92%, and 57%, respectively. Genome and transcriptome-based analysis revealed that nine ring-cleaving dioxygenases were differentially entailed in the degradation of the following six PAHs: phenanthrene, naphthalene, pyrene, biphenyl, anthracene, and fluoranthene. Besides, it was found that the dominant intermediate metabolites during naphthalene, phenanthrene, and pyrene biodegradation were salicylaldehyde, 1,2-naphthalenediol, salicylic acid, and catechol, implying that the degradative pathway in *Cycloclasticus* sp. strain P1 is common to the three PAHs tested (Wang et al. 2018). More recently, out of 20 bacterial isolates from PAHs-contaminated sediments, only 3 (*Pseudomonas aeruginosa*, *Pseudomonas* sp., and *Ralstonia* sp.) were successfully tested for their ability to degrade a mixture of 20 mmol/L pyrene, phenanthrene, and fluoranthene thanks to different catabolic genes identified in their genomes, especially *C12O* and *C23O* that encode catechol 1,2-dioxygenase and catechol 2,3-dioxygenase, respectively (Sangkharak et al. 2020). Another recent study isolated from compost mixed with crude oil sludge 55 fungal isolates belonging to *Fusarium*, *Aspergillus*, *Bionectria*, *Exophiala*, *Galactomyces*, *Mucor*, *Penicillium*, *Geotrichum Trichoderma*, *Trichurus*, and *Doratomyces*, and 36 bacterial isolates pertaining to the following genera: *Stenotrophomonas*, *Pseudomonas*, *Bordetella*, *Brucella*, *Bacillus*, *Achromobacter*, *Advenella*, *Klebsiella*, *Mesorhizobium*, *Mycobacterium*, *Ochrobactrum*, *Pusillimonas*, and *Raoultella* and holding the *C230* genes. It was found that the entire fungal isolates and almost all the bacterial isolates were capable of metabolizing 17 PAHs compounds, identified in the crude oil sludge, as sole source of carbon and energy. The isolates pertaining to *Fusarium* sp., *Galactomyces*, *Bacillus*, *Pseudomonas* sp., *Aspergillus*, *Bionectria*, *Pusillimonas*, *Mucor,* and *Achromobacter* were the best effective in degrading PAHs. Interestingly, some PAHs (benzo[a]pyrene, benzo[a]anthracene, dibenzo[ah]anthracene, benzo[b]fluoranthene, benzo[k]fluoranthene, indeno[1,2,3,cd]pyrene, and chrysene) were even completely degraded (mineralized) within 4 months of composting (Obi et al. 2020).

12.3.1.2 Oxidation by Methane Monooxygenase

Methane monooxygenases (MMO), which catalyze the conversion of methane to methanol through oxidation reaction, have been particularly highlighted in a particular group of bacteria called methanotrophic bacteria (or methanotrophs) that use methane as sole substrate for growth and energy production (Ro and Rosenzweig 2018). The methanotrophs harbor two forms of MMO: membrane-associated (particulate) (pMMO) form and cytoplasmic (soluble) (sMMO) form, the latter is efficient in oxidizing a large number of organic compounds including PAHs (Pandey et al. 2014). Several studies have dealt with investigating the potential of using methanotrophic bacteria in environmental remediation. sMMO initiates the degradation of PAHs by inserting into their C-C bonds single oxygen atom, the other oxygen atom is transformed into H_2O through reduction by NADH + H^+ (reduced nicotinamide adenine dinucleotide). In a study by Rockne et al. (1998), a marine methanotrophic enrichment from Eagle Harbor in Puget Sound (Washington) was used in phenanthrene, anthracene, pyrene, and fluorene degradation. It was found that phenanthrene, fluorene, and anthracene were completely degraded within 6, 11, and 15 days, respectively, while pyrene was not transformed at all, very likely because of its low solubility in the culture medium. In a more recent study, it was clearly showed that sMMO from *Methylosinus trichosporium* OB3b was capable of significantly oxidizing naphthalene in the presence of 1μM copper (II) chloride and methanobactin from *Methylocystis* sp. strain SB2 (Ul-Haque et al. 2015).

12.3.1.3 Oxidation by Cytochrome P450 Monooxygenases

Cytochrome P450 monooxygenases are found in practically all living organisms, including prokaryotes, viruses, fungi, mammals, and plants, in which they are categorized into ten different classes (Finnigan et al. 2020). They are harbored by most eucaryotes, mainly by non-lignolytic fungi and, at a lesser extent, by bacteria (Prince and Drake 1999). Several PAHs have been shown to be metabolized through various P450 microbial systems. In a study by Luo et al. (2016), a P450 monooxygenase (CYP108J1) from *Rhodococcus* sp. P14 showed oxidizing activity toward 10μg/mL high-molecular-weight PAHs, namely phenanthrene, anthracene, and benz[a]anthracene used as the sole source of carbon, through the appearance of their respective metabolites 1-hydroxyphenanthrene, 9,10-anthracenedione, *o*-hydroxybiphenyl, and benz[a]anthracene-7,12-dione. Tao et al. (2020) have attempted to improve the catalytic efficiency of P450 monooxygenase (CYP116B3) belonging to *Rhodococcus ruber* DSM 44319 using site-directed and saturation mutagenesis strategies. The mutant CYP116B3 with three mutations (E88C/N199Q/Q209A) engineered by the authors significantly enhanced 14-fold the oxidation of naphthalene to generate 1-naphthol compared to the control (wild type). In another study, Ostrem Loss et al. (2019) tested the capability of 13 different fungal strains belonging to the following species, namely *Aspergillus nidulans*, *A. fumigatus*, *A. oryzae*, and *A. flavus* to degrading 200μM BaP (classified as a Group 1 carcinogen to humans). All the four species tested showed degradative capabilities with different degrees. *A. oryzae* and *A. nidulans* were the most effective degraders with 95% and 92% efficiencies, respectively, obtained within 7 days cultivation. Besides, the authors identified and characterized a P450 monooxygenase (CYP BapA), which is required for BaP degradation by *A. nidulans* and whose genetic expression is controlled by two fungal NF-κB-type *velvet* regulators (VeA and VelB).

12.3.2 Anaerobic Biodegradation of PAHs

Under anaerobic conditions, PAHs oxidation has been described extensively within sulfate- and nitrate-reducing bacteria tested either in pure or mixed cultures. Carboxylation or methylation reaction is known to be the key step in the biodegradation of PAHs under anaerobic conditions. As examples of studies dealing with anoxic PAHs biodegradation, the one of Rockne et al. (2000) who succeeded in degrading (mineralizing) 15.6μM naphthalene in the presence of nitrate used as the sole electron acceptor by two pure cultures of bacterial isolates phylogenetically related to *Pseudomonas stutzeri* and *Vibrio Pelagius*. Kumar et al. (2009) reported the application of central composite design for the optimization of the biodegradation of a mixture of five PAHs (fluoranthene, naphthalene, phenanthrene, fluorene, and pyrene) by a consortium of mesophilic sulfate-reducing bacteria in batch experiments. The results revealed the following optimum conditions: acidic pH between 4 and 6.5, ethanol concentration <35 mg/L and biomass concentration >65 mg/L provided the maximum PAHs biodegradation values of 43% (naphthalene), 78% (fluorene), 76% (phenanthrene), 81% (fluoranthene), and 81% (pyrene) within 27 days of cultivation for an initial concentration of 50μg/g of soil for each PAH. In a more recent study, Zhang et al. (2019) tested different electron acceptors including nitrate, sulfate, iron, and bicarbonate for the biodegradation of phenanthrene by different bacterial communities enriched from petroleum contaminated soil, coking sludge, and domestic sludge. It was found that the degradation rates of phenanthrene under different redox conditions were in the order nitrate > sulfate > methanogenic > iron. Moreover, it was verified that carboxylation reaction represents the first step during anoxic phenanthrene biodegradation followed by ring reduction and cleavage.

12.4 Mechanisms of Phytoremediation of PAHs

Phytoremediation is a set of techniques applied to the treatment of polluted soils using plants and associated microorganisms to immobilize (via accumulation or sequestration), extract or transform (biodegradation, volatilization) pollutants (Yan et al. 2020). A new way of treating polluted soils, phytoremediation has aroused over the past 10 years of growing interest. If we currently have technologies valid for the treatment of water and soils, on the other hand, the strong interactions with the organo-mineral make it difficult to extract or inactivate soil pollutants from available technologies, without altering the properties of the medium. Thus, due to their potential to act on soil pollutants, plants are viewed with a new look. With the Sun as a source of energy, a root system "Prospector" and a moderate impact on the intrinsic properties of the soil, this type of approach appears economically and ecologically very attractive for managing contaminated sites. The rhizosphere is particularly involved in the degradation of PAHs. Plants adsorb, accumulate, or volatilize these compounds, they also increase the microbiological activity of the rhizosphere in the soil (Guo et al. 2017; Dai et al. 2020).

The use of phytoremediation for the treatment of sites polluted by PAHs shows satisfactory results. Some plants have been proposed as candidates for the phytoremediation of soils polluted by PAHs: sunflower (*Helianthus annuus*), Indian mustard (*Brassica juncea*), fescue (*Festuca arundinacea*), alfalfa (*Medicago sativa*), ryegrass (*Lolium perenne*), etc. (Ndubueze 2018; Dai et al. 2020; Reddy et al. 2020). Mechanisms of phytoremediation of PAHs can be summarized as follows:

12.4.1 Penetration and Mobility of PAHs in the Plant

The fate of a PAH arriving at a barrier depends on the physicochemical properties of the compound (molecular weight, dissociation constant, etc.), and the affinity of the various constituents of the barrier for the molecule considered. Thus, the greater this affinity, the more the PAH will be stored in this compartment, and the less chance it will have of progressing in the plant unless the adsorption capacities of the barrier are saturated.

12.4.1.1 The Cuticle

The cuticle is present on all non-lignified aerial structures. It is composed of a surface layer, the epicuticle, made up of waxes, and a deeper layer made up of cutin and waxes. Waxes are made up of a complex assembly of monomers including long-chain alkanes, alcohols, ketones, aldehydes, acetals, and acids (Racovita and Jetter 2016). Cutin, another compound of the cuticle, is a bio-polyester, a low-polar polymer made up of two families of hydroxylated and epoxidized fatty acids. These two families are respectively made up of C16 fatty acids and C18 fatty acids (Bakan and Marion 2017). Waxes constitute a hydrophobic pole. Cutin is a relatively hydrophilic polymer therefore capable of excluding the passage of PAHs.

Thus, the architecture of the cuticle determines two entry routes for xenobiotics, depending on their polarity. PAHs, very lipophilic molecules, can reach cell walls via waxes. They are also highly likely to be retained there, just as at the level of the cutin, by Van der Waals bonds or covalent bonds. The composition of the cuticle will therefore govern the passage of xenobiotics. This can vary according to the stages of development of the plant because the composition of the cuticle changes over time (Li et al. 2017).

12.4.1.2 Suberin

Suberin is the constituent polymer of protective barriers for underground parts, injured surfaces, and certain internal organs, such as the Caspary frame. It is a polymer close to lignin, rich in phenolic and aromatic functions. Due to its polyester nature, suberin behaves as a weak cation exchanger. The suberin constitutes, although it is close to lignin which is a hydrophilic polymer, one of the lipophilic poles of the cellular limits at the level of the roots, capable of allowing the adsorption of the PAHs to the root surfaces (Fismes et al. 2002; Guarino et al. 2019).

12.4.1.3 Pecto-Cellulosic Walls

They are present in all cells, including root hairs and stomatic cells. These walls can behave like a physical trap for molecules, thanks to the meshes of the constituent polymers, or like a chemical trap by binding to the constituents. PAHs are first adsorbed on the surface of the pecto-cellulosic walls and then diffuse inside the plant cell (Kang et al. 2010).

12.4.1.4 The Plasma Membrane

This barrier regulates the mobility of water and solutes in the plant. The plasmalemma is made up of a lipid bilayer in which proteins are fully or partially embedded. The geometry of this membrane is variable. The two properties governing the passage of chemical species through the plasma membrane are lipophilicity, expressed by the water-octanol partition coefficient (log K_{ow}), and the dissociation constant pK_{a}. PAHs have log K_{ow} values greater than 3 and are not ionized. Therefore, these molecules are unlikely to cross the plasma membrane. The mobility of molecules having crossed the plasmalemma would thus be reduced and confined to the apoplasm of the xylem vessels (Achten and Andersson 2015; Schriever and Lamshoeft 2020).

12.4.2 Root Absorption of PAHs

The entry of PAHs into the root can take place by passive-type processes, based on transpiratory flow and the phenomenon of diffusion, or by active processes, involving specialized structures or not (Fismes et al. 2002; Zhang et al. 2017). In the first case, the entry phenomenon can be correlated with the physicochemical properties of the molecule considered. In the case of PAHs, the literature does not mention the existence of a transporter; the entry of these compounds into the root is probably done by a ripple effect following the water flow.

12.4.3 Transfer of PAHs from the Roots to the Aerial Parts

Many authors have demonstrated the transfer of PAHs in plants (Sonowal et al. 2018). These studies indicate the passage of PAHs in the plant as much by the root route as by the foliar route, followed by transfer and mobility in the plant; the transfer is based on the physicochemical properties of the molecules. The transfer of molecules from the root to the aerial parts occurs via passage through the xylem. The higher the lipophilicity of the compound, the more difficult the access to the transpiratory flow is, even if there is an equilibrium of concentration at the level of the root (Fismes et al. 2002). On the other hand, the pollutant is in contact with the walls of the xylem. Consequently, translocation to the leaves will depend on the capacity of the wall constituents to retain pollutants. These will reach the leaves when the sorption capacity of the vessels is saturated (Castello et al. 2014). Assuming that the mobility of PAHs in the plant is passive, depending on the transpiratory flow, the relationship between the water flow and the transport of pollutants is described by the TSCF (Transpiration Stream Concentration Factor) which corresponds to the ratio between the amount of pollutant in the aerial parts per milliliters of water transpired and the amount of pollutant in the external solution. The TSCF is generally less than 1 for xenobiotics, which is indicative of passive transport. It can be related to the log K_{ow} (Schriever and Lamshoeft 2020). Since PAHs have log K_{ow} values greater than 3, they are unlikely to be transferred to aerial parts of plants (Dettenmaier et al. 2009).

12.4.4 Passage of PAHs into the Plant from the Leaves

PAHs are semi-volatile compounds present in the atmosphere in gaseous form. They are therefore likely to penetrate the plant following contamination of the leaves by dry or wet deposition of particles (Zha et al. 2018). The foliar penetration route could thus be a major route of contamination of plants by PAHs due to:

1. The total area of the foliage.
2. The affinity of the constituents of the surface of the leaves for lipophilic compounds.
3. The capacity to penetrate in gaseous form at the level of the stomata (Lehmann and Or 2015; Terzaghi et al. 2020).

12.4.5 Fate of PAHs in the Plant

In plant cells, PAHs seem to follow two main pathways: either they are passively retained by the parietal compounds, or they enter a process of biotransformation.

The metabolism of PAHs in the plant takes place in three phases:

Phase I: is an oxidation phase, during which the molecule is functionalized. This step is catalyzed by enzymes of the cytochrome P450 monooxygenase type (heme proteins located in the endoplasmic reticulum) and peroxidases (Chroma et al. 2002; Kumar et al. 2012).

Phase II: proceeds by conjugation to various water-soluble molecules, including sugars, amino acids, ellagic acid, and glutathione, through the action of glutathione transferases.

Phase III: aims to eliminate the xenobiotics in its conjugated form. Two processes seem to co-exist: either incorporation into plant constituents such as lignin, or compartmentalization in vacuoles. The enzymes, that catalyze the polymerization of lignin, are nonspecific, so aromatic and reactive molecules such as PAH metabolites can enter the process (Koh et al. 2000; Alagić et al. 2015; Jafari et al. 2018).

12.5 Conclusion

Although research efforts dealing with PAHs have gained a wide interest in the last several decades leading to a great advance in knowledge of physiological, biochemical, and microbiological processes of their decomposition, supplementary studies into the genetic/epigenetic regulation mechanisms are still needed. It was demonstrated that many phylogenetically diverse microorganisms are able to use them as a source of carbon and energy for growth. The screening of new and more effective microbial strains and plants species from nature remains a focal topic for researchers and engineers to decontaminate the environment from residues of PAHs. On the other hand, implementation of innovative process engineering will greatly help expanding the currently limited application of bioremediation to PAHs in real field conditions. Further efforts should also be made to target factors that may limit the biotransformation of PAHs in the environment. This involves in particular having a better understanding of the kinetics and stoichiometry of microbial degradative reactions.

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Biogenic Nanoparticles and Strategies of Nano-bioremediation to Remediate PAHs for a Sustainable Future

13

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Abstract

Polyaromatic hydrocarbons (PAHs) are considered as dangerous contaminants in water and soil, which are highly toxic, and also carcinogenic to living organisms including humans. The concerns on the PAHs removal are increased due to the difficulties in their removal from contaminated water and soil. Bioremediation technology is the most promising, cost-effective, and eco-friendly approach to

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remove the hydrocarbons by using potential microorganisms. Nevertheless, the existing bioremediation technology has important limitations, such as, poor efficiency of microbial communities in the field, and lesser bioavailability of pollutants. To overcome these issues, advanced nano-biotechnology could be used. In recent studies, functionalized biogenic nanomaterials have shown possible PAH removal efficiency by adsorbing/desorbing them. Also, nano-sized photocatalysts can be used for photocatalytic oxidation of adsorbed or separated PAHs. Combining these integrated approaches will make a significant impact on the bioremediation of PAH contaminants. Nano-bioremediation could play an important role in mobility, micelle formation, and increasing bioavailability, which will assist in the removal/utilization of PAHs by biological (i.e., using microorganisms) or physicochemical (i.e., photocatalysis) methods.

Keywords

Polyaromatic hydrocarbons · Bioremediation · Biodegradation · Biosurfactant · Eco-friendly · Bioavailability

13.1 Introduction

Globalization along with enormous anthropogenic actions leads to the accumulation of toxic contaminants/pollutants into the environment. These toxic contaminants are very harmful to living organisms including human beings (Behera et al. 2018). Common toxic contaminants which are making major issues to the environment are hydrocarbons (majorly from crude oil), non-degradable plastic, heavy metal accumulations, polycyclic aromatic hydrocarbons (PAHs), etc. Most of these contaminants are classified as carcinogenic and mutagenic to most living organisms (Sajid et al. 2021). Among the hydrocarbons, PAHs are classified as the most dangerous pollutants due to their impact on the environment and health issues (Sarma and Prasad 2015).

PAHs are classified as persistent organic hydrocarbons with two or more fused aromatic rings. PAHs are frequently encountered naturally or by man-made actions (Sarma et al. 2016a, b). The main source of PAHs in environments are crude oil spill, apart from this PAHs are entered into the environment by inadequate incineration of many organic materials including coal, petrol, wood, natural gas, garbage, used lubricating oil, waste incineration, petroleum spill/discharge, etc. (Sarma and Prasad 2016; Muangchinda et al. 2018; Sarma et al. 2019). PAHs from used motor oil makes an enormous impact on the environment due to inappropriate dumping into the soil. A study by Paneque et al. (2020) describes, 16 types of PAHs were identified and all these PAHs are considered to be most toxic to nature since their disposal is closely relevant to human activities, which makes the chance of inhalation by human beings very common and may perhaps cause serious health problems. Partial burning of organic materials gives out about 100 different types of PAHs, which are the primary pollutants. PAHs are found in varying levels in soil from 1μg to 300 g/kg, depending on the sources of the pollutants (Bamforth and Singleton 2005).

The life-threatening concerns regarding the PAHs is their ubiquity in air, soil, water, and aquatic sediments along with huge dwell periods in the environment (Mrozik et al. 2003). PAHs are solely responsible for the numerous health issues in human beings including cancer, nausea, anemia, abdominal pain, etc. (Chaudhary et al. 2018). Also they cause numerous antagonistic changes to aquatic living matters, including causing several adverse effects to aquatic organisms, including growth reduction (Christiansen and George 1995), endocrine modification (Meador et al. 1995), DNA mutation (Caliani et al. 2009), and deformities in larvae and embryos (Carls et al. 2008). Removal of PAHs is very difficult due to their high hydrophobicity nature, particularly in aquatic atmospheres; they habitually get adsorbed over the particulate matter for prolonged periods (Behera et al. 2018).

13.2 Remediation Technologies of PAHs: Overview

Over the years, many approaches (physical, chemical, and biological) including conventional methods to advanced technologies are used to treat the hydrophobic PAHs contaminations from soil and water sources. In brief, incineration and in-situ thermal desorption an physical methods used for the treat coal tar, wood treatment waste by volatilizing or destroying them from contamination (Gan et al. 2009). This incineration technique working at high temperatures (900–1200 °C) destroyed more than 90% of the PAHs. The major drawback of this technology is the requirement of high energy for the incinerator off-gas control devices, and also a long period (in most cases more than 3 years) along with high operational cost (Islam et al. 2012). The next one is solvent extraction/washing; this method is highly preferable for the high molecular weight (HMW) PAHs. This method is not successful as expected, since it has few drawbacks including the high hydrophobic nature of HMW PAHs, which makes it very difficult to wash, slow desorption, and low bioavailability (Pourfadakari et al. 2019). To overcome these issues, surfactants are utilized to increase bioavailability, but which makes harmful effects on the environment. Another widely used technique is chemical oxidation. This is in-situ processing technology and preferable for both low molecular weight (LMW) and HMW PAHs. In this process, PAHs are degraded after reacted with oxidants which are injected into the soil (Lemaire et al. 2013). For this purpose, various chemical oxidants have been tested including ozone and Fenton's reagent, $KMnO_4$, H_2O_2, peroxy-acid, etc. (Cheng et al. 2016).

Among all these existing approaches, bioremediation is considered as a best alternative technology for the removal of hydrophobic PAHs. Bioremediation is accomplished great attention among the scientific and industrial sectors since it is a sustainable and green approach to treat PAHs contaminated environment (Azubuike et al. 2016). In general, bioremediation techniques are carried out in two ways such as in-situ (bioaugmentation, biostimulation, phytoremediation, and land farming) and ex-situ (using bioreactors) (Kuppusamy et al. 2016a, b). In the case of the in-situ method, degrading organisms is influenced by the physicochemical properties of the environments. At the same time for the ex-situ approach, all the parameters and conditions can be controlled, which enhances the degradation rate (Gan et al. 2009). Many factors such as cost-effectiveness, efficiency, contaminants types, complexity, time duration, and availability of resources are the major aspects that need to be considered for the selection of appropriate bioremediation methods for the removal of PAHs from the contaminated environments.

13.3 Integrated Bioremediation Approaches

Nevertheless, bioremediation is limited successive since it is a time-dependent method, high cost, low bioavailability, long duration and it is not an ideal approach for the highly contaminated environments with HMW PAHs. So, these limitations can be overcome by applying integrated approaches such as physical-chemical (for example, solvent extraction along with chemical oxidation), physical-biological (for example, solvent extraction along with bioremediation), chemical-biological (for example, chemical oxidation along with bioremediation), biological-biological (for example, enhanced bioremediation with biostimulation) and physical-chemical-biological (for example, soil washing with chemical oxidation and bioremediation) (Kuppusamy et al. 2016c). Few integrated approaches are discussed briefly here.

13.4 Electrokinetic Remediation of PAHs

One of the potential and effective integrated approaches being investigated in recent time is electrokinetic remediation. This approach is mostly applied for the treatment of the least hydraulic permeability soils. In this method, a low-intensity direct current is applied to the contaminated soil samples using appropriate electrodes. Contaminants with ionic charges transported to the oppositely charged electrode through electromigration. Besides, electroosmotic flow offers a driving power for the migration of soluble pollutants (Reddy et al. 2006). Regarding the PAHs degradation using the electrokinetic, it is not a well-established technique. The low bioavailability with the hydrophobic nature of PAHs makes it hard to separate them from soil environments using the conventional electrokinetic method. However, this problem can be rectified using the solubilizing agents (i.e., surfactants, co-solvents). At the same time, using these harmful solvents and chemical surfactants might make an adverse impact on the soil environments (Kuppusamy et al. 2016c).

13.5 Enzymatic Treatment of PAHs

Another useful approach implemented for PAHs removal is enzymatic treatment. The catalytic activity of the enzymes is an eco-friendly approach and efficient as compared to the chemical catalysts with higher reaction rates, stable at different temperature and pH ranges (Mohan et al. 2006). In a study, Wu et al. (2008) extracted laccase enzyme from a fungus *Trametes* sp. and used it to oxidize 15 priority PAHs-polluted field soils with the presence of 2,20-Azino-bis-3-ethylbenzthiazoline sulfonate as mediator. Outcomes from this study illustrated that laccase played a major role in the conversion of the toxic PAHs into the less toxic intermediate products (for example, anthracene converted as anthraquinone). If the quantity of enzyme is increased, the degradation rate is also enhanced. Recently, Perini et al. (2020) also performed laccase activity on the degradation of anthracene, benzo(a)pyrene, and naphthalene. The addition of the laccase doubled the degradation rate and converted toxic hydrocarbons into less toxic products. One of the major limitations of this method is financial constraints. This cost factor can be overcome by using advanced biotechnological approaches such as immobilization, and optimization of production conditions.

Apart from these two integrated approaches, other two methods are also existing. The first one is phytoremediation, which can be implemented using combinations of physical, chemical, and biological methods. This approach can be executed with landfarming followed by introducing potential microorganisms to degrade the PAHs and finally growing contaminants-tolerant plants in that soil. It’s considered to be a good approach but removal efficiency is comparatively very less (Huang et al. 2004; Kuppusamy et al. 2016c). Another similar approach is vermi-remediation, this method is preferable for some special case, where contaminated soil has a lesser pore size (below 1μm) and there penetration of degrading microorganism is a much difficult process. In this case, PAHs are not bioavailable. In such cases, introducing earthworms makes a huge impact in the enlarging of the pore sizes, which permits degrading microorganisms to access the PAHs efficiently (Ma et al. 1995).

13.6 Nano-bioremediation

In recent times, these integrated approaches are greatly improved by introducing advanced nanotechnology and its innovations. In this technology, nano-sized materials are introduced into the contaminants to alter their physicochemical properties. This technology is implemented with other technology such as chemical methods (increasing bioavailability with the addition of surfactants) and biological methods (biodegradation). Still, many factors need to be considered in executing this approach such as the selection of toxic-free nanomaterials, biomolecules for the functionalization, and other factors. Because many nanomaterials are synthesized using highly toxic reducing agents, which need to be avoided. This problem can be sorted out by the selection of toxic-free green and biologically synthesized nanoparticles. Biogenic nanoparticles are the preferably best choice for green and sustainable nano-bioremediation.

13.7 Biogenic Nanomaterials: Synthesis, Properties, and Importance

Nanomaterials including metal and metal oxide nanoparticles (silver, gold, zinc, copper, nickel, graphene, etc.) are widely used in many applications due to their physicochemical properties. Two broad approaches such as top-down and bottom-up are most widely used for synthesis of nanomaterials. In the top-down method, large-sized bulk materials are reduced using physical methods (i.e., sonication, mechanical milling, etc.). This method is time-consuming and very difficult to obtain a uniform size of nanomaterials (Suganeswari 2011). In the bottom-up method, nanomaterials are formulated from a molecular base. Most of the common synthesis methods such as co-precipitation and sol-gel process fall under this category only. Most commonly using reducing agents such as hydrazine and sodium borohydride are classified as a highly toxic chemical, which could be accumulated as hazardous products in the environments (Wu et al. 2011; Sadhasivam et al. 2020). This problem can be overcome by utilizing eco-friendly reducing and capping agents. In recent times, it is well documented that biological reducing and capping agents such as plant extract and microbial metabolites (from bacteria, fungi, yeast, algae, etc.) is a potential alternative to the chemical reducing agents (Owaid 2019). Nanomaterials synthesized using these reducing agents are widely used in many industrial and environmental applications (Patil and Kim 2018). In this chapter, the biogenic synthesis of nanomaterials and their impact on the bioremediation of the PAHs contaminated environments are documented. For this purpose, the biogenic synthesis of nanomaterials using various biological sources is discussed in detail. In biogenic synthesis, nanomaterials can be synthesized by intracellular or extracellular methods. Comparatively, the extracellular method is easier and highly feasible, since microbial products such as proteins, amino acids, reductase enzymes, and peptides are serving as reducing and capping agents (Subbaiya et al. 2017). It is very easy to collect these kinds of bacterial metabolites from the growth medium by using a simple centrifugation method (Fatemi et al. 2018). At the same time, the intracellular method is less preferable since it requires several steps to acquire contamination-free nanoparticles, particularly this process is started with cell lysis, repetitive centrifugation/washing to separate cell debris and nanomaterials (Patil and Kim 2018).

13.8 Bacteria-Mediated Synthesis of Biogenic Nanomaterials

As earlier said, different biological sources are being tested as eco-friendly reducing agents to synthesis of different nanomaterials. The most widely tested biological source is bacterial metabolites. In most cases, the bacterium can be easily culturable and within a short time bacterium will reach their maximum growth state. Wide ranges of bacterial strains and their metabolites are employed as simple and sustainable reducing agents. Recently, Suriyaraj et al. (2019) used the *Acinetobacter* strain a zirconium resistant extremophilic bacterial strain for the synthesis of crystalline zirconium dioxide. This synthesis process is simply done by adding the starting materials into the growth medium and metabolites released into the growth medium reduced the $ZrOCl_2$ to ZrO_2. Similarly, Jha et al. (2009) synthesized TiO_2 using the *Lactobacillus* bacterial strain. The membrane-bound oxidoreductase plays important role in the bio-reduction of oxide nanoparticles.

Diverse bacterial strains are being tested for the biosynthesis of the nanomaterials. For instance, Fayaz et al. (2011) used a thermophilic bacterium *Geobacillus stearothermophilus* for the biogenic synthesis of silver (Ag) and Au nanoparticles. This strain and their metabolites reduced metal nanoparticles without any aggregation, which might be due to the production of the capping protein in the growth medium. A study by Zhang and Hu (2018) used a marine bacterium *Bacillus* strain for the biogenic synthesis of Palladium (Pd) and gold (Au) nanoparticles. Starting materials are added in the growth medium, the reduction process is occurred along with the production of the bacterial metabolites in the growth medium. In this approach, obtained nanoparticles have a uniform size (below 40 nm). A study by Srivastava and Mukhopadhyay (2013) reported the biological synthesis of selenium nanoparticles using the non-pathogenic bacterium *Zoogloea ramigera*. Selenium oxyanions were added in the growth medium as an electron acceptor along with bacterial strain. Selenium NPs were formed extracellularly with uniform size and shape. A protein secreted by this bacterium was bound over the membrane surface and it was belonging to the oxidoreductase which is playing a key role in the reduction of $SeO_3{}^{2-}$ into Se^0. Also described that electrostatic interaction of proteins traps $SeO_3{}^{2-}$ ions over the surface of proteins and which leads to the reduction of Se nanoparticles. Similarly, Presentato et al. (2018) also synthesized biogenic Se nanoparticles using bacterium *Rhodococcus aetherivorans*. Similarly, Wadhwani et al. (2018) used *Acinetobacter* strain for the biogenic synthesis of Au and Se nanoparticles. This bio-reduction process was mediated by the lignin peroxidase enzyme produced by *Acinetobacter* strain. In another study, Tiwari et al. (2016) used a copper-resistant *Bacillus* strain isolated from copper mine for the synthesis of copper nanoparticles (Cu NPs). Similarly, Ag NPs were biologically synthesized using *Salmonella typhirium* cell extract in bright conditions (Ghorbani 2017).

13.9 Fungi-Mediated Synthesis of Biogenic Nanomaterials

Different types of fungus are found in the environments and some of them are used for the synthesis of various types of nanomaterials. Recently, Ganesan et al. (2020) used *Periconium* species an endophytic fungus for the biosynthesis of the zinc oxide (ZnO) nanoparticles. In this study, *Periconium* biomass extract was obtained from dried biomass and the crude extract was used for the reduction purpose. A similar study by Clarance et al. (2020) used *Fusarium solani* another endophytic fungus for the biogenic synthesis of Au NPs. Polypeptides and proteins secreted by *Fusarium solani* play a key role in the reduction of the Au NPs. In a study, ligninolytic fungi *Trametes trogii* was used for the biogenic synthesis of the Ag NPs. This strain produces several ligninolytic enzymes, which are playing an important role in the bio-reduction of Ag NPs (Kobashigawa et al. 2019). Similarly, biogenic Ag NPs were synthesized using an extracellular extract of two white-rot fungi, namely *Ganoderma enigmaticum* and *Trametes ljubarsky* (Gudikandula et al. 2017). In a study, fungus *Aspergillus oryzae* was used for the fermentation of the lupin. This fermented lupin extract is directly used for the biogenic reduction of Se NPs (Mosallam et al. 2018). A study by Vago et al. (2016) used filamentous fungus belonging to the genus *Aspergillus*, *Penicillium,* and *Trichoderma* for the successful reduction of Au NPs.

13.10 Algae-Based Biogenic Nanomaterials Synthesis

Algae are classified as a photoautotrophic member of eukaryotic organisms; different types of algae are spread over in seawater globally. Algae is a potential resource for nanomaterials synthesis since it is enriched with secondary metabolites (proteins, pigments, etc.). These characteristic features make them nano-biofactories for the metallic nanoparticle's synthesis (Khanna et al. 2019). Also, algae are easily available, easily cultivable, eco-friendly, and least cost. Still, very few numbers of algae species were only explored for the biogenic synthesis of nanomaterials. Polysaccharides extracted from marine algae, namely *Pterocladia capillacae, Jania rubens, Ulva fasciate,* and *Colpomenia sinuosa* were used for the reduction of Ag NPs (El-Rafie et al. 2013). An interesting study by Pytlik et al. (2017) described the usage of *Stephanopyxis turris* a unicellular diatom for the biogenic synthesis of Au NPs. This diatom was reduced Au NPs both extracellularly and intracellularly. Similarly, Gonzalez-Ballesteros et al. (2017) used *Cystoseira baccata* a brown alga for the biogenic synthesis of Au NPs. Interestingly, synthesized Au NPs are found below 10 nm only. This brown alga extract was obtained by applying the conventional reflux method and the obtained extract was directly used for the bio-reduction. Similarly, Colin et al. (2018) synthesized Au NPs using algae *Egregia* species. In another study, iron oxide nanoparticles were biologically synthesized using brown algae *Colpomenia sinuosa* and red algae *Pterocladia capillacea* extracts (Salem et al. 2019). The polysaccharides present in these algae act as reducing and capping agents. Recently, Fatima et al. (2020) synthesized biogenic Ag NPs using *Portieria hornemannii* a red alga.

Apart from these biological sources (bacteria, fungi, and algae), some of the other microbial sources (for example some species of yeast) are also being tested for the biogenic synthesis of metal nanoparticles. Overall, these biogenic nanoparticles are considered as eco-friendly, stable at diverse environmental conditions; synthesis procedure is very simple; also there is no requirement of any harmful chemical compounds. This feature makes them a supreme candidate for many of the interdisciplinary applications from medicine to environment.

13.11 Principles/Strategies of Nano-bioremediations

In the bioremediation process, nanomaterials itself play a major role in the removal of contaminants. In some cases, nanomaterials are functionalized to perform some auctions; for this purpose, some basic methods are followed such as covalent coupling of nanomaterials surface with the ligand, the non-covalent coupling of nanomaterials surface with ligand, adsorption, and co-encapsulation. In a covalent coupling, either the nanomaterials or biomolecule binds directly through a dative bond or external bound ligand attaches nanomaterials with biomolecule using a covalent bond. In continuation into the initial interaction, some other interaction might be possible like irreversible bond formation to reversible and transient interaction based on modification, fixation, etc. (Basak et al. 2020). In the non-covalent coupling biomolecules, nanomaterials are functionalized by self-assembly redox enzyme-protein complexes (Diaz et al. 2018), dock and lock mechanism (Gong et al. 2019), etc. Another famous approach is encapsulation, in this method nanomaterials remain coated inside the capsules and also moveable. This approach is very useful for materials with higher oxidation, leaching, etc. (Gross et al. 2015). The final approach is adsorption, this method is recent technology with more advantages, more likely being applied for the nano-remediation for the affected environment.

13.12 Nano-bioremediations of PAHs

Many functionalized nanomaterials are tested for the bioremediation of PAHs contaminants from the soil and water sources. Figure 13.1 describes the overall process and steps involved in the nano-bioremediation of PAHs-polluted water. This figure clearly illustrated how integrated approaches are combined in an appropriate step for the successful removal of PAHs. In a study, Laveille et al. (2010) reported how functionalized nanomaterials react with PAHs removal. In this study, the authors used mesoporous silica to immobilize hemoglobin (Hb), since free Hb has the aptitude to oxidize about 11 different types of PAHs. But the problem associated with their use in the real-time application is their sensitivity/activity beyond pH 5 (Hb is highly active at pH 5). Most of the wastewater contaminated with PAHs are ranged from pH 6.5 to 8.5. To overcome this issue, Hb was immobilized with mesoporous silica nanoparticles using a simple adsorption method (300 mg/g). In that study also pointed out an interesting factor that free Hb activity is decreased to 47% at pH 7. At the same time, functionalized Hb showed 82% PAHs removal, which makes clear that functionalization Hb in silica nanoparticles leads to higher stability towards a broad range of pH, temperature, solvents, etc. Jin et al. (2016) used green synthesized iron nanoparticles (Fe NPs) along with bacterial strain for the removal of phenanthrene and naphthalene from aqueous solution. Initially, authors tried biodegradation alone for the removal of both PAHs, for their effort naphthalene was easily degraded completely by strain *Bacillus fusiformis.* At the same time, that capability of phenanthrene degradation by the same strain was not up to mark as they

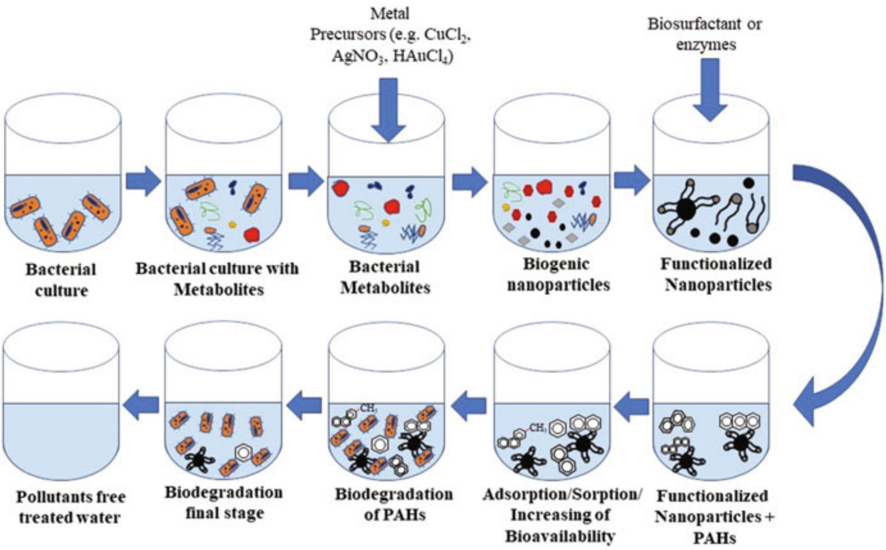

Fig. 13.1 Different steps involved in synthesis of biogenic nanoparticles and functionalization for the effective PAHs bioremediation

expected (only 28.9% removed). Further to improve the degradation efficiency tea extract reduced Fe NPs were included in the degradation systems and monitored the removal efficiency. They achieved 100% phenanthrene removal successfully using an integrated approach with bacterial strain and Fenton-like oxidization using Fe NPs.

The impact of the biosurfactant and iron nanoparticle on indeno(1,2,3-cd)pyrene (InP) biodegradation by a yeast isolate was described in an interesting report by Ojha et al. (2019). In that study, seven different isolates of yeast were applied for the biodegradation of InP. Isolates *Candida tropicalis* NN4 showed higher degradation efficiency among other isolates. Also, strain NN4 has a higher capability of biosurfactant production among other strains. Besides, iron NPs were green synthesized using mint plant leaves. Further, different reaction conditions are applied to obtain maximum removal of InP (for example with and without iron NPs and sophorolipid type of biosurfactant). Further, they added that 20 mg/L of iron NPs along with sophorolipid addition increased InP degradation up to 90%. At the same time increasing iron NPs concentration beyond 20 mg/L leads to a decrease in the degradation activity, it might be due to that higher concentration of iron NPs highly toxic to the yeast cells and may perhaps reduce their growth and development.

The use of single-walled carbon nanotubes (SWCNT) for the environmental cleanup specifically for the removal of phenanthrene in the sediment sample was done by Cui et al. (2011). In this study, they used *Mycobacterium vanbaalenii* strain for the degradation of phenanthrene. The addition of SWCNT leads to the enhanced removal of phenanthrene, it was due to that SWCNT have a higher attraction towards phenanthrene like highly hydrophobic contaminants, and the presence of SWCNT enhanced the bioavailability of phenanthrene. Also, the large surface area and high pore volume of SWCNT played important role in the sorption of phenanthrene. At the same time inclusion of dissolved organic matters (tannic acid, humic acid, and peptone) reduced the surface area by attachment of polar functional groups over the SWCNT, which reduces the sorption of phenanthrene. In a similar study, Wannoussa et al. (2015) subjected biphenyl for the biodegradation using *Rhodococcus erythropolis* with the inclusion of different metallic nanoparticles such as silver (Ag NPs), copper (Cu NPs), cobalt (Co NPs), and palladium (Pd NPs). The addition of nanoparticles alone into the degradation system leads to the agglomeration, so all the metallic NPs were anchored into the inside of microporous SiO_2 as Ag/SiO_2, Cu/SiO_2, Co/SiO_2, and Pd/SiO_2. The bacterial cultures included with Co/SiO_2 with the concentration of 10^{-4} M showed 50% higher biphenyl degradation, also improved growth and development of *R. erythropolis* strain. Further, the authors added that thermal treatment also playing a key role in the stimulating effect of biphenyl removal, since calcinated Co NPs show more effective degradation efficiency than as prepared Co NPs. Also, they summarized that the addition of Cu^{2+} or Ag^{+} ions makes a negative impact on the biphenyl biodegradation since they are toxic to the *R. erythropolis* bacterial strain than their respective metal nanoparticles anchored inside SiO_2. But the addition of Co^{2+} ions or Co NPs anchored on SiO_2 enhanced the activity of catechol 1,2-dioxygenase (a key enzyme playing a major role in the aromatic biodegradation pathway), their activity was inhibited in absence of those nanoparticles and their respective ions. This observation infers that the activity of nanoparticles and their ions are specific to each reaction condition.

In 2018, Mandal et al. (2018a) used a yeast consortium (YC04) with the combination of *Rhodotorula* sp., *Hanseniaspora valbyensis*, and *Debaryomyces hansenii* for the biodegradation of benzo[ghi]perylene with aid of ZnO nanoparticles biosurfactant. The addition of the ZnO NPs and biosurfactant into the degrading system shows improvement in the degradation efficiency with 62%. Later on, the same research group in another work used a similar concept to degrade benzo[a]pyrene. For this, they used yeast consortium (YC01) as said above but *Hanseniaspora valbyensis* was replaced with strain *Hanseniaspora opuntiae,* and the remaining two strains are the same. Yeast consortium (YC01) degraded 82.6% of benzo[a]pyrene within the 6 days of incubation period with the inclusion of biosurfactant and ZnO NPs. To obtain this much degradation efficiency growth conditions such as pH (7.0), temperature (30 °C), shaking condition (130 rpm), ZnO NPs (2 g/L), and inoculum (3%) concentrations are optimized (Mandal et al. 2018b).

In interesting integrated bioremediation, naphthalene was subjected to biodegradation using *Bacillus fusiformis*. In this study, *B. fusiformis* alone degraded about 99% of the naphthalene in 96 h of reaction time. At the same time, 59.4% of chemical oxygen demand only removed, which means that the remaining degraded metabolites still exist in the solution. For this reason, nanoscale zero-valent iron (nZVI) was applied as heterogeneous catalyst material to enhance Fenton-like oxidation of degraded products after *B. fusiformis*-mediated biodegradation process takes place (Yu et al. 2015). In a similar study, Gholami et al. (2019) used magnesium peroxide (MgO_2) nanoparticles for the nano-bioremediation of naphthalene. For this purpose, MgO_2 was encapsulated in the permeable reactive barrier (PRB) and a degradation study was conducted for 50 days and found almost complete removal within the 20 days incubation period. Microorganism responsible for this much degradation was conformed using next-generation sequencing and found *Pseudomonas putida* and *Pseudomonas mendocina*, their growth is stimulated by the addition of MgO_2 NPs in the bioremediation systems. Shanker et al. (2017) used a green approach to synthesis of iron hexacyanoferrates (FeHCF) nanoparticles. A natural surfactant rich plant *sapindus-mukorossi* was used for the synthesis of FeHCF with a size of 10–60 nm. Further, synthesized nano-sized FeHCF was used for the photocatalytic degradation of hazardous PAHs including phenanthrene, anthracene, fluorene, benzo(a)pyrene, and chrysene in both water and soil conditions. Under the solar light irradiation with 25 mg/L of catalyst concentration almost all the PAHs are fractioned into the less toxic small molecules. The degradation ranges were differed based on the PAHs for instance anthracene and phenanthrene, which were removed in the range of 80–90%. At the time benzo(a)pyrene, chrysene, and fluorene were degraded in the range of 70–80%.

Adsorption-synergic biodegradation effect of phenanthrene over the surface of multi-walled carbon nanotubes (MWCNT) buckypaper was established by Tarafdar et al. (2018). In their study, they used *Bacillus thuringiensis* bacterial strain for the degradation of phenanthrene. In general, MWCNT is highly toxic to many bacterial species but the addition of phenanthrene reduced contacts between bacterial cells and MWCNT. At the lower layer cells have to contact with MWCNT and they are disrupted, whereas at the upper layer bacterial cells are developed as a biofilm. About 93.81% of phenanthrene was degraded in presence of *Bacillus thuringiensis* and MWCNT buckypaper. This MWCNT buckypaper acts as a biological carrier or matrix which strongly supports microbial growth.

In a recent study, Wang et al. (2019) described an efficient integrated nano-bioremediation process. In that study, microbe consortium (MC) developed from sewage sludge was adjourned in the microcapsule (MI) interior space, further nano-sized photocatalyst $Ag_3PO_4@Fe_3O_4$ was anchored on the membrane of MI. These entire arrangements are called an MI-MC-photocatalyst compound system (MCS). The biocompatibility test confirmed that $Ag_3PO_4@Fe_3O_4$ makes a slight impact on soil microbe activity. This MCS degraded about 944.1 mg/kg of PAHs in 30 days, it was 49.83% higher than the control system. The addition of MCS makes a huge impact on the soil texture and microbial diversity in the contaminated areas. It also enhanced some enzyme activity more specifically dehydrogenase and hydrolase. Also, the soil toxicity was greatly decreased, which permits the germination of some seeds on the treated soil. This study clearly describes the role of photocatalyst and biodegradation process on the removal of some high molecular weight PAHs. This kind of approach and techniques facilitate sustainable environments.

Researchers from China tested the impacts and effectiveness of nano bamboo charcoal (NBC) towards the biodegradation rate of phenanthrene by bacterial strain *Sphingomonas* sp. GY2B. The addition of NBC enhanced 10.29–18.56%

degradation within 24 h of incubation and completely removed in 48 h. The addition of NBC at 20–50 mg/L enhanced the growth of *Sphingomonas* species. At the same time, increasing the concentration of NBC over 200 mg/L inhibited the growth of *Sphingomonas* strain and notable it makes a small impact in the solubilization of phenanthrene while included in low concentrations (She et al. 2016). Later on, another group of researchers focused on the role and impact of stearic acid-modified montmorillonite on the biodegradation of phenanthrene by the same bacterial strain *Sphingomonas* sp. GY2B. In the biodegradation system surface and colloidal characters of stearic acid-modified montmorillonite were altered in presence of bacterial strain, and found about 98% degradation efficiency in 2 days (Ruan et al. 2018).

Recently, Pourfadakari et al. (2019) used biosurfactants for the sorption of PAHs followed by electrokinetic oxidation for efficient removal of separated hydrocarbons. For this purpose, a halotolerant strain *Pseudomonas aeruginosa* PF2 was used for the synthesis of the rhamnolipid type of biosurfactant. This biosurfactant solution was used for the sorption of three PAHs such as anthracene, pyrene, and phenanthrene in a soil sample. Further, these desorbed PAHs were subjected to electrokinetic oxidation using the magnetite nanoparticles modified graphite. Specific pH, contact time, voltage, and electrolyte concentration are playing a key role in the removal efficiency in electrokinetic oxidation. As said, among the tested broad range of these parameters effective outcome was observed at a pH value of 5, 6 h of contact time, and applied voltage was 3 V with 25 mg/L of electrolyte concentration. These conditions show more than 99% removal efficiency of all three tested PAHs. Similarly, another recent report by Baragano et al. (2020) describes the use of commercial magnetite nanoparticles for the immobilization of PAHs in soil samples. Different concentration of nanoparticles was used such as 0.2%, 1%, 2%, and 5%. About 89% of PAHs are immobilized with 0.2% magnetite nanoparticles. After this treatment, the accumulation of iron content in the soil samples is unavoidable but the toxicity of the soil was reduced greatly.

More recently, Qin et al. (2020) used combinations of photocatalysis and biodegradation as integrated approaches for the removal. For this purpose, Cu, N-TiO_2 was coated over the polytetrafluoroethylene carriers. Microorganisms used for the biodegradation purpose were cultivated from two types of petroleum-contaminated soil (A0 and B) and biofilms are developed over the Cu, N-TiO_2 coated polytetrafluoroethylene carriers, which was further used for the photocatalytic degradation of phenanthrene. Later on, high-throughput sequencing of the 16S rRNA gene was done to find out microbial diversity in both biofilm samples and found *Lysinibacillus* as a dominant genus in the A0 sample, but in sample B genus *Pseudomonas* is more dominant. These nanocarriers enhanced the development of diverse groups of microbial strains in the biofilm sample and also these strains have actively participated in the biodegradation of phenanthrene.

Apart from these nanoscale functionalized materials, some micro-scaled materials are also used for integrated bioremediation studies. In a study, Imam et al. (2021) used rice straw biochar for the immobilization of laccase a ligninolytic enzyme, and used for the anthracene degradation. Biochar used in that study was treated with acid to make carboxyl functionality, which leads to a twofold increase in their surface area. This immobilized laccase enzyme was completely removed about 50 mg/L of anthracene in 24 h of incubations. Another study by Yang et al. (2017) used graphene oxide/Ag_3PO_4 composites as photocatalyst for the removal of three PAHs, namely phenanthrene, naphthalene, and pyrene using visible light irradiation. Within a few seconds to minutes, all the PAHs are completely removed in the solution. This much photocatalytic degradation was facilitated by superoxide radicals, photogenerated holes, and hydroxyl radicals. In a very similar manner, Cai et al. (2019) describe the use of new integrated technology called visible-light photocatalysis and biodegradation (VPCB). Efficient photocatalyst Mn_3O_4/MnO_2-cubic Ag_3PO_4 with exposed facets (MnO_x-cAP) was used in that study for the biodegradation of phenanthrene. This photocatalyst shows extraordinary degradation efficiency with 96.2% of phenanthrene removal within 20 min of reaction time. Further, elimination and mineralization were enhanced by introducing the VPCB sponge with biofilm with enriched microbial strains belonging to *Sedimentibacter, Shewanella, Acinetobacter, Comamonas,* and *Pseudomonas*. The intermediate compounds formed during the photocatalytic degradation were utilized by bacterial strains present in the biofilms. This kind of integrated approach also gives promising outcomes in PAH removal. Table 13.1 summarized the functionalized nanomaterials used for the treatment of PAHs contaminants with the mode of auction and removal efficiency.

13.13 Factors Influencing the Biogenic Nano-bioremediation Process

It's very important to choose the correct nanomaterials for the degradation of specific hydrophobic contaminants. In some cases, the addition of some nanomaterials makes an adverse impact on the bioremediation process. For example, Zhang et al. (2018) tested the impact of carbon nanomaterials (CNM) for the mineralization and degradation of phenanthrene. In the initial period, maximum mineralization rate and mineralization efficiency were positively associated with the bioavailability of phenanthrene. Notably, the addition of phenanthrene enhanced the growth of fungi and bacteria communities and catabolic gene biomarker *nidA*. The addition of CNM suppressed the sorption rate and also makes an adverse effect on the biomass of bacterial, fungal cells, and *nidA*. These findings suggest that the selection of appropriate sorption or nano-carrier is a key feature for the successful bioremediation process.

In a study, Chaudhary et al. (2018) used silica nanoparticles (SiO_2 NPs) functionalized with four various types of cationic surfactants. These functionalized silica nanoparticles are used for the removal of naphthalene a simple and white crystalline common PAHs found in most of the contaminated areas. Among the used cationic surfactants, cetyl pyridinium bromide functionalized SiO_2 NPs showed an 85% removal percentage. This removal efficiency is 35% higher than as prepared SiO_2 NPs. In this specific study, the authors used chemical surfactants, for

Table 13.1 Different functionalized nanomaterials used for PAHs bioremediations

S. no.	Nanomaterials	Functionalized compounds/ materials	PAHs types	Mode of auction	Removal (%)	Reference
1	SiO_2 NPs	Cetyl pyridinium bromide (cationic surfactants)	13 PAHs	Adsorption	85	Chaudhary et al. (2018)
2	Mesoporous silica	Hemoglobin	11 PAHs	Adsorption	82	Laveille et al. (2010)
3	Iron NPs	Sophorolipid (biosurfactant)	Indeno(1,2,3-cd)pyrene	Biodegradation	90	Ojha et al. (2019)
4	Fe NPs	*Bacillus fusiformis*	Phenanthrene and naphthalene	Biodegradation	100	Jin et al. (2016)
5	Single-walled carbon nanotubes	*Mycobacterium vanbaalenii*	Phenanthrene	Sorption	85–95	Cui et al. (2011)
6	Ag/SiO_2, Cu/SiO_2, Co/SiO_2, and Pd/SiO_2	*Rhodococcus erythropolis*	Biphenyl	Biodegradation	80	Wannoussa et al. (2015)
7	ZnO	Rhamnolipid + yeast consortium (YC04)	Benzo[ghi]perylene	Biodegradation	62	Mandal et al. (2018b)
8	ZnO	Rhamnolipid + yeast consortium (YC01)	Benzo[a]pyrene	Biodegradation	82.67	Mandal et al. (2018a)
9	Nano bamboo charcoal	*Sphingomonas* species	Phenanthrene	Biodegradation	100	She et al. (2016)
10	Stearic acid-modified montmorillonite	*Sphingomonas* species	Phenanthrene	Biodegradation	98	Ruan et al. (2018)
11	Magnetite nanoparticles modified graphite	Rhamnolipid	Pyrene, anthracene, and phenanthrene	Desorption	99	Pourfadakari et al. (2019)
12	Magnetite nanoparticles	–	PAHs	Immobilization	87	Baragano et al. (2020)

sustainable remediation this chemical surfactant can be replaced with biosurfactant (Parthipan et al. 2017a, b, c; Parthipan et al. 2018). Biosurfactants usage in the nano-bioremediation is less documented.

Many physicochemical factors are influencing the removal percentage including the adsorbent concentration, pH, naphthalene concentration, etc. In this study, it was highlighted that increasing adsorbent dose increases the removal percentage, but concentration exceeds above 25 mg/L, adsorption steadiness of naphthalene was attained. The availability of large surface area due to the restriction in growth of nanomaterials may perhaps play a major role in this enhanced removal in presence of cationic surfactant. Similarly, decreasing the pH of the reaction condition also enhanced removal efficiency. Also, many studies have proven that physicochemical properties including temperature, pH, the concentration of catalysts or nanomaterials are playing a major role in the successful remediation of PAHs contaminated environmental samples. For this reason, some studies focus on the optimization of these parameters before proceeding to the bioremediation process (Laveille et al. 2010; Mandal et al. 2018b; Pourfadakari et al. 2019b). Also, biosurfactants are the ideal option to replace the chemical surfactant.

13.14 Conclusions and Future Directions

As discussed earlier, PAHs are considered as most toxic compounds persistent in the environment due to natural and anthropogenic reasons. The toxic nature is very harmful to aquatic organisms, even human beings are also affected due to their toxic nature. Removal of PAHs is a very basic and important necessity to make this environment eco-friendly and sustainable. Their higher hydrophobicity nature makes them very complicated compounds in the bioremediation process. Conventional bioremediation techniques are not effective and time taking process. Increasing bioavailability at the PAHs contaminated area may perhaps be useful for the PAHs utilizing/degrading microorganisms. For this purpose, integrated approaches are tried for the successful removal of PAHs from soil and water sources. Many biological molecules/compounds are playing a key role in the solubilization or degradation of PAHs, but direct delivery of these biomolecules is having many troubleshoots (active or inactive to specific pH, temperature, and other physicochemical conditions that may influence their activity). To overcome these problems, functionalized nanomaterials are introduced for the adsorption or sorption of PAHs. In some cases, nano-sized photocatalysts are used for the photocatalytic oxidation of adsorbed or separated PAHs.

However, in a sustainable point of view, very limited studies are dealing with the use of eco-friendly biogenic nanomaterials for the integrated bioremediation approaches. Still, many active biogenic nanomaterials are available or can be synthesized for nano-bioremediation. Combining nanotechnology with biotechnology will promote the expansion of "sustainable-bio-nanotechnology" approaches for the cleanup of PAHs contaminated environments. For better understanding and improvement of PAHs remediation, few technologies or approaches are needed to be considered. For instance microbiological and molecular techniques can be used for the identification of potential biosurfactant producing microorganisms. Since, biosurfactants can be used as sole compounds to enhance the bioavailability of PAHs. Highly sensible, low cost, quick detecting device or biosensors are needed to be developed to detect PAHs level and types in the environmental samples, because current methods are time-consuming analytical methods which need to be developed. Overall, very little research work is only done on the concept of biogenic nano-bioremediation, this is one of the advanced integrated approaches for the removal of PAHs pollutants. More researches need to be focused on this approach to develop sustainable and environmentally friendly bioremediation technology.

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Value-Added Products from Agroindustry By-product: Bagasse

14

Aparna B. Gunjal

Abstract

The agroindustry by-products are generated in huge amount in all countries. Bagasse is by-product generated in huge amount from the sugar factories. This bagasse is either incinerated or disposed directly to the landfills, which creates environmental problems, i.e., pollution. The management of this agroindustry by-product bagasse can be done by converting into different value-added products viz., chemicals (xylooligosaccharides, organic acids, and enzymes); biofertilizer; materials (paper products, particleboard, bioplastics, textile fibers, bricks, bio-char, and bagasse ash); energy (methane, power and producer gases production, charcoal and activated unburnt carbon); fuels (bioethanol and biogas); and animal feed, which is focused in detail in this review. The management of bagasse by conversion into value-added products will be eco-friendly. This will also contribute to a significant sustainable environmental approach in the management of bagasse and also reduce the pollution. In addition, the value-added products obtained from bagasse will have industrial applications which are significant and highlighted in this review.

Keywords

Eco-friendly · Pollution · Soil conditioner · Biofuels · Bioplastics · Feed · Bio-char

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14.1 Introduction

Sugarcane production as compared to other crops is more globally. India ranks second in sugarcane production in the world (15% of the total sugarcane production in the world) (Bahurudeen et al. 2015). In 2018–2019, the world sugar production from sugarcane was 138.89 million metric tons. From this, the sugar production in India was 33.29 million metric tons (www.statista.com). In 2011–2012, 342.56 million tons of sugarcane was produced (Naik et al. 2013). The sugarcane production has enhanced 8 times over the last 50 years (Bahurudeen et al. 2015). Bagasse is agroindustry by-product obtained from the sugar factories (Bezerra and Ragauskas 2016; Munir et al. 2009). It is the sugarcane fiber which remains after extraction of juice from the sugarcane. The processing of 1 ton of sugarcane gives 280 kg of bagasse (Moreira 2004). In India, 80 million tons of bagasse is produced annually. Bagasse is lignocellulosic in nature. So, bagasse mainly comprises cellulose (45–55%), hemicellulose (20–25%), lignin (18–24%), and pectin (0.6–0.8%) (Yadav et al. 2015). It has about 45–50% moisture content. Cellulose is most crystalline. They are tightly packed due to strong intermolecular hydrogen bonds. The cellulose exists in three types viz., α, β, and γ. The α-cellulose is called pure cellulose. The β and γ cellulose together is known as hemicellulose (Marthur 1975). The hemicellulose is linked with cellulose. The third main component present in bagasse is lignin which has high molecular weight. The bagasse generated is usually incinerated or disposed directly to the landfills. This causes immense environmental pollution which is a serious issue. The state-wise bagasse generated in different states in India is represented in Fig. 14.1. Bagasse can be made to various useful products by solid state fermentation (SSF). SSF involves fermentation by the microbial process without water. In SSF, there are many substrates viz., paddy husk, pressmud, bagasse, sawdust, wheat straw, coconut shell, etc. which can be

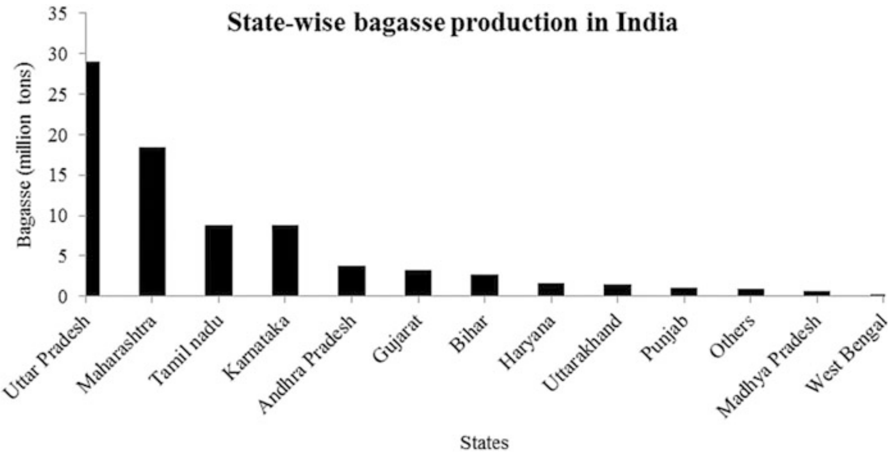

Fig. 14.1 State-wise bagasse generated in different states in India. (*Source: Indian Sugar Mills Association 2010)

used for the fermentation (Romanoschi et al. 1997). SSF generates various products having industrial and agricultural applications (Gunjal et al. 2018). The wastes can be used to generate products such as compost, soil conditioner, enzymes, animal feed, single cell protein, mushroom production, antibiotics, and plant growth promoting substances (Lizardi-Jimenez and Hernandez-Martinez 2017). The bioconversion of agroindustry by-products for various value-added products is significant and fruitful (Sindhu et al. 2016). There is a report on energy efficiency of biorefinery schemes using bagasse as substrate (Restrepo-Serna et al. 2018). This will be eco-friendly and cheap and also the value-added products obtained from bagasse will have various applications. The objective of the review here is to focus on the value-added products from agroindustry by-product, bagasse (Yang-Rui 2012). This will help in the management of bagasse and also value-added products obtained from bagasse will have immense industrial and agricultural applications.

14.2 Sugarcane Processing for Generation of Bagasse

Sugar is consumed in daily life and is important. It can also help to bring up new industries. Sugarcane is source of energy. Sugar industries provide employment and convert raw material into value-added products. Sugarcane is source of biomass for generation of biofuels. The environmental sustainability and social issues are concerned with sugar production. From the 700 sugar factories in India, about 314, 324, and 62 factories are under cooperative, private, and government sector, respectively (Patil et al. 2016). The juice is extracted from sugarcane and concentrated to get juice. The juice is further processed to produce sugar granules. After separation of the molasses by centrifugation, the fibrous material is shredded to get bagasse.

14.3 Value-Added Products from Bagasse

The different value-added products from bagasse are viz., chemicals (xylooligosaccharides, organic acids, and enzymes); biofertilizer; materials (paper products, particleboard, bioplastics, textile fibers, bricks, bio-char, and bagasse ash); energy (methane, power and producer gases production, charcoal and activated unburnt carbon); fuels (bioethanol and biogas); and animal feed. Each of these value-added products from bagasse is described below along with their applications.

14.4 Chemicals

Bagasse is a good raw material for xylooligosaccharides production (Jaypal et al. 2013). Xylose can be used in dehydration process; the useful products viz., xylitol, furfural, and hydroxymethylfurfural (HMF) are obtained (Chatterjee et al. 2010; Chheda and Dumesic 2007). Xylitol has immense uses in medical as well as food

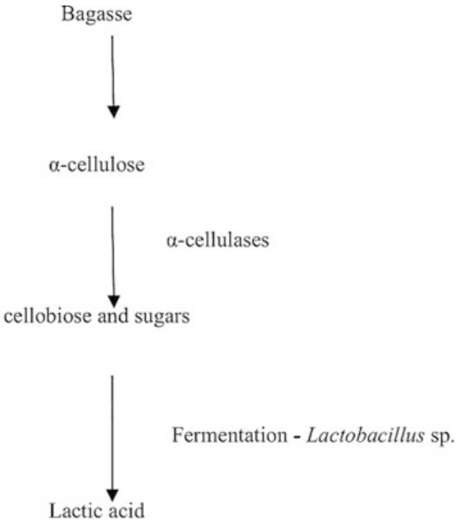

Fig. 14.2 Lactic acid production with bagasse as substrate

industries (Prakasham et al. 2009). Xylitol is also used in making chewing gums, gum drops, and bakery food. These compounds can be further processed to get important compounds. This furfural has excellent application as solvent for refining lubricating oils, and resins used for molding powders (Murugan et al. 2013).

There is a report on production of organic acids from sugarcane bagasse by hydrothermal pretreatment of bagasse using batch fermentation (Soares et al. 2018). Bagasse has attracted attention for the use in making of other acids viz., acetic acid; citric acid (Berovic and Legisa 2007); glutamic acid (Borges and Pereira 2010); lactic acid (Fig. 14.2), itaconic acid (Dodds and Gross 2007). Lactic acid production from bagasse has been reported (Adsul et al. 2007; van der Pol et al. 2016). There is a report where bagasse is used as substrate in SSF (Chandel et al. 2012) for the production of citric acid (Kumar et al. 2003) and lactic acid (Garg and Hang 1995) where the microorganisms used in SSF were *Aspergillus niger* and *Rhizopus oryzae,* respectively.

Bagasse has been used for the production of many enzymes by SSF (Parameswaran 2009). The enzymes production viz., cellulase and xylanase with bagasse as substrate by SSF has been extensively studied (Singh et al. 2010). The enzymes production using SSF with bagasse as a substrate has been reported (El-Bakry et al. 2015; Parameswaran 2009). These enzymes have various applications viz., in food industries, bakery, animal nutrition, cosmetics, medical field, and in research (Li et al. 2012).

14.5 Biofertilizer

Biofertilizers are formulations which contain beneficial microorganisms that colonize the roots of plants and improve the plant growth by direct and indirect mechanisms. The formulations of biofertilizers are made by combination of beneficial microorganisms and carrier-based inoculants. Bagasse is used as a carrier in the preparation of formulations of biofertilizers. Bagasse can be used by co-composting with other wastes to obtain useful biofertilizer. This biofertilizer is also called as "soil conditioner" which will help in the plant growth and yield, and ultimately benefit the farmers. There is a report on co-composting landfill leachate with sugarcane bagasse for biofertilizer production (Shaarani et al. 2018). Bagasse is excellent substrate in SSF to produce substances which improve the crop growth viz., hormones like indole acetic acid (IAA); gibberellins and cytokinins, enzymes, siderophores (iron chelating compounds), etc. The use of bagasse as a substrate for production of plant growth promoting substances by SSF is shown in Fig. 14.3. Bagasse by composting process can be made to a valuable biofertilizer. This biofertilizer which is rich in major and minor nutrients is essential for the plant growth (Sidana and Farooq 2014).

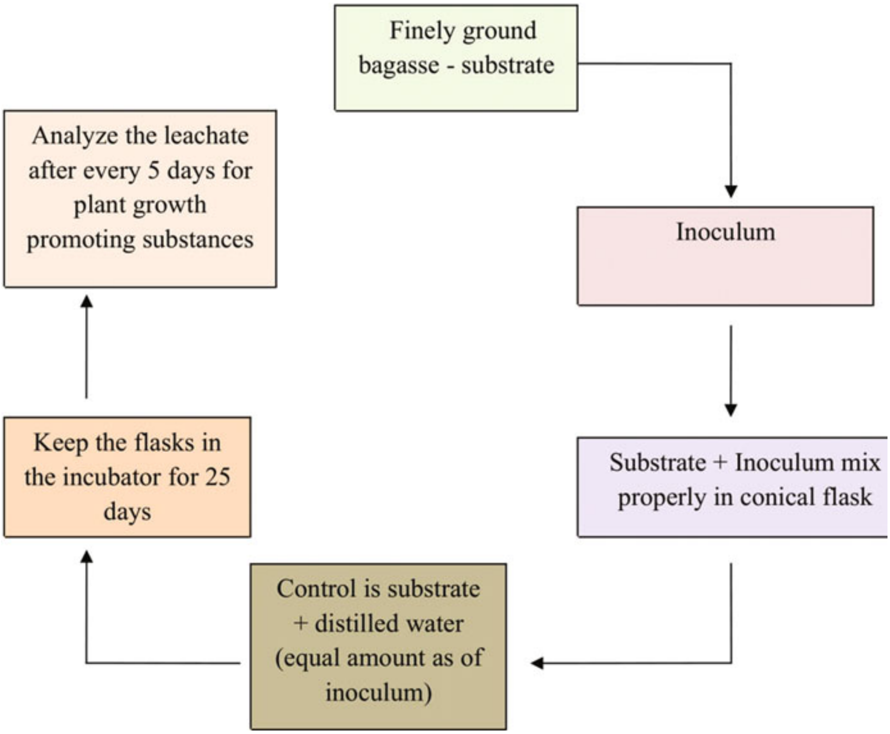

Fig. 14.3 Bagasse as a substrate for plant growth promoting substances by SSF

14.6 Materials

Bagasse is used as a raw material for various paper products (Catosse et al. 2009; Kumaraguru et al. 2014; Martinez-Hernandez et al. 2017; Poopak and Reza 2012; Rainey 2009). There is a study on use of bagasse for pulp and paper (Elballa et al. 2017).

Bagasse particleboard is used as raw material for laminated floor and furniture applications. The particleboard from bagasse has excellent mechanical properties and is also economical.

The bioplastics have immense applications. Bagasse after pretreatment can be used for the production of bioplastics (Getachew and Woldesenbet 2016; Jian and Heiko 2008; Wu 2011; Zaid and Jamil, 2015). These bioplastics are polyhydroxyalkonates (PHA) biopolyesters. These bioplastics can be used as alternate to petroleum-based non-degradable plastics which causes pollution and is harmful to the environment. The use of bioplastics from bagasse will be very cheap and eco-friendly (Getachew and Woldesenbet 2016).

The cellulose present in bagasse is used to make textile fibers which have high tensile strength and also water-holding capacity. The fibers are used for making nonwoven products which are easily biodegradable and eco-friendly (Chiparus 2004).

Bagasse in combination with quarry dust and lime can be used to make bricks which are eco-friendly and stronger (Alavez-Ramirez et al. 2012). These bricks have more advantage in comparison to clay bricks. These bricks have applications in making eco-friendly construction materials and thus help in sustainability.

Bio-char can be produced from anaerobically digested bagasse by pyrolysis at 600 °C in nitrogen environment (Inyang et al. 2010). The bio-char can be used as:

- Soil amendment to improve soil properties and texture.
- Contaminant remediation barrier.
- Adsorbents for the bioremediation of heavy metals or toxic compounds from contaminated water. Bagasse ash have good silica content which can be used in buildings for cement (Ganesan et al. 2007). Bagasse ash thus can be excellent alternate for cement in concrete.

14.7 Energy

Methane (CH_4) is sustainable source of energy. There is a report which shows production of CH_4 from sugarcane bagasse by hydrothermal pretreatment of bagasse using batch fermentation (Soares et al. 2018). This CH_4 gas can be used to generate electricity.

Sugarcane industries use boiler systems for steam generation. Bagasse when burned in presence of oxygen produces carbon monoxide and hydrogen gases. These gases have uses in turbine engines and boilers for steam generation and power (Basu 2010). Co-generation of electricity by incineration of bagasse can

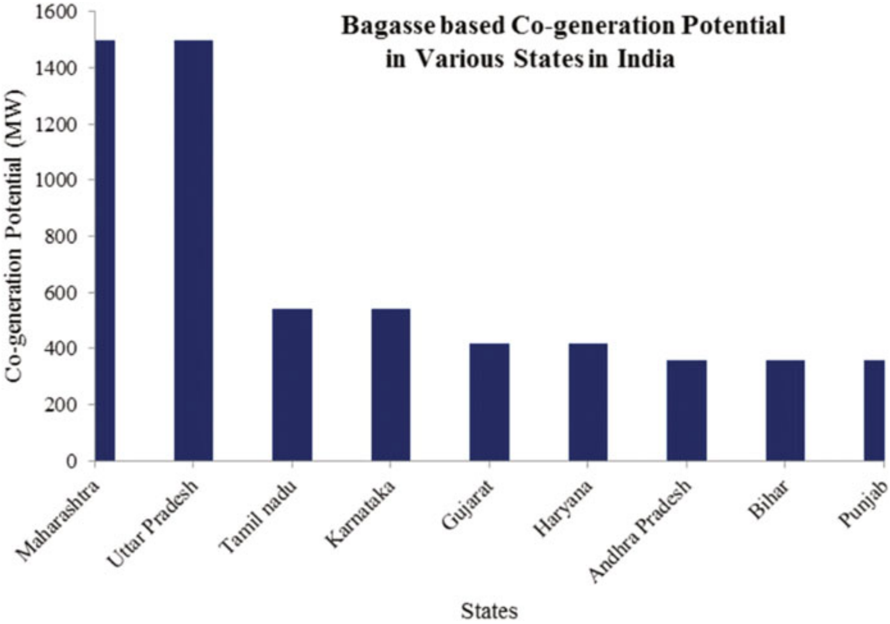

Fig. 14.4 Bagasse based co-generation in various states in India. (*Source: Singh 2010)

yield 130 kWh/ton bagasse (Seabra and Macedo 2011). In 2015, 474 TWh (2%) of the global electricity was from biomass resources (IEA 2017). Bagasse (about 280 kg) can generate 500–600 kg steam per ton of sugarcane (i.e., 2 kg steam/kg bagasse) (Yang et al. 2013).

Pyrolysis is heating bagasse without oxygen to produce charcoal, bio-oil, and producer gases. The bagasse based co-generation in various states in India is shown in Fig. 14.4. This is good alternative to the conventional fossil energy. The bagasse based co-generation will be economical and reduce fuel consumption and pollution (Sahu et al. 2015). Bio-char contains 85% carbon, and remaining amount consist of hydrogen and oxygen. Bio-char when burned generates power which has energy. Bio-oil is dark brown/black liquid which is used as fuel in power plants. Bio-oil is a clean fuel and when mixed with petroleum refinery feedstocks can produce petroleum grade refined fuels. The producer gases also have applications in gas turbines for heat generation and power (Basu 2010; Nigam and Pandey 2009; Parameswaran 2009).

There is a report on sugar mills in Brazil generating around 412 kWh electricity per ton bagasse, 330 kWh heat, and 16 kWh mechanical energy (Turdera 2013).

The activated unburnt carbon (AUC) is prepared from bagasse by deashing and steam activation. The AUC can decolorize oils, fats, and beverages. It is also to make refined sugar and also used as fuel in boilers (Batra et al. 2010; Mobarak et al. 1982). The charcoal from bagasse can be used to make briquettes (Clement 2007; Martinez-Hernandez et al. 2017; Teixeira et al. 2010).

14.8 Fuels

The fuels as value-added product from bagasse include bioethanol and biogas. There is a growing concern over high cost fossil fuels. The conversion of lignocellulosic wastes into bioethanol or any biofuels is economical and is renewable alternative (Rubin 2008). The world production of bioethanol in 2015 was around 97×10^6 m^3. From this total production, United States (56×10^6 m^3) and Brazil (27×10^6 m^3) account for major production of bioethanol which is nearly 85% (Alternative Fuels Data Center 2016). Brazil is the major exporter of bioethanol. Ethanol is major energy source in countries viz., Brazil, United States, China, and India. The production of ethanol in the world in 2013 was around 89 GL, where 74% availability of ethanol was from Brazil and the USA (Bhatnagar et al. 2016). Bagasse has been reported to have application in bioethanol production (Bezerra and Ragauskas 2016; Cardona et al. 2010; Chandel et al. 2012). Pretreatment of bagasse is necessary to separate cellulose, hemicellulose, and lignin. There is a report on bioethanol production from bagasse (Antunes et al. 2014; Iram et al. 2018; Khattab and Watanabe 2019; Tyagi et al. 2019).

Biogas can be produced with bagasse as a substrate which involves anaerobic digestion (Carvalho et al. 2005). Bagasse is decomposed into simple sugars by acidogenesis. After this process, fatty acids present are decomposed to carbon dioxide (CO_2) and hydrogen (H_2). These final products by acetogenesis form hydrogen sulfide (H_2S). This H_2S can be removed by scrubbers. The final step involves conversion of methane acetate to CH_4 which is major constituent of biogas (Carvalho et al. 2005). This biogas can be used as fuel and in cooking. Fermentation of bagasse for biohydrogen production can yield about 70.6% gas. This will be very cheap, eco-friendly and also reduce greenhouse emissions (Zhang et al. 2007).

14.9 Animal Feed

Bagasse has important component, i.e., cellulose and ruminants can easily digest this cellulose due to cellulase enzyme. Also, it is good source of proteins and hence, can be recycled into protein-rich food. Bagasse can be excellent substrate for the production of animal feed (Parameswaran 2009). Complete feed for ruminants is prepared by mixing bagasse with molasses as major components. The percentage of bagasse and molasses can be varied depending upon the ruminants to make complete animal feed (Diwan et al. 2020). Single cell protein (SCP) is a good protein-rich palatable feed and alternative to costly soymeal and fishmeal. SCP is cheap and can be easily converted to animal and human feed (Mtui 2009).

14.10 Biosorbents

Bagasse also has important application to remove toxic metals (Joseph et al. 2009; Moubarik and Grimi 2015; Sarker et al. 2017; Vera et al. 2019), dyes, organic compounds (da Silva et al. 2019; Siqueira et al. 2020), etc. from aqueous solutions, waste waters, etc. The AUC from bagasse has been used in melanoidins adsorption (Kaushik et al. 2017).

14.11 Conclusion and Future Prospects

The different important value-added products from the agroindustry by-product bagasse mentioned in the review are very useful, cheap, non-toxic, and eco-friendly. These value-added products have various industrial and agricultural applications which will be very helpful. It also maintains sustainable approach which is essential. The value-added products from bagasse can be termed as "green products" as they do not contribute to any pollution. This is the main important factor which is focused in this review. Bagasse is also available easily in huge amount. This bagasse instead of burning or directly disposing to the landfills when converted to value-added products will help in the management of agroindustry by-product waste. The aspect of wastes turning to wealth will be a clean technology. This will also contribute to a significant sustainable environmental approach in the management of bagasse. The reduction in environmental pollution is the need of the time.

The agroindustry by-product bagasse can be used to generate novel products which will have immense industrial applications. The study in this area needs to be carried out on a large scale with other agroindustry by-products viz., sawdust, peanut shell, corncob, etc. Further research is needed to study more additional new uses of bagasse which will have more applications in the medical field.

Conflict of Interest Statement The author declares there is no conflict of interest.

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Bio-prospecting of Fruits Waste for Exopolysaccharide Production by Bacteria

15

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Abstract

Exopolysaccharides (EPS) are secondary metabolites which are produced by organisms utilizing various carbon sources. They can be found in microorganisms, plants, and animals. EPS are able to perform different fundamental biological functions. There is large amount of carbon consumption required for production of EPS, which makes the production process costly. However, cheaper alternative substrates are there, which may lead to higher EPS production. A large amount of wastes is produced by food industry every year. Management of such waste is a critical issue for food industries. Because of their high organic water content, disposal of such waste has a notable problem both in terms of costs and environmental pollution. Normally disposal of such waste is done in traditional ways, such as land filling and composting; it is used as low quality animal feed, incineration and land spreading. Dumping of these wastes can have several adverse effects like it may produce greenhouse gases. It is one of the source of secondary pollution, it emits foul smell, such waste is easily susceptible to microbial attack, and the process like land spreading results

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in contamination of underground water table due to its runoff in rainy seasons; landfills and land spreading create breeding grounds for many human pathogens which can be a reason of epidemics, and thus causing negative impact on human health. The industries experience losses as these wastes require pre-treatment and transportation costs for dumping into landfills, which is costly. Generally it is believed that such waste can be used as biofertilizer or as fodder for cattle. But all of these wastes cannot be always used as fodder or biofertilizer due to high fibre content, however, it is observed that these waste can easily be utilized by microbes as a source of energy and metabolism, thus being useful in producing secondary metabolites like EPS. Unpleasant effects of agro-industrial wastes can be handled with the help of environmental biotechnology, which is an emerging field nowadays. The use of agro-industrial wastes for production of valuable bio-products can minimize various environmental hazards. For these reasons, such wastes may offer probable solutions to the actual need of a sustainable development which would accomplish the increasing demand of energy which is required for the production of EPS and would lower the overall cost of the production at higher level.

Keywords

Bio-prospecting · Bioprocess · Exopolysaccharide · Fermentation · Fruit waste · Bacteria

15.1 Introduction

Extracellular polymeric substances are produced by both prokaryotes and eukaryotes. One group of these polymeric substances are exopolysaccharide (EPS); they are the secondary metabolites that are produced by organisms by utilizing carbon source. Several bacteria, algae, fungi and yeasts are known to produce exopolysaccharide. Exopolysaccharide is also investigated from autotrophic acidophiles, hydrothermal vent bacteria, methanogens, halobacteria, as well as from microbes present in groundwater and sewage sludge. Extracellular polymeric substances of microbial origin are also scattered in marine environments. They are found in dissolved forms, colloids, in association to particulate matter, which includes bio-films, cell aggregates, microbial mats, detritus, etc. (Bhaskar and Bhosle 2005). EPS can be found in various plants and animals as well. EPS are able to perform various biological functions. They are reserve energy substances and are structural elements which maintain mechanical shape and rigidity of the living cells such as cellulose, hemicelluloses and pectin in plant and microbial cell wall, chitin in arthropod exoskeletons. Many microbes produce EPS as strategy of growing, surviving under adverse conditions and adhering to solid surface. EPS enables the microbes to survive continuously at high cell densities in a stable mixed population of biofilm communities. Biofilms are mainly composed of EPS and microbial cells. In biofilm, EPS is considered primary matrix material as it accounts for 50–90% of the total organic carbon. The collective behaviour of microbes has significance in bioengineering, agriculture, biotechnology, environmental sectors, industrial, medicine, dentistry, geosciences and many other disciplines. In response to selective pressures, microbes produce EPS naturally. EPS may hinder the mass transportation of antibiotics through biofilm by directly binding to these agents and thus contributes to the antimicrobial resistance properties of bio-films (Donlan 2002).

Depending on their position, they may be endopolysaccharides or exopolysaccharides. They are classified into homopolysaccharide that has only one type of sugar moiety, e.g. alternan, cellulose, curdlan, dextran, levan, mutan, pullulan and heteropolysaccharide that has two or more sugar moiety, e.g. gellan and xanthan (Zannini et al. 2016). The process of its synthesis involves complex enzyme system. They are organic macromolecules that are formed by polymerization of simple or identical building blocks like monosaccharides, amino sugars, uronic acids linked by glycosidic bonds, amino acids linked by peptide bonds, nucleic acid, phospholipids and humic substances, which may be arranged as repeating units within the polymer molecules. EPS may also contain low molecular weight non-polymeric substituent, which significantly changes structure and physiochemical properties. Thus EPS carry organic substituent such as succinyl, acetyl or pyruvyl group or inorganic substituent like sulphates. Polysaccharide chains vary in molecular weight from 10^3 to 10^8 kDa and it also contains functional and species-specific sub-unit (Sutherland 1997). The charge carried by the polysaccharide mainly depends upon the components of the repeated units. It is observed that polysaccharides are usually negatively charged, sometimes neutral or rarely positively charged. Furthermore, polysaccharides may be hydrophilic but can also have hydrophobic properties. The behaviour of the polysaccharide at the cell surface or at an interface is determined by hydrophobic nature of polymer. Angle of bonds determines the shape of EPS; it also manages the relative orientation of adjacent sugar residues in chain. In solution EPS have an ordered helical confirmation and may be single, double, or triple helix. Intermolecular hydrogen bond stabilizes their helix. Such confirmation makes polysaccharides semi-rigid and thus the molecules can shunt large volume of solutions. Intermolecular interaction stabilizes the helix and effects properties of EPS such as viscosity, solubility and gel formation. Poor intermolecular interaction will solubilize the EPS. Presence of side chain mainly influences the interaction. EPS gets affected with change in environmental factors in terms of molecular mass and composition; this turn can affect their ability to interact with other polymers and cations.

15.2 Bacterial EPS

Structural diversity of bacterial polysaccharides is found to be many folds higher in comparison to plant. This is due to variations in condensation linkages, monosaccharide composition, molecular weight and non-sugar decorations, and this all leads to their various biological functions. This diversity adds up to diverse physical and rheological properties of microbial EPS and thus opens up its commercial applications in various sectors (Becker 2015). Apart from structural diversity, microbial polysaccharides are preferred over plant and algal polysaccharides because the microbial EPS are produced in short period of time and in higher amount, the production process in fully controlled, microbial EPS is energy efficient, there is no effect of seasonal variation during production and have the possibility of utilizing agricultural waste.

EPS are considered as a probable alternative to chemical polymers because of their ease of extraction, biodegradability, biocompatibility, high efficiency, improved physical properties, reproducible physicochemical properties, edibility and non-toxicity to both human and environment. Vast number of bacterial EPS are reported over last decades; their structure, composition and functional properties have extensively been studied (Table 15.1). Due to their bioactive role and their extensive range of applications, substantial interest has aroused with regard to isolation and identification of new bacterial polysaccharide. Due to their unique physical properties and structure, EPS are used in textile, food, detergents, beverages, agricultural, pharmaceutical, drug delivery, biotechnology, paper, petroleum, paint, medical, cosmetic industries and in the formulation of the culture media.

Polysaccharides are involved in several mechanisms such as stress tolerance associated to food process, conformation, pre-biosis, pro-biosis, viscoelasticity, polyelectrolyte adherence and biocompatibility, thus some of their applications include their use as binders, coagulants, emulsifiers, film formers, gelling agents, lubricants, stabilizers, pro-biotic, suspending and thickening agents. The natural environment and ecological niches from which microorganisms have been isolated mainly determine the physiological role of EPS. A new approach to encounter EPS with novel properties might entail investigating different EPS producers from different environments.

In spite of great diversity in molecular structures of bacterial EPS, only a few have been industrially developed. There are different roles of bacterial EPS such as hiding the bacterial surface for protection, as protective agents against environment, bonding agent for interactions with other bacterial surfaces or substratum, in the rhizosphere communities as substances for bacterial aggregation, as signalling molecules and as a structure stabilizer in biofilm. Still various physiological functions of EPS remains unidentified and thus only few of them have been industrially used (Suresh Kumar et al. 2007; Badel et al. 2011).

15.3 Mechanism and Regulation of EPS Synthesis

Production pathway and utilization of substrate molecule differ depending upon the type of EPS that is produced (Patel et al. 2010). Three phases are involved in EPS biosynthesis: (a) assimilation of a carbon substrate, (b) intracellular synthesis of the polysaccharide and (c) its exudation out of the cell.

Extracellular glycosyltransferase catalyses the polymerization reaction for synthesis of homopolysaccharides. In this reaction, there is transfer of a monosaccharide

Table 15.1 Types of exopolysaccharide and example of EPS producing bacteria

Exopolysaccharide	EPS producing bacteria
Glycogen	Archaea and several bacterial species
Xanthan	*Xanthomonas* species
Levan	*Halomonas smyrnensis* AAD6T, *Zymomonas mobilis*, *Lactobacillus* species, *Bacillus* species, *Bifidobacter* species, *Halomonas* species., *Paenibacillus polymyxa, Streptococcus* species, *Leuconostoc mesenteroides*
Dextran	*Leuconostoc* species., *Bacillus* species, *Lactobacillus* species, *Weissella cibaria*
Mutan	*Lactobacillus* species, *P.damnosus, Streptococcus mutans, S. sobrinus, S. thermophiles, S. salivarius*
Reuteran	*Lactobacillus reuteri*
Alginate	*Bacillus* species isolated from marine environment, *Pseudomonas* species *Azotobacter* species
Glucomannan	*Rhizobium leguminosarum, R. meliloti*
Curdlan	*Alcaligenes faecalis, Cellulomonas flavigena, Agrobacterium* species, *Paenibacillus jamilae, Cellulomonas* sp.
Hyaluronan	*Pseudomonas aeruginosa, Pasteurella multocida, E. coli*
Gellan	*Sphingomonas* species, *S. paucimobilis*
Alteran	*Leuconostoc mesenteroides*
Insulin	*Streptococcus mutans*, *Lactobacillus species*, *Leuconostoc citreum* CW28
Welan	*Alcaligenes* species
β-Glucan	*Bacillus* spp., *Pediococcus* spp., *Streptococcus* species
α-D-glucans	*Leuconostoc pseudomesenteroides*
Fructans	*Streptococcus salivarius, Leuconostoc* species
Pyruvated galactan	*Methylbacterium* sp. (isolated from a Finnish paper machine) (Lembre et al. 2012)
Cellulose	*Salmonella*, *E. coli,* some Gram-positive bacteria
Polygalactan	*L. lactis* subsp. *lactis* H414 (Ruas-Madiedo et al. 2002)
K30 antigen	*Escherichia coli*
Colanic acid	*Enterobacter* spp., *Escherichia coli, Salmonella* spp., *Shigella* spp.
FucoPol	*Enterobacter* A47

unit from a disaccharide and this leads to increase in the length of the polysaccharide chain. The same enzyme causes reassignment of a sugar residue from activated donor molecules which are nucleoside mono-phosphate sugars, lipid phosphate sugars and sugar 1-phosphate to specific acceptor molecules. This leads to biosynthesis of glycosidic bonds.

In the case of hetero-polysaccharides, glycosyltransferases relocate sugar residues to a lipophilic carrier continuously; this leads to the assembly of repeating units in the cytoplasm. Once it is formed, the sugar chain is transferred outside the cells and polymerized. There is genetic and enzymatic control on the level of the release of the polymers, their chemical modification such as acetylation or pyruvylation reactions, and addition of phosphate or sulphate substituent (Finore et al. 2014).

For the production of polymers in bacteria by utilization of carbohydrates, four general mechanisms are known:

1. Wzx/Wzy-dependent pathway: At the inner membrane, individual repeating are linked to an undecaprenol diphosphate anchor (C55), they are then assembled by number of glycosyltransferases and translocated across the cytoplasmic membrane by flippase, which is a Wzx protein. Wzy protein (polymerase) carries out the polymerization at the periplasmic space (Islam and Lam 2014). Polysaccharide co-polymerase and the outer membrane polysaccharide Transport of the polymerized repeat units from the periplasm to the cell surface is dependent upon additional protein(s) assigned to as polysaccharide copolymerase (PCP) and the outer membrane polysaccharide export (OPX) families (Cuthbertson et al. 2009; Whitney et al. 2011).
2. ATP-binding cassette (ABC) transporter-dependent pathway: Synthesis of capsular polysaccharide (CPS) (Whitney and Howell 2013) occurs through ABC transporter-dependent pathway where assembly occurs through action of glucosyltransferases at the cytoplasmic face of the inner membrane. The export across the inner membrane and translocation to the cell surface occurs with the help of tripartite efflux pump, which is composed of ABC transporters. CPSs produced through this pathway carry a conserved glycolipid at the reducing terminus which is made up of phosphatidylglycerol and a poly-2-keto-3-deoxyoctulosonic acid linker.
3. Synthase-dependent pathway: Secretion of complete polymer strands across the membranes and cell wall occurs through this pathway. A single synthase protein performs the polymerization as well as the translocation process. For the assembly of homopolymers requiring only one types of sugar precursor, such pathways are often utilized.
4. Extracellular synthesis, polymerization reaction: Various enzymes carry out transfer of a monosaccharide from a disaccharide to a growing chain of polysaccharide in the extracellular environment (Ates 2015). In a particular linkage pattern like α and β, the sugars of polysaccharides get then assembled (Dave et al. 2020).

15.4 Alternative Substrates for EPS Production

The main troubles with large-scale production of EPS are their high substrate cost and costs related with their downstream processing, which makes the production an expensive affair. The amount and composition of EPS produced by a bacterium are determined genetically, but still there is high influence of media components and cultivation conditions. Large amount of carbon consumption for production of EPS makes the process costly. However, cheaper alternative substrates are there which may lead to higher EPS production. High carbon containing food waste mainly generated from fruits and vegetables processing industry, household kitchen waste, dairy waste, cafeteria, fruit juice vendors can be a very good source for EPS production at higher level. All these sectors produce mammoth amounts of wastes constituted by pulps, seeds and peels (Kanimozhi et al. 2018). Getting rid of these wastes is a huge problem both economically and environmentally. The nature of waste is also such that it has limited application as biofertilizer and fodder. However, microbes can use such waste as a source of energy and metabolism, thus being useful in producing secondary metabolites.

Environmental biotechnology provides alternative options to handle the adverse effects of fruits wastes. The use of fruits wastes for production of priceless by-products like EPS through microbial fermentation is not only economically important but can also decrease several environmental hazards. For these reasons, such waste may offer possible solutions to the need of a sustainable development, thus satisfying the increasing demand of energy required for EPS production and thereby lowering the overall production cost.

Research has found that such fruits residues are very good alternate substrate for EPS production, mainly due to their potential advantages like biocompatibility, non-toxicity, easy and cheap availability; above all such wastes are easily utilized by microbes. It is observed that the conversion of such substrate is up to 70%. Fruit wastes consist of hemicellulose, carbon, cellulose, lignin, vitamins, ash, moisture, nitrogen, etc. and these constituents can be biochemically digested by bacteria to produce commercially important product such as EPS (Sadh et al. 2018). Use of such waste in medium formulation makes the medium cheaper and simpler; and by utilizing such medium for EPS production the extraction and purification of EPS also becomes simple (Hafez et al. 2007; Leroy and De Vuyst 2016).

Availability and utilization of simple and cheap technologies might attract the researchers and industrialist to isolate novel microbial strains with novel EPS production ability with novel application. Intelligent screening of bacteria for EPS is necessary for its further exploration towards commercialization. Wastes attained after processing of vegetables and fruits are exceedingly seasonal and perishable; such wastes are massive problem to the pollution monitoring agencies and processing industries (Arun et al. 2015). An attempt is made for use of several fruits waste for large scale production of EPS which might have commercial application. These concepts could include new pre-treatment methods and resources as well as modified downstream processing and fermentation techniques (Castillo et al. 2015). The entire process of utilizing the waste for EPS production might lead towards Green revolution.

For production of diverse products, industries are reluctant in utilizing fruits waste as substrate. This may be due to various problems that might be faced in handling such waste, dewatering it, pre-treatments, discarding and many more. Bio-processing may play an important role by providing adequate pre-treatment, coagulation, dewatering and modification of alternatives. Currently, these fruits wastes are allowed to decay naturally in the fields; they are dried and then burned. For production of bacterial EPS, processes like submerged fermentation and solid-state fermentation make appropriate use of such waste as substrate or raw material. In addition, the use of such wastes is an environmentally friendly method of managing waste because their disposal presents an added cost to processors, and direct disposal into soil or landfills causes serious environmental problems. Investigations are going on for development of value-added process for production of EPS on large scale using such waste with the goal that the production would be cheap and there would be reduced use of non-renewable substrates and other resources which may lead to an economical outcome.

15.5 Bio-processing of Fruits Wastes

Bio-processing involves the complete use and conversion of substrates by microorganisms for the production of EPS. This may also solve the environmental problem of pollution. Through industrial development and new innovations, different bioprocesses are employed in the utilization of such waste residues in various products. By making use of various physical processes and harsh chemicals for synthesis of valuable products from waste resources, the process becomes very expensive, hazardous and non-renewable proposition. Making use of microorganisms to synthesize value-added bio-chemicals from biomass is a promising alternative to harsh chemical synthesis and physical processes. The process is not hazardous, there is use of renewable source, and the process is less expensive. It is very much important to lower production costs. Different ways to reduce production costs could engage using low-priced substrates like agro-industrial waste, optimization of fermentation conditions and downstream process or developing recombinant strains by mutagenesis or genetic manipulation which could give higher yield.

Bio-processing mainly involves bioconversion, where there is biotransformation of organic matter present into the waste to energy containing fuels and value-added products through microbes. Two steps are involved in bioconversion of solid waste to valuable product. First step mainly engrosses breakdown of complex carbohydrates into simple reducing sugar by using various pre-treatment methods, and in the second step, by fermentation of the reducing sugars needed products are obtained (Lange 2007; Lee et al. 1997; Sun and Cheng 2002). Several industrially important biochemical products have been produced via bio-processing techniques which makes use of different biological wastes as substrates (Gunasekar et al. 2014). The main goal of these technologies is to use the waste to develop value-added products, thus reducing environmental pollution and solving issues which are associated with waste disposal (Cheng et al. 2012; Göksungur, et al. 2011). Furthermore, it will also assist in the positive development of a biologically built economy.

Bio-processing can be carried out by both solid-state fermentation (SSF) and submerged fermentation (SmF). SSF is the method which has shown high productivity from low-cost carbon and nitrogen source; it is simple and is mainly preferred by industries as it is less costly as well. There are various other advantages of this process too. It generates less effluent, no sophisticated fermentation equipments are required and above all, substrate can directly be used as feed in fermentation with no requirement of pre-treatments (Yang et al. 2001). There are less chances of contamination during the process and the downstream process is very simple requiring no machines.

15.6 Methods of Fermentation

15.6.1 Solid-State Fermentation (SSF)

In SSF, the microbes are grown on solid substrate, there is absence of free moving water but to maintain the growth and metabolism enough moisture is maintained (Rahardjo et al. 2006). Inert and organic materials can be used in SSF. Inert material acts only in attachment places, whereas organic material functions as a source of nutrients so they are also termed as support substrate. Using support substrates has several advantages; for example, there is a reduced production cost because these substrates provide some nutritive substances to the microbes. Above all it has similarity to the natural habitat of several microorganisms. Biological wastes are a good example of such material. SSF has been used for the production of EPS; yield attained by this method is compared to those obtained from conventional submerged cultivation (Thomas et al. 2013). Agro-industrial wastes like pomaces and lignocellulosic biomass are commonly used for production of EPS in SSF (Özcan and Öner, 2015). Making use of mango peels as substrate cost-effective EPS with fructose, mannose and glucose production is carried out from *B. licheniformis* in SSF (Asgher et al. 2020). Stredansky and Conti (1999) made use of grape pomace, citrus peels and apple pomace as solid substrate to produce xanthan from *Xanthomonas campestris* strains in SSF.

Disadvantages of SSF to commercial application include limited knowledge of the operation and design of large-scale bioreactors; there are various problems faced in controlling important culture parameters such as heat removal and mass transfer, difficult to control the process parameters such as nutrient conditions, pH, moisture, heat, tedious downstream process requiring removal of the matter, cells and then extraction. Various types of bioreactor have been used in SSF processes, which mainly include rotating drums, stirred bioreactors, packed beds and gas–solid fluidized beds.

15.6.2 Submerged Fermentation

Use of submerged fermentation (SmF) method of fermentation requires various steps before the process of fermentation begins. The waste needs to be crushed by a mechanical mixer. Hydrolyzed by thermo-chemical pre-treatment using sulphuric acid or alkali solution with incubation at higher temperature, i.e. autoclaved, can be incubated at room temperature or can be subjected to boiling as well (Li et al. 2017). Enzymatic hydrolysis of such waste is also an alternate but it is costly (Vavouraki et al. 2013, 2014). There is a need to build up environment-friendly, cost-effective and novel alternative approaches for effective pre-treatment of waste (Philippini et al. 2020). Filtration is the next step for removal of insoluble matters. The hydrolysate thus attained is used for fermentation after autoclaving it. Certain time the hydrolysate attained needs to be diluted to attain proper ratio of nutrients present in it, one also needs to analyse the amount of carbon and nitrogen present into it and maintain the pH of the attained hydrolysate before being used for fermentation in specially designed bioreactors. The bioreactors are sophisticated and are equipped with well-controlled operative systems. It is a onetime investment for production by industries as these reactors can be operated under batch, continuous and fed batch mode with automation. Downstream processes are simpler as it mainly includes removal of cell from the fermented broth and then extraction of the product. Such hydrolysate attained after pre-treatment can easily replace synthetic medium used for EPS production.

There are various other advantages of SmF like easier product purification and in short period one can achieve high yield. In liquid culture, the control of the fermentation is simpler with reduced fermentation time. The use of submerged culture can also benefit the production of many secondary metabolites and decrease production costs by reducing the labour cost which is involved in SSF. However, the method of SmF is less used as there is more effluent generation, process requires pre-treatment which is time consuming and also requires complex fermentation equipments. It is observed that by SSF there is improved product characteristics as compared to the product attained by SmF.

Different types of fermenter made up of autoclavable material are designed to carry out SmF. For example, continuous stirred tank fermenter, bubble column fermenter, tower fermenter, two-stage airlift fermenter, etc. Pineapple fruits waste attained from the pineapple processing industries is successfully used for bacterial EPS production using submerged fermentation at laboratory scale (Pyar et al. 2014). Use of grapes skin waste as source for production of pullulan using fungal strain is also reported (Israilides et al. 1994**).** Successful use of sugarcane juice as source of carbon for xanthan production making use of SmF at laboratory scale is also reported (Faria et al. 2011). As a biomass resource fruit juices are also a good substitute. For bacterial cellulose production, Hungund et al. (2013) made use of various fruit juices including pomegranate, pineapple, water melon, sugarcane juice, muskmelon, tomato and orange and also molasses, coconut water, and coconut milk as cheap and alternative carbon sources by *Gluconacetobacter persimmonis*. Survase et al. (2007a, b) used various dilutions of sugarcane molasses, sugarcane juice and coconut water which were not subjected to any pre-treatment methods before their use for the scleroglucan production by filamentous fungi *S. rolfsii* MTCC2156. An attempt to use coconut water and sugarcane juice for EPS production by *Lactobacillus confusus* cultures (Seesuriyachan et al. 2011) is also reported. Residue from apple juice processing is used as an alternate source of carbon for xanthan gum production where it is observed that there is ten times higher production of EPS in comparison to medium containing sucrose as carbon source (Druzian and Pagliarini 2007). An attempt to produce EPS by making use of apple peels, pineapple pulp and mixed fruits waste containing pulp, seeds and peels of various fruits attained from fruits juice vendor from *Bacillus* species and *Xanthomonas campestris* isolated from fruits waste itself (Vaishnav et al. 2016) at laboratory scale and scale up to 5 L fermentation medium is also reported. The production was 1.4 and 1.2 folds higher respectively by both the strains in comparison to the synthetic medium used for EPS production (Vaishnav and Dave 2017). Plenty of information is reported in various literatures and research articles regarding use of fruits waste for production of various organic acids (Ahmad and Langrish 2012), but reports of making use of fruits waste for EPS production are very few. Table 15.2 briefly describes the EPS produced by bacteria making use of different fruits and its different parts as well as the method of fermentation applied.

15.7 Substrate for Exopolysaccharide Production

The extensive research and development mainly aims on determining how to reuse fruits waste and convert it into useful product. It also aims for an alternate to current disposal method. The improper disposal is environmentally and ecologically not advantageous. Certain times due to constrains in transport fruits are not exported, the cost of the fruits spikes, and so they remain unused resulting in to spoilage upon long storage. Such wastes have large amount of organic matter that is rich in bio-molecules which are easily bio-convertible. Cultivation of microorganisms on these wastes may be a valuable process that is capable of converting these materials, into value-added product which otherwise are considered to be wastes. Processes with techno-economic feasibility are used by scientists and researchers nowadays to convert waste into valuable products. Industrial production of EPS is costly affair as more than half of production cost is covered up by the substrate used. By making use of this waste one can easily lower the production cost to around 40% (Suresh Kumar et al. 2007). Regarding fruit wastes, more attention has been paid to wastes in form of pomace, peels and juice. These wastes are organic in nature and are rich in sugars and are thus easily used by the microorganisms. This makes such wastes very suitable to be exploited as raw materials in the industrial production of EPS under SSF and SmF conditions.

15.7.1 Pomace

Pomace is the remainder produced after the extraction of juice, flavours and concentrates from fruits. Pomace consists of peel, core and pulp, which are usually used as animal feed or fertilizer. Pomace is also directly converted into snacks, cereals and pet foods via extrusion process (Paraman et al. 2015). The presence of carbohydrates and other bio-molecules is in very high concentration, due to which pomace can no longer be considered to be waste. It is rich in dietary fibres, polyphenols, bioactive compounds and natural antioxidants that make it an attractive source for human diet supplements (Kanimozhi et al. 2018). It contains a lot of health-promoting ingredients as well as value-added products such as organic acids, enzymes, alcohols, bio-fuels, bio-adsorbents, flavours and pigments. Among all the types of pomace, apple pomace and grape pomace are largely used. Pomace has a large potential for bioconversion into several value-added products in an economically feasible way. Content of pomace is crude fibre, pectin and minerals such as K,

Table 15.2 Biomass and type of fermentation used for production of microbial EPSs

EPS	Microbial strain	Biomass	Types of fermentation	References
Levan	*Paenibacillus polymyxa NRRL B-18475*	Sugarcane syrup	Smf SmF	Han and Watson (1992)
Levan	*Zymomonas mobilis ATCC 31821*	Sugarcane syrup	SmF	de Oliveira et al. (2007)
Scleroglucan	*Sclerotium rolfsii MTCC 2156*	Sugar can juice	Smf	Survase et al. (2007a, b)
	Lactobacillus confusus	Coconut water	SmF	Kuntiya et al. (2010)
		Coconut water		Seesuriyachan et al. (2011)
Bacterial cellulose	*Gluconacetobacter xylinus PTCC 1734*	Date syrup	SmF	Moosavi-Nasab and Yousefi (2011)
Xanthan	*Xanthomonas campestris*	Sugar cane juice	SmF	Faria et al. (2011)
Bacterial cellulose	*Gluconacetobacter persimmonis*	Watermelon juice	SmF	Hungund et al. (2013)
Bacterial cellulose	*Gluconacetobacter persimmonis*	Orange juice	SmF	Hungund et al. (2013)
Gluco dextran	*Bacillus species SRA4*	Mixed fruits pulp, apple peels, pineapple fruits waste	SmF	Vaishnav and Dave (2017) Vaishnav et al. (2020)
Xanthan	*Xanthomonas campestris*	Apple juice	SmF	Druzian and Pagliarini (2007)
Xanthan	*Xanthomonas campestris*	Mixed fruits pulp, apple peels, pineapple fruits waste	SmF	Vaishnav and Dave (2017)
Galactose and mannose containing EPS	*Shewanella chilikensis*	Ripe plantain peels	SmF	Nwosu et al. (2019)
Xanthan	*X. campestris* PD 656	Apple pomace	SSF	Stredansky and Conti (1999)
Xanthan	*X. campestris*	Grape pomace	SSF	Stredansky and Conti (1999)
Heteropolymeric nature of EPS with mannose glucose, and fructose	*B. licheniformis*	Mango peels	SSF	Asgher et al. (2020)

Mg, Fe and Mn and thus it is a common substrate for microbial fermentation in SSF (Shalini and Gupta 2010).

15.7.2 Fruits Peel

Tons of waste in form of fruits peel is produced by the fruit processing industries, which are involved in canning of fruits pulp and fruits juice. Peels are wealthy source of biologically active compounds, and enzymes such as, peroxidase, protease, polyphenol oxidase, carotenoids, vitamins C and E, dietary fibres, and carbohydrate (Ahmad and Langrish 2012). These fruits wastes are either allowed to decay naturally, or are burnt. However, these wastes are also rich in sugars like fructose, sucrose and glucose as well as pectin and cellulose (Giese et al. 2008) and due to their organic nature, they are easily used by microorganisms as substrates for the production of industrially relevant compounds like EPS through its microbial conversion (Ahmad and Langrish 2012). Globally citrus fruit production is very high. Almost half of these citrus fruits are squeezed to juice, and the leftover includes peel, segment membranes and other by-products. Customarily it is used as a cattle feed which is of low value to cover the transportation and production costs. So, a large fraction of citrus waste remains deposited leading to environmental and economical disadvantages, and also leading to health problem of human beings (Kim et al. 2011). Stredansky and Conti (1999) have reported the use of citrus fruits waste for xanthan production using *Xanthomonas campestris*. Production and optimization of xanthan gum from pineapple peels using *Xanthomonas campestris* through SmF is reported by Amenaghawon et al. (2015). These fruits peels can be sundried, pulverized and then powdered. Such powders can be stored for longer period of time used for EPS production along with addition of nitrogen source and essential salts.

15.7.3 Fruits Juice

Fruits juice is mainly generated by canning industries. Fruits juice mainly consists of high concentration of sugar like sucrose and organic acids. They can be a cheaper source for the large-scale production of bacterial EPS. Palm date juice by-product is one of the most widely cultivated crops in the Mediterranean region; it has been used for xanthan production using *Xanthomonas campestris* at laboratory level by Salah et al. (2011). Sugarcane juice is rich in carbon source with high concentration of sucrose and it is a good substrate for production of EPS at cheaper level (Hungund et al. 2013). Several fruit juices including orange, muskmelon, pineapple, water melon, pomegranate, coconut milk and coconut water are used for the production of EPS using *Gluconacetobacter persimmonis* GH-2. Spoiled fruits juice can also be a substrate for polysaccharide production. Reports are there of cellulose synthesis by strain *Gluconacetobacter xylinus* ATCC 53582 on medium containing juice of spoiled fruits like plums, grapes, green pineapple and apples (Jozala et al. 2015).

The shelf life of the fresh fruits juice is short and so if during the fruit processing or during the storage the spoilage could result into economical loss but one can easily make up to it by making use of spoiled juice of EPS production.

15.8 Applications of EPS

Application of microbial EPS began in 1960s and since then there has been an amazing increase in their commercial use. Exploiting the biological, chemical and physical properties of microbial EPS leads to its successful commercial application (Dave et al. 2016). Depending upon properties of EPS, its applications have been observed in variety of fields. Table 15.3 briefly reports the application of EPS.

15.9 Disadvantages of Using Fruits Waste as Substrate

On such cheaper substrate, bacteria through various metabolic pathways can produce various undesirable products along with EPS (Kanimozhi et al. 2018). This might cause structural changes in biopolymer. Unused part of the waste may get accumulated in the broth and thus may act as inhibitor leading to lowering of the overall production. So to decrease the risk of impurities to the final product and attaining high purity and high quality product one has to make use of high and good quality substrate. Therefore, the use of wastes or by-products might not be an option or, if they are used, higher investment must be put in downstream procedures. Media components and cultivation conditions do influence the composition of EPS and it also influences the amount of EPS production. Growth conditions do not have significant effect on EPS structure but its content in substituent groups can differ widely, and thus it changes the properties of the polymer. However, there are exceptions to this behaviour. Downstream processing of this product is also the area which requires good research and modification in the conventional methods used for extraction and purification.

15.10 Improvement in Strategy

To avoid the accumulation of the unused part of waste in the broth, one can extract the sugar content of the waste by applying various pre-treatments and then subjecting the content to filtration and then autoclaving, by following such strategy one can avoid the production of undesirable product formation as well as can get rid of the accumulation.

To obtain consistent and high polymer yields, the large-scale production processes should be standardized, that too under controlled conditions, keeping in consideration the strain used for production. In order to accomplish superior quality and consistency of product yield, it is essential to regularly monitor and control the EPS production processes. The methods developed and used by the industry for this

Table 15.3 Properties and application of Bacterial EPS

Properties	Uses	Polymer
Biological properties		
Antitumor and immunomodulating	Antitumor agent	β-D-Glucans
Antithrombotic, antiseptic and regenerative	Heparin analogues	*E. coli* K5 EPS
		Bacterial cellulose
Antiadhesive	Eye and joint surgery	Hyaluronic acid (*Streptococcus* EPS)
Chemical properties		
Protein	Enzyme substrates	*E. coli* K4 and K5 EPS
Polymer	Oligosaccharides preparation	Curdlan, Pullulan, scleroglucan
Physical properties		
Emulsion stabilization	Foods, thixotropic paints, drug stability	Xanthan, FucoPol
Fibre strength	Acoustic membranes	Bacterial cellulose
Film formation adhesion	Food coating, biodegradable film formation, biosorption, bioaugmentation	Pullulan
		FucoPol
		Scleroglucan
Flocculent	Water clarification, ore extraction	Various polymers
Foam stabilization	Beer, fire fighting fluids	Xanthan
Gelling agent	Cell and enzyme technology as well as food	Gellan, Curdlan, Pectin, alginate, FucoPol
	Oil recovery	Xanthan and curdlan
Retention of ice crystals in size	Ice creams and ice lollies	Propylene glycon alginate
Heat reversible gel formation	Microbial solid media, jellies, synthetic meat feels	Agar, Carrageenan
Hydrating agents	Cosmetics and pharmaceuticals	Hyaluronic acid
		FucoPol
Suspending agent	Food, paper coating agrochemical pesticides and sprays	Xanthan
Non reactivity with dyes	Textile print thickeners	Alginate, Xanthan
Inhibitor of crystal formation	Frozen foods, pastilles and sugar syrups	Xanthan, Gum arabic
Shear thinning and viscosity control	Jet printing, oil drilling	Xanthan
Complex formation with milk products	Milk drinks	Carrageenan

purpose cannot be easily implemented in processes for the production of microbial EPS as it is hard to link key process factors. Although some real-time monitoring techniques have been attempted, most often EPS production monitoring depends on off-line analytical methods that requires regular removal of samples from the bioreactor (Seviour et al. 2011). Downstream process can be made simpler by optimizing the required parameters like cell separation, pigment removal, etc. Different extraction methods and purification techniques can also be experimented and applied. This processing stage optimization is important and can be modified depending on the desired purity as well as the area in which EPS is to be applied (Castillo et al. 2015). These processes are incredibly simple and more often than not result in high product yields.

With advancement in new technologies, improvisation in research and development, and extensive trials more and more industries would go for EPS production using such many low-cost and promising wastes and by-products.

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Plant Growth Promoting Rhizobacteria as Bioinoculants for Plant Growth

16

Aparna B. Gunjal and Balasaheb P. Kapadnis

Abstract

Agriculture is very important for food production. The use of chemical fertilizers to increase the plant growth is harmful to the humans and environment. The use of biological approach is the best way to increase the plant growth and yield. The bioinoculants include biofertilizers, biopesticides, and organic decomposers. These bioinoculants improve the productivity of many plants by helping in the availability of minerals such as phosphorus, potassium, iron; production of ammonia; plant growth hormones; enzymes; antimicrobial compounds; etc. The plant growth promoting rhizobacteria; nitrogen-fixing bacteria; phosphorus and potassium solubilizing, mycorrhiza inoculants have helped in the increase of yield of rice, wheat, sugarcane, tomato, cauliflower, sunflower, etc. The chapter here mentions the carrier for preparation of bioinoculants; types of bioinoculants; the advantages, market demand, and preparation of bioinoculant; and types of bioinoculant formulation. The chapter also focuses on mechanisms behind the plant growth and sustainable approach in agriculture for the plant growth. The effect of bioinoculants on the plant growth and in control of plant diseases is also taken into consideration. The use of bioinoculants in agriculture is getting wider attention and can be widely applied in the fields for many crops, thus ultimately helping the farmers. The use of plant growth promoting rhizobacteria as

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bioinoculants due to their several immense advantages will gain more importance, and completely replace the use of chemical fertilizers, in the near future.

Keywords

Sustainable · Plant growth promoting rhizobacteria · Economical · Carrier · Eco-friendly · Biological

16.1 Introduction

Agriculture is very important for the availability of cereals, pulses, vegetables, and fruits. The use of chemicals in agriculture has disadvantages viz., toxic, costly, harmful to the environment, causes pollution, and not safe to consume the vegetables, fruits, and pulses (Chandini et al. 2019; Farnia and Hasanpoor 2015; Pretty and Bharucha 2015). The alternate to chemical fertilizers is biological approach, i.e., use of microorganisms, which is eco-friendly, economical, safe and easy to use (Suyal et al. 2016). The biomass of microorganisms is also easily available in abundant amount. Plant growth promoting rhizobacteria (PGPR) are associated with the roots of various plants. The PGPR increase the growth of plants and crops by direct as well as indirect mechanisms (Backer et al. 2018; Kundan et al. 2015). These include production of enzymes: indole acetic acid (IAA); gibberellins; iron chelating compounds called siderophores; phosphorus (P) solubilization; ammonia (NH_3); antibiotics; 1-aminocyclopropane carboxylate deaminase (ACC); antimicrobial activity (antibacterial and antifungal); nitrogen fixing (N-fixing); etc. (Backer et al. 2018). The PGPR include species of *Pseudomonas*, *Bacillus*, *Azospirillum*, *Azotobacter*, *Arthrobacter*, *Alcaligenes*, *Serratia*, *Rhizobium*, *Enterobacter*, *Burkholderia*, *Beijerinckia*, *Klebsiella*, and *Xanthomonas* (Kalita et al. 2015). Rhizosphere is the hotspot for many soil microorganisms (Kalita et al. 2015) and is divided as endorhizosphere, rhizoplane, and ectorhizosphere (Nazir et al. 2018).

With this aspect, the chapter here mentions the PGPR bioinoculants for sustainable agriculture. The advantages of biological approach are more eco-friendly, safe, economical, and very easy. Another advantage is that the biomass of microorganisms can be available in abundance.

16.2 Carrier for the Preparation of Bioinoculant

A carrier is a solid or semi-solid material which sustains the growth of microorganisms for a given period of time. For the preparation of bioinoculants, a carrier is required. A good carrier must have the characteristics viz., moisture absorption capacity, easy to use, non-toxic, economical, easy to autoclave, readily available, and good pH buffering capacity (Nehra and Choudhary 2015). It should also have good adhesion property to the seeds and have good stability. The various

Table 16.1 Various important bioinoculants

Inoculants	Examples	References
PGPR	*Serratia*, *Pseudomonas*, *Bacillus*, *Acetobacter*, *Burkholderia*, *Xanthomonas*, *Enterobacter*, *Erwinia* sp.	Gupta et al. (2015)
Symbiotic nitrogen-fixing bacteria	*Rhizobium, Sinorhizobium*, *Azoarcus*, *Mesorhizobium*, *Frankia*, *Allorhizobium*, *Bradyrhizobium*, *Azospirillum*, *Azorhizobium* sp.	Turan et al. (2016)
Non-symbiotic nitrogen-fixing bacteria	*Azoarcus* sp., *Gluconacetobacter diazotrophicus*, *Azotobacter* sp.	Vessey (2003)
Phosphorus solubilizing microorganisms	Bacteria—*Bacillus*, *Pseudomonas*, and *Rhizobium* sp. Fungi—*Penicillium*, *Aspergillus* sp. Actinomycetes—*Streptomyces* sp.	Kalayu (2019)
Potassium solubilizing microorganisms (PSMs)	*B. mucilaginosus*, *B. edaphicus*, and *B. circulans*	Meena et al. (2016)
Mycorrhiza	*Glomus versiforme*, *Gigaspora margarita*, *G. mosseae*	Begum et al. (2019)

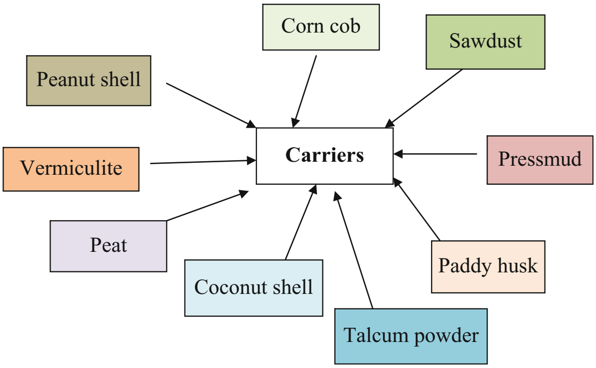

Fig. 16.1 Different carriers used in preparation of bioinoculants

bioinoculants include PGPR; nitrogen-fixing bacteria; phosphorus, potassium solubilizing microorganisms; and mycorrhiza (Pathak and Kumar 2016) and are represented in Table 16.1. The various carriers used for the preparation of bioinoculants are peat, lignite, vermiculite, talcum powder (Nehra and Choudhary 2015), paddy husk, pressmud, sawdust, corn cob, peanut shell, coconut shell, etc. The different carriers used in preparation of bioinoculants are represented in Fig. 16.1. The bioinoculants *Trichoderma harzianum* and *Pseudomonas fluorescens* have shown increase in the pomegranate fruit quality, fruit length and diameter (Reddy et al. 2017).

16.3 Various Important Bioinoculants

Rhizobia bioinoculants occupy 79% demand, PSB 15%, and mycorrhiza 7% (Owen et al. 2014). The various important bioinoculants are shown in Fig. 16.2. The bioinoculants include biofertilizers, biopesticides, and organic decomposers. *Azospirillum* is a Gram negative and motile bacterium which belongs to the order Rhodospirillales. It colonizes the roots of plants (e.g., wheat, rice) and forms symbiotic association. *Azospirillum* helps the plants by nitrogen fixation and production of plant growth hormones (Abd El-Lattief 2016).

16.4 Advantages of Bioinoculants

The main advantages of bioinoculants are (Ahemad and Kibret 2014; Bhardwaj et al. 2014):

- Eco-friendly
- Economical
- Easy to use
- Non-toxic
- No use of chemicals
- Help in absorption of nutrients by the plants
- Helps in the plant growth by direct and indirect mechanisms
- Improves soil fertility

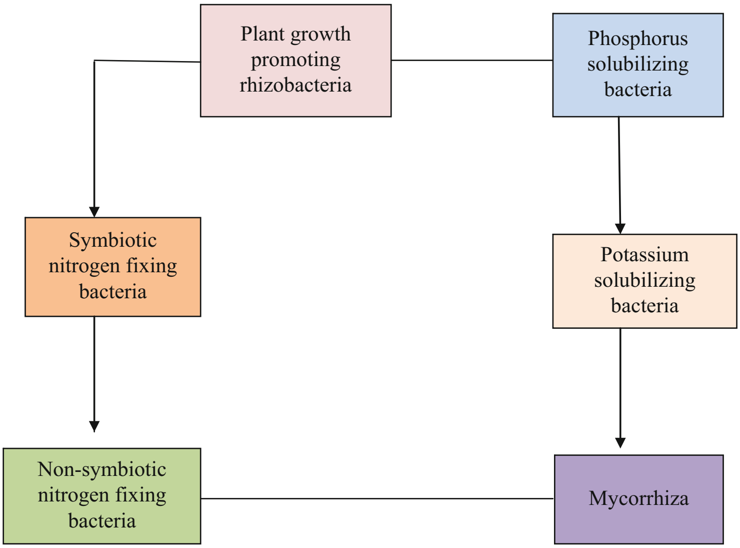

Fig. 16.2 Various important biofertilizers

16.5 Market Demand of Bioinoculants

The bioinoculants market was about USD 1.57 billion in 2018 in the global world. From this, North America had the biggest market (27.7%). This bioinoculants market is thought to increase more by 10.1% in the coming time. The advantages of bioinoculants help to enhance the market of bioinoculants worldwide.

16.6 Different Types of Bioinoculants

16.6.1 Biofertilizers

The different types of biofertilizers include plant growth promoting rhizobacteria, phosphorus and potassium solubilizing microorganisms, mycorrhiza, symbiotic and non-symbiotic nitrogen-fixing bacteria. The PGPR are the beneficial bacteria found near the rhizosphere region of the plants. Phosphorus and potassium solubilizing microorganisms convert the insoluble form of phosphorus and potassium to soluble form which can be readily taken by the plants. Mycorrhiza is the symbiotic association between the plants and fungi. In this symbiotic association, the plants prepare the food, i.e., carbohydrates (sugars) which are made available to the fungi and the fungi helps in the absorption of water and nutrients to the plants (Chen et al. 2018). The symbiotic and non-symbiotic nitrogen-fixing bacteria help to make available the major element, i.e., nitrogen, to the plants.

16.6.2 Biopesticides

The bioinoculants can be used as biopesticides which are safe and do not impose any toxic effects on the crops (Pathak and Kumar 2016). The main biopesticides which have gained wide attention are *Trichoderma, Bacillus thuringiensis,* and *Azospirillum*. Biopesticides are used to control the insects and pests on the crops. The biopesticides are target specific and required in small amount. They are safe to use, naturally biodegradable, economical, easy and do not cause any environmental pollution. The biopesticides therefore are used as alternate to the chemical fertilizers. The biopesticides when used with Integrated Pest Management (IPM) improve the yield of many crops (Kawalekar 2013). Biopesticides include viz., microbial pesticides, biochemical pesticides, and plant-incorporated protectants (PIPs) (Thakur et al. 2020). The biopesticides can be combined with organic manures for sustainable agriculture (Kaushik et al. 2019).

16.6.3 Organic Decomposers

The decomposing microorganisms convert the complex organic matter into very simpler form, so that it can be readily utilized by the plant for their growth. The organic decomposing microorganisms include *Trichoderma*, *Aspergillus* sp., etc.

16.7 Methods of Application of Bioinoculants

The different methods for applying bioinoculants into soil include (Mahanty et al. 2016):

- Seed inoculation.
- Sprinkle method.
- Slurry is prepared using bioinoculants and adhesive. The seeds are coated with this slurry using some ground material viz., lime.
- Seed pelleting.
- Seedling root dip.

16.8 Preparation of Bioinoculant

16.8.1 Preparation of Inoculum

The inoculum of PGPR is prepared by growing the PGPR in suitable media at 28 °C for 48 h on a rotary incubator shaker. The cell count is determined which has to be 1×10^8 cells/mL. After this, the inoculum (10 mL) and sterilized carrier (10 g) are mixed properly with calcium carbonate [$CaCO_3$] (1 g) to prepare the slurry. The $CaCO_3$ serves as adhesive.

16.8.2 Disinfection of Seeds

The seeds are disinfected with 95% ethanol for 5 min and then by 0.2% mercuric chloride ($HgCl_2$) for 3 min. The disinfected seeds are further washed three times with sterile distilled water to remove any traces of the $HgCl_2$ (Sachdev et al. 2009). The disinfected seeds are coated with slurry and used for the growth of plants. The preparation of bioinoculants is represented in Fig. 16.3.

16.9 Types of Bioinoculant Formulation

The types of bioinoculant formulations are peat, liquid, granules, and freeze-dried powders.

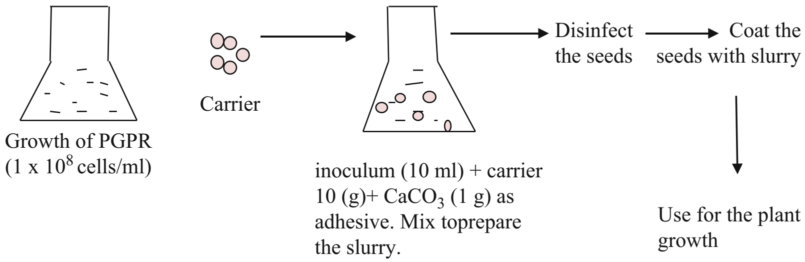

Fig. 16.3 Preparation of bioinoculants

16.9.1 Peat

Peat is a carrier used for PGPR. It contains nutrients and therefore can allow the growth of beneficial microorganisms (Bashan et al. 2014). Peat must be economical, readily available, safe, and easy to autoclave and have good water-holding capacity (Malusa et al. 2012).

16.9.2 Liquid Bioinoculants

Liquid bioinoculants are in aqueous form (e.g., broth cultures or polymer-based suspensions). The mode of application of liquid bioinoculants on seeds or soil is easy (Herrmann and Lesueur 2013). These liquid bioinoculants have good amounts of minerals, nutrients, and also cell protectants which improves the plant growth (Sahu and Brahmaprakash 2016). They have long shelf-life as compared to solid-based formulations and can be used on a small scale (Mahanty et al. 2016).

16.9.3 Granules

Granules are prepared using small marble, calcite, or silica grains with inclusion of adhesive. The granules are coated with PGPR (Bashan et al. 2014). Granules are safe, easy to use and apply (Bashan et al. 2014).

16.9.4 Freeze-Dried Powders

The bioinoculants in freeze-dried powder form are prepared by making use of soil or organic/inert carrier (Bashan et al. 2014).

16.10 Mechanisms for the Plant Growth

16.10.1 Mechanism for the Plant Growth by Plant Growth Promoting Rhizobacteria

The PGPR improve the plant growth by production of plant hormones, iron chelation, enzymes production, etc. There is a report where IAA production from *Serratia* sp. isolated from chickpea nodules enhanced the grain biomass of chickpea in mineral-deficient areas (Zaheer et al. 2016). PGPR strain *Promicromonospora* sp. SE188 due to production of plant growth hormone enhanced the shoot length (Kang et al. 2014). The use of PGPR as bioinoculants can be due to colonization of the plant roots, exudation by the plant roots, the type of soil, and moisture content in the soil.

16.10.2 Mechanism of Inorganic Phosphorus Solubilization by PSMs

The organic acid produced in the periplasmic space is the reason for solubilization of phosphorus (Zhao et al. 2014). This organic acid such as gluconic acid produced causes a decrease in pH which gives out H^+ ions, with accumulation of cation. Gluconic acid chelates the cations bound to phosphate and this helps to make phosphorus available to the plants (Alori et al. 2017).

16.10.3 Mechanism of Organic Phosphorus Solubilization by PSMs

The enzymes are involved in the mechanism of solubilization of organic phosphorus. One enzyme is phosphatase. Another enzyme is phytase that helps in the breakdown of phytate with the release of phosphorus which can be readily used by the plants.

16.10.4 Mechanism of Biological Nitrogen Fixation

The diazotrophs help to fix the nitrogen by biological way. The nitrogenase enzyme helps in biological nitrogen fixation (BNF), where nitrogen (N_2) is reduced to produce NH_3 (Geddes and Oresnik 2016).

The reaction is as follows:

$$N_2 + 8H^+ + 8e^- + 16ATP \overset{\text{nitrogenase}}{\rightarrow} 2NH_3 + H_2 + 16ADP + 16Pi \qquad (16.1)$$

16.10.5 Mechanism of Arbuscular Mycorrhizal Fungi

Arbuscular mycorrhizal fungi (AMF) improve plant yield by the uptake of nutrients and minerals in exchange for carbon required for photosynthesis from hosts (Smith et al. 2010) and also reduce stress.

16.11 Advantages of Plant Growth Promoting Rhizobacteria

- Beneficial microorganisms.
- Help in the yield of plants by various mechanisms.
- PGPR microorganisms are easy to cultivate.
- Help to improve the fertility of soil.
- They show antimicrobial activity and thus help to control bacterial and fungal pathogens.
- Help the plants to tolerate biotic and abiotic stress (Hassen et al. 2016).
- Very fruitful in agriculture.

16.12 Sustainable Approach in Agriculture for the Plant Growth

The sustainable approach is very important for the plant growth. It is safe to the environment which is important and also very easy. Also, it will be safe to the humans (Zerihun et al. 2019). The bioinoculants are very useful in sustainable agriculture to increase the crop growth and productivity. This will be helpful in the field of agriculture and will also maintain the balance of the ecosystem (Zandi and Basu 2016).

16.13 Effect of Bioinoculants on the Plant Growth, a Sustainable Approach

There are several reports where PGPR bioinoculants have improved the yield of many plants and crops. There is a study on PGPR as potential bioinoculants for the growth of *Pennisetum clandestinum* (Poaceae) (Manoj and Kaushal 2018; Romero-Perdomo et al. 2019). The PGPR bioinoculants have immense use in sustainable agro practice (Odoh 2017). There is a report where *Bacillus* and *Pseudomonas* bioinoculants have shown increase in seed germination, root and shoot length, dry weight, and chlorophyll content (Padder et al. 2015). The use of *Azotobacter chroococcum* and PSB bioinoculants has shown increase in the no. of leaves per plant in combination of farm yard manure (FYM) + Vesicular Arbuscular Mycorrhizae (VAM) (Pathak et al. 2013). Study is done where *Bacillus* and *Pseudomonas* sp. bioinoculants have shown 23.5% and 21%, respectively, enhancement in the yield of green pod (Rafique et al. 2018). The bioinoculants have shown increase in the growth of crops viz., beans, cotton, legumes, pea, rice, and soybean.

The phosphate solubilizing bacteria (PSB) are also used to prepare various inoculants (Bashan et al. 2014). The PSB single or in combination can increase the growth of various plants (Ruzzi and Aroca 2015). This will reduce the use of chemicals, leading to sustainable agriculture practices. There is a report where bioinoculants (*Bacillus* sp. JN700924 + *Bacillus megaterium* ATCC 14581 + *Pseudomonas monteilii* HQ 995498 and AMF *Glomus intraradices*) in different combinations have improved the yield of *Ocimum basilicum* (Varma et al. 2016). Bioinoculants serve as good biofertilizer, biostimulator, and biocontrol agent (Tallapragada and Seshagiri 2017). There is a report where halophilic bioinoculants helped in nutrient mobilization in saline soil, thereby improving the growth of plants (Tripathi et al. 2015). There is a study on bioinoculants as prospective inputs for achieving sustainability (Gupta et al. 2020). *Azotobacter* bioinoculant has shown to enhance the biomass of plants as well as seed germination (Chennappa et al. 2017). Bioinoculants shape the microorganisms community in the rhizosphere region, which enhanced soil nitrogen turnover and soil fertility (Sharma et al. 2020). Also, there is a report on increase in the yield of rice plant with the use of *Rhizobium leguminosarum* inoculant in Australia (Kecskes et al. 2013). There is a study where *Azotobacter* has been found to provide 15–93 kg N/ha for *Paspalum notatum* (Youssef and Eissa 2014). There is a report on increase in the plant height, root length, stem diameter, and dry weight of *Zea mays* due to *Azotobacter* and *Azospirillum* bioinoculants (Iwuagwu et al. 2013).

16.14 Bioinoculants for Control of Plant Diseases

The bioinoculants help to control the fungal diseases in plants (Babalola and Glick 2012). *Colletotrichum coccodes*, *Fusarium* sp., *Trichoderma harzianum*, *Aspergillus fumigatus*, *Aspergillus niger*, *Penicillium funiculosum*, *Penicillium aurantiogriseum*, *Penicillium citrinum*, and *Trichoderma koningii*, etc. are reported to exhibit activity against the fungal phytopathogens (Olanrewaju et al. 2017). *Bacillus amyloliquefaciens*, *Amphibacillus xylanus*, *Bacillus subtilis*, etc. are reported to show activity against *Aspergillus flavus*. *Pseudomonas* sp. is also studied to control *Fusarium* wilts (Alori and Babalola 2018). *Bacillus* sp. due to production of volatile compounds can help to control diseases in many plants (Alori and Babalola 2018). All these bioinoculants are reported to control plant diseases caused by bacteria and fungi.

16.15 Solid State Fermentation for the Production of Plant Growth Substances Using PGPR

The solid state fermentation (SSF) can be used for the production of plant growth promoting substances using agroindustry by-products substrates viz., bagasse, pressmud, corn cob, sawdust, paddy husk, peanut shell, coconut husk, wheat straw, etc. These agroindustry by-products are wastes, so they can be converted to value-added products such as compost, soil conditioner, enzymes, and plant growth hormones by the SSF using PGPR. This is very eco-friendly, economical and also helps in the management of agroindustry by-products.

16.16 Conclusion

The bioinoculants will be useful in agriculture for various plant growths and will also increase the yield of plants. The bioinoculants will not have any disadvantages and will be eco-friendly, economical, and easy to use. The use of chemical fertilizers will be minimized, which is very necessary. This will also lead to sustainable approach in agriculture and also organic farming. The bioinoculants used to enhance the crop growth and productivity will be alternate to other chemical fertilizers and thus help the farmers. The bioinoculants can also be used as biopesticides and for control of bacterial and fungal diseases in plants. This will be really fruitful in the field of agriculture.

16.17 Future Prospects

- New bioinoculants should be developed and studied.
- The bioinoculants (using the consortium of microorganisms) should be studied on a large scale by doing the field experiments.
- Molecular aspects of PGPR in plant growth promotion also need to be considered. More research needs to be done in this area.

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17 Microbial and Enzymatic Bioconversion of Tannery Wastes: Progress Toward a Circular Economy in the Leather Industry

Rania Ouertani, Mouna Mahjoubi, Habib Chouchane, Ameur Cherif, and Mohamed Neifar

Abstract

Leather industry has been considered as one of the most highly polluting industries, because of the generation of solid, liquid, and gaseous wastes containing organic and inorganic matters. During leather processing, huge amounts of untanned and tanned solid wastes are discarded. Nearly 800 kg of solid wastes are generated while production of only 200 kg of finished leather. Generally, 90% of water used in leather manufacturing is generating as wastewaters rich in toxic and hazardous compounds. However, these wastes accumulates in nature and contributes to a global pollution over the years. For this reason, the tannery sector needs to implement new greener strategies and solutions to provide a cleaner, more sustainable and more competitive industry. This chapter discusses the possible solutions for utilizing and bioconverting leather industry wastes such as, the bioremediation of dye and chromium contaminated wastewaters, the enzymatic digestion of chrome shavings, and the bioconversion of organic wastes as fleshings and keratin rich wastes to renewable energy and biofuels.

Keywords

Leather industry · Solid waste · Wastewater · Waste treatment · Cleaner technologies

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17.1 Introduction

The leather industry is an old industrial sector which produces a variety of products such as leather goods, shoes, gloves, clothes, and furniture. It is an industry which is based on the use of co-products as raw material and therefore the valorization of food industry wastes, particularly from meat processing (Bizzi et al. 2020). This industry is considered as one of the most important sectors in the development of the world economy by producing each year more than 400,000 tons of heavy leather worldwide (Bouacem et al. 2016; Beghetto et al. 2017). Leather manufacturing is comprised of three main steps: beamhouse operations, tanning operations and finishing operations (China et al. 2020; Villalobos-Lara et al. 2020). During these processes, several baths were used for hides and skins treatment. These baths which are full of chemicals and organic wastes were unfortunately generated in nature and caused a serious damage to the environment and human health. Among these wastes, we found chromium, salts, sulphide, hair, fats, fatliquors, dyes, etc. (Zhao and Chen 2019; China et al. 2020; Tang et al. 2021). This chapter provides a review of the treatment and valorization of leather industry wastes including the bioremediation of synthetic dye and hexavalent chromium contaminated wastewaters and the biotransformation of solid wastes mainly chrome shavings and keratin rich wastes into valuable and useful products.

17.2 Leather Making Process

Once animals were slaughtered, fresh hides and skins will be used as raw material and transported to leather industry where they will be arranged depending on their species, size, weight and quality. After selection, hides and skins were conserved to avoid any microbial contamination (Kahsay et al. 2015).

Leather manufacturing is a long process aimed to transform raw hides and skins into resistant finished leather, and this through several operations. However, each operation requires inputs such as water and chemicals and leads to outputs such as wastewater, organic and mineral pollutants (Jahan et al. 2014; Elabed et al. 2019) (Fig. 17.1).

The process started with beamhouse operation which consists of the preparation of raw hides and skins for tanning by the elimination of all components other than collagen including the elimination of salt used in conservation, the removal of blood and all other dirt attached to the skin (Nazer and Siebel 2006; Dixit et al. 2015). Soaking enzymes generally proteases and carbohydrases could be also implicated in the degradation of fats and proteins (other than collagen) and also make the rehydration easier. Once cleaned, hides and skins will be treated with sodium sulphide in order to remove hair and epidermis. The liming operation implicates the use of huge amounts of water with approximately 4 to 6 L/kg of skin/hide (Rao et al. 2003; Nazer and Siebel 2006). After liming, pickling is performed for fibres dehydration using sulphuric acid and sodium chloride and also implicates the use of large quantity of water (about 80–100%) (Thanikaivelan et al. 2004).

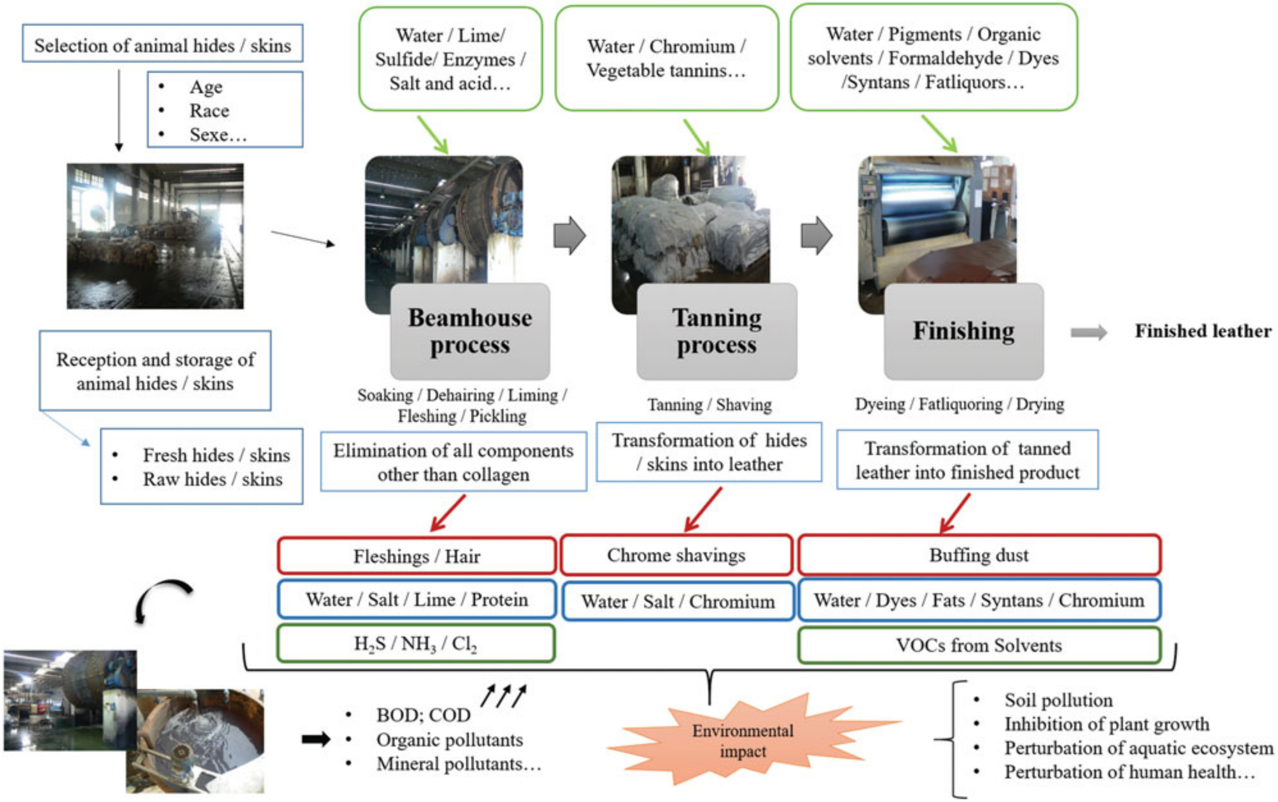

Fig. 17.1 Leather making and wastes generated from the leather industry. The inputs are illustrated in light green and the outputs in red (solid waste), blue (liquid waste) and dark green (gaseous waste)

The second and the most important operation in leather manufacturing is tanning. This irreversible operation leads to the transformation of hides and skins into resistant leather. During this process, the leather acquires specific properties as stability and heat resistance (Duraisamy et al. 2016; Nur-E-Alam et al. 2018). Tanning could be performed either by chrome tanning or by vegetable tanning, but the most used method in 60–70% of leather industries is chrome tanning using chromium salts. This tanning agent induces the formation of a complex with collagen molecules through covalent bonds and stabilizes collagen against microbial attacks (Oruko et al. 2020). However, only 60% of chromium salts are fixed to collagen while the other 40% are discharged in tanning effluent (Erdem 2006; China et al. 2020).

Finally, the finished operation took place to ameliorate the final aspect of the product. It generally consists of giving a more uniform appearance and softness for leather and hiding surface imperfections by the use of dyes and the injection of fats and oils. This step also leads to the generation of wastewater full of organic and mineral pollutants (Thanikaivelan et al. 2004, 2005).

17.3 Characterization of Leather Industry Wastes

Despite its importance, the leather industry is known of being involved in a huge environmental pollution. This industry is generating hazardous liquid and solid wastes containing organic and mineral pollutants such as proteins, lipids, sulphides, heavy metals, solvents, buffing dust, syntans, salt, acids and dyes (Fig. 17.1). Gaseous wastes were also generated during leather process which leads to the emission of toxic and poisonous compounds mainly hydrogen sulphide, ammonia and volatile organic compounds (VOCs) derived from different solvents used in leather manufacturing (Hashem et al. 2015a, b; Sivaram and Barik 2019). As shown in Table 17.1, these wastes are characterized with a high chemical oxygen demand (COD); biochemical oxygen demand (BOD); total suspended solids (TSS), dyes and chromium concentrations (El-Sheikh et al. 2011; Chowdhury et al. 2015; Nur-E-Alam et al. 2018). According to Nur-E-Alam and its collaborators in 2018, the amount of COD and BOD in wastewaters could exceed 21,000 and 12,000 mg/L, respectively. Chowdhury and collaborators in 2015 also revealed that chromium concentration in tannery effluent could reach 2000 mg/L.

17.4 Bioremediation of Leather Industry Wastewaters

17.4.1 Microorganisms Involved in Synthetic Dye Decolourization and Degradation

Generally, dyeing process is performed after tanning using mainly acid, basic or direct dyes (Table 17.2). Mixing different dyes could be also used to provide a particular colour for finished product. During this process, different dyes were not

Table 17.1 Characteristics of leather industry effluents

COD (mg/L)	BOD (mg/L)	pH	TSS (mg/L)	Sulphides (mg/L)	Sulphate (mg/L)	Cr (mg/L)	Dyes (mg/L)	References
5000–5500	3000–3500	8–9	2500–3000	–	1800–2000	80–100	–	Krishanamoorthi et al. (2009)
3200	1000	7	1240	–	–	2075	1820	Chowdhury et al. (2015)
2840	1359	7.9	203	–	2400	–	–	Cooman et al. (2003)
13,000–14,000	5000–8500	–	1200–9000	90	–	–	–	El-Sheikh et al. (2011)
12,840	4464	8.3	1250	–	–	10.348	–	Jahan et al. (2014)
3000–4000	1500–2000	8–11	2000–3000	50–100		60–100	–	Zhao and Chen (2019)
3640	1520	7.6	1040	210	–	10	–	Prakash et al. (2014)
1378	983	6.3	3985	–	756	3.13	–	Ahamed et al. (2014)
2787	508	4.1	–	–	2848	596	–	Elabed et al. (2019)
4335	1800	3.39	–	–	5100	–	–	Jain et al. (2006)
3700	1470	7.4	2690	440	–	22	–	Apaydin et al. (2009)
3980	920	7.5	6800	–	4000	9.81	1760	Chowdhury et al. (2013)
2490–21,060	1700–12,600	6.2–8.2	2395	–	–	10–616	–	Nur-E-Alam et al. (2018)

COD chemical oxygen demand, *BOD* biochemical oxygen demand, *TSS* total suspended solids, *Cr* chromium

Table 17.2 Different classes of dyes used in leather industries

Dye class	Examples	Characteristics	References
Acid dyes	• Acid blue 113	• Dyeing leather, nylon, Wool, silk…	Huang et al. (2015), da Fontoura et al. (2017), Rocha et al. (2017), Shanmugam et al. (2019)
	• Acid blue 161	• Soluble in water	
	• Acid black 210	• Stable in alkaline medium	
	• Acid black ATT		
Basic dyes	• Malachite green	• Dyeing leather, textile, silk, ceramic, wood, paper…	Kumar and Chopra (2017), Ali and Muthuraman (2020)
	• Methylene blue	• Soluble in water and alcohol	
	• Crystal violet		
Direct dyes	• Direct red 28	• Dyeing leather, cotton, silk, plastic…	Sauer et al. (2006), Huang et al. (2015), Hernández-Zamora et al. (2015)
	• Direct black 38	• Soluble in water	
	• Direct violet N		

totally fixed to leather. Wastewater containing the excess of all these colouring agents is discharged in the environment (Sharma and Malaviya 2016). Therefore, it requires an intensive treatment before discharge. Until today, various studies have confirmed the toxicity of synthetic dyes particularly azo-dyes for microorganisms, plants, animals and even for human. Their complex structure makes them less susceptible to biodegradation. Therefore, they represent a huge risk to human health, being implicated in many diseases as diarrhoea, skin irritation and even cancer especially in liver and lungs (Selvam et al. 2003; Sen et al. 2016; Sivakumar and Anbalagan 2016).

Dyes are known with their recalcitrant and complex structure. However, many studies have described the use of microorganisms and their effectiveness in dye removal and degradation during the treatment of industrial wastewaters (Table 17.3). These studies highlighted the contribution of bacterial strains, yeasts and fungi in bio-decolourization through different enzymatic and non-enzymatic mechanisms (Sen et al. 2016; Ajaz et al. 2020).

It’s been reported that the most efficient bacteria that have the ability to degrade industrial dyes belong to the genera of *Bacillus* and *Pseudomonas*. Many researchers have identified various bacterial species with a high dye removal yield such as *Bacillus cereus* (Tripathi and Garg 2014), *Pseudomonas aeruginosa* (David et al. 2015; Mishra and Maiti 2019) and *Pseudomonas stutzeri* (RS1D) (Khan et al. 2019).

Table 17.3 Microorganisms implicated in decolourization of dye contaminated wastewaters

Microorganisms	Dye	Conditions of incubation	Dye (mg/L)	% and time of decolourization	References
Bacillus cereus RMLAU1	Tannery dyes	Shaking (150 rpm); pH 7; 35 °C	–	42%/24 h	Tripathi and Garg (2014)
Pseudomonas aeruginosa	Tannery dye	Shaking (110 rpm); pH 7; 30 °C	–	92.77%/96 h	David et al. (2015)
Lactobacillus paracasei CL1107	Acid black ATT	Shaking (170 rpm); pH 7; 37 °C	100	92.3%/7 days	Huang et al. (2015)
P. aeruginosa 23N1	Reactive orange 16	Shaking (150 rpm); pH 10; 40 °C	50	95.1%/48 h	Mishra and Maiti (2019)
Shigella sp.	Crystal violet methylene blue	Shaking (150 rpm); 30 °C	–	64.3%/6 days 65.7%/6 days	Kumar and Chopra (2017)
P. stutzeri (RS1D)	Black dye	pH 7; 35 °C	1000	100%/10 days	Khan et al. (2019)
Listeria monocytogenes RS2E				100%/9 days	
Trametes versicolor	Black dycem TTO	Shaking (135 rpm); 25 °C	150	86–89%/ 10 days	Baccar et al. (2011)
Aspergillus flavus SPFT2	Tannery dye	Shaking (150 rpm); pH 5.3; 30 °C	–	61.5%/6 days	Sharma and Malaviya (2016)

Many other genus such as *Shigella* and *Listeria* were also widely studied (Kumar and Chopra 2017; Khan et al. 2019).

Until now, the mechanism of dye biodegradation in yeasts species is still unclear and needs more investigations (Pajot et al. 2011; Martorell et al. 2012). Meanwhile, it has been revealed that several yeasts are involved in dye removal (Pajot et al. 2011; Jafari et al. 2013). For example, Pajot and collaborators, in 2011, have reported that *Pichia membranifaciens* HP-3098 and *Candida sorbophila* HP-3094 were able to degrade 200 mg/L of Vilmafix Blue RR-BB and Vilmafix Red 7B-HE dyes with a yield exceeding the 95% in just 36 h.

Other than bacteria and yeasts, ligninolytic fungi have been widely used in dye removal and degradation (Pajot et al. 2011; Singh et al. 2015; Sen et al. 2016; Neifar et al. 2016; Singh and Singh 2017; Ajaz et al. 2020). Among these fungi, Trametes versicolor reported by Baccar and collaborators in 2011. It was able to remove around 89% of Black Dycem TTO dye (150 mg/L) in just 10 days. Devi and collaborators, in 2013, have also described the effectiveness of the application of the fungal strains *Aspergillus niger*, *Aspergillus flavus* and *Penicillium* spp. in the decolourization of azo-dyes. The efficiency of *Aspergillus* genus was also confirmed by Sharma and Malaviya in 2016 by the application of *Aspergillus flavus* SPFT2 in the treatment of tannery effluent.

The decolourization using bacteria and fungi was achieved through the utilization of pool of oxidative enzymes such as azoreductases (EC 1.7.1.17), laccases (EC 1.10.3.2) and peroxidases (EC 1.11.1) (Neifar et al. 2011; Abdel-Hamid et al. 2013; Sen et al. 2016).

Bacterial azoreductases are the most used in azo-dyes decolourization due to their extremophilic properties. These enzymes are able to break down the azo bond even under saline and alkaline conditions. *Bacillus megaterium, Bacillus subtilis, Pseudomonas aeruginosa,* and *Bacillus latrosporus* were investigated in several projects, being able to degrade synthetic dyes through azoreductase activities (Chen 2006; Singh et al. 2015; Ajaz et al. 2020).

Microbial laccases were produced by either bacteria, yeasts or fungi (Wakil et al. 2017, 2019; Yang et al. 2017; Ponomareva et al. 2019; Unuofin et al. 2019; Arregui et al. 2019). These oxidoreductases were involved in bioremediation strategies in the decolourization of dyes in textile and leather industries wastewaters (Dubé et al. 2008; Pajot et al. 2011; Jafari et al. 2013; Bharagava et al. 2018). It has been found that the white rot fungi such as *Trametes versicolor* (Couto and Herrera 2006), *Thelephora* sp. (Selvam et al. 2003), and *Pleurotus ostreatus* (Bharagava et al. 2018) were able to degrade dyes using laccases. Other ligninolytic enzymes were involved in dye removal including lignin peroxidase and manganese peroxidase. Bharagava and collaborators, in 2018, have described the biotechnological potential of *Aeromonas hydrophila*. This bacterial strain was able to remove 99% of 100 mg/L of crystal violet dye in just 48 h. Pajot and collaborators, in 2011, also reported the important role of peroxidases in dye removal. *Pichia membranifaciens* HP-3098 and *Candida sorbophila* HP-3094 were able to degrade more than 95% of dyes through peroxidases enzymes. Also *Thelephora* sp. uses lignin peroxidase and manganese peroxidase for the decolourization of orange G, congo red and amido black 10B (Selvam et al. 2003).

In addition to dyes biodegradation, several microorganisms are able to generate electricity via microbial fuel cell technology (Huang et al. 2011; Khan et al. 2015; Ezziat et al. 2019; Aarthy et al. 2020; Xu et al. 2020). Lai and collaborators, in 2017, have described the efficiency of *Ganoderma lucidum* BCRC 36123, a dye decolourizing and laccase producing white rot fungus in electricity generation using microbial fuel cell technology. It shows a rate of decolourization of acid orange 7 of 90% with the generation of maximal power density which reached 13.38 mW/m^2. Moreover, it was found that the application of the bacterial strain *Stenotrophomonas* sp. based microbial fuel cell leads to the production of electricity (power density was around 370 mW/m^2) with total decolourization of the reactive black 5 dye (Galai et al. 2015).

17.4.2 Microorganisms Involved in Hexavalent Chromium Reduction and Removal

Chromium wastes come mainly from tanning effluents and chrome shavings (Nigam et al. 2015; Shakilanishi et al. 2017; Abebaw and Abate 2018). Chromium is considered among the most toxic and dangerous heavy metals due to its mutagenicity and carcinogenicity (Joutey et al. 2015; Ezziat et al. 2019; Ouertani et al. 2020a). In fact, this heavy metal exists in nine forms of valence ranging from −II to +VI, thus, the most abundant forms are trivalent chromium (Cr III) and hexavalent chromium (Cr VI) (Smith et al. 2002; Garg and Tripathi 2013; Sanjay et al. 2020). The soluble Cr VI is considered as the most toxic form, meanwhile, the stable Cr III is the less toxic form. Therefore, several research projects have focused on the efficiency of the reduction of Cr VI into Cr III using various microorganisms. It has been reported that microbes mainly bacteria, yeasts and fungi were used in bioremediation of leather industry wastewaters through chromium removal (Table 17.4). The best studied chromium reducing microorganisms are *Rhizobium leguminosarum* (Raaman et al. 2012), *Bacillus cereus* NCr4 (Tamindžija et al. 2019), *Chelatococcus daeguensis* TAD1 (Li et al. 2016), *Pseudomonas putida* SKG-1 (Garg and Tripathi 2013) and *Thermus scotoductus* strain SA-01 (Opperman and Van Heerden 2007). Furthermore, it was investigated in the study of Ouertani and collaborators in 2020a, that *Microbacterium metallidurans* TL13, a bacterial strain isolated from a tannery wastewater, was able to remove more than 70% of 500 mg/L of Cr VI and to promote plant growth under metal stress.

Apart from bacteria, the application of yeasts and fungi was extensively reported in literature. *Saccharomyces cerevisiae* was used in the bioremediation of heavy metal contaminated effluents through the accumulation of more than 99% of Cr VI (90 mg/L) (De Rossi et al. 2018). Other yeast species were also used in chromium bioremediation mainly *Candida tropicalis* strain HE650140 (Bahafid et al. 2013), *Yarrowia lipolytica* NCIM 3589, *Yarrowia lipolytica* NCIM 3590 (Rao et al. 2013), *Pichia jadinii* M9 and *Pichia jadinii* M10 (Fernández et al. 2012).

As for fungal strains, researchers have confirmed the effectiveness of these microorganisms and their biotechnological potential in chromium detoxification (Table 17.4). Long and collaborators, in 2018, have revealed that *Penicillium oxalicum* SL2 has the capacity of removing 98% of chromium with an initial concentration of 200 mg/L. Other fungi were investigated, mainly, *Aspergillus niger* MTCC 2594 (Mala et al. 2006), *Rhizopus Oryzae* (Sukumar 2010) and *Trichoderma viride* (Wang and Wang 2013).

As reported by Ouertani and collaborators in 2020a, *Microbacterium metallidurans* TL13 isolated from a tannery wastewater can reduce chromium by several mechanisms. Chromium gets into the cell via the sulphate permease leading to the activation of the efflux system (chromate transporter). Cr VI was then reduced into Cr III through enzymatic pathway which involved thioredoxin reductase, superoxide dismutase, catalase and glutathione peroxidase. It has been also found that TL13 could remove chromium by forming an exopolysaccharides (EPS)-chromium complex.

Table 17.4 Selected microorganisms involved in chromium bioremediation

Strain	Origin	Condition	Cr VI initial concentration (mg/L)	% of Cr VI removal	Mechanism of Cr removal	Biotechnological potential	References
Bacteria							
Rhizobium leguminosarum	Tannery effluent contaminated soil	pH 7; 30 °C	150 (0.5 mM)	74.7	Chromium reduction	Bioremediation of tannery effluent contaminated soils	Raaman et al. (2012)
R. sp. PGS				30.7			
Bacillus cereus NCr4	Serpentine soil	pH 7.2; 26 °C	40 (0.2 mM)	98.5	–	Bioremediation of chromium contaminated soils	Tamindžija et al. (2019)
Serratia marcescens ZD-9	Sediment of a tannery effluent outfall	pH 8; 30 °C	20	80	Polysaccharides	Detoxification of tannery effluents	Xu et al. (2018)
Chelatococcus daeguensis TAD1	Biofilm from a biotrickling filter		10	98	Chromium reduction	Bioremediation of chromium contaminated wastewaters	Li et al. (2016)
Streptomyces sp. MC1	Sugarcane	pH 7; 30 °C	50	75	Chromium reduction	Bioremediation of chromium contaminated soils	Polti et al. (2009)
S. M7	Wastewater sediment	pH 7; 30 °C	25	49.5	Chromium reduction	Plant growth promotion in chromium contaminated soils	Simon Sola et al. (2017)
Pseudomonas putida SKG-1	Dairy sludge	pH 8; 30 °C	100	98	Chromium reduction	Bioremediation of industrial wastes contaminated with chromium and pentachlorophenol	Garg and Tripathi (2013)
Thermus scotoductus strain SA-01	Groundwater in a south African gold mine	pH 7; 80 °C	20	20	Chromium reduction	Bioremediation of industrial wastes	Opperman and Van Heerden (2007)

Microbacterium metallidurans TL13	Tannery wastewater	pH 9; 30 °C	500	71.68	Thioredoxin reductase, superoxide dismutase, catalase/peroxidase, and glutathione	Bioremediation of tannery wastewater, plant growth promotion in heavy metal contaminated soils	Ouertani et al. (2020a)
Yeast							
Candida tropicalis strain HE650140	Chromium contaminated site	pH 7.2; 30 °C	50	100	Chromium reduction	Bioremediation of chromium contaminated soils	Bahafid et al. (2013)
Yarrowia lipolytica NCIM 3589	–	pH 2; 30 °C	100	83	Chromium reduction	Bioremediation of chromium contaminated wastes	Rao et al. (2013)
Y. lipolytica NCIM 3590			100	88			
Saccharomyces cerevisiae	Commercial lyophilized yeast	pH 5; 60 °C	90	99.66	Bioaccumulation (biosorption)	Bioremediation of heavy metal contaminated effluents	De Rossi et al. (2018)
Pichia jadinii M9	Textile effluent	pH 3; 30 °C	50	63	Chromium reduction	Bioremediation of textile effluents	Fernández et al. (2012)
P. anomala M10			50	56			
Fungi							
Penicillium oxalicum SL2	Indoor air	pH 7; 30 °C	200	98	Chromium reduction	Detoxification of chromium contaminated environment	Long et al. (2018)
Aspergillus niger MTCC 2594	–	pH 9; 28–32 °C	3.6	78	Bioaccumulation (biosorption)	Detoxification of chromium from tannery wastewaters	Mala et al. (2006)
A. niger	Effluent of leather tanning	pH 6; 30 °C	500	75	Bioaccumulation (biosorption)	Detoxification of tannery effluents	Srivastava and Thakur (2006)
Rhizopus Oryzae	–	pH 7; 37 °C	400	91.15	Chromium reduction	Biotreatment of industrial wastewaters	Sukumar (2010)

(continued)

Table 17.4 (continued)

Strain	Origin	Condition	Cr VI initial concentration (mg/L)	% of Cr VI removal	Mechanism of Cr removal	Biotechnological potential	References
Trichoderma viride	Wastewater	pH 7; 30 °C	50	66.16	Bioleaching	Bioremediation of heavy metal contaminated wastewaters	Wang and Wang (2013)

The studies of Gangadharan and Nambi (2015), Fei et al. (2017) and Uddin et al. (2020) reported the role of chromium reduction combined to the electricity production. Microorganisms used in microbial and enzyme fuel cells were able to use wastewater as a culture medium and to break the organic and inorganic matters to produce electricity. Wang and collaborators in 2008 showed that 100 mg/L of chromium VI reduced into chromium III could produce about 150 mW/m^2 in 150 h. Gangadharan and Nambi (2015) revealed that the reduction of almost total concentration of chromium (100 mg/L) is coupled with the generation of about 767.01 mW/m^2 of power density, in 48 h. The study of Fei and collaborators, in 2017, has succeeded to remove more than 80% of Cr VI with maximal power density up to 132.1 mW/m^2 in 48 h.

17.5 Bioconversion of Leather Industry Solid Wastes

17.5.1 Valorization of Leather Industry Fleshings Wastes

During the liming-fleshing process, a significant amount of limed fleshings solid wastes were discarded. These wastes represent around 55% of untanned wastes and characterized with a high lipids content including fats and oils (Hashem et al. 2015a, b; Sandhya et al. 2016). Therefore, the biotransformation of these wastes is considered as an energy saving method for the production of a renewable energy. However, these fleshings wastes were explored in terms of valorization leading to the extraction of fats and oils and the production of biofuels. As reported by Kubendran et al. (2017), da Fontoura et al. (2017), Fazal et al. (2018), Li et al. (2019), these lipid containing wastes are useful for biodiesel production. Kubendran and collaborators in 2017 have succeeded to extract 70% of fats contained in the fleshings wastes leading to biodiesel production. Another study by Hashem and collaborators in 2017 has indicated that fats extracted from leather industry fleshings are useful for soap manufacturing.

17.5.2 Biotransformation of Chrome Shavings Waste into Valuable Products

Just after tanning, the wet blue leather should be shaved to give desired shape and thickness. The excess of wet blue (small and thin pieces) were directly eliminated. Meanwhile, the chrome shavings wastes were generated (Rigueto et al. 2020; Kandasamy et al. 2020). Chrome shavings solid waste consists of collagen (nearly 75–90%), chromium, fats, oils, etc. (Pati et al. 2014; Shakilanishi et al. 2017; Li et al. 2019).

The bioconversion of these chromium containing wastes is achieved through two steps (Kocurek et al. 2017) (Fig. 17.2). Firstly, the denaturation under alkaline conditions of collagenous wastes which leads to the gelatin extraction useful in many applications such as cosmetics and agrifood (gelatin making). Then, the

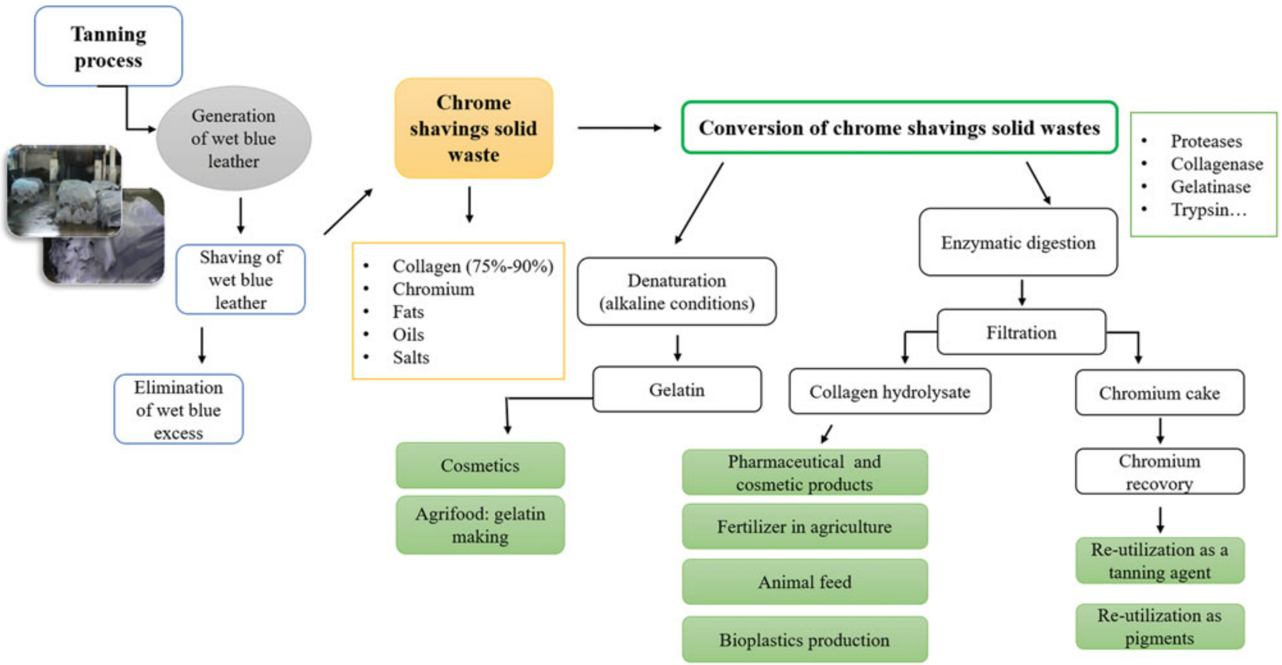

Fig. 17.2 Conversion of chrome shavings wastes and potential applications

second step which consists of the enzymatic digestion. Proteases, trypsin, collagenase and gelatinase are among several enzymes that could be involved in this hydrolysis (Shakilanishi et al. 2017; Li et al. 2019; Gomes et al. 2020). After filtration, chromium remains in the filter (chromium cake or chromium sludge) could be extracted and recovered for further reutilization as a tanning agent in leather process or a pigment (Kocurek et al. 2017; Li et al. 2019). Hence, the dechromed protein hydrolysate or collagen hydrolysate is a mixture of small peptides and amino acids including glycine, proline and hydroxyproline that represents around 50% (Shanthi et al. 2013; Abebaw and Abate 2018). In fact, collagen hydrolysate has been applied in biomedical sector, in cosmetics, in agriculture as a biofertilizer, in livestock production as an animal feed, in bioplastics production, biogas production and many other applications (Pati and Chaudhary 2015; Epure et al. 2018; Gomes et al. 2020; Kandasamy et al. 2020).

In an earlier study in 2017, Shakilanishia and collaborators have used the collagen hydrolysate as a substrate for bacterial strain *Bacillus cereus* VITSN04 to enhance the production of keratinolytic enzymes useful in dehairing during leather making. Another study of Pati and Chaudhary in 2013 had confirmed that the high value collagen hydrolysate could be also used as a substrate for microorganisms to produce biogas. Also, a recent project of Epure and collaborators in 2018 focused on the importance of the collagenous hydrolysate application in the agriculture field as a biofertilizer. The effectiveness of this valuable product has been proven on cereal seeds such as wheat and oat. The results show a remarkable increase in the germination yield.

17.5.3 Valorization of Finished Leather Solid Wastes

Animal hides and skins are biodegradable resources that can be damaged by several factors such as parasitic diseases, microbial putrefication, animal husbandry, bad conservation, mechanical damage or human practices, which leads to their rejection (Kahsay et al. 2015). Furthermore, these factors affected the value of hides and skins, thus the production of low quality leather. However, a large quantity of leathers are discarded which leads to considerable financial loss for the leather industry (Teklay et al. 2019).

Hides and skins are made up of three layers: the epidermis, the dermis and the hypodermis. The dermis which is the major component of leather consists primarily of collagen (Lacouture 2006). This specific structure makes it susceptible to microbial attack (Zhang et al. 2015; Pal and Suresh 2016; Duarte et al. 2016; Bhagwat and Dandge 2018). Biocides and specific enzyme inhibitors are added during industrial processing to improve leather protection by the prevention of the growth of collagenolytic microorganisms. However, this treatment shows certain limitations concerning microorganisms whose already secreted their collagenases (Oppong et al. 2006). Several studies have been focusing on the valorization and the recycling of finished leather solid waste (Senthil et al. 2015; Teklay et al. 2017). Since recent years, in 2015, Senthil and others researchers have used the leather solid waste for the making of blended fabrics. They have used enzymes especially collagenases to biodegrade these collagen rich wastes.

17.5.4 Biotransformation and Valorization of Keratin Rich Wastes

Keratinous wastes were generating in huge quantities during the dehairing process. Bovine hair and ovine wool are the major sources of keratin in leather industry wastes (Brandelli et al. 2010; Verma et al. 2017; Li et al. 2019). Keratin is a fibrous protein known for its highly stable, recalcitrant and insoluble structure due to the presence of disulphide bonds. However, it mainly contains cysteine residues which explains its high sulphur content. It also contains other amino acids such as proline, valine, glycine, lysine and serine (Gopinath et al. 2015; Thankaswamy et al. 2018; Ghaffar et al. 2018). Keratin is classified into two groups depending on its structure: hard keratin with high sulphur content and flexible or soft keratin with a lower sulphur content. Other keratinolytic sources exist in nature such as feathers, human hair, nails, horns and hooves (Laba and Rodziewicz 2014; Kumawat et al. 2016).

Hair and wool containing 90–95% of keratin, are both classified as hard keratins which makes them hard to degrade. The use of physico-chemical methods for keratin degradation causes a huge problem for the ecosystem (Bhari et al. 2019). However, the development of enzymatic strategies using microorganisms and their applications for bioremediation and bioconversion of leather industry keratinous wastes into valuable products become a green solution for contamination (Sharma and Gupta 2016; Zhang et al. 2020).

Keratins are resistant to most proteases, therefore, they are generally degraded by keratinases leading to the production of small peptides and amino acids (Vidmar and Vodovnik 2018). Keratinases (EC 3.4) are able to catalyse the cleavage of the insoluble, recalcitrant and highly stable keratin substrates through the hydrolysis of sulphide bonds. They could easily break down different keratin sources. These enzymes are among proteases that can tolerate different temperatures and pH (Pissuwan and Suntornsuk 2001; Brandelli et al. 2010; Vidmar and Vodovnik 2018).

Keratinolytic enzymes are widespread in nature and could be produced by various microorganisms including fungi and bacteria (Laba and Rodziewicz 2014; Paul et al. 2016; Verma et al. 2017). Microbial keratinases generally belong to the metalloproteases family (EC 3.4.24) but they may belong to serine-proteases family (EC 3.4.21). They could also belong to serine-metalloproteases and their inhibition occurs by the same inhibitors as serine-proteases, and metalloproteases. Keratinase mechanism is still not well characterized (Pissuwan and Suntornsuk 2001; Gupta et al. 2013; Lange et al. 2016).

Bacterial keratinases have been widely described (Table 17.5). It has been proven that the most efficient keratinases are produced by *Bacillus* genus due to their physico-chemical properties (Brandelli et al. 2010; Gopinath et al. 2015; Verma et al. 2017). Among the most studied *Bacillus* species are: *Bacillus subtilis* (Cai and Zheng 2009; Mazotto et al. 2010; Kumar et al. 2010; Daroit and Brandelli 2014), *Bacillus licheniformis* (Hu et al. 2013; Huang et al. 2017; Abdel-Fattah et al. 2018)

Table 17.5 Various keratinase producing microorganisms

Strain	Enzyme class	Enzyme physico-chemical properties	Substrate	Condition of keratin degradation	Biotechnological potential	References
Bacteria						
Bacillus pumilus AR57	Serine keratinase	pH 7–10 (optimum pH 9); T°: 37–50 °C (optimum 45 °C)	Chicken feather, human hair, goat hair,	shaking for 48 h (150 rpm); 37 °C	Degradation of poultry wastes; cosmetic application	Jagadeesan et al. (2020)
Streptomyces sp.	Serine metallo-keratinase	pH 9–12 (optimum pH 11); T°: 25–55 °C (optimum 45 °C)	Chicken feather, hair	Incubation 5 days at 30 °C	Feather degradation, dehairing in leather industry	Tatineni et al. (2008)
Geobacillus stearothermophilus AD-11	Recombinant keratinase (recgeoker)	pH 5–11 (optimum pH 9); T°: 20–90 °C (optimum 60 °C)	Wool, collagen, casein, gelatin	Shaking for 18 h (180 rpm); 37 °C	Degradation of leather industry wastes	Gegeckas et al. (2015)
B. altitudinis RBDV1	Recombinant keratinase (kbalt)	pH 6–11 (optimum pH 8); T°: 35–95 °C (optimum 85 °C)	Chicken feather, human hair, gelatin, casein, nails	Incubation 18 h at 37 °C	Degradation of keratinous wastes	Pawar et al. (2018)
B. polymyxa B20	Serine keratinase	Optimum pH 8; optimum T°: 50 °C	Chicken feather, pig bristle, wool, human hair	Shaking for 4 days (170 rpm); 30 °C	Degradation of keratinous wastes	Laba and Rodziewicz (2014)
B. cereus B5esz		Optimum pH 7; optimum T°: 45 °C				
Micrococcus luteus B1pz	Serine keratinase	Optimum pH 9.4; optimum T°: 55 °C	Lamb’s wool, ostrich feather, pig bristle, chicken feather, human hair	shaking for 15 days (170 rpm); 30 °C	Degradation of keratinous wastes	Łaba and Szczekala (2013)
S. gulbargensis DAS 131	–	pH 7–9 (optimum pH 9); T°: 30–45 °C (optimum 45 °C)	Chicken feather, human hair, human nail	Shaking for 7 days (250 rpm); 30 °C	Poultry waste degradation, application in leather industry	Syed et al. (2009)

(continued)

Table 17.5 (continued)

Strain	Enzyme class	Enzyme physico-chemical properties	Substrate	Condition of keratin degradation	Biotechnological potential	References
Meiothermus taiwanensis WR-220	Serine keratinase	pH 4–11 (optimum pH 10); *T*°: 25–75 °C (optimum 65 °C)	Chicken feather	Incubation 2 days at 65 °C	Degradation of keratinous wastes	Wu et al. (2017)
B. halotolerans 4 BC	–	pH 4–12 (optimum pH 10); *T*°: 30–100 °C (optimum 80 °C)	Chicken feather, bovine hair, keratin azure	Shaking for 10 days (120 rpm); 37 °C	Degradation of keratinous wastes, dehairing in leather industry	Ouertani et al. (2020b)
B. subtilis (MTCC9102)	–	Optimum pH 7; optimum *T*°: 37 °C	Horn meal	Incubation 48 h at 37 °C	Bioremediation and application in pharmaceutical industries	Kumar et al. (2010)
B. licheniformis ALW1	–	pH 7–9 (optimum pH 8); *T*°: 30–65 °C (optimum 65 °C)	Turkey feathers, wool, hair, nail, horn	Incubation 48 h at 37 °C	Biodegradation of feather wastes	Abdel-Fattah et al. (2018)
Fungi						
Doratomyces microsporus	Serine keratinase	Optimum pH 9; optimum *T*°: 50 °C	Human and porcine nails, casein, gelatin	Shaking for 6 days (120 rpm); 30 °C	Cosmetic application	Gradišar et al. (2000)
Aspergillus oryzae	Metallo-keratinase	pH 7–9 (optimum pH 8); *T*°: 30–45 °C (optimum 45 °C)	Chicken and duck feather, sheep wool	Incubation 5 days at 30 °C	Industrial application, animal feed	Farag and Hassan (2004)
Myrothecium verrucaria	Serine keratinase	Optimum pH 9; optimum *T*°: 40 °C	Chicken feather, human hair and nail, sheep wool	Shaking for 4 weeks (120 rpm); 28 °C	Degradation of keratinous wastes	Moreira et al. (2007)

Trichoderma atroviride F6	Serine keratinase	pH 4–11 (optimum pH 8–9); *T*°: 26–70 °C (optimum 50–60 °C)	Chicken feather	Shaking for 5 days (150 rpm); 30 °C	Poultry waste degradation	Cao et al. (2008)
Penicillium spp.	Metallo-keratinase	Optimum pH 6; optimum *T*°: 28 °C	Feather, bovine hair, horn, nails	Shaking for 5 days (200 rpm); 30 °C	Poultry waste degradation, application in leather industry	El-Gendy (2010)
Chrysosporium keratinophilum IMI 338142	–	Optimum pH 7–8; optimum *T*°: 40 °C	Feather, human hair, bovine hair	Incubation 15 days at 40 °C	Bioremediation of keratinous wastes	Singh (2003)
A. flavus	–	Optimum pH 8.5	Feather	Shaking for 40 days (120 rpm); 28 °C	Bioremediation of soils contaminated with keratinous wastes	Kim (2003)
A. fumigatus						

and *Bacillus pumilus* (Kumar et al. 2008; Jaouadi et al. 2014; Jagadeesan et al. 2020). Other bacterial strains have been also reported such as *Meiothermus taiwanensis* (Wu et al. 2017), *Caldicoprobacter algeriensis* (Bouacem et al. 2016), *Bacillus cereus* (Arokiyaraj et al. 2019), *Bacillus aerius* (Bhari et al. 2019) and *Brevibacterium luteolum* (Thankaswamy et al. 2018).

Apart from bacteria, fungi are also considered as an efficient keratinase producing microorganisms. They have the ability to degrade different types of keratins such as feather, hair and wool. Keratinases produced by *Doratomyces microsporus* (Gradišar et al. 2000), *Aspergillus oryzae (*Farag and Hassan 2004), *Trichoderma atroviride* (Cao et al. 2008), *Chrysosporium keratinophilum* (Singh 2003) and many other species have been described and characterized in many researches.

Since several years, keratinases producing microorganisms have been considered as great candidates for bioremediation and degradation of keratin rich wastes leading to the production of valuable organic matters (amino acids) (Paul et al. 2016; Ghaffar et al. 2018). These keratin-based products present a big industrial interest with biotechnological potential and could be widely applied specially in agriculture (biofertilizers), animal feed, pharmaceutical industries, cosmetics, leather industries, etc. (Verma et al. 2017; Zhang et al. 2020).

It has been proven that keratin hydrolysate extracted from bovine hair could serve as an ecological biofertilizer for a durable agriculture. It contributed in the amelioration of soils quality due to its high carbon and nitrogen content. Several hair-based organic fertilizers are already available and commercialized. Instead of fertilizers production, keratin meal also served as nutritional animal feed (Sundar et al. 2011; Sharma and Gupta 2016; Ghaffar et al. 2018). Thankaswamya and collaborators, in 2018, used *Brevibacterium luteolum* (MTCC 5982) for the conversion of keratinous wastes (about 80%) from the leather industry into hair meal in just 72 h and under alkaline conditions. Another study by Hussain and collaborators in 2020, revealed the use of keratins extracted from leather industry solid wastes for the fabrication of sponges useful for the removal of chromium from wastewaters. Furthermore, in 2011, Barbosa and his group have succeeded to develop a process for the fabrication of biodegradable films using bovine hair wastes.

Besides the use of bovine hair for the preparation of renewable products, it have been demonstrated that ovine wool presents similar properties which allows its use as a biofertilizer or animal feed. It contains amino acids, lipids, salts and carbohydrates, hence, it was able to improve soils characteristics. Wool keratin also was also integrated in cosmetical products for skin, hair and nail care (Sundar et al. 2011; Zhang et al. 2020).

17.6 Conclusion

The leather industry contributed in an enormous environmental pollution being responsible for the generation of three types of wastes: liquid, solid and gaseous. In this chapter, we reported the characterization of different wastes and their potential valorization using green processes. Microorganisms and enzymes were involved in bioremediation of wastewaters, particularly dyes biodegradation and chromium detoxification. Enzymatic digestion of chrome shavings, reutilization of finished leather wastes and bioconversion of keratin rich wastes for the production of valuable products were also investigated. The biovalorization of leather industry wastes and the use of an eco-friendly, energy saving and non-polluting alternatives instead of chemicals during leather manufacturing will contribute to an ecological and green industry by the decrease of environmental pollutions and risks.

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