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Biosynthetic Technology and Environmental Challenges

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Biosynthetic Technology and Environmental Challenges

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Preface

Biomass is a renewable organic material and a continuous source of sustainable energy. The bio-based economy is getting more and more interest, and there is a need for innovative and low-emissions economy for the production of industrially important chemicals through a green approach. The biorefinery concept development is based on the techno-economic viability of the proposed process considering raw materials, process options, mass and energy management, market constraints, etc. Stable technologies are required for the proper management of industrial waste. Collaboration between biotechnologists, microbiologists, environmental scientists, biochemical engineers, molecular biologists and modellers is required for sustainable environmental development.

The first international conference on 'Sustainable Energy and Environmental Challenges' (SEEC-2017) was organized under the auspices of 'International Society for Energy and Environmental Sustainability' (ISEES) by the 'Center of Innovative and Applied Bioprocessing' (CIAB), Mohali, held from 26 to 28 February 2017. ISEES was founded at IIT Kanpur in January 2014 with the aim of spreading knowledge in the fields of energy, environment, sustainability and combustion. The Society's goal is to contribute to the development of clean, affordable and secure energy resources and a sustainable environment for the society and to spread knowledge in the above-mentioned areas and spread awareness about the environmental challenges, which the world is facing today. ISEES is involved in various activities such as conducting workshops, seminars and conferences in the domains of its interest. The Society also recognizes the outstanding works done by the young scientists and engineers for their contributions in these fields by conferring them awards under various categories.

This conference provided a platform for discussions between eminent scientists and engineers from various countries including India, USA, South Korea, Norway, Malaysia and Australia. In this conference, eminent speakers from all over the world presented their views related to different aspects of energy, combustion, emissions and alternative energy resources for sustainable development and cleaner environment. The conference started with four mini-symposiums on very topical themes, which included (i) New Fuels and Advanced Engine Combustion,

(ii) Sustainable Energy, (iii) Experimental and Numerical Combustion and (iv) Environmental Remediation and Rail Road Transport. The conference had 14 technical sessions on topics related to energy and environmental sustainability and a panel discussion on 'Challenges, Opportunities and Directions of Technical Education & Research in the Area of Energy, Environment and Sustainability' to wrap up the three-day technical extravaganza. The conference included 2 plenary talks, 12 keynote talks, 42 invited talks from prominent scientists, 49 contributed talks and 120 posters. A total of 234 participants and speakers attended this three-day conference, which hosted Dr. V. K. Saraswat, Member NITI Ayog, India, as a chief guest for the award ceremony of ISEES. This conference laid out the road map for the technology development, opportunities and challenges in this technology domain. The technical sessions in the conference included Advances in IC Engines and Fuels; Conversion of Biomass to Biofuels; Combustion Processes; Renewable Energy: Prospects and Technologies; Waste to Wealth—Chemicals and Fuels; Energy Conversion Systems; Numerical Simulation of Combustion Processes; Alternate Fuels for IC Engines; Sprays and Heterogeneous Combustion of Coal/Biomass; Biomass Conversion to Fuels and Chemicals—Thermochemical Processes; Utilization of Biofuels; and Environmental Protection and Health. All these topics are very relevant for the country and the world in the present context. The Society is grateful to Prof. Ashok Pandey for organizing and hosting this conference, which led to the germination of this series of monographs, which included 16 books related to different aspects of energy, environment and sustainability. This is the first time that such voluminous and high-quality outcome has been achieved by any Society in India from one conference.

The editors express their sincere thanks to the authors for submitting their work in a timely manner and revising it appropriately at a short notice. We are grateful to the reviewers for reviewing various chapters of this monograph and providing their valuable suggestions to improve the manuscripts. We acknowledge the support received from various funding agencies and organizations for the successful conduct of the first ISEES conference SEEC-2017, where these monographs germinated. These include Department of Science and Technology, Government of India (special thanks to Dr. Sanjay Bajpai); TSI, India (special thanks to Dr. Deepak Sharma); Tesscorn, India (special thanks to Sh. Satyanarayana); AVL India; Horiba, India; Springer (special thanks to Swati Mehershi); CIAB (special thanks to Dr. Sangwan).

The involvement of scientific knowledge, biofuels and biorefinery industries in future can be the replacement of chemical market with bio-derived bulk and fine chemicals. The book gives detailed information on biosynthetic approaches for the production of various industrially important chemicals and products and the environmental challenges for its production under biorefinery approach. There are several books on industrial biotechnology or on waste remediation; however, there is hardly any comprehensive book covering these two interrelated topics in a single book. This is a state-of-the-art book providing interdisciplinary aspects on the subject matter, which deals with 'green processes' for production of chemicals, fuels and processes involving biotechnological interventions for remediation.

The book shall include chapters on different aspects of biosynthetic technology from well-known experts in the field, both from within India and internationally. Some of the topics covered shall include bioprocesses for the production of 2,5-furandicarboxylic acid; production, characterization and applications of microbial poly-γ-glutamic acid; recent advances in composting of organic and hazardous waste: a road map to safer environment; computational modelling and prediction of microalgae growth focused towards improved lipid production; perennial energy crops on drained peatlands in Finland; environmental assessment of biorefineries; oil palm biomass and its kinetic transformation properties; management of agro-industrial wastes with the aid of synthetic biology; plant biosynthetic engineering through transcription regulation: an insight into molecular mechanisms during environmental stress; bioremediation by microalgae: current and emerging trends for effluent treatments for value addition of waste streams; recovery of nutraceuticals from agri-food industry waste by lactic acid fermentation; advances and tools in engineering yeast for pharmaceutical production; biosynthesis and technological advancements of biosurfactants, etc. This book shall provide a synthesis of scientific literature on biosynthetic technology and related environmental challenges for researchers and academicians working in this area globally.

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Thiruvananthapuram, India — Binod Parameswaran
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About the Editors

Dr. Sunita J. Varjani is Scientific Officer at Gujarat Pollution Control Board, Gandhinagar, Gujarat, India. She holds M.Sc. degree in Microbiology (2009) and Ph.D. in Biotechnology (2014). Her major areas of research are Industrial and Environmental Microbiology/Biotechnology and Molecular biology. She has authored 35 publications, including 1 book, 19 book chapters/reviews and 15 original research papers. She has won several awards and honours, including Young Scientist Award at AFRO-ASIAN Congress on Microbes for Human and Environmental Health, New Delhi, (2014) and Best Paper Awards for oral presentations at national and international conferences in 2008, 2012 and 2013. She is a member of the editorial board of Journal of Energy and Environmental Sustainability.

Dr. Binod Parameswaran is currently working as Scientist in the Microbial Processes and Technology Division of CSIR-National Institute for Interdisciplinary Science and Technology, Trivandrum, India. He obtained his Ph.D. in Biotechnology from Kerala University, Trivandrum, in 2008, and after that, he did his postdoctoral studies at Korea Institute of Energy Research, Daejeon, South Korea, in the area of lignocellulosic biofuels. He has nearly 85 publications including research papers, reviews and book chapters. In 2001, he received Young Scientist Award from International Forum on Industrial Bioprocesses (IFIBiop), and in 2014, he

received Kerala State Young Scientist Award. His research interests include bioprocess and bio-products including biofuels, biopolymers, biochemicals of industrial importance and Enzyme Technology.

Dr. Sunil Kumar is Senior Scientist in Solid and Hazardous Waste Management Division of CSIR-NEERI, Nagpur. He has extensive experience in the field of solid and hazardous waste management. His major research interests include a wide range of environmental science and engineering fields, which include municipal solid waste management, hazardous waste management and environmental impact assessment. He is Honorary Director of Institute of Chartered Waste Managers, Jaipur, India. He has more than 80 publications/communications, which include three books and eight book chapters. He has provided industrial consultancy for various projects in Indian/international industries. He is an editorial board member of Bioresource Technology and Associate Editor of Journal of Hazardous, Toxic and Radioactive Waste, USA, and Environmental Chemistry Letter.

Prof. Dr. Sunil K. Khare is presently working as Professor of Biochemistry at the Department of Chemistry, IIT Delhi. He received his Ph.D. from IIT Delhi, carried out postdoctoral research at National Food Research Institute, Tsukuba, Japan, and has been DBT Visiting Scientist at Northern Regional Research Laboratory, Illinois, USA. He has been working in the area of solvent-tolerant and halophilic class of extremophiles and their enzymes, especially understanding the structural and molecular bases of their stability. The applications of these extremozymes in various biotechnological and industrial bioprocesses have far-reaching implications. His current noteworthy contributions have been in differential proteomics of solvent-tolerant and halophilic class of extremophiles and deciphering nanotoxicity mechanisms in microbial and plant systems. The work has led to 135 publications with high impact, 2 patents, 12 book chapters to his credit, besides a large number of conference publications. A large number of culture deposits in Microbial Type Culture Collection, India, and GenBank submissions to National Centre for

Biotechnology, USA, have also been made. The h-index for his work is 32 with a total of 3609 citations. He has guided 14 Ph.D. and 40 master's students. Several awards have been conferred on him, and noted amongst them are United Nations Amway Award; Fellow United Nations University; Fellow International Forum on Industrial Bioprocesses, France; Fellow Biotech Research Society, India; and Member National Academy of Science, India. He is General Secretary of the Biotech Research Society, India, Joint Secretary of Association of Microbiologists of India, member of many national committees and professional societies including DBT committees, DST task force, Academic council member of AMU, Central University of Haryana, Indraprastha University and Visiting Faculty for University of Blaise Pascal, France.

Part I
General

Chapter 1
Introduction to Biosynthetic Technology and Environmental Challenges

Sunita J. Varjani, Parameswaran Binod, Sunil Kumar and Sunil K. Khare

Abstract Bio-based processes and products are getting more and more acceptance nowadays mainly because of the environmental friendly process. Many current petroleum-derived products would be replaced by less expensive and better performing products based on renewable materials in near future. This will help for achieving economic and environmental sustainability. Bioeconomy is now emerging as a major industrial breakthrough and new biomass-based products are emerging due to the advancement in technologies. These potential benefits of bio-based products could justify future public policies that encourage a transition to renewable raw materials for production of organic chemicals, fuels and materials. Biosynthetic approaches for production of various industrially important chemicals and products through microbial and plants routes have been discussed in this book. The environmental challenges for its production under biorefinery approach and the various methods for addressing the environmental issues have been discussed in detail.

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Keywords Biochemicals · Biofuels · Biosurfactants · Biopolymers
Biopharmaceuticals · Prebiotics · Nutraceuticals · Biorefinery
Waste management

Lignocellulosic biomass is one of the most abundant renewable resources available on earth. Huge amount of lignocellulosic biomass is generated from agricultural and food industries as a waste material, commonly known as agro-industrial waste. It has low value for industries and also a big problem as environmental pollutant, therefore its proper management is needed. Several technologies have been applied to recover maximal quantity of valuable product from agro-industrial waste but applications of emerging synthetic biology in production of high value product seem to be more promising for its management. Agro-industrial waste is a good source of carbon and energy for the growth of microorganisms. The lignocellulosic biomass can be converted easily to fermentable sugars and these sugars can be converted to valuable products such as biofuels, bioactive molecules, rare sugars, prebiotic oligosaccharides, biochemicals and many more by applying engineered enzymes or organisms.

The complete utilization of lignocellulosic biomass for various products leads to the emergence of biorefinery concept. This also leads to more advanced interdisciplinary area of techno-economic analysis. The development of the biorefinery concept is based on the techno-economic feasibility of the proposed process considering raw materials, process options, mass and energy integration, market constraints, etc. Nevertheless, along with all these considerations, the environmental assessment must be taken into account at the same time, and concepts like sustainability, land uses, environmental impacts and other related issues must be included in the biorefinery design. A detailed information on this aspect based on different case studies covering a wide range of raw materials and products is also presented in this book. Comparing technological options for biorefinery development is usually done based on economic criteria, but environmental aspects have gained a key role in addition to economic factors for the selection of the best production scheme. Although different approaches, methodologies and data bases are available, the combined consideration of economic and environmental factors can be used as a right tool for decision-making.

Microorganisms such as bacteria, fungi, yeasts and algae are used for production of various chemicals of industrial and pharmaceutical importance. Biopharmaceuticals are important part of modern medical biotechnology and current commercial market of pharmaceutics show annual growth rates between 7 and 15%. The microbial production systems offer several advantages in production of pharmaceutically important proteins due to its unicellular nature, easy gene manipulation, cost-effective and fast growth. Recently major industries focus on biological method for production of chemicals such as 2,5-Furandicarboxylic acid, 1,3 propanediol, biosurfactants, etc. Biopolymers are another important entity of commercial importance. Poly γ-glutamic acid is a promising biodegradable polymer from bacterial sources with intense applications in the field of medicine,

wastewater treatment, food and cosmetics, etc. Another polymer having wide application is Pullulan. This microbial pullulan acts as the promising biomaterial that is currently used for packaging of readily oxidized food materials, controlled drug delivery, tissue engineering and can also function as artificial molecular chaperones. Biosurfactants also constitute an important compound having wide application. The realm of surfactant activity widespread to different industries including food, environmental remediation, textiles, fuel extraction, biotechnology, antimicrobials and many more. Recent developments in the production of these chemicals and polymers are described in this edition of book.

The enormous amount of by-products produced in food and agricultural sector and current attention towards sustainability is attracting researchers to look into possibilities of its utilization in recovery of nutraceuticals. Nutraceuticals are food or food products that confer health and medical benefits, and are instrumental in prevention and treatment of diseases. The agri-food industry by-products are an excellent source of proteins, lipids, fibre and other bioactive compounds. Among different methods used for extraction of these bioactive compounds (nutraceuticals), fermentation by lactic acid bacteria is one of the economical and eco-friendly approaches. During fermentation, chemical changes induced in organic substrate by action of microorganisms aid in formation of bioactive compounds, either by a process of hydrolysis of large polymers to simple molecules or transformation of substrates. One of the chapters in this book discusses the role of lactic acid fermentation in transformation/hydrolysis of by-products for efficient recovery of nutraceuticals. The bioprocess of nutraceuticals recovery from waste by lactic acid fermentation with better efficiency adds value to food waste, reduces environmental pollution and has positive impact on the economy.

Prebiotics constitute non-digestible food ingredient that promotes growth of beneficial microorganisms in the intestines. The oligosaccharides are highly stable and are used as dietary supplement possessing prebiotic, antioxidant activity and potential immune-modulating properties. A detailed information on manno-oligosaccharides has been included in one of the chapters in the book.

Plants also constitute an important source for biosynthetic products. They are well responding to environmental factors and endow rapid adaptation for survival under multiple environmental stress. These various environmental perturbations not only affect productivity of plant but also cause different variations in plant morphology and anatomy, growth, development and metabolism as well. During commencement of environmental stress, majority of biological processes are directly affected, such as photosynthesis, transpiration, respiration, stomatal conductance, pigment concentrations, energy and metabolism. To improve plant secondary metabolism production, it is essential to understand its biosynthetic pathway at the transcription level.

Energy crops such as Miscanthus, switchgrass, etc., represent important sources for biofuel production. The cultivation of perennial bioenergy crop on a drained peatland is an interesting approach. Peatlands are globally important because of their high carbon content. While the use of peatlands as an economic resource was deemed necessary, the fact that such land use (drainage) leads to environmental

problems is increasingly being realized the world over since the 1990s. Hence, continued attempts are being made to find suitable land use options for drained peatlands after their intended land use. Simply abandoning these lands is considered environmentally untenable. Therefore, several options have been suggested as after-use options: intensive forestry, rewetting, restoration to a functional peatland, creation of artificial wetlands, use of cutover peatlands for agriculture and cultivation of energy crops, etc. Decision on which option to use at a given drained peatland is complex. As an after-use option on a cutover peatland, the cultivation of a perennial bioenergy crop on a drained peatland in eastern Finland was explored during 2004–2011. The long-term measurements of greenhouse gas exchange from this study site showed that the benefits from bioenergy crop cultivation vary strongly depending on the climatic conditions during the crop cultivation phase.

Another plant-based residue is the oil palm biomass from oil palm mills which need a proper waste utilization application. Pyrolysis is the commonly used method to utilize this waste. The kinetics of this process and the recently available kinetic models such as lumped and distributed models are discussed in detail in one of the chapters in this book.

Cultivation of microalgae has appeared as an emerging alternative approach for removing pollutants and heavy metals present in the water bodies. Biomass production in the alga depends on rapid utilization of the organic content and other nutrients present in the effluent and can be considered as an attractive and eco-friendly means for treating waste streams, other than removing the pollution load, algal cultivation adds value to the process by production of commercially valuable products such as fuels and various chemicals from biomass. The recent developments and perspectives in bioremediation of waste streams by algae for removal of various pollutants for value addition of waste have been discussed in the book. Various computational modelling methods applied to microalgae growth in various environmental conditions have been reviewed. The possibility and potential of employing these models for better lipid production have also been highlighted, as better predictability of models can lead to better transgenic algal platform.

Increasing amount of waste due to urbanization, lifestyle and shift of huge masses from rural to urban largely contribute for the need of waste management. This developed a need of categorizing the waste into municipal waste, industrial waste, agricultural waste, hospital waste, etc., which would make it easy for the proper disposal, reuse and recycling of waste so determined effort should be taken for management of waste. Vermicomposting is the biocomposting process of organic waste by earthworms and bacterial action directing the stabilization of organic matter. The final product produced in this process is known as vermicompost and it assists in enrichment of soil as well as useful for sustainable agriculture. This technology is a boon for recycling of the solid waste generated from various sources including aquatic weeds. Vermicompost production from water hyacinth (*Eichhornia crassipes*) and its application to a commercial crop groundnut (*Arachis hypogaea*) for total yield and biomass production have been described in one of the chapters. Deliberation on the variations of life cycle computation of solid

waste management that involved to different global warming potential of composting is also a topic of discussion in this book.

Another important issue in waste management is handling and disposing the hazardous wastes as antibiotics residue, municipal solid waste and agricultural waste. These wastes contain lots of organic matter and nutrients, while also contain various kinds of toxic materials or elements (e.g. heavy metals, pathogens and antibiotics). The improper disposal of these wastes would result in environmental pollution and potential risk of human health. One chapter in this book discusses benefit and challenge of composting of organic and hazardous waste. It also discusses current method to promote the compost quality and reduce the environmental risk.

Water bodies are subject to a considerable pressure from sewage and industrial wastes. Monitoring methods adopted so far have helped in assessment level of contaminants in water but not interaction of these pollutants with living organisms. Water quality testing programs use two traditional methods for water quality assessment that includes physico-chemical parameters and bio-monitoring. Looking at the limitations of these two traditional methods, a new method known as 'biomarkers of pollution' should be adopted. Evaluating various biomarkers in sentinel species can be of great help in environmental monitoring program as they forecast various risks and hazards associated with the habitats of aquatic animals. The major advantage of biomarkers is that bioavailability or potential exposure to toxicants can be demonstrated which is not possible in chemical analysis.

The topics are organized in five different sections: (i) General, (ii) Biosynthetic approaches and products and (iii) Environmental assessment and waste management.

Part II
Biosynthetic Approaches and Products

Chapter 2
Management of Agro-industrial Wastes with the Aid of Synthetic Biology

Lokesh Kumar Narnoliya, Jyoti Singh Jadaun and Sudhir Pratap Singh

Abstract Biomass is the renewable organic material and it can serve as a continuous source of sustainable energy by passing through proper channel. Lignocellulosic biomass is one of the most abundant renewable resources available on earth. Huge amount of lignocellulosic biomass is generated from agricultural and food industries as a waste material, commonly known as agro-industrial waste. It has low value for industries and a big problem as environmental pollutant, therefore its proper management is needed. This agro-industrial waste could be used to generate other valuable products which aid on its value as well as manage agro-waste substances. Thus, several technologies have been applied to recover maximal quantity of valuable products from agro-industrial waste but applications of emerging synthetic biology in production of high-value products seem to be more promising for its management. Synthetic biology is a combination of engineering and biology which is helpful in designing novel biochemical pathways, organisms, or redesign existing, genetic circuits, biological modules, and natural biological systems. Generally, bacteria or yeast biological systems grow easily and are able to produce altered enzymes very efficiently with desired modifications in their genome. These engineered organisms are able to convert agro-industrial waste into valuable products. Nowadays, several valuable products are produced by using synthetic biology approach from industrial waste generated from agro-food industries such as sugarcane bagasse, apple pomace, and citrus peel. Here, we will discuss about agro-industrial waste, biosynthetic tools, and case studies of application of synthetic biology to produce valuable products from agro-industrial waste such as production of prebiotics, nearly calorie-free sugars, and bioactive compounds.

Keywords Agro-industrial waste · Synthetic biology · Lignocellulosic waste Prebiotic oligosaccharides · Biofuels

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1 Introduction

In India, agriculture has the main socioeconomic impact on business development of the country. More than 50% population are still completely dependent on agriculture for their livelihood. After harvesting crops, there is a huge amount of agricultural residues left in the fields, which remain unused and often burnt causing serious environmental hazards. This leftover organic material contains significant quantity of carbon, which can be used in production of alternative sources of food, feed, and energy being a low-cost raw material for value-added molecules (Matos-Moreira et al. 2012; Kulkarni et al. 2015; Díaz et al. 2017). Thus, development of biosustainable and renewable resource technology for management of agro-industrial waste has immense importance with respect to environmental concerns. Lignocellulose and starch are the most abundant agro-industrial waste as well as renewable organic biomass on the earth, which are produced every year in all over the world through photosynthesis in wide area from the forest to sea (Da Silva 2016).

Lignocellulosic waste could be decomposed by microbes and later transferred into environment which finally becomes a part of carbon cycle operating in ecosystem, where it is converted into CO_2 and H_2O. However, natural decomposition of lignocellulosic waste is a time-consuming process, it will take so many years for degradation of even currently present amount of lignocellulosic waste. Furthermore, the value of natural lignocellulosic feedstock is in demand due to recycling economy and sustainable development of energy sources. The research aspects on lignocellulosic biology are flourishing rapidly and this area is expanding for biosynthesis of bioactive compounds from biomass (Hongzhang 2014). Lignocellulosic biomass consists of mainly three types of polymers such as cellulose, hemicellulose, and lignin, which are strongly intermeshed and bonded by covalent cross-linkages and non-covalent forces (Singh et al. 2012). Microbial organisms degrade lignocellulosic biomass mainly through two enzyme systems: hydrolytic and ligninolytic. Hydrolytic enzymes are responsible for cellulose and hemicellulose degradation, whereas ligninolytic enzymes degrade lignin and open phenyl rings (Dhillon and Kaur 2016). In recent years, several technologies have been developed to manage lignocellulosic biomass. Synthetic biology is a combinational branch of engineering and biology containing several disciplines such as biotechnology, molecular biology, genetic engineering, systems biology, molecular engineering, evolutionary biology, and computer engineering. Synthetic biology is used to design novel biological pathways, organisms, or redesign existing, genetic circuits, biological modules and natural biological systems. Microbes, especially bacteria, are the key organisms used as bioengineered system for biosynthesis of valuable compounds from agro-industrial wastes (Cameron et al. 2014; Hongzhang 2014).

Array of genes encoding beneficial enzymes from various sources is available which could be transferred into targeted microbes via synthetic biology approach. The enzymes from microbial factories are useful bio-tool for treating the agro-industrial waste to generate high-value bioactive substances. Bacteria has a simple genomic structure, their genetic manipulation can be controlled efficiently,

and they can be grown easily and rapidly to produce huge amount of desirable enzyme or bio-product. Substantial efforts have been made to produce high-value biomolecules from agro-industrial wastes including agriculture waste, food wastes, fruit waste etc., by employing synthetic biology approaches. Synthetic biology has received significant attention since last two decades, after forthcoming of computational era (Cameron et al. 2014; Andrianantoandro et al. 2006). Synthetic biology is successful in altering the biochemistry and physiology of an organism by using modern bioengineering technologies, so they can perform new tasks or existing tasks more efficiently according to need. Reports are available showing applications of synthetic biology in transforming agro-industrial residues and by-products into products such as prebiotics, zero calorie sugars, and herbal medicines.

2 Source of Agro-industrial Waste

The source of agro-industrial waste is the agriculture residues generated from agriculture and fruit industries wastes, such as sugarcane bagasse, fruit pomace, wheat bran, rice straw, corn cob, and wheat straw (Dhillon and Kaur 2016). Agro-industrial wastes are the highly abundant and cheapest natural carbon source and their disposal is a big concern for environment. These wastes are mostly disposed in landfills causing soil pollution and gradually these are drained out in water prompting water pollution. Soil microbes degrade these wastes, generating intolerable and awful smell, thus generating air pollution. Use of the inexpensive agro-industrial wastes and surplus by-products in production of valuable products has double benefit of providing additional livelihood opportunities to farmer as well as addressing environmental issues. This approach gives opportunity to develop products from less-valued materials and the green solution worthwhile for environment protection.

Food and agriculture-based industries generate waste residues after vegetable and food processing steps, grain industries produce spent grains including breweries, distilleries as well as fruit, sugar cane, pulses, oil-based industries also produce huge amount of waste. Broadly, we categorized total waste into three categories based on their production sources such as agriculture, fruit, and food waste.

2.1 Agriculture Wastes

It is generated as a result of agriculture practices either in the field or during processing of agricultural products in the industries. For example, huge vegetative biomass waste is generated during the cultivation of crops such as rice, wheat, corn, barley, oats, rye, etc., after grain harvest. Agriculture waste comprises the largest proportion of total agro-industrial waste. In agriculture, more than 50% part of plant is directly not in use as food and is generally attempted to be used for animal feed,

however, a major proportion of this biomass is left as waste. Half of the world's population consumes rice as a staple food and rice cultivation generates rice straw and rice husk as major waste materials, which have negligible commercial values. Rice straw is produced as half to same quantity as rice produced and fifth part of harvested rice is husk. The quantity of rice crop waste is often more than rice grain and it is considered as poor animal feed due to presence of silica, which is not liked by animals (Singh and Sidhu 2014; Dhillon and Kaur 2016). The chemical composition of rice straw is 42–49% of cellulose, 26–32% of hemicellulose, 12–16% lignin, 15–20% of ash, and 9–14% silica (Garay et al. 2009). In India, most of the farmers burn the rice crop leftover on their field for preparation of next crop which results in excessive air pollution (Binod et al. 2010).

Wheat straw consists of approximately 40% of cellulose, 20% of lignin, 20% of xylose, and 3–5% arabinose. It is generally used for animal feed and has been explored for production of compressed agricultural fiber, resins, paper, and bioethanol (Kristensen et al. 2008). Another food crop is corn in which after harvesting of corn seeds, the rest of the plant becomes waste and corn cob is the main post-harvest waste. The application of corncob is not much more explored, except its use as feed for poultry farms, however, significant opportunities are being discussed in different publications to produce valuable products from this abundant biomass. Recently, corncob has been demonstrated as feedstock for production of furfural chemical and bioethanol (Jerry et al. 2016). There are three main steps to produce bioethanol from agro-industrial waste, physicochemical pretreatment, cellulose saccharification, and finally monosaccharide fermentation (Jeffries and Jin 2004).

2.2 *Fruit and Vegetable Wastes*

Fruits and vegetables residues belong to the highest wastage rates at consumer and retail levels. Vegetable wastes can be used as low-cost feedstock for production of fermentable sugar through various treatments (Díaz et al. 2017). The vegetable wastes, such as cabbage leaves, cauliflower leaves, tomato pomace, baby corn, etc., have been explored to be used for production of value-added product through aid of synthetic biology technique. The fructose sugar present in the cauliflower foliage can be converted into nearly zero calorie rare sugar, D-psicose (Patel et al. 2016). Recently, dual production of bioethanol and D-allulose from vegetable residue has also been demonstrated (Song et al. 2017).

In the past decades, consumption of fruit juices has increased rather than consumption of whole fruits, which led to rise in fruit processing industries. Fruit processing wastes include citrus fruit skins, pineapple residues, sugarcane bagasse, apple pomace, and other fruit residues. Now fruit waste became a problem in big cities as huge municipal solid waste which has negative impacts on environment (Cheok et al. 2016). Currently, fruit wastes are managed by landfilling or incineration. However, this approach is limited by emission of methane and carbon

dioxide gases during landfilling, and incineration and simultaneously generates pollutant and other toxic substances (Dhillon and Kaur 2016; Deng et al. 2012). Suitable biotechnological solutions are required to address the abundant fruit and vegetable residues.

Huge amount of apple pomace (30% of original fruit) is produced in apple industry which is mainly subjected to landfill (Vendruscolo et al. 2008). Its use as animal feed is currently avoided due to high sugar content, low digestibility, low vitamin, and mineral content. However, its pretreatment with enzyme complex, containing amylase, cellulase, and proteases makes it a superior feed grade (Dhillon et al. 2013; Vendruscolo et al. 2008). Further, several valuable products have been demonstrated to be produced using apple pomace as feedstock, such as organic acids, high protein feed, bioethanol, aroma compounds, antioxidants, phenolics, fibers, etc. Bio-synthetically engineered cells and enzymes can efficiently transform these low-cost residues into high-value bio-products (Vendruscolo et al. 2008). The sugars present in fruit pomace wash can be transformed into high-value functional sugar, e.g., D-fructose to D-psicose (Patel et al. 2016), and the remaining fibrous material may be used in other food or feed products. Banana is a popular fruit rich in nutrients such as sugars, minerals, antioxidant molecules, and dietary fibers. However, banana cultivation generates about 10–40% waste in the form of peel, pseudostem stalk, leaves, and banana fruit skin (Sharma et al. 2017). Thus, banana is also a potent source of lignocellulosic waste which could be valorized by transforming it into animal feed or utilizing the biomass into bioethanol production (Velásquez-Arredondo et al. 2010). Bioprocessing of banana stem juice led to the development of a health drink (CSIR-CFTRI). By employing suitable enzymes, the nutritive scale of the drink may be improved (Sharma et al. 2017). After citrus juice extraction, pulp residues remain which is made up of peel, internal tissue, and seeds. Citrus pulp consists of water and soluble sugars which make it digestible. It can be used as feedstock for ruminants but due to high fiber content, it is not liked by monogastric animals (Dhillon and Kaur 2016). Pineapple pomace is produced after processing of pineapple juice and it accounts $\sim$60% weight of total harvest. It contains soluble sugars, fibers, and pectin which can be easily digested so it is used as ruminants feed. Other fruits also generate significant level of lignocellulosic waste such as melon peels and pulp, mango peels and seed kernels, carrot pulp, sugar beet pulp, and grape. Fruit waste is composed of lignocellulose biomass which can be used to produce valuable products such as biofuel, food materials (prebiotics and zero calorie sugars), bioactive compounds, enzymes, etc. (Sharma et al. 2016, 2017). Engineered enzyme or microbial systems (bacteria or yeast) are developed by synthetic biology which is directly able to convert agro-industrial waste into commercially important products in efficient manner with low cost and minimum time (Fig. 1). Thus, it upgrades the value of raw material (agriculture or fruit or food) and helps in the agro-industrial waste management.

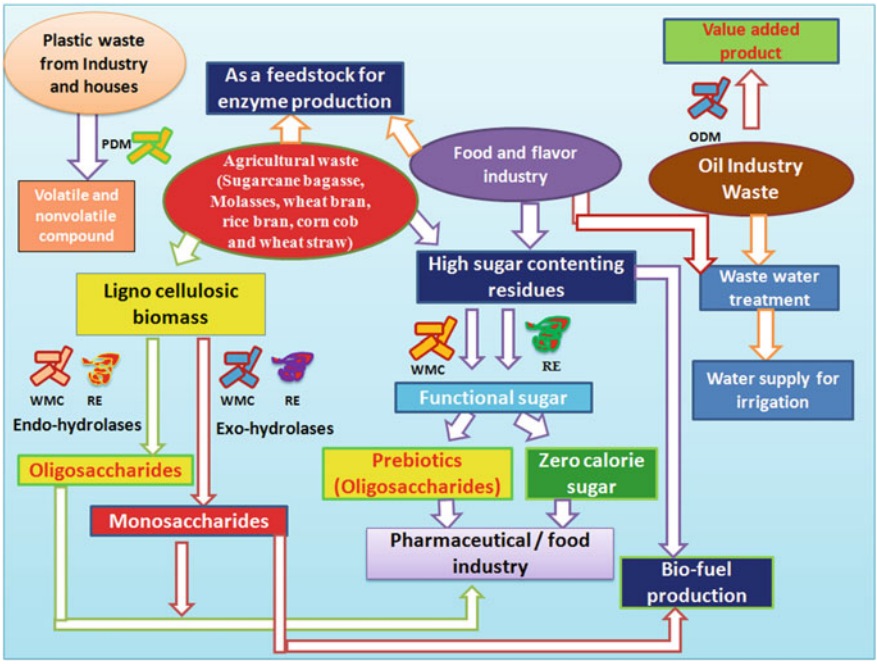

Fig. 1 Different routes of bioprocessing of agro-industrial waste through synthetic biology approach. *WMC* whole microbial cell, *RE* recombinant enzyme, *PDM* plastic degrading microbes, *ODM* oil-degrading microbes

2.3 Other Wastes

Food wastes are produced by households, food manufacturing industries, and food service sectors. A huge quantity of waste is produced in the form of liquid and semisolid by food processing industries, bakery industries, milk-based industries, livestock, and poultry farms, appropriate management of which is a challenge for scientists, engineers, and industrialists (Ravindran and Jaiswal 2016). The residues produced from grain processing (maize, barley, wheat, triticale, sorghum, rye, and oats) and sugar industries (cane and beet) are majorly used for bioethanol production; whereas, oilseed wastes (rapeseed, jatropha, canola, palm oil, soybeans, castor, and neem) are used for production of biodiesel. Production of liquid biofuel as bioethanol and biodiesel also produces massive amount of by-products as waste. Bioethanol waste directly can be used as animal feed and biodiesel by-products are used for animal nutrition as a protein source (Dhillon and Kaur 2016). The pretreatment of lignocellulosic biomass by enzymatic hydrolysis is able to release maximum quantity of sugar which could be used in making isomers and oligomers of health significance. Nowadays, industrial ecology concepts are considered as

leading principle for eco-innovation aimed to "zero waste economy" in which waste materials of industries are again used as raw material for new products.

3 Synthetic Biology and Its Applications for Management of Agro-industrial Waste

Synthetic biology combines both engineering and biology streams at a single platform. Biological transformation involves use of native or engineered organism or biocatalyst for conversion of agro-industrial waste into precious chemicals or products. Biological transformation is more favorable mode of conversion because chemical conversion needs high temperature and pressure (Brar et al. 2014). Generally, engineered organisms are used in fermentation techniques to generate value-added products from waste. There are a lot of fermentation-based products in market like chemicals, biofuels, biopolymers, organic acid, amino acids, antibiotics, and pharmaceuticals. Nowadays, utilization of biocatalyst is significantly increased toward transformation of renewable resources due to their high specificity, selectivity, high yield, and less by-product production (Brar et al. 2014). Role of synthetic biology came in notice when system biology and genetic engineering disciplines are evolved up to significant status. System biology explores omics such as genomics, transcriptomics, proteomics, and metabolomics which provide valuable gene/enzyme information rapidly which could be used in enzyme engineering. Genome or transcriptome resource of any living system will provide genetic information related to novel enzymes or a new source of enzyme which might have enhanced activity (Sangwan et al. 2013; Narnoliya et al. 2017).

These newly identified enzymes can be directly used or they can be engineered for better performance as per demand. Engineered enzymes are able to catalyze direct or preprocessed agro-industrial waste into valuable products. Enzyme engineering comes under genetic engineering discipline which uses biotechnology, molecular biology, and metabolic engineering branches of biology. Engineered enzymes acquire higher catalytic efficiency, thermal and pH tolerance than wild enzymes for better functionality to convert agro-industrial biomass into precious products such as prebiotics, calorie-free sugars, bioactive compounds, biofuel, lipids, biosurfactant, pigments etc. These modified enzymes either alone or in combination with other enzymes may be integrated in genome of a suitable host organism to perform better biotransformation of waste substances. Although biological transformation through synthetic biology approach provides high-value products from agro-industrial waste but still there are problems associated with downstream and upstream processes such as pretreatment, sterilization, aeration, agitation, product recovery, and temperature control which are needed to be resolved. Since, last decade utilization of agro-industrial waste increased in a notable manner as renewable resource alternative of petroleum fuels and some other industrial process.

Example of such utilization of agro-industrial waste is production of catechol and adipic acid from glucose present in agro-industrial waste through recombinant *Escherichia coli* (Draths and Frost 1994). Engineering of *Saccharomyces* sp. which is able to produce bioethanol from glucose and xylose present in lignocellulosic waste was a good step for conversion of low-value substrates to high-value products (Sedlak and Ho 2004). Algae is used for biodiesel production through lipid extraction, and the remaining algal biomass can be explored for carbohydrates, crude glycerol, pigments, proteins, and others products (Brar et al. 2014). Likewise, citrus fruit waste are used to produce ethanol, fibers, essential oils, carotenoids, cellulose as well as bioactive molecules like hesperidin and hesperetin which possess significant activities such as anti-inflammatory, anticarcinogenic (Simmons 2016). Brewery waste and apple pomace are the good sources of ligninolytic enzymes such as laccases, manganese peroxidase, and lignin peroxidase which are responsible for lignin degradation (Gassara et al. 2010).

4 Case Study on Production of Valuable Products from Agro-industrial Waste

4.1 Productions of Biofuels from Agro-industrial Waste

The sources of fossil fuel are limited and world population is continuously increasing so in upcoming few years, we may face energy crisis. Fossil fuels also generate pollution, therefore new and cleaner alternative energy sources are required for the future. Biofuels provide a good source of renewable energy and are also safer for environmental concern (Balat and Balat 2009). For growing population, we need more food as well as fuel so we have to increase the proportion land area for crop production but for fuel production, we also need land. Land is becoming a limiting factor in fuel production and biofuel is the only alternative source for accessing the fuel requirement. Thus, there is little bit imbalance situation between "food and energy" therefore new technologies are emerged which will be able to produce biofuels from agro-industrial waste (Jeihanipour and Bashiri 2015). The cost of agro-industrial waste is almost zero so the raw material for biofuel has no limitations. The biodiesel conversion from agro-industrial waste is carried out by transesterification of branched triglycerides into smaller straight-chain esters (Leiva-Candia et al. 2014).

Biofuels are classified into four categories based on their production technology as first to fourth generation biofuel. First generation biofuel used food crop (sugar, starch, vegetable oils) and second generation used nonfood crops (wheat straw, corn, wood, solid waste) as feedstock and produced bio-alcohols, biodiesel etc. Third generation used algae to produce vegetable oil and biodiesel, and the fourth generation biofuel used vegetable oil and biodiesel to generate Biogasoline (Demirbas 2009). Brazil and the USA are the leading countries which produce

bioethanol. Several microorganisms such as bacteria, fungi, yeast, and algae contain the ability to store lipids under specific cultivation conditions. Agro-industrial waste is good source for growing such microorganisms because it contains different types and high level of carbon source (Fig. 1). For enhancing efficiency of these microbes, synthetic biology can give a solution. System biology provides information regarding responsible pathway and enzymes which can be modifying by recent genetic engineering techniques. Some yeast species retain high oil content compared to algae or bacteria and their growth rate is also fast therefore they can be easily used for biofuel production. Some oleaginous yeast strains such as *Rhodosporidium* sp., *Lipomyces* sp., *Cryptococcus* sp., and *Rhodotorula* sp., accumulate significant level of lipids especially when glucose is used as carbon source (Hu et al. 2009).

A well-known yeast *Saccharomyces cerevisiae* genus was used for bioethanol production. A remarkable level (more than 50 g L^{-1}) of bioethanol production was achieved by recombinant *S. cerevisiae* from glucose and xylose as source which was extracted from agro-industrial waste after pretreatment process (Alfenore and Molina-Jouve 2016). Agro-industrial wastes such as molasses, cheese whey, crude glycerol, meat product waste, and waste oil possess significant quantity of carbon and these yeast species can use these sources as substrate for growing. *Candida* sp. uses molasses as carbon source and could be able to store 60% (w/w) lipid content. *Cryptococcus curvatus* strain uses crude glycerol, cheese whey, and molasses as carbon source and produces significant level of lipids (Leiva-Candia et al. 2014). For yeast, lignocellulosic waste is not a suitable food without pretreatment. It can be pretreated by enzymatic, acidic, basic, thermal, or combination of these methods. Therefore, sweet sorghum bagasse, wheat straw, cassava starch, Jerusalem artichoke extract, corn cob, and rice straw could be used as potent carbon source for lipid storages microbes (Leiva-Candia et al. 2014; Jeihanipour and Bashiri 2015; Alfenore and Molina-Jouve 2016). A number of sources available such as cassava bagasse, raw residual coconut milk, raw residual pineapple juice, grape and sugar beet pomaces, rice straw and corn stalks, sugar cane molasses, soy molasses, corn fiber xylan, etc., were used for biofuel production through engineered organisms as mentioned in Table 1.

E. coli is the most common host for genetic manipulation studies and possesses suitable system such as to grow in salt condition, anaerobic condition, and utilize array of carbohydrates substrates, polyols, and fatty acids (Clomburg and Gonzalez 2010). Glycerol dissimilation pathway and *Zymomonas mobilis* ethanol production pathway was introduced in *E. coli* by aid of synthetic biology and this metabolic engineered *E. coli* produces 20–40 g L^{-1} ethanol (Durnin et al. 2009; Yomano et al. 2008). Several pathways responsible for synthesis of isobutanol, 1-propenol, 1-butanol, 2-methyl-1-butanol, 3-methyl-1-butanol, isopentenol, and fatty alcohols were introduced in *E. coli* and significant yield of respective alcohol was obtained (Clomburg and Gonzalez 2010). Besides *E. coli*, *S. cerevisiae* and *Z. mobilis* were also engineered and extensively used for ethanol production. *E. coli, Pichia stipitis* and *Klebsiella oxytoca* can use pentose sugar for ethanol production. Xylose

Table 1 Production of biofuels from agro-industrial waste materials using engineered organism

Category of valuable products	Source of waste	Organism used for biotransformation	Nature of obtained product	Reference
Biofuel	Wastewater media	Green algae	Lipid production	Woertz et al. (2009)
	Hydrolysate of cassava starch	*Rhodotorula mucilaginosa*	Lipid	Li et al. (2010)
	Raw residual coconut milk, raw residual pineapple juice	*Saccharomyces cerevisiae*	Bioethanol	Domínguez-Bocanegra et al. (2015)
	Grape and sugar beet pomaces	*S. cerevisiae*	Bioethanol	Rodríguez et al. (2010)
	Rice straw and corn stalks	*Aspergillus niger* EMCC 72 and *Trichoderma viride*	Bioethanol	El-Tayeb et al. (2012)
	Sugar cane molasses	*Zymomonas mobilis*	Bioethanol	Cazetta et al. (2007)
	Soy molasses	*Clostridium beijerinckii BA101*	Butanol	Qureshi et al. (2001)
	Cassava bagasse hydrolysate	*Clostridium acetobutylicum*	Butanol	Lu et al. (2012)
	Corn fiber xylan	*C. acetobutylicum*	Butanol	Qureshi et al. (2006)

catabolism pathway was successfully engineered in *S. cerevisiae* and elevated ethanol level was observed by using the biosynthetic organism (Dellomonaco et al. 2010).

Although ethanol is the main source of biofuel market due to its easy production and existing market but other long-chain fuels like isobutanol, fatty acid, and isoprenoid derived fuels have better fuel properties and these may be finer source for future (Liao et al. 2016). Currently, significant research is going on worldwide and hopefully we will able to generate stable, environment-friendly, and economical biofuel production technology. Therefore, the ultimate success of sustainable biofuels can be visualized from a synthetic biology standpoint through the discovery and design of new enzymes, pathways, or organisms which could be coupled with efficient, innovative, and inexpensive processing technologies (Fig. 1).

4.2 Production of Zero Calorie Sugars from Agro-industrial Waste

Recently, trend of packed or fast food is increasing and in preparation of all these kind of food or drink materials usually table sugar is added as sweetener. Intake of high sugar content in diet leads to problem of obesity which is initial symptom for generation of all other health-related issues like cardiac arrest, diabetes, blood pressure, etc. Therefore, an alternative strategy is needed which could provide almost same level of sweetness but with low calories. Rare sugars or nearly zero calorie sugars seem to be the best solution for this problem. Rare sugars are the monosaccharides present in a very small amount in nature as natural products (Granström et al. 2004; Patel et al. 2016). The International Society of Rare Sugars (ISRS) has classified all the sugars in two forms as normal and rare sugars. Only seven sugars; glucose, fructose, xylose, galactose, mannose, ribose, and L-arabinose are abundantly present and they fall under normal sugar category whereas 20 hexoses and 9 pentoses are rarely occur in nature so these are described as rare sugars (Beerens et al. 2012).

Although, derivatives of rare sugars are available which are generated by deoxygenation and secondary transformations, such as amination or methylation. Extraction of rare sugars from natural sources is not easy task because of their low abundance although production of rare sugars by biological reactions is not very much favored by metabolism due to their less or no physiological activity (Beerens et al. 2012; Izumori 2006; Granström et al. 2004). Some important examples of rare sugars are D-psicose (D-allulose), D-tagatose, L-xylose, D-allose, L-allose, D-sorbose, and L-glucose. Generally, there are three types of enzymes which catalyze interconversion of monosaccharides into rare sugars are keto-aldol isomerases (EC 5.3.1), carbohydrate epimerases (EC 5.1.3) and oxidoreductases (EC 1.1). Rare sugars have potential application in foods and beverages, as sweeteners, seasonings and used for preparation of high-valued pharmaceutical products. Only rare sugar such as D-allulose and D-tagatose can serve as very low-calorie sweetener, and therefore very useful in diabetes (Beerens et al. 2012). Tagatose contains low glycemic index therefore it is suitable for diabetic patients and it is in clinical trials phase III (Espinosa and Fogelfeld 2010). D-Allulose is used as nearly calorie-free sweetener in food industries and serves as diabetic and obesity control agent in pharmaceutical industries. D-Allose possesses different properties such as having inhibitory effect for carcinogenesis and cancer proliferation and contains anti-inflammatory, antioxidant, cryoprotectant, and immunosuppressant properties so it can also be useful in surgery and transplantation (Lim and Oh 2011).

As available literature supports the opinion that rare sugars can only be produce by biochemical reactions so synthetic biology could be effective method which can provide engineered enzyme or organism to perform the biochemical reaction with higher stability and better kinetic efficiency. Agro-industrial and food wastes are the rich sources of carbohydrates so it can serve as good source for rare sugar production. Starch, whey, or hemicellulosic waste could be used as raw material for

commercial production of rare sugars. Apple, kinnow, and pineapple pomace contain 25–40 g D-fructose per kg of its fresh weight and banana pseudostem and cauliflower foliage have 1–6 g per kg of its fresh weight. By using an engineered enzyme, about 25–30% of the D-fructose present in the fruit and vegetable wastes has been converted into D-allulose. This accounts to production of 6–14 g kg^{-1} D-allulose from fresh weight of fruit pomace, and about 0.25–2 g kg^{-1} of fresh weight of banana pseudostem and cauliflower foliage (Patel et al. 2016).

Spent coffee grounds contain arabinose and galactose in an amount of less than 10 and 20%, respectively. *Thermotoga neapolitana* derived L-arabinose isomerase enzyme has been expressed in a host strain *Corynebacterium glutamicum* and it is used to produce tagatose (Lee et al. 2014). Whey, produced in huge amount from food industries, contains good quantity of lactose which can be easily used for production of galactose by β-galactosidase enzyme and this galactose could serve as raw material for tagatose production by L-arabinose isomerase enzyme. Several L-arabinose isomerase enzymes have been isolated from different sources such as *Thermotoga* spp., *Streptococcus* spp., *Lactobacillus* spp., *Bacillus* spp., *Geobacillus* spp., etc., and using synthetic biology approach they can be immobilized or engineered which can able to provide 70% yield in batch reactors at pilot scale production. Alginate immobilized *Lactobacillus plantarum* cells converted 33–38% galactose into D-tagatose using lactose hydrolyzed whey permeate (Jayamuthunagai et al. 2017). L-Rhamnose isomerase, D-tagatose epimerase, and fructokinase enzymes are transferred in *E. coli* and two redundant hexose kinases removed from *E. coli* genome which could phosphorylate D-allose to produce D-allose rare sugar (Bosshart 2014). Thus, agro-industrial waste could also be used for rare sugar production using engineered enzyme or organism (Fig. 1). Synthetic biology offers an opportunity to produce the high-value rare sugars for health benefits. This approach can be clubbed with the bioethanol production. This will minimize the bioethanol production cost from biomass.

4.3 *Production of Prebiotic Oligosaccharides from Agro-industrial Waste*

Prebiotics are the food components which stimulate the growth and activities of microbes residing in gastrointestinal tract especially in colon to enhance digestion. Generally, these microorganisms are from bifidobacteria and lactobacilli group. Prebiotics are nondigestible carbohydrate substances and chemically stable at low pH and high temperature (Younis et al. 2015). Oligosaccharides are the key ingredients of prebiotics but resistant starch and fermentable fiber also act as prebiotics and enhance growth of colon bacteria. Oligosaccharides such as lactulose, raffinose, stachyose, fructooligosaccharides, gluco-oligosaccharides, xylooligosaccharides, malto, and isomalto oligosaccharides act as prebiotics. Oligosaccharides are synthesized from plants such as chicory inulin, enzymatic hydrolysis such as

oligofructose from inulin and chemical synthesis by trans-glycosylation reactions. Enzymatic hydrolysis is the most common approach used at pilot scale to produce oligosaccharides. Currently, synthetic biology provides engineered enzymes or organism which can be able to generate oligosaccharides with significant yield (Fig. 1).

The raw material is the main component of any product formation process and it defines the cost of final product therefore its cost should be always least. Agro-industrial waste is the key source of carbohydrates especially polysaccharides and it could serve as potent starting substrate for prebiotic oligosaccharides production. A dextransucrase enzyme was isolated from *Leuconostoc mesenteroides* MTCC 10508 strain and used for oligosaccharides production using cane molasses as feedstock and starch industry corn fiber residue as a source for acceptor molecules. The production of oligosaccharides was approximately 124 g kg^{-1} of fresh molasses with degree of polymerization three to six. In this process, fructose was released as by-product which converted into D-allulose using enzyme with D-psicose 3-epimerase activity (Sharma et al. 2016).

The β-galactosidase enzyme from *Streptococcus thermophilus* was introduced in a food-grade *L. plantarum* WCFS1, which breaks down lactose of whey permeate and along with this also have significant transgalactosylation activity to produce oligosaccharides. A total of 90% lactose conversion and 50% galacto-oligosaccharides yield obtained through this system (Geiger et al. 2016). The *Bacillus* sp. TS 47 pectate lyase and *Geobacillus thermodenitrificans* JK1 xylanase enzyme were immobilized to produce pectic oligosaccharides and xylooligosaccharides. Immobilized pectate lyase enzyme uses pectin from apple pomace and xylanase uses xylan from beech wood, distiller's grains, and wheat bran to provide pectic oligosaccharides and xylooligosaccharides (Kieraite et al. 2015). Extracellular β-fructofuranosidase was isolated from *Sclerotinia sclerotiorum* and immobilized covalently on alginate and chitosan for its altered transfructosylating activity. Immobilized enzyme has stability range for pH 4–7 and temperature from 4 to 70 °C. Chitosan cross-linked enzyme activity remains ~90% after 1 week thus, continuous production of fructooligosaccharides from beet molasses in reactor was enhanced. The catalytic efficiency was 8–21-fold improved after alginate and chitosan immobilization, respectively (Mouelhi et al. 2015).

4.4 Production of Bioactive Molecules from Agro-industrial Waste

Bioactive molecules are the compound resulting from secondary metabolism having low molecular weight, eliciting pharmacological effects in human being and animals. Secondary metabolites are not directly involved in growth and development but these are required for survivability and fecundity of plants. Generally, bioactive molecules are synthesized from primary metabolites or intermediate

products of primary metabolites. Naturally, bioactive molecules are the key component of plant defense system, playing an important role in the adaptation of plants against unfavorable environment but they have strong pharmaceuticals properties therefore these are used as herbal medicines (Okino Delgado and Fleuri 2016).

Generally, they are classified in three main categories, terpenes, alkaloids, and phenolics. More than 200,000 bioactive molecules from natural sources (plant, animal and microbes) have been isolated and their chemical and functional properties are also identified. Nowadays, herbal medicines have a remarkable position in pharmaceutical or medicinal market and more than 50% herbal medicine are in clinical use (Shakya 2016). The market of plant-based medicine is increasing continuously due to their less side effects, safety, stability, and effectiveness but their supply is quite difficult from their natural sources. Due to their structure complexity, chemical synthesis is impractical, thus still we depend on their biological sources. Therefore, new sources or technologies are required which could produce significant quantity of bioactive phytochemicals to supply harbal medicines. Agro-industrial waste is a potent source for production of bioactive molecules through chemical or enzymatic process but they are not sufficient to extract maximal product.

Several reports are available regarding extraction of bioactive molecules from agro-industrial waste but the yield is not significant. Several bioactive compounds are isolated from citrus waste such as carotenoids, limonene, amines (histamine, serotonin, and sipenephrine), ascorbic acid, flavonoids, quinones, phenolic acids, and polyphenols. Some industrial enzymes like proteases, lipase, and peroxidases have also been extracted in remarkable quantity from citrus waste (Galaverna et al. 2008; Okino Delgado and Fleuri 2016). India is the largest mango producer in world, so huge quantity of mango waste is produced every year. Array of commercial important enzymes and bioactive molecules such as protease, polyphenol oxidase, peroxidase, pectinases, carotenoids, tocopherols, sterols, terpenes (xenthones, lupeol) vitamins, fiber, and carbohydrates have been isolated from mango waste (Ajila et al. 2007; Okino Delgado and Fleuri 2016). *Pseudomonas putida* was genetically engineered by introducing several genes and a number of strains are developed which are able to produce bioactive molecules such as quinoline, pyrroles, benzaldehyde, phenazine, phthalate, andrimid, bushrin, zafrin, pyrrolidinedione, and phloroglucinol from cheap precursor available in agro-industrial waste (Romanenko et al. 2008; Kahlon 2016).

Phenol was produced by modified *P. putida* strain using glucose as source which can be easily isolated in huge quantity from waste (Wierckx et al. 2008). Engineered *P. putida* KT2440 strain was able to produce myxochromide S with significant yield almost five times higher yield from its parent strain, *Stigmatella aurantiaca* (Stephan et al. 2006). The production of itaconic acid was enhanced ($\sim$5 to 10%) by introducing cadA (cis-aconitate decarboxylase) and mfsA (major facilitator super-family transporter) genes into the *Aspergillus terreus* genome (Huang et al. 2014). Tannase gene from *A. oryzae* was overexpressed in yeast *Pichia pastoris* and elevated production (7000 IU/L) of tannase was obtained (Zhong et al. 2004). Thus, there are huge opportunities in the field of bioactive molecule production from agro-industrial waste using engineered organism.

5 Future Aspects

Agricultural practices and industries involved in food processing produce too much agro-industrial wastes, which was difficult to handle through traditional manners. Actually, now we do not have enough land for its landfilling and it also causes soil pollution and influences soil fertility. Still, if we are hoping that it will be biodegraded by itself, meanwhile major part of earth will be covered by the waste product. Consequently, environment will not favour our survival on this planet. Thus, proper management of agro-industrial waste has become a major challenge. We should focus on development of cheap and useful technologies with efficient conversion of low-value waste or useless products into high-value or useful product. With this aim, biosynthetic technologies are needed for transformation of waste products into valuable products. Synthetic biology approach has remarkably presented some successful examples showing the total conversion of zero value products into rare available biomolecules. Agro-industrial waste can be transformed into valuable products by applying engineered enzymes or organisms, and these valuable products include biofuels, bioactive molecules, rare sugars, prebiotic oligosaccharides, and many more. Still, stable technologies are required for proper management of agro-industrial waste. This is just starting of research in this area, further gradual progress will lead us to achieve our target of "zero waste concept".

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Chapter 3
Advances and Tools in Engineering Yeast for Pharmaceutical Production

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Abstract Production of recombinant pharmaceutical protein is a multibillion-dollar industry and plays a crucial role in treatment of many diseases. Among the several potent microbial production systems, yeast offers several advantages in production of pharmaceutically important proteins due to its unicellular nature, easy gene manipulation, cost-effectiveness and fast growth and incorporates post-translational modifications in heterologous proteins. *Saccharomyces cerevisiae* is the widely used heterologous host for the production of medically important proteins and drugs; however, several non-conventional yeast species including *Hansenula polymorpha*, *Pichia pastoris*, *and Yarrowia lipolytica* are also gaining much attention as alternative heterologous hosts for the industrial production of therapeutic proteins. In this chapter, most recent advances in glycoengineering of yeast for successful therapeutic pharmaceutical production, current progress in humanization of yeast and various interventions in the secretory mechanisms and pathways in yeast for the improvement of the production of pharmaceutical proteins are overviewed. In addition, emerging genetic, omics, systems and synthetic biology tools and other technologies to enhance the efficiency of yeast pharmaceutical proteins are precisely discussed. The use of synthetic biology tools in yeast for the production of pharmaceuticals is clearly entering a new phase right now. Combination of yeast systems biology data with synthetic biology will open new vistas to better production, improved glycosylation and secretory mechanism. The application of currently available synthetic biology tools like CRISPR/Cas9 in yeast pathway engineering is also discussed.

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1 Introduction

Biopharmaceuticals are important part of modern medical biotechnology and current commercial market of pharmaceutics are $70–80 billion and annual growth rates between 7 and 15% are expected (Walsh 2010; Goodman 2009). As a heterologous protein production host, yeast has been used to synthesize a wide range of products including terpenoids, sterols, organic acids, biofuels, derivatives of sugars, fatty acids, terpenes, different aromatics, peptides and other engineered therapeutic proteins. Above all, the heterologously produced biopharmaceutical proteins are one of the rapid-growing and attractive classes of biomedicine. Recombinant vaccines, interferons, interleukins, hormones like insulin, glucagon, human growth hormone (hGH) and erythropoietin (EPO) and other therapeutic proteins are examples for protein biopharmaceuticals. Antibodies represent the large group of protein pharmaceuticals (Wriessnegger and Pichler 2013; Liu et al. 2013).

Unlike the traditional *E. coli*, yeast (*Saccharomyces cerevisiae*, *Pichia pastoris*) based expression systems offer the advantages of inexpensive cultivation, easy induction, efficient secretion and a protein synthetic machinery closer in functioning to the higher order animals which has advantages in production of several proteins of interest in pharma and diagnostic industries and requires efficient post-translational modification. Mammalian cell lines like Chinese Hamster Ovarian cell line offers the advantages of human-like N-glycosylation and gave better pharmacokinetics (Mattanovich et al. 2012). However, as compared to other widely used expression hosts, they need complex growth media and are more sensitive to growth conditions than *E. coli or yeast*. Their growth rate is relatively slow and is highly susceptible to viral contamination (Kim et al. 2014). The prokaryotic protein production host *E. coli* uses simple media components, easy cultivation conditions and attains high growth density in minimum time with high biomass production rate and protein yield. However, *E. coli* has the drawbacks of inefficient folding of proteins, low protein secretion capacity and lack of post-translational modifications (Swartz 2001). Yeast is generally recognized as safe (GRAS) organism and has a good tolerance to acidic pH conditions and other fermentation inhibitors (Nielsen 2013; Borodina et al. 2014). The complete genome sequence of *S. cerevisiae* was the first-sequenced eukaryotic genome (Goffeau et al. 1996), currently the complete genome sequences of many industrially important yeast species are available in the public domain (Dujon et al. 2004; De Schutter et al. 2009; Ravin et al. 2013), facilitating the function level genomics approaches based on various genomics and proteomics technologies. Systems biology-based approaches could enhance our knowledge of yeast metabolic and physiological networks, which can help to identify current bottlenecks in recombinant protein production. The advent of novel technologies and tools in synthetic biology

facilitated quick and efficient engineering metabolic pathway and secretion machinery to improve heterologous protein production, based on the data generated from systems-level approaches. Thus, synthetic biology and other genome engineering tools are expected to revolutionize the construction of 'yeast cell factories'

Table 1 Characteristics of yeast cell factories and biopharmaceuticals produced

Host	Characteristics	Produced pharmaceuticals	References
S. cerevisiae	• Well-studied model organism • Easy genetic manipulation to both classical genetics and modern recombinant DNA techniques • Well-established expression vectors (episomal, integrative, copy number regulated) • Availability efficient mutant strains • Established fermentation and downstream processing techniques • GRAS status	Human serum Albumin Human transferrin Insulin precursor Glucagon Hepatitis vaccines GM-CSF (S)	Nevoigt (2008), Camirand et al. (1991), Goffeau et al. (1996)
Pichia pastoris	• A Crabtree-negative yeast allowing for high dilution rates and high biomass yields in fermentation processes • Can grow rapidly on inexpensive media at high cell densities (up to 150 g DCW L^{-1}) • Highly efficient and stable chromosomal integrating vectors and host strains large-scale production of human and industrial proteins • Highly efficient signal peptides for secreted expression • GRAS status • Tightly regulated, methanol-inducible *AOX* promoter • A lesser extent of hypermannosylation compared to *S. cerevisiae* • No terminal α-1,3-linked mannose residues	Insulin Tat-p53 (cancer drug) Human Papillomavirus vaccine Drug for artherosclerosis	De Schutter et al. (2009), Ahmad et al. (2014), Bretthauer (2003)
Yarrowia lipolytica	• An oleaginous yeast • GRAS status • Efficient in secreting a variety of proteins via co-translational translocation and efficient	Pancrelipase	Dujon et al. (2004), Song et al. (2007)

(continued)

Table 1 (continued)

Host	Characteristics	Produced pharmaceuticals	References
	secretion signal recognition similar to higher eukaryotes • Low hypermannosylation compared to *S. cerevisiae* and no immunogenic terminal α-1,3-mannose residues		
Kluyveromyces lactis	• GRAS status • Under aerobic conditions, the Crabtree effect is less dominant in *K. lactis*, which helps it to attain high cell densities during aerobic cultivation • strong, lactose-inducible *LAC4* promoter • Terminal *N*-acetylglucosamine and no mannose phosphate	Human Interferon β Human serum albumin Interleukin 1-b	Madhavan and Sukumaran (2016), Van Ooyen et al. (2006) Dujon et al. (2004)

for the production of biopharmaceuticals with improved yield and quality (Vogl and Glieder 2013). The characteristics of various yeast cell factories used for recombinant protein production and some biopharmaceuticals produced in them are described in Table 1.

In this chapter, we focus on new developments in the area of pharmaceutical production by reengineered yeast species by employing new metabolic system and synthetic biology tools, secretion pathway engineering, etc.

2 Secretory Pathways in Yeast and Engineering

Bacteria are known for their efficiency in producing pharmaceutically important heterologous proteins; however, they lack machinery to perform folding, trafficking and secretion of eukaryotic proteins as these are the main post-translational modifications carried out in a eukaryotic system. Here comes the importance of 'Yeast' as it is able to produce and do the post-translational modifications and release heterologous eukaryotic proteins in their indigenous and biologically efficient form. Yeast cells are generally recognized as safe organisms and are predominant hosts for biopharmaceutical production. *S. cerevisiae* is one of the well-described and commonly used eukaryotic host systems since from the budding of recombinant protein production (Martinez et al. 2012). *Hansenula polymorpha*, *Pichia pastoris*, *Yarrowia lipolytica*, *Schizosaccharomyces pombe* and *Kluyveromyces lactis* are also used as unconventional source for recombinant protein manufacturing (Kim et al. 2015).

The secretory expression is one of the important criteria for facilitating the subsequent protein purification process. On the other hand, there are several barriers that obstruct the secretory expression of heterologous proteins in yeast. Since the protein expression level is low in eukaryotic systems, several experiments have been done to engineer yeast for enhanced protein production, which mainly includes optimization of fermentation process (Caspeta et al. 2015), selection of the expression vectors systems (Partow et al. 2010), identifying the signal sequence for extracellular targeting (De Pourcq et al. 2012a, b, c) and engineering host strains for better protein-folding and post-translational modification (Idiris et al. 2010a, b). Nonetheless, nearly all of these efforts effectively work only for one or a few proteins which could not be extrapolated as a common way for manufacturing diverse collection of recombinant proteins (Hou et al. 2012). Apart from this, the quantity of protein produced by yeast is found to be lower (100–1000 fold) than the theoretically estimated range (Robson 2007; Schroder 2007). Hence, there is an urgent prerequisite of improving the recombinant protein expression in yeast, especially focusing the secretion system. One of the effective strategies to overwhelm the defects in yeast secretion pathways is genetic modification of yeast strains. This strategy is aided by prevailing postgenomic and system biology technology. As evident, there are many cross-linked factors that complicate the process of modulation of the yeast secretion system (Idiris et al. 2010a, b). This section focuses on latest approaches and their leverage for efficient engineering of yeast strains for potent protein secretion.

2.1 The Secretory Pathway

Before maturation and targeting to specific location, secreted proteins undergo many post-translational modifications through a common corridor called 'the secretory pathway'. A simple schematic representation of secretory pathway implemented by yeast is shown in Fig. 1 with major bottlenecks involved in heterologous protein expression. However, the pathway is somewhat complex and is estimated that more than 160 proteins that are responsible for different post-translational processes like folding, glycosylation, etc., are involved in the accomplishment of the pathway (Nielsen 2013). The protein folding is mainly controlled by endoplasmic reticulum (ER) through the ER-resident protein-folding machinery (Anelli and Sitia 2008). Just after synthesis from ribosome, the secretory proteins are translocated to ER through translocon protein Sec61protein seen on the ER membrane. Thereafter, the incipient proteins are obliged to the BiP or calnexin proteins for their correct folding. The folding usually involves signal sequence processing, disulfide bond formation, N-glycosylation, glycosyl-phosphatidyl-inositol addition, degradation and sorting.

Only accurately folded and compiled proteins are transported to Golgi complex through vesicles, where it undergoes further modifications to being shifted to extracellular matrix, vacuoles or other organelles. In the mean time, misfolded

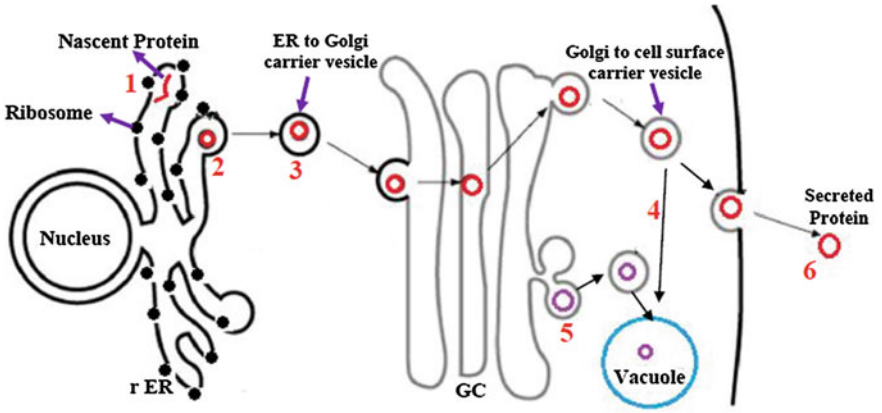

Fig. 1 An illustrative diagram showing yeast secretory pathway and distinctive barriers in the secretory pathway of heterologous proteins in yeast. The barriers are denoted with numbers 1–6. 1 Ineffective translocation, 2 Misfolding, aggregation, UPR and ERAD, 3 Intracellular degradation, 4 ALP pathway and missorting, to vacuole, 5 CPY pathway and missorting, to vacuole and 6 Degradation of extracellular secreted proteins

proteins are identified by the quality control system in ER and are redirected to degradation process via BiP complex (Yoshida 2007). Eventually, if the misfolded protein binds to the BiP complex for a prolonged time, the unfolded protein response (UPR) gets induced, this activates proteolysis and inhibits the transcription and translocation of the target protein. Due to the stringent quality control mechanisms in the ER, protein folding often has an inclination to become the most rate-limiting obstacle in heterologous protein secretion. Thus, genetic alteration of the ER protein folding and control mechanism in ER has become the most useful approach in the existing strain engineering technique.

2.2 *Endoplasmic Reticulum as a Target for Enhancing Recombinant Protein Secretion*

The efficiency of protein expression and quality of protein can be enhanced through engineering of the protein secretory machinery. The initial stage of secretion mechanism is the relocation of target protein through ER (as explained earlier) into the secretion pathway, depends upon secretion signal peptides for the translocation of proteins into ER (Kim et al. 2015). Therefore, the capability of leader sequence portrays a significant part in the secreted protein production. The synthetic prepro-leader sequence exhibited more efficient protein secretion when correlated to the prepro-leader sequences of native and alpha-mating factor (Swartz 2001). Misfolded proteins cause a luminal burden in ER which further induces UPR that accordingly reduces elevated stress in ER. Hence, by ER luminal environment

manoeuvring, betterment of the secretion efficiency can be enhanced by the overexpression of chaperones and redox enzymes in ER (Payne et al. 2008). For instance, the overexpression of the activated mutant of heat shock factor 1 initiates heat shock response (Hou et al. 2012) and Hac1p (Guerfal et al. 2010) contributes to enriched protein secretion mechanism. A few studies have reported a 5-fold increase in secretion of human erythropoietin (Robinson et al. 1994) and a 26-fold increase in bovine prochymosin (Harmsen et al. 1996) in *S. cerevisiae*, through the overexpression of the chaperone *BiP*; and a 7-fold increase in human leukocyte elastase inhibitor secretion by overexpressing polyubiquitin gene *UBI4* in *S. cerevisiae*. However, overexpression of BiP halts the action of other chaperone mechanisms, as it increases the strength of binding between BiP and its target proteins, which drives to endoplasmic associated degradation (ERAD) rather than discharge of protein (Kauffman et al. 2002).

A different concept to encourage the secretory pathway effectively is by manipulating the UPR pathway which involves Hac1p, PDI, PPI and ERAD genes. On the basis of different studies, many targets in the UPR pathway have been identified for improving protein secretion (Hou et al. 2012) and are achieved by reducing ER stress and its subsequent impairment caused by heterologous protein production results in augmented secretion of the protein. Heterologous overexpression of *Hac1* improves protein secretion of Bacillus amylase (2.4-fold), endogenous invertase (2-fold) and recombinant α-amylase (70%) (Valkonen et al. 2003). The production of recombinant human transferrin (rTf) was enhanced by introducing deletion of *YPS1* and *HSP150* and *PDI1* overexpression in *S. cerevisiae* which in turn results in improved yield and quality (Finnis et al. 2010). The overexpression of UPR pathway genes is protein or host-specific and regulatory effects between chaperones, hence Schröder (2008) recommended that co-expression of multiple chaperones and folding partners in the ER lumen may improve the expression of different heterologous proteins. Single and multiple gene introductions of *S. cerevisiae* chaperones to *P. pastoris* have improved recombinant protein secretion. However, a synergistic effect was seen in multiple gene introductions as the protein secretion was much higher in multiple genes than the single gene introduction (Zhang et al. 2006).

2.3 Glycoengineering of Yeast

Glycoengineering modifications play significant roles in proper folding, half-life, and pharmacokinetic properties of recombinant proteins (Fidan and Zhan 2015). Initial glycosylations occur during translocation that helps with proper protein folding, protects from proteases and provides beckon for quality control. Yeast is able to carry out N-linked and O-linked glycosylation. N-linked glycosylation is accomplished by adding a 14 sugar glycan tree to the asparagine residue of the recognition sequence (Bause 1983). The N-linked glycosylation is completed by the ER-resident oligosaccharyl transferase (Burda and Aebi 1998).

O-mannosyltransferases catalyse O-linked glycosylation at the hydroxyl groups of serine and threonine (Strahl-Bolsinger et al. 1999) and this O-linked glycosylation happens prior to N-linked glycosylation, showing that N-linked asparagine glycosylation and O-linked serine/threonine glycosylation possibly will be in competition (Ecker et al. 2003). Interestingly, as indigenous high-mannose yeast N-glycans interact with components of immune system especially with human C-type lectins, it is not appropriate for medical use without modifications (Hamilton and Gerngross 2007).

The pharmacokinetic characteristics, potency, immunogenicity and half-life of therapeutic proteins are greatly affected by the structure and the number of mannose glycans present (Rabinovich et al. 2012; Piirainen et al. 2014). Hence, the so-called glycoengineered yeast strains have been developed with the capability to produce human-like glycans rather than yeast-specific ones. In glycoengineered yeasts, N-glycan humanization generally occurs in three steps. Eliminating the yeast hypermannolysation is the first stage which is achieved by disrupting or deleting glycotransferase genes while expressing mannosidase genes, ensuing human-like $Man_5GlcNAc_2$ glycoform (De Pourcq et al. 2012a) or deletion of ALG3 gene to obtain human-like $Man_3GlcNAc_2$ glycoform (De Pourcq et al. 2012b). In the second stage, a glycoform with GlcNAc terminal was generated using *N*-acetylglucosamine transferase, followed by the addition of second GlcNAc sugar to mannose resulting in $GlcNAc_2$–$Man_3GlcNAc_2$ (Fidan and Zhan 2015). In the last step, heterologous synthetic genes are introduced to produce sialylated glycoproteins (Hamilton et al. 2006).

In the same way, O-linked glycosylation reduces the pharmacokinetic properties of proteins as these proteins interact with human mannose lectins (Rabinovich et al. 2012). To overcome this issue, the PMT gene was disrupted to reduce the possession of O-glycan, however this technique will not eliminate all O-glycans as this disruption is usually interrupted by the O-mannosyltransferases available in yeast. As an alternative, O-glycans were reengineered of in yeast through mimicking mammalian O-glycosylation which mainly involves the action of a-1,2-mannosidase and b-1,2-*N*-acetylglucosaminyltransferase enzymes to produce the human-like sialylated glycoforms (Hamilton et al. 2013) or through including genes encoding *Bacillus subtilis* UDP-Gal/GalNAc 4-epimerase, human UDP-Gal/GalNAc transporter, human ppGalNAc-T1 and Drosophila melanogaster core 1 b1–3 GalT (Amano et al. 2008) which introduces mucin-type O-glycosylation in *S. Cerevisiae*. Even though, glycoengineering of yeast to remodel N- and O-glycosylation helps to optimize the pharmacokinetic characteristics of recombinant proteins, more studies are necessary to completely humanize N- and O-glycosylation pathways in yeast and retain the consistency of glycosylation (Lepenies and Seeberger 2014).

3 Protein Trafficking as a Target for Enhancing Recombinant Protein Secretion

Through the engineering of protein expression and folding mechanisms in yeast, the recombinant proteins can be folded precisely in ER, even then the secretion of the required protein is regularly deprived because the protein secretion machinery takes place in more than a few cell compartments (Fig. 1). ER, Golgi, trans-Golgi network, endosome and either cell membrane or vacuole are the cell compartments that are involved in the secretory machinery of yeast for protein trafficking (Ellgaard and Helenius 2003). Sec1p, Sly1p, Vps45p and Vps33p are some of the responsible proteins at each vesicle trafficking steps and overexpression of these proteins resulted in 70% rise in on the whole α-amylase production (Hou et al. 2012). After exact folding of newly synthesized proteins in the ER lumen, they are gathered into discrete vesicles, which are further targeted to a precise acceptor cubicle. On the whole, secretory effects are secured by several intracellular membrane proteins that determine each traffic step and correct vesicular destination. As a result, genetic augmentation of traffic pathway is needed mainly on the terms of disorganized traffic or missorting, which frequently marks in intracellular build-up of the target proteins for secretion.

Co-translational and post-translational pathways are availed by nascent proteins for cytosol to ER targeting. The indigenous ribosomal protein usually utilizes signal recognition particle (SRP) and its receptor in co-translational translocation pathway (Kida et al. 2007); however proteins have reasonable hydrophobic signal sequences which helps them to flee from detection by the SRP during their production in cytosol (Rapoport 2007). In both translocation secretory pathways, protein targeting specificity is predominantly accorded by the hydrophobic core of the secretory signal sequences and its synergy with SRP in cytosol (Kida et al. 2009). Hence, several types of N-terminal secretory signal sequences have been established for each host system and yeast prepro-sequences like mating pheromone α- factor signal MFα1 are used regularly (Fuller et al. 1989). The yield of heterologous proteins of *Escherichia coli* phytase, human lysosomal acid lipase and interleukin-6 was enhanced in fission yeast *S. pombe* using a prepro-secretory signal peptide P4 (Giga-Hama 1997). Preprotoxin signal peptide—viral K28 has been reported to be used as signal peptide for the secretion of GFP in four yeast species, *Candida glabrata*, *P. pastoris*, *S. cerevisiae* and *S. pombe*, indicating the possibility of the viral signal peptides as exceptional gears to speed up the recombinant protein production (Eiden-Plach et al. 2004).

Even though the proteins intended for secretion are folded correctly into their indigenous structure inside the ER lumen, they failed to secrete completely in some cases. This vacuolar missorting is mainly caused by the vacuolar protein sorting (vps) receptor Vps10p, which recognize and targets vacuolar carboxypeptidase Y to the vacuole (Iwaki et al. 2006; Idiris et al. 2010a, b). A single vps10 deletion is not sufficient to block completely the vacuolar missorting pathway due to the presence of the alkaline phosphatase pathway (Conibear and Stevens 1998). Therefore

further systematic analysis of gene functions of the vacuolar missorting pathway is necessary. Even though, some studies have showed the possible traffic modification in yeast engineering, it seems that complete blocking of genes is because of the complexity of membrane trafficking mechanisms affecting the viability of cells.

4 Protein Degradation as a Target for Enhancing Recombinant Protein Secretion

Yeast produces abundant number of proteases that disintegrate the expressed protein thereby reducing the yield and virtue of the interested protein. The disintegration occurs during the pathways involved in endocytosis to vacuole and from post-Golgi sorting to vacuole (Tyo et al. 2014). Various techniques have been adapted to avoid disintegration which mainly includes modification of growth conditions and addition of protease inhibitors (Gonzalez-Lopez et al. 2002; Kang et al. 2000). However, these techniques are of host specific and have limitations. Accordingly, many protease-deficient yeast strain, as genetic alteration of the host proteases minimizes host-specific degradation (Chung and Park 1998; Komeda et al. 2002; Jønson et al. 2004). Multiple deletions of YPS1, YPS2, YPD3, YPS6 and YPS7 genes have done in *S. cerevisiae* for the efficient secretory production of recombinant proteins (Kim et al. 2014). This technique efficiently reduces the cleavage of recombinant parathyroid hormone protein (Cho et al. 2010) proving that the removal of proteases potentially increases the efficiency of protein secretion.

5 Synthetic Biology Tools for Improving Pharmaceutical Production in Yeast

Different pathways for alcohols, lipids, terpenoids and polyketides have been constructed synthetically by introducing individual gene parts from different organisms and transferred into *E. coli* host (Singh 2014). This approach can be implemented for several other molecules. The atremisin production is a classic example for the success of synthetic biology approach (Paddon et al. 2013). Another pioneering work in the area of synthetic biology was the synthetic pathway design for D-hydroxy phenyl glycine—the essential building block for the penicillins and cephalosporins (Müller et al. 2006). They constructed the synthetic pathway by incorporating hydoxy mandelate synthase and a hydroxyl mandelate oxidase from *Streptomyces coilicolor* and *Amycolatopsis orientalis* and hydroxyl phenyl glycine aminotransferase from *Pseudomonas putida*.

The widely accepted employment of synthetic biology in health sector is the microbial synthesis of different pharmaceutical like artemisinin, the antimalarial drug. This drug is now produced large quantities in several heterologous hosts like *E. coli* and *S. cerevisiae* (Paddon et al. 2013). Last few years witnessed dramatic increase in the production of pharmaceuticals with the help of synthetic biology tools like taxol (Jiang et al. 2012), farnesene (Zhu et al. 2014) and many others.

6 Role of System Biology in Synthetic Biology of Yeast

System biology is the union of theoretical and experimental findings. The experimental concepts include high throughput analysis of various omics technologies. All these omics approaches help to improve the understanding of the complex cellular networks and circuits (Lee et al. 2011). Information obtained from these approaches can be integrated into a biological database for further use. This database can be further used for several computational modelling and simulation—the theoretical system biology (Jung et al. 2010). In summary, the massive volume of biological data generated allows the theoretical system biology for the mathematic modelling and computer simulation. System biology and synthetic biology have already successfully been applied to metabolic engineering for strain development and increasing the production of target molecule (Stephanopoulos 2012). The system biology tools can be used to identify the problems to increase the target molecule. Sanchez and Nielsen (2015) discussed how we can integrate different omics data into genome-scale metabolic models. Recently, full proteome of *S. cerevisiae* has been characterized by Picotti et al. (2013) and also structural alterations in proteins also have been profiled (Feng et al. 2014) and both will aid in the future developments of metabolic models and synthetic regulatory network construction. Recently targeted mutation for enhancing succinic acid in *S. cerevisiae* has been identified (Otero et al. 2013). The genes encoding succinate dehydrogenase (SDH3) and glycine biosynthetic genes were deleted and this led to the forced production of glycine from glyoxylate, and as a result cells will produce succinate. Further conversion of succinate is not possible due to the deletion of SDH3 and succinate starts accumulates. The growth rate of recombinant stain was improved by adaptive laboratory evolution. Transcriptome analysis of this strain identified a possible target isocitrate lyase (coding gene is ICL1). The overexpression of this ICL1 resulted in the increased production of succinate and resulted in the improved production (80 fold) of succinate. This provided a real insight into the importance of system and synthetic biology for the production of biochemicals. The transcriptional level regulatory systems in yeast is highly complex and more than 80% of the transcriptional control elements are interacting (Osterlund et al. 2015).

6.1 Various Steps Synthetic Biology Technique

6.1.1 Pathway Design

Because of the difficulties in marketing new chemicals, most biotechnological production has centered on molecules with well established market. Initially, most of the microbial production system has been focussed on the high-value low-volume products like drugs and pharmaceuticals, in which the value in the commercial market could produce an immediate impact (Baeshen et al. 2014). Later the scenario has been changed to low-value high-volume products like biofuels, industrial enzymes, etc. The selection of a molecule is based on economic consideration and commercial market.

Target Gene Identification and Metabolic Pathway Construction

After the selection of a target molecule, the best production pathway for that molecule has to be identified. Previously, the pathway identification involves manual selection and was based on very little gene protein expression data. Advanced technologies in next-generation sequencing and recent developments in bioinformatics analyses have changed the scenario of gene discovery for synthetic biology and metabolic engineering by enabling a large genome data. Many computational tools are in public domain to use this genome data for gene mining, identification of gene clusters, etc. The recent introduction of computer-assisted programs and database based on biological reaction and availability of huge genome data has automated the identification process and implemented several scoring methods for pathway selection (Medema et al. 2014).

Biosynthetic gene cluster consists of several genes arranged in an operon or more than one operon and their expression is tightly regulated by several regulators at transcriptional and translational level. Most of these regulators are overlapping with or situated in neighbouring genes make the individual engineering of each genes difficult and they are under the tight control of several transcription factors and regulators (Temme et al. 2012). The concept of genetic parts implies that they are modular parts with well-defined function. This can be applied to biosynthetic gene cluster by 'refactoring' in which the natural genetic information are modified and rewritten in a way to make the gene manipulation more easy (Chan et al. 2005). In this process, several non-essential genes, UTR regions, regulatory elements like promoter, RBS and transcription regulators are eliminated and several crucial genes are codon-randomized to remove any undercharacterized regulatory gene (Carrera and Jaramillo 2013). The newly designed coding DNA sequences are synthesized in vitro and introduce to regulate the transcription and protein biosynthesis. This technique was tested in phage and in bacteria (Durante Rodríguez et al. 2016). Another example for refactoring is the replacement of natural control element (promoter) in the spectinabilin gene cluster of *Streptomyces orinoci* with highly

characterized and strong promoter increased the heterologous production of spectinabilin to an amount of 105 µg/l (Shao et al. 2013).

The key importance of synthetic biological approach to pathway design is several possible roots of different pathways considered for the production of a single compound. Some computational tools are designed for the alteration of the metabolic regulatory pathway by using gene knockouts or by the introduction of new catalytically active enzymes. Others involved in finding entirely new metabolic pathway other than the first one (Prather and Martin 2008). One important tool for metabolic pathway prediction is From Metabolite to Metabolite (FMM)—freely available software (Medema et al. 2012). This includes the compilation of the KEGG map and KEGG ligand data to obtain a combined pathway. The list of softwares available for pathway design is enlisted in Table 2.

Synthetic Genetic Circuits

Synthetic genetic circuits are essential for controlling the target molecule production (Kalir et al. 2001). In natural condition, the natural molecule production is

Table 2 Tools for pathway design and construction

Tools	Description	Reference
FMM (from metabolite to metabolite)	Finds the biosynthetic intermediates between two molecules	Chou et al. (2009)
DESHARKY	Identifies biosynthetic pathways that match to the native metabolic pathway of a particular host	Rodrigo et al. (2008)
Optstrain	A computational tool that give suggestions on the optimization of the host's metabolic pathway network for the addition of new metabolic pathway	Pharkya et al. (2004)
IMG (Integrated microbial genomes)	Tool for the comparative and evolutionary analysis of microbial genome	Mavromatis et al. (2009)
AntiSMASH	Identification, pathway annotation and comparative analysis of secondary metabolite biosynthesis genes	Medema et al. (2012)
KEGG	Collection of metabolic pathways, enzymes and genes	Kanehisa and Goto (2000)
RBS calculator	Ribosome binding site design software	Na and Lee (2010)
Gene composer	Software gene cassette design, codon optimization and gene assembly	Medema et al. (2011)
Biojade	Tool for design, modelling and simulation of genetic circuits	Becker et al. (2007)
COBRA toolbox	Metabolic modelling	Cvijovic et al. (2010)
BioMet tool box	Analysis tool for gene knockouts and flux analysis	Kalir et al. (2001)

controlled by different regulatory networks containing DNA, RNA, interacting proteins, transcription factor, all these components work together for the production of a target compound. In this context, synthetic genetic elements circuits help to regulate the target molecule production by artificially connecting different inputs and outputs of different regulatory factors to control the cell mechanism to produce target molecule. Synthetic genetic circuits can be used to create different cascades, genetic on/off switches, logic gates, oscillators and different feedback motifs to control a synthetic pathway. Most of the natural biosynthetic clusters involved in the production of natural molecules and their natural regulation are not enough to produce the desired molecule at high titres (Brophy and Voigt 2014).

6.1.2 Pathway Construction

Combinational prospecting of new natural products from genome requires information about biosynthetic gene cluster. The derived knowledge about biosynthetic gene clusters has to be converted into gene constructs for increasing the production capability. This requires simultaneous screening of different designed DNA constructs and then increasing the titre of the production (Kakule et al. 2014).

Most of these are achieved by combinatorial biosynthesis. That is a pathway can be engineered by changing the combination of genes involved in a biosynthetic gene cluster to increase the product yield (Sánchez et al. 2005). Currently, there are two types of pathway construction strategies: (a) De novo synthesis: here the whole gene clusters are chemically synthesized (Kosuri and Church 2014). (b) DNA assembly: here the different genes are assembled together to construct large cassette. Several gene assembly methods are currently available like Gibson assembly, isothermal assembly, golden gate assembly, etc.

6.1.3 Alteration of Host Metabolism—Metabolic Flux Control

The engineering of host metabolism to redirect the metabolic flux in favour of the newly introduced biosynthetic pathway is extremely important. The newly introduced pathway normally depends on the cellular machinery for the generation of initial building blocks as well as the necessary cofactors like ATP, NADH and NADPH (Chubukov et al. 2014). Cells normally sense the increased demand for all these factors and normalize their internal metabolism. Sometimes this imposes extra burden on cell and resulted in stressed condition and leads to decreased expression of introduced biosynthetic gene cluster (Wu et al. 2015). To resolve all these problems, several stoichiometric—metabolic models are applied for considering the whole metabolic network and for studying the effect of host cell metabolism during the introduction of new biosynthetic gene cluster (King et al. 2015).

Table 2 summarizes various synthetic biology tools at different modification levels that have recently been used for heterologous protein production by yeast. Synthetic biology tools for the assembly of genes of multigene pathways include

Gibson assembly, gateway recombination, DNA assembler and other gene assembly techniques like BioBrick™ and ePathBrick (Gibson et al. 2009; Endy and Knight 2008). Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR), yeast oligo-mediated genome engineering, CRISPR-associated (Cas) systems, Transcription Activator-Like Effector Nucleases (TALENs) and Zinc Finger Nucleases (ZFNs) are the important genome editing and engineering tools. Recent technique, CRISPR–Cas9 systems revolutionized the field of genome editing which efficiently and quickly modifies several endogenous genes in different biomedically important cell types and in other organisms in which the genetic manipulation is challenging. This technology has greatly helped for the genome engineering of model yeasts (*S. cerevisiae* and *Schizosaccharomyces pombe*) and resulted in the increased homologous recombination efficiency and further genetic manipulation.

6.1.4 CRISPR–Cas

Yeast synthetic biology witnessed a huge transformation with the advent of CRISPR–Cas (Clustered Regularly Interspaced Short Palindromic Repeat) (Cong et al. 2013). CRISPR-mediated RNA-guided control of gene using nuclease-deficient Cas9 (dCas9) was experimentally proved to regulate the expression of genes inside the yeast, with the help of synthetic single stranded-guide RNAs (sgRNAs) which specifically target the genes to silence. This technique helped to introduce a series of simultaneous targeted genome integrations and double-stranded breaks in *S. cerevisiae* genome easily and efficiently (Bao et al. 2015). The dCas9 was fused to Mxi1, another protein that involved in the formation of histone deacetylase Sin3p homolog, a component of gene repressor complex in yeast. Further, this technique has been used to repress the *TEF* promoter of *S. cerevisiae* about 10-fold (Gilbert et al. 2013). This technique has also been applied to improve industrially important non-conventional *K. lactis* strain, where muconic acid production was improved by incorporating six genes at three targeted loci (Horwitz et al. 2015).

7 Conclusions

Modern innovations in yeast have revolutionized the field of heterologous protein production. Yeast systems are established end-to-end platforms for the discovery, development, and manufacture of biopharmaceuticals. Notable developments include glycoengineering systems, humanized strains, established synthetic biology tools, display technologies and libraries, and high throughput and scalable fermentation and downstream processing platforms to aid the use of yeast in all aspects of research and development. These new technologies have a high potential to boost the production of biopharmaceuticals.

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Chapter 4
Plant Biosynthetic Engineering Through Transcription Regulation: An Insight into Molecular Mechanisms During Environmental Stress

Rakesh Srivastava, Krishan Mohan Rai and Rashmi Srivastava

Abstract Transcription is not only essential for the synthesis of coding and non-coding RNA, but also extremely significant in regulating gene expression with the coordination of several protein complexes in development, differentiation, phenotype, metabolism, exposure to different challenging environmental conditions, and many other cellular pathways. Further extensions of these studies in plant biology will be very helpful in dealing and maintaining a balance between challenging environmental stress, plant resistance, plant productivity, and yield. Different plant secondary metabolites are induced by various developmental, hormonal, and environmental cues and facilitate the plant to fight and cope up with stress conditions. Interestingly, recent advances suggest that the biosynthesis pathway of secondary metabolites is tightly regulated at the transcriptional stages. Keeping in view studying both the molecular mechanisms at a single platform, the aim of this chapter is to understand plant secondary metabolite biosynthetic pathways at the transcriptional level under unfavorable or stressful environmental conditions.

Keywords Transcription · Promoter engineering · Transcription complex Transcription factors · Secondary metabolites

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1 Introduction

Plants are sessile in nature which instigates them to resist well and endowed with rapid adaptation for survival under multiple environmental stresses. These various environmental perturbations not only affect the productivity of plant, but also cause different variations in the plant morphology and anatomy, growth, development, and metabolism as well. Due to environmental stress, majority of biological processes is directly affected, such as photosynthesis, transpiration, respiration, stomatal conductance, pigment concentrations, energy, and metabolism. Consequently, with secondary stresses such as ion uptake nutritional stress, and oxidative stress, strongly influence the plant development and growth (Saher et al. 2005; Chaves et al. 2009; Compant et al. 2010; Tullus et al. 2012). Several molecular, cellular, and environmental factors have a dramatic effect on the yield of plant biosynthetic product. Moreover, plants need to strongly counteract and adapt rapidly towards environmental stress to increase their productivity at the cellular and molecular levels. Environmental stresses, such as drought, heat or both, not only decrease the plant's growth, but also prominently decrease plant biosynthetic products and yield. A key question is that how to increase the production of plant biosynthetic products while considering the adverse effects of global changes in the environmental factors.

Genomics and proteomics technologies have discovered several plant genes and their downstream pathways, which elaborate their role in the gene regulation, signaling, and resistance in plant stress mechanism. These pathways explain about the different mechanisms to fine-tune the plant gene regulation and strengthen them to survive in different environmental stress conditions. During the alteration in environmental agitations, the defense- and protection-related pathways undergo huge gene expression reprogramming through transcription regulation. The progression of high-throughput tools leads to the finding of numerous genes in plants with changed expression in feedback to environmental perturbations. Genes with improved expression in different aspects of environmental changes are frequently essential for fine adaptation to stress and provide plant tolerance and resistance. Environmental stress, such as chilling, drought, salinity, high temperature, and waterlogging, leads to harmful effects on the growth of plants and decreases crop yield, and, in extreme phase, results in the death of the plant. Abiotic stress is one of the main factors of crop damage at the global level, decreasing yields for most main crop plants by over 50% (Bray et al. 2000).

Since several plant genomes are sequenced using the new high-throughput genomic and proteomic technologies, several reports have been published to improve our understanding of how the plant genome works to facilitate and synchronize the specific program of transcription regulation. Accumulating studies have suggested several molecules, for instance, transcription factors, activators, or cofactors, act as potential candidates or common players that are participated in cross-talk between abiotic and biotic stress-signaling and biosynthetic pathways in the course of the changing environmental stresses (Srivastava et al. 2014b).

The reaction to the wide-ranging environmental stress in the plant development can be prepared promptly by intense genomic reprogramming which results in improved alternative transcriptome and gene regulation programs.

In the era of high-throughput technologies, epigenomics, and nanotechnology, little is known about the functions of the plant transcriptional complex in response to biotic and abiotic stress at the molecular level. Plant secondary metabolites have highly significant social and economic value for human uses such as agrochemicals, food and chemical flavors, nutraceuticals, pharmaceuticals, biofuels, and biomass (Tatsis and O'Connor 2016; Ramakrishna and Ravishankar 2011; Vaishnav and Demain 2011). Moreover, plant secondary metabolites participate in plant growth, reproduction, energy production, or several other primary functions; interestingly, they also significantly facilitate plant responses to biotic and abiotic environmental stresses (Theis and Lerdau 2003; Zhao et al. 2005; Zhai et al. 2017; Ramakrishna and Ravishankar 2011). However, most secondary metabolites have been produced in fewer amounts during native condition; therefore, different approaches based on genetic and metabolic engineering have been successfully introduced to enhance the production of secondary metabolites enormously. Nevertheless, information related to regulation of their production by different transcription machineries or complexes present on the plant promoter is still lacking. Transcription machinery is an important player for coordinating the interaction between transcription factor and promoter, thereby tightly controlling the gene expression of target genes. This chapter mostly highlights how transcription machinery, transcription factors, and promoter augment the production of the secondary metabolites.

2 Promoter Engineering and Transcription Regulation in Plants

RNA polymerase II (RNAPII) is a multisubunit holoenzyme accountable for the gene transcription of the genetic information encoded in the form of DNA sequence (Smale and Kadonaga 2003). The precise spatial and temporal gene transcription necessities should be regulated efficiently, which is a requirement for the accomplishment of different biological and cellular processes, environmental stress response, several diseases signaling pathways, morphology, and differentiation (Maston et al. 2006; Srivastava et al. 2014a, b). The regulation of transcription eukaryotic protein-coding genes are highly dynamic and an orchestrated process, which involves the coordinated action of various proteins, several protein complexes and transcription factors (Maston et al. 2006; Shandilya and Roberts 2012).

The promoter is present in the upstream of the coding gene and 5′ UTR (untranslated region) regions, which is accountable for controlling transcription enormously. The promoter is comprised of a core promoter and the proximal promoter. The core promoter elements (CPEs) function as the docking site for the

general transcription factor (GTF) and responsible for preinitiation complex (PIC) assembly. The CPEs contain two most indispensable elements: the TATA box, the binding region for the TBP (TATA-binding protein) and the Initiator element (INR), transcription start site (TSS) of the protein-coding genes (Maston et al. 2006; Srivastava et al. 2014b; Kiran et al. 2006). The core promoter sequences comprise of 40 bp upstream and/or downstream of the TSS along with several other key elements such as Downstream Promoter Element (DPE), Downstream Core Element (DCE), TFIIB-Recognition Element (BRE), and Motif Ten Element (MTE) (Juven-Gershon and Kadonaga 2010). The proximal promoter is the region further upstream from the CPEs and contains multiple binding sites for activators or repressor protein factors. These regulatory motifs present at the promoter facilitate binding of the sequence-specific DNA-binding transcription factors in order to activate or repress transcription (Lodhi et al. 2008; Ranjan et al. 2009; Srivastava et al. 2014a, b; Chaturvedi et al. 2007). Moreover, these regulatory motifs ensure the gene to be selectively regulated in response to various environmental stresses in a specific cell or tissue. Various kinds of co-regulators and transcription factors are specific in their action and bind specifically to the regulatory motif of the promoter, which is regulated in response to various external environmental stimuli or internal cellular conditions. The distal (upstream) regulatory elements, such as enhancers, silencers, insulators, and locus control regions, present thousands of base pairs away from the TSS, and are also found in the intronic region and downstream of the gene as well (Maston et al. 2006). These upstream regulatory elements sequences can considerably suppress or enhance transcription and are also important for tissue-specific expression. These sequences often contain repeats of the similar elements as in the proximal promoter. The distinct core promoters architecture also lead to alterations in the gene expression (Kiran et al. 2006; Srivastava et al. 2014a). The promoter modulation is regulated by cofactors, transcription factors, activators, and suppressors which bind to cis-regulatory elements of the promoter regions (Priest et al. 2009). Accumulating evidence suggest that many different types of cis-regulatory elements have been discovered in the response to environmental stimuli. The different types of web-based and bioinformatics methods have been used to identify potential plant cis-elements in the regulatory sequences of the target genes (Higo et al. 1999; Lescot et al. 2002). Different types of plant cis-regulatory elements are identified in response to environmental stress, which are listed in Table 1.

Engineering the biosynthesis of plant secondary metabolites is critical for maximizing product formation. To address this matter, promoters play a fundamental role in metabolic engineering and biotechnological prospects because gene expression is operably associated with them and promoter elements drive transcriptional control at any phases of plant development or to a specific cell or tissue or an external/internal signal under defined situations. Promoters are classified into various classes, such as constitutive promoters (active in most or all tissues/parts), inducible promoters (controlled by chemical or physical signal), a development stage-specific promoters (regulated at certain development phases), cell-, tissue-, and organ-type specific promoters (targeted to a specific location to plant region),

Table 1 Cis-regulatory motif for different environmental stresses present in the plant promoter

ID	Stress	Origin species	Sequence	Reference
14BPATERD1	Water stress	*Arabidopsis*	*CACTAAATTGTCAC*	Simpson et al. (2003)
ABREATRD22	ABA, dehydration	*Arabidopsis*	*RYACGTGGYR*	Iwasaki et al. (1995)
ABRELATERD1	ABA, Early responsive to dehydration	*Arabidopsis*	*ACGTG*	Simpson et al. (2003)
ABREZMRAB28	ABR; freezing tolerance	*Arabidopsis; Zea mays; Oryza sativa*	*CCACGTGG*	Suzuki et al. (2005)
ACEATCHS	ACE; UV-A; UV-B;	*Arabidopsis*	*GACACGTAGA*	Hartmann et al. (1998)
ACGTABREMOTIFA2OSEM	ABA; ABRE; DRE;	*Arabidopsis; Oryza sativa*	*ACGTGKC*	Hattori et al. (2002)
ACGTROOT1	ABE; cold tolerance	*Glycine max; Nicotiana tabacum*	*GCCACGTGGC*	Salinas et al. (1992)
ASF1MOTIFCAMV	Xenobiotic stress	*Nicotiana tabacum; Arabidopsis*	*TGACG*	Despres et al. (2003)
CBFHV	Low temperature	*Hordeum vulgar*	*RYCGAC*	Xue (2002)
CCAATBOX1	Heat shock element	*Glycine max*	*CCAAT*	Rieping and Schoffl (1992)
COREOS	Oxidative stress;	*Oryza sativa*	*AAKAATWYRTAWATAAAAMTTTTATWTA*	Tsukamoto et al. (2005)
DRE1COREZMRAB17	ABA; drought	*Zea mays*	*ACCGAGA*	Busk et al. (1997)

(continued)

Table 1 (continued)

ID	Stress	Origin species	Sequence	Reference
DRECRTCOREAT	Drought	*Helianthus annuus; Oryza sativa; Zea mays*	*RCCGAC*	Dubouzet et al. (2003)
DREDR1ATRD29AB	Drought; water stress; dehydration; low temperature; high salt; oxidative stress	*Arabidopsis Populus spp.*	*TACCGACAT*	Kasuga et al. (1999)
HBOXCONSENSUSPVCHS	Stress; wounding	*Nicotiana tabacum; Phaseolus vulgaris*	*CCTACCNNNNNNNCT*	Loake et al. (1992)
HSELIKENTGLN2	Heat shock	*Nicotiana tabacum*	*AGGAATTCCT*	Ohme-Takagi and Shinshi (1990)
LTRE1HVBLT49	Low temperature	*Hordeum vulgare*	*CCGAAA*	Dunn et al. (1998)
LTREATLTI78	Low temperature	*Arabidopsis; Hordeum vulgare*	*ACCGACA*	Nordin et al. (1993)
LTRECOREATCOR15	Cold; drought; low temperature	*Arabidopsis; Brassica napus*	*CCGAC*	Baker et al. (1994)
MYBATRD22	Dehydration; water stress	*Arabidopsis*	*CTAACCA*	Abe et al. (1997)
MYCATERD1	Water stress	*Arabidopsis*	*CATGTG*	Simpson et al. (2003)
MYCATRD22	Dehydration; water stress	*Arabidopsis*	*CACATG*	Abe et al. (1997)
MYCCONSENSUSAT	Cold	*Arabidopsis*	*CANNTG*	Abe et al. (2003)

(continued)

Table 1 (continued)

ID	Stress	Origin species	Sequence	Reference
T/GBOXATPIN2	Wounding	*Lycopersicon esculentum; Arabidopsis*	*AACGTG*	Boter et al. (2004)
TATCCAOSAMY	Sugar starvation	*Oryza sativa*	*TATCCA*	Lu et al. (2002)
TCA1MOTIF	Stress	*Hordeum vulgare; Nicotiana tabacum*	*TCATCTTCTT*	Goldsbrough et al. (1993)
TGA1ANTPR1A	Xenobiotic stress	*Nicotiana tabacum*	*CGTCATCGAGATGACG*	Strompen et al. (1998)
WBOXNTERF3	Wounding	*Nicotiana tabacum*	*TGACY*	Nishiuchi et al. (2004)
WINPSTPIIIK	Wounding	*Solanum tuberosum* (*potato*)	*AAGCGTAAGT*	Palm et al. (1990)
WRKY71OS	Wounding	*Oryza sativa; Petroselinum crispum*	*TGAC*	Zhang et al. (2004)

bidirectional promoters (two genes are controlled in opposite directions). However, the endogenous or native promoter is limited to maximizing the plant biosynthetic product and hence restricts transcriptional output to a particular stage. Promoter engineering has great prospect and acts as a molecular tool for biosynthetic engineering to challenging environments in an attempt to maximize the plant biosynthetic product. Promoter engineering increases the transcriptional capacity and regulates the expression levels and expression patterns of transgenic genes in a different type of cell or tissue. Numerous effective promoter engineering approaches have been described for the generation of unique and new synthetic promoters (assembly of enhancer, CPEs and cis-regulatory elements that can increase the transcriptional capacity in any cell or tissue of interest). These are classified into different groups: (1) Generation of randomized promoter mutagenesis libraries, (2) Hybrid promoters generation, (3) Manipulation of nucleosome architecture occupancy and accessibility, (4) *de novo* synthesis of the promoter (Peremarti et al. 2010; Venter 2007; Liu and Stewart 2016; Blazeck and Alper 2013). In the perception of various environmental stimuli and perturbations, promoters engineering is essential to modulate the dynamical process of the gene expression for metabolic engineering of the biosynthetic pathway in response to a particular state or variation in the environment are impeccable. These engineered promoters are regulated by one or more signal such as various chemicals, environmental stress (drought, high temperature, salt, water stress and others), hormones (auxin, abscisic acid, gibberellin, ethylene, jasmonic acid, nitric oxide, salicylic acid, strigolactones, and others), and biotic stress (insects, microbes, nematodes, and others). Such engineered promoters are not only effective in reducing the genetic load to plants, but also directly or indirectly guard and protect the environment in this way.

3 Transcriptional Machinery at the Promoter

Gene expression is regulated at multiple levels in response to environmental stress. Synchronization of cis-regulatory and core promoter elements are essential to regulate expression of growth and stress-regulated genes. Knowing how transcription factors and PIC bind to cis-regulatory and core promoter elements are not only essential, but also the harmonization between them in stress-regulated gene expression. General cofactors are normally associated with gene regulation to accelerate the interaction between gene-specific transcription factors and general transcription machinery components. These general cofactors include Mediator complex associated with RNAPII and TAFs found in TFIID complex (Thomas and Chiang 2006). Moreover, TBP-containing and non-TBP-containing complex also affects transcription during development and stress condition (Baumann et al. 2010; Juven-Gershon and Kadonaga 2010).

3.1 RNA Polymerase II and Its Modifications

In most eukaryotes, transcription of nuclear genes is achieved by the three different RNA polymerases (RNAP), such as RNAPI, RNAPII, and RNAPIII, however, two more RNAP, viz., RNAPIV and RNAPV are also reported in plants. Furthermore, the RNAPII holoenzyme participates in the transcription of protein-coding genes. It also takes part in the formation of other RNAs such as CUTs (cryptic unstable transcripts), lncRNA (long non-coding RNA), miRNA (microRNA), snRNA (small nuclear RNA), snoRNA (small nucleolar RNA), SUTs (stable unannotated transcripts), and XUTs (Xrn1-dependent unstable transcripts) (Dieci et al. 2007; van Dijk et al. 2011; Neil et al. 2009). RNAPII has a unique repeated disordered domain at the carboxy-terminal domain (CTD), which emerges from the core region of enzyme catalytic part (Cramer et al. 2001). The RNAPII CTD comprises tandem Tyr1–Ser2–Pro3–Thr4–Ser5–Pro6–Ser7 ($Y_1S_2P_3T_4S_5P_6S_7$) heptapeptide repeats that are highly conserved among yeast, humans, and plants. The RNAPII CTD of *Arabidopsis* comprises 15 consensus and 19 non-consensus heptapeptide repeats, whereas *Oryza sativa* contains 12 consensus and 17 non-consensus heptapeptide repeats. RNAPII CTD is indispensable for cell viability, dynamically modified, and roles as a scaffold to coordinate the association of several proteins. The modification of the RNAPII CTD has critical roles in transcription cycle development. The RNAPII CTD modification on the consensus repeats mainly include phosphorylation, isomerization between cis/trans conformations, glycosylation. Other modifications also occur on CTD at non-consensus repeat sequences, includes acetylation methylation and ubiquitylation (Sims et al. 2011; Schroder et al. 2013; Daulny et al. 2008). Accumulating evidence suggest that RNAPII CTD modification is associated with different types of histone modification and to other cellular and stress processes (Srivastava and Ahn 2015). Only a few reports have revealed that modifications of the RNAPII CTD are related with stress stimuli, suggesting their importance in stress-signaling pathways. The variations in environmental conditions such as high temperature or heat, osmotic stress, pH, starvation, toxic agents, or radiations change RNAPII CTD modifications and lead to important alterations in transcription regulation (Miguel et al. 2013; Liu and Tao 2013; Baugh et al. 2009). Though, it remains unclear and needs to be investigated on how the plant RNAPII CTD modifications associate and acclimatize to variations in challenging environmental stress at the cellular and molecular levels for metabolite accumulation or end product of a biosynthetic pathway.

3.2 TFIID Complex

TFIID is a multisubunit protein basal transcription complex and plays an important role in PIC assembly (Thomas and Chiang 2006). It has a role in promoter recognition and its binding to the promoter is considered as the key rate-limiting

step in regulating the core promoter activity (Smale and Kadonaga 2003; Thomas and Chiang 2006). The *Arabidopsis* TFIID complex is comprised of TBP (TATA-binding protein) and 15 different subunits of TBP-Associated Factors (TAFs) (Lago et al. 2004). Studies on TAFs suggest substantial structural and functional conservation among mammals, plants, and yeast (Lago et al. 2004; Srivastava et al. 2015). In budding yeast, genome analysis of TAFs temperature-sensitive mutants has suggested that TAFs control approximately 70% expression of the gene (Lee et al. 2000). As compared to yeast and human, the information of plant TAFs is limited and their roles in stress conditions, development, and differentiation are being increasingly published (Mougiou et al. 2012; Benhamed et al. 2006; Bertrand et al. 2005; Furumoto et al. 2005; Lago et al. 2004, 2005; Tamada et al. 2007; Gao et al. 2006; Kubo et al. 2011; Srivastava et al. 2015). *Arabidopsis* TAFs are associated with plant development and influence the organization of shoot apical meristems, development of leaf, floral organs formation, pollen tube, and light regulation (Gurley et al. 2007). TAF1A regulates light-controlled genes by modification of histones acetylation to H3 and H4 at the target promoters (Benhamed et al. 2006; Bertrand et al. 2005). *Arabidopsis* TAF10 mutant is more affected than the wild type to NaCl stress, whereas TAF10 overexpression improved seed germination rate during osmotic stress (Gao et al. 2006). A mutation in *Arabidopsis* TAF12B results in failure to induce a subset of ethylene-regulated genes in etiolated seedlings (Robles et al. 2007). A recent study reveals that TAF1 plays an important function in genotoxic stress and DNA damage response in plants (Waterworth et al. 2015). These studies suggest that plant TFIID components mediate different functions in gene regulation to plant development and environmental stress response, however, their role in biosynthetic and metabolism pathway is still unknown. Metazoan and yeast TAFs play a significant role in the cell metabolism, but plant TAFs are less characterized in the same aspect, so it is interesting to examine the role in TAFs in plant metabolic pathway.

3.3 The Mediator Complex

The Mediator complex was first identified in the yeast, during the study when GTFs components and purified activators were not enough for regulation of transcription in vitro (Kelleher et al. 1990; Flanagan et al. 1991). Mediator complex has a compact and roughly triangular form with four different modular structures designated as a head module, middle module, kinase module, and tail module (Dotson et al. 2000; Tsai et al. 2014). The Mediator intermediates regulatory signals to the RNAPII and basal transcriptional machinery to facilitate the PIC assembly. The Mediator complex is composed of 29 subunits in *Homo sapiens* and 25 subunits in budding yeast *Saccharomyces cerevisiae* (Poss et al. 2013). In plants, Mediator complex has been first obtained and characterized in *Arabidopsis thaliana* using cell suspension culture (Backstrom et al. 2007). Interestingly, most of the plant Mediator components are conserved between yeast and human. The *Arabidopsis*

Mediator complex is comprised of 33 subunits, where 10 subunits are present in the head module, 6 are found in the middle module, 7 constitute the tail and 4 subunits in the CDK module (Yang et al. 2016). In addition, some plant-specific Mediator subunits are also associated with the complex, such as MED34, MED35, MED36, and MED37 (Mathur et al. 2011). However, MED1 is not present in rice and *Arabidopsis*, but present in red algae (Backstrom et al. 2007; Mathur et al. 2011). The Mediator complex is not merely participating in the transcription initiation process, but several recent reports indicate that it is also involved in other phases of transcription such as promoter escape, co-transcriptional process, transcription elongation, transcription termination (Poss et al. 2013; Allen and Taatjes 2015). These evidence suggest that Mediator contributes to both the general and specific functions in the gene regulation and significantly important for almost every process of transcription of eukaryotic genes.

Recent report suggest that several plant Mediator complex subunits have been connected with many signaling pathways, including plant development and growth, for instance, embryo, flower organ development, meristem pattern, root development, non-coding RNA processing, regulation of genomic stability, plant immunity, and defense pathway, stresses such as cold and drought tolerance (Yang et al. 2016; Samanta and Thakur 2015). *Arabidopsis* Mediator complex subunits MED2, MED14, and MED16 play a significant role in cold acclimation-induced freezing tolerance (Hemsley et al. 2014). MED16 mutant also shows reduced sensitivity to osmotic stress tolerance in *Arabidopsis* (Boyce et al. 2003). *Arabidopsis* MED25 participates in abiotic stress response pathway to control plant development (Elfving et al. 2011). The roles of Mediator subunits are also critical to plant metabolic and biosynthetic pathways. For example, *Arabidopsis* MED5a and MED5b are necessary for regulating phenylpropanoid biosynthetic genes and downstream products (Bonawitz et al. 2012). The metabolites formed from the pathway of the phenylpropanoid are important for plant development and is also useful for human health. *Arabidopsis* MED16 and MED25 positively regulate iron homeostasis iron deficiency metabolism, supporting the molecular mechanism by the Mediator complex for plant metabolic responses to iron deficiency stress (Yang et al. 2014). Although only a few reports have been revealed for Mediator complex that regulates the biosynthesis of plant metabolite, it has high potential to become an ideal target for plant biosynthetic pathway engineering.

3.4 Other Complex that Modulates the Promoters During Transcription

A number of different multi-protein complexes are noted in yeast and human cells on the promoter, which directly or indirectly control transcription regulation, for instance TBP-free TAF-containing complex (TFTC), Spt-Ada-Gcn5 acetyltransferase (SAGA), Spt3-TAF9-GCN5L acetylase (STAGA), SAGA-like (SLIK),

TFTC-related PCAF/GCN5 complexes, and nucleosomal acetyltransferase of histone 4 (NuA4) complex have numerous and vital roles in transcription regulation of RNAPII genes. These complexes are involved in transcription primarily either due to their histone-modifying activity or coactivator function of TAFs. However, much information about these complex subunits in yeast, human, and metazoan species are prevalent, there is only a little evidence in the case of plants hence it still remains to be thoroughly investigated.

The histone-modifying SAGA complex plays various functions in transcription regulatory processes such as RNAII recruitment, transcription progression, nucleosome removal, and nuclear export of transcribed mRNA. In addition, SAGA complex also participates in the different cellular developmental process and environmental stresses such as DNA damage, starvation, and heat-regulated pathways directly or indirectly in yeast and human (Baker and Grant 2007). Huisinga and Pugh (2004) also described that yeast SAGA complex is engaged in the genes upregulation in carbon starvation response (Huisinga and Pugh 2004). Recently, plant SAGA complex subunits are identified and suggested the contribution of the SAGA complex in plant gene regulation and environmental stress conditions such as high temperature and salt stress (Srivastava et al. 2015, 2016). The SAGA complex has two important distinct enzyme functions, a histone acetyltransferase, and deubiquitinases. *Arabidopsis* SAGA complex components histone acetyltransferase GCN5 and coactivator ADA2B mediate major activity in cold responses and disruption of these proteins displayed a failure of several cold-regulated gene expression (Stockinger et al. 2001; Vlachonasios et al. 2003). The disruption of SGF29A shows salt stress tolerance, but the stress-related gene expression is decreased, for example, COR78 (cold-regulated 78) and RD29b (responsive to desiccation 29b) in the mutant of SGF29A during salt stress (Kaldis et al. 2011). Another histone acetyltransferase NuA4/Tip60 is also a multisubunit chromatin remodeling complex that plays a vital role in transcription regulation, RNA processing, DNA damage response, stress response, and metabolism in eukaryotes (Sapountzi and Cote 2011). Recently, *Arabidopsis* homologs of NuA4 are identified and defined its role in H4 acetylation in the promoter regions of major flowering regulator genes (Bieluszewski et al. 2015). *Arabidopsis* HAM1 and HAM2 two homologs of yeast ESA1 histone acetyltransferase and the catalytic component of the NuA4 complex (Bieluszewski et al. 2015). *Arabidopsis* lacking HAM1 and HAM2 expression showed more DNA damage after UV-B radiation stress treatment, indicating the functions of these proteins in DNA damage repair exposed to environmental perturbations (Campi et al. 2012).

4 Transcriptional Regulation of Plant Metabolite Biosynthetic Pathway

Plants produce a large number of secondary metabolites, which significantly function as signaling molecules for several cellular and biological pathways and protect the plant in response to diverse environmental adverse conditions and development stage. Plant secondary metabolites are different in chemical structure and nature. Biosynthesis of secondary metabolites is influenced by cell or tissue or organ type, different developmental stage, and environmental cues (both abiotic and biotic). On the basis of chemical composition, plant secondary metabolites are broadly categorized into two groups: nitrogen-containing compound and nitrogen lacking compound (Patra et al. 2013). The nitrogen-containing compounds are extremely diverse such as alkaloids, amines, and non-protein amino acid. The nitrogen-lacking compounds are further grouped into two classes: terpenoids and phenolics. Plant terpenoids are derived from five-carbon isoprene units and play a specific role as chemical interaction molecules and protect from abiotic and biotic environment conditions. Plant phenolics are aromatic benzene ring compounds with one or more hydroxyl groups used primarily for protection against abiotic and biotic stress. Phenolics provide a significant function in plant development, mainly in the biosynthesis of lignin, flavonoid, and pigment (Patra et al. 2013; Yang et al. 2012).

As discussed above, biosynthetic pathway for plant secondary metabolite is controlled and influenced by several factors such as abiotic and biotic environmental stress or spatial and temporal manner (Pandey et al. 2016; Srivastava et al. 2014b; Pavarini et al. 2012). These factors directly or indirectly orchestrate transcriptional regulation of secondary metabolite biosynthesis pathway and thereby their accumulation. The transcription factor, sequence-specific DNA-binding factor, that bind to cis-regulatory elements present generally to the promoter of target genes and mediate the transcription process by the assembly of transcription machineries such as PIC assembly and several chromatin modifiers (Srivastava et al. 2016, 2014b). These transcription factors are perfect targets for biosynthetic pathway engineering as they transfer signal from environmental stress or developmental-specific signal to the target promoter for activating or repressing the gene expression. In plants, several transcription factor families have been investigated for controlling biosynthetic pathway for plant secondary metabolite. These transcription factor families include AP2/ERF (**AP**etala2/**E**thylene **R**esponse **F**actor), bHLH (**B**asic **H**elix-**L**oop-**H**elix), bZIP (**B**asic Leucine **Zip**per domain), DOF (**D**NA-binding with **O**ne **F**inger), MYB (**MY**elo**B**lastosis), NAC (**N**AM, **A**TAF1/2, and **C**UC2), SPL (**S**QUAMOSA promoter binding **P**rotein-**L**ike), WRKY and Zinc finger (Yang et al. 2012; Liu et al. 2015). This transcription factor can act as transcriptional activators to enhance or improve the production of secondary metabolite for example, several R2R3-MYBs have been described to regulate expression of the flavonoid, glucosinolate, and phenylpropanoid pathway genes (Zhong et al. 2010; Zhao and Dixon 2011; Mehrtens et al. 2005; Stracke et al. 2007; Liu et al. 2015). PsAP2, a transcription factor of an AP2/ERF family from

Papaver somniferum, interacts with both DRE and GCC box element and highly increased in response to wounding stress. Overexpressing PsAP2 augmented both abiotic and biotic stresses tolerance in transgenic tobacco plants (Mishra et al. 2015). The AP2/ERF family ORCA2 and ORCA3 transcription factors bind to the JERE (jasmonate and elicitor-responsive element) on the secondary metabolite biosynthetic gene strictosidine synthase promoter. ORCA2 and ORCA3 are upregulated by plant stress hormone jasmonic acid and have been reported as regulators for terpenoid indole alkaloids biosynthesis (Menke et al. 1999). Jasmonic acid-responsive AP2/ERF family from *Artemisia annua* AaERF1 and AaERF2 interacts with the CRTDREHVCBF2 (CBF2) and RAV1AAT (RAA) regulatory element found in both ADS and CYP71AV1 promoters resulting in augmented metabolites artemisinin accumulation, which is an effective component for malaria treatment (Yu et al. 2012). Overexpression of the bHLH transcription factors TSAR1 or TSAR2 in hairy roots of the *Medicago truncatula* leads to increase in the transcript of triterpene saponin biosynthetic genes and intensely enhanced the triterpene saponins accumulation. TSAR1 and TSAR2 mediate HMGR1 (3-hydroxy-3-methylglutaryl-coenzyme a reductase1) transactivation by direct interaction to the N-Box of the HMGR1 Promoter (Mertens et al. 2016). *Gossypium arboretum* GaWRKY1, coding for a Leu zipper-containing WRKY protein, acts as a transcriptional activator of the cotton (+)-δ-cadinene synthase gene CAD1-A, contributing to cotton sesquiterpene biosynthesis. In suspension cells, both *GaWRKY1* and *CAD1-A* genes expression and sesquiterpene aldehydes biosynthesis were strongly activated by a fungal elicitor and methyl jasmonate or other environmental stimuli (Xu et al. 2004). Thus, it is a useful technique to use appropriate transcription factors for plant secondary metabolic biosynthetic engineering.

5 Conclusion

To improve the plant secondary metabolism production, it is essential to understand its biosynthetic pathway at the transcription level. Therefore, engineering the transcriptional regulatory mechanism, especially promoter or transcription factors, promises to be of high potential in engineering plant secondary metabolism. The transcription complex mediates the coordination between the engineered promoter or the native promoter with transcription factor at the TSS of the promoter. Since, reports suggest that transcription complexes control the secondary metabolic biosynthetic pathway, therefore the production and accumulation of these metabolites in response to a different kind of environmental stress are valuable. Cis-regulatory motifs present in the promoter have also been recognized and well established to enhance the production of plant secondary metabolic pathway. Thus, engineering the coordination between promoter, transcription factors, and transcription complex assembly at the promoter can potentially be used for fine-tuning the plant metabolic biosynthetic pathway.

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Chapter 5
Oil Palm Biomass and Its Kinetic Transformation Properties

Muhammad Fakhrul Syukri Abd Aziz and Zainul Akmar Zakaria

Abstract Malaysia generates millions of tons of oil palm biomass from oil palm mills which need a proper waste utilization application. One of the most commonly method used to utilize the wastes is by using pyrolysis method. This chapter discusses some current applications of biomass pyrolysis. Moreover, kinetic transformation properties of biomass, biomass pyrolysis reaction kinetics, and parameter estimation have also been discussed as the purpose of kinetic modeling is to find a good correlation between reaction rate (k), conversion rate (α), and reaction temperature (T). In order to interpret the biomass pyrolysis behavior, activation energy (E) and pre-factor (A) were needed and calculated based on Arrhenius equation. Last but not least, the recently available kinetic models such as lumped and distributed models are also listed.

Keywords Oil palm biomass · Pyrolysis · Kinetics · Modeling

1 Introduction

Malaysia is blessed with abundant of agricultural resources. In the previous years, agricultural industry has become the major of few other contributors for Malaysia's economy sector. Malaysia is the world's second major producer of palm oil, after Indonesia, and is one the biggest contributor to the country's economy. In 2016, Malaysia had an increase of 1.7% of total plantation, with total plantation area of 5.74 million hectares compared to 5.64 ha in the previous year. The state of Sabah has the largest total planted area in Malaysia with 1.55 million hectares followed by Sarawak with total hectarage of 1.51 million hectares. Meanwhile, Peninsular Malaysia with the 11 states contributed around 2.68 million hectares of palm

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plantation area (MPOB 2017). Biomass is a popular source of renewable energy, can be converted into fuel, and used to generate electricity or other forms of power. It usually refers to plant-based materials which are used other than for food or feed. This lignocellulosic biomass is often used directly in producing heat and power by combustion, as well as indirectly after it has been converted into biofuel. Lignocellulosic biomass contains three main components; lignin, cellulose, and hemicellulose, followed by other subcomponents; protein, pectin, ash, and extractives.

2 Current Application of Oil Palm Biomass in Malaysia

Currently, many researches focused on biomass as another source of power generation. Figure 1 shows some initiatives of using biomass as renewable energy.

2.1 Bioproduct

Bioproducts are materials, chemicals, and energy derived from renewable biological resources. Oil palm biomass utilization as bio-fertilizer is a common way to reuse the waste as a beneficial product. For example, shredded OPF and OPT are being returned to the oil palm estate as mulch (Geng 2014). Moreover, Wu et al. (2009) also reviewed the potential application of POME as fertilizer, fermentation media, and live food for animals and aquaculture organism. In POME, high concentrations of carbohydrates, lipids, protein, nitrogenous compounds, and minerals can be found. These compounds could be used as fermentation media for the production of various metabolites such as bio-insecticides, polyhydroxyalkanoates,

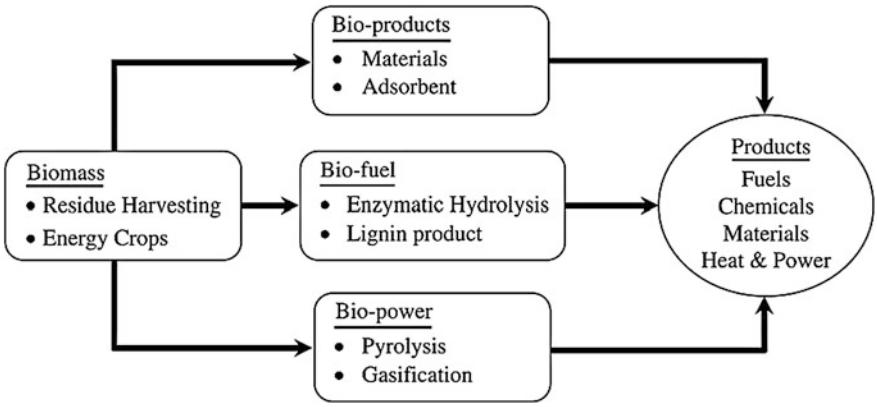

Fig. 1 Biomass initiatives as renewable energy (Sumathi et al. 2008)

antibiotics, organic acids, and enzymes. According to Saidu et al. (2011), mesocarp fiber (MF) of oil palm also served as good substrate for cultivation of oyster mushroom. Last but not least, activated carbon also can be produced from EFB, OPF, and OPT (Alam et al. 2007; Salman and Hameed 2010; Ahmad et al. 2007). They also have been used as adsorbents (Shuit et al. 2009). Moreover, OPF can also be used as a component in the formulation of ruminant animals' feed (Zahari et al. 2003).

2.2 *Biofuel*

A type of fuel that is produced through contemporary biological processes, such as agriculture and anaerobic digestion is known as biofuel. It is a liquid or gaseous fuel that is derived from renewable sources such as biomass and generally used for power plants and transport sectors. The depletion of petroleum source makes biofuel, such as biohydrogen and bio-methanol, a feasible alternative as fuel for the transportation sector. Besides its much cheaper pricing and sustainable supply, biofuel resulted in a near-zero air pollution (Ibrahim et al. 2014). The oil palm biomass can also be converted to bioethanol (Shuit et al. 2009). Bioethanol is one of the products of fermentation from sugar or starch rich biomasses including oil palm. Yamada et al. (2010) stated that oil palm trunk (OPT) has become a promising source of sugars as good feedstock for bioethanol. The sap from OPT core also gives a great potential biofuel and value-added chemical as it can be converted into bioethanol (Wan Asma et al. 2010). Furthermore, utilization of POME with a greater beneficial return to culture microalgae for biodiesel and bioethanol production was discussed by Lam and Lee (2011) in a review study.

Bio-oil contains a huge amount of oxygenated molecules which made it unstable, viscous, and corrosive but it depends on biomass feedstock and pyrolysis condition (Demirbas 2011). There are so many names for bio-oil such as pyrolysis oil, pyroligneous acid, and wood liquids. It has dark-brown color with strong acrid smell (Abnisa et al. 2011). Solid biomass can be converted into liquid bio-oil through pyrolysis, which is easier to transport, store, and upgrade (Demirbas 2011). Pyrolysis of lignocellulosic biomass produced bio-oil which is a complex group of chemical as it contains organic acids, ketones, phenolic compounds, furans, aldehydes, and many other compounds (Chen and Lin 2016). In some previous studies, production of bio-oil from oil palm biomass has concluded that the bio-oil has potential as fuel source and chemical feedstocks (Sukiran et al. 2009; Khor et al. 2009; Abnisa et al. 2013). The generated bio-oil may be directly used for boiler or furnace and also can be considered to be used for vehicles after refinement process (Khor et al. 2009).

Study by Abnisa et al. found that the oxygenated contents of bio-oil from pyrolysis process were quite high (71.40%). High content of oxygenated compounds makes bio-oil less attractive to be used as transport fuels due to decrement of high heating values. This makes it imperative to remove these compounds using

methods such as hydrotreating–hydrocracking process which would not just increase the heating value but also reduce the corrosive properties (Abnisa et al. 2011). According to Sumathi et al. bioethanol and bio-oil can be produced by using EFB as a feedstock through fermentation or hydrolysis, and pyrolysis, respectively (Sumathi et al. 2008). Last but not least, solid by-product generated from pyrolysis also found to be potentially used as alternative fuel for energy production (Salema and Ani 2012).

2.3 Bioenergy

Bioenergy or bioenergy is renewable energy made available from materials derived from biological sources and it is the biggest source of national renewable energy (Sumathi et al. 2008). The production of bioenergy from lignocellulosic biomass provides another alternative for biomass conversion. A review on electricity generation in Malaysia by Shafie et al. (2012) stated that oil palm based biomass including EFB, PKS, OPF as well as other biomasses have great potential for this sector. There also a study by Lam and Lee (2011) discovered the potential of POME as an alternative source of biomethane and biohydrogen for renewable energies generation. Furthermore, oil palm biomass also can produce hydrogen gas as renewable energy (Mohammed et al. 2011).

3 Kinetic Transformation Properties of Biomass

Biomass is a carbonaceous resource of renewable energy and is abundantly available around the world. The awareness in producing energy from biomass has increased drastically in the past few decades due to environmental issues which is increasing of carbon dioxide content in atmosphere, associated with the depletion of fossil fuel resources. Bio-oil from biomass pyrolysis is a suitable and eligible replacement for the fossil fuels (Asadieraghi and Daud 2015). Pyrolysis is a thermochemical process of decomposition of organic material or biomass such as wood, garbage and industrial waste. It is a most known and promising industrial technology as it efficiently converts biomass into advanced fuels, bio-oil, solid charcoal, and gaseous products (Cai and Liu 2016; Asadieraghi and Daud 2015). Understanding the kinetics of biomass pyrolysis could help in describing the physicochemical phenomena occurred inside the pyrolysis vessel. The kinetics also can predict the products distribution, mass loss behavior, chemistry of the solid decomposition, and thermal transfer between wall of vessel as it provides valuable information for reactor design and optimization of pyrolysis process in industry (Wang et al. 2016; Reverte et al. 2007). The purpose of kinetic modeling is to find a good correlation between reaction rate (k), conversion rate (α), and reaction temperature (T). Thus, a solid understanding of kinetics of biomass pyrolysis is very

crucial in developing design tools for a bigger scale pyrolysis including industrial scale pyrolysis process and reactor design.

4 Biomass Pyrolysis Reaction Kinetics and Parameter Estimation

The Arrhenius equation is commonly used in determining the pyrolysis reaction kinetics. Activation energy (E), pre-factor (A), and kinetic equation or reaction model ($f(\alpha)$) were calculated to interpret the biomass pyrolysis behavior (Wang et al. 2016). A solid body thermal degradation needs solving of heat and mass balance equation in order to calculate the temperature and gas composition at each location inside the material. The calculation of mass and heat source terms comes from the chemical reactions progress which is computed from reaction schemes considering some kinetic parameters (Reverte et al. 2007). Biomass thermal degradation under non-isothermal condition can be represented as follow (Zhang et al. 2006; Khan et al. 2011):

$$\frac{\mathrm{d}\alpha}{\mathrm{d}T} = \frac{A}{\beta}\exp\left(-\frac{E}{RT}\right)(1-\alpha)^n \tag{1}$$

Based on above equation, α—sample mass fraction reacted, T—temperature, A—frequency factor, E—activation energy, R—universal gas constant and n—reaction order.

α can be defined as:

$$\alpha = \frac{m_0 - m}{m_0 - m_\infty} \tag{2}$$

where m_0—initial weight, m—actual weight, m_∞—final weight.

By integrating Eq. (1), it becomes:

$$\frac{1-(1-\alpha)^{1-n}}{1-n} = \frac{A}{\beta}\int_0^T \exp\left(-\frac{E}{RT}\right)\mathrm{d}T \tag{3}$$

In Eq. (3), $\int_0^T \exp\left(-\frac{E}{RT}\right)$ has no definite solution. Thus, integrating the function by neglecting high order terms, it becomes:

$$\frac{1-(1-\alpha)^{1-n}}{(1-n)} = \frac{ART^2}{\beta E}\left[1-\frac{2RT}{E}\right]\exp\left(-\frac{E}{RT}\right) \tag{4}$$

Equation obtained below is in logarithmic form:

$$\ln\left[\frac{1-(1-\alpha)^{1-n}}{T^2(1-n)}\right] = \ln\left[\frac{ART^2}{\beta E}\left[1-\frac{2RT}{E}\right]\exp\left(-\frac{E}{RT}\right)\right] \tag{5}$$

As $RT/E \ll 1$, it can be assumed that $1–2RT/E \sim 1$ (Ahmed and Gupta 2009), so the final form for nth order reaction can be derived as:

$$\ln\left[\frac{1-(1-\alpha)^{1-n}}{T^2(1-n)}\right] = \ln\left[\frac{AR}{\beta E}\right] - \frac{E}{RT} \tag{6}$$

For the first order reaction, the equation becomes:

$$\ln\left[-\frac{\ln(1-\alpha)}{T^2}\right] = \ln\left[\frac{AR}{\beta E}\right] - \frac{E}{RT} \tag{7}$$

Plotting left-hand side term of Eqs. (6) and (7) against $1/T$ will get nth and first order reactions, respectively. The resulting straight line poses a slope of E/R and intercept of $\ln(AR/\beta E)$. Activation energy and frequency factor can be determined using the slope and intercept values (Ahmed and Gupta 2009).

In conjunction with that, it is important to consider some factors such as kinetic model, reaction scheme, and reaction rates before analyzing the kinetic parameters. The kinetic parameters should be distinctive and independent of operating conditions (Reverte et al. 2007). The determination of activation energy, reaction model, and other kinetic parameters such as pre-factor, and reaction order have been discussed by Wang et al. in their research about kinetics of biomass pyrolysis (cellulose, hemicellulose, and lignin) using model-fitting method (Wang et al. 2016). Furthermore, in a previous study by David et al. uses six reaction schemes to analyze thermal degradation of cardboard but it comes out with no conclusion which one is the right scheme (David et al. 2003). Moreover, literature reviews have been made by (Antal and Varhegyi 1995; Conesa et al. 1995) on how many kinetic laws and parameters have been proposed by previous researches about cellulose thermal degradation.

5 Kinetic Models

Kinetic models consist of some different types of reaction models developed to differentiate the reaction occurs during biomass degradation. These models can be categorized to two groups based on their mechanism of reaction through pyrolysis process named as lumped and distributed models (Sharma et al. 2015).

5.1 Lumped Model

The first group of kinetic models is lumped model where both individual components of biomass and reaction products are lumped into three categories; gas, tar, and char. There were some proposed kinetic schemes respecting the biomass degradation and volatile products. The lumped model is then separated to study the homogeneous species decomposition of biomass and its individual components.

There are some models developed for lignin, cellulose, and hemicellulose decomposition. Bradbury et al. proposed a three-reaction model for the degradation of cellulose through pyrolysis (Bradbury et al. 1979). The model was developed from Broido model which had an assumption that an initiation reaction will lead to active cellulose formation which can decompose two competitive first order reaction (Broido and Nelson 1975; Bradbury et al. 1979). The products of reaction can be classified into two yield types which are char with gaseous fraction and volatiles. The Broido model stated that dehydration reactions will occur at low temperature (493 K) and can cause anhydrocellulose formation which will be converted exothermally into gas and char (Broido and Nelson 1975). Meanwhile, at higher temperature (553 K), depolymerization reactions will occur and may lead to levoglucosan formation which then converted to volatile tars. The three-reaction model for cellulose pyrolysis was affected by two aspects; at low temperatures, the reaction tends to cross-linking and conversion of cellulose into char. Second, at high temperature and low pressure, the reaction favors cellulose cracking reactions to gases (Agrawal 1988). Moreover, a model proposed by Diebold claimed that formation of char, carbon dioxides, and water was due to chain cleavage and active cellulose dehydration (Diebold 1994). But then, active cellulose vaporization may lead to primary vapors formation which undergo dehydration, decarbonylation, decarboxylation, and cleavage reactions afterward forming secondary gases such as CO, H_2, CO_2, CH_4 higher olefins, and also produce secondary tar (Fig. 2). Lin et al. had another study about the mechanism of cellulose conversion into anhydrosugar and levoglucosan. It was stated that the conversion reaction will further undergoes some series of reactions; polymerization, dehydration, and condensation which may lead to gas and char formation (Lin et al. 2009). Despite that, the model proposed by Diebold seems contradicted to this in terms of energy requirement and product

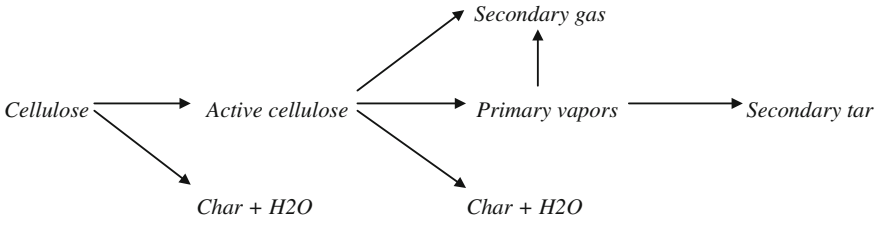

Fig. 2 Decomposition of cellulose through pyrolysis (Diebold 1994)

formation. This is because majority of char is produced from depolymerization of fragmented species and volatile anhydrosugars instead of active cellulose (Diebold 1994).

Kinetics of decomposition of hemicellulose (xylan) can be described using two-stage mechanism isothermally. The process was carried out in inert condition. The reaction was illustrated in Fig. 3 as A represents hemicellulose (xylan), B represents intermediate product of reaction with a reduced degree of polymerization, and V1 and V2 represent volatiles formed during reactions (Di Blasi and Lanzetta 1997).

For lignin, Antal et al. have proposed a model named semi-global multistep model. The model stated that at higher temperature above 773 K, there were lignin monomers which may lead to secondary decomposition and condensation reaction (Antal 1983). Furthermore, the lignin also might be converted into reactive gas and vapors at a very high temperature which subsequently led to secondary char formation by condensation. Concurrently, at low temperature below 773 K, char and gas formation occurred caused by dehydration reactions. Caballero et al. in his research has reviewed about the thermal degradation kinetics of lignin as a function of distribution of solid decompositions. Moreover, Adam et al. (2013) came with a lumped model for primary and secondary pyrolysis of Kraft lignin and stated that longer reaction time will trigger the production of lower molecular weight components and decrease the higher molecular weight compounds (Fig. 4).

There are also some models by some researchers (Shafizadeh and Chin 1977; Thurner and Mann 1981; Font et al. 1990; Samolada and Vasalos 1991; Di Blasi and Branca 2001) developed by considering biomass as a homogeneous and heterogeneous mixture separately. Only primary thermochemical degradation of biomass is considered for biomass of homogeneous species. A study by Yang et al. on degradation of cellulose, hemicellulose, and lignin showed that the products of

Fig. 3 Decomposition of hemicelluloses; A—xylan (major component in hemicellulose), B—intermediate reaction product with a reduced degree of polymerization and V1, V2—volatiles prroduced during reactions (Di Blasi and Lanzetta 1997)

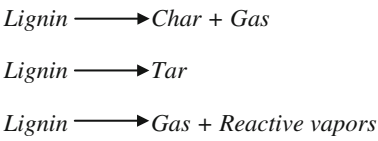

Fig. 4 Various degradation products from the degradation of lignin through pyrolysis (Antal 1983)

degradation were same which are main gaseous products which are CO, CO_2, CH_4, and organics but with different yields for each component (Yang et al. 2007). Moreover, lignin was notably had higher decomposition temperature ranged from 433 to 1173 K. However, Thurner and Mann (1981) with their investigation on kinetics of wood pyrolysis found that gas consists mainly of CO_2, CO, O_2, and $C3^+$ compounds with a small quantity of CH_4, C_2, H_4, and C_2H_2 while tar contains a lot of levoglucosan. A detailed chemistry for the char, liquid, and gaseous formation from pyrolysis process of wood was discussed by Shafizadeh and Chin with their model (Shafizadeh and Chin 1977). The decomposition of lignin and cellulose was found to occur at 523–773 K and 598–648 K respectively while hemicellulose was analyzed to be decomposed at 498–598 K which was the least stable component among others. Besides, it was also discovered that lignin formed a charred residue while hemicellulose and cellulose produced volatile products. Lastly, investigation on product yields variation and kinetic rates by Di Blasi and Branca was mainly because of some aspects such as operating temperatures, experimental setups, and different heating rates (Di Blasi and Branca 2001).

There were also some models developed by assuming biomass as heterogeneous mixture of different components. However, these models neglect the effect of secondary tar degradation mechanism on yield of products. There were many studies regarding calculation of kinetic parameters for devolatilization of components (cellulose, hemicellulose, lignin) from various species of biomass using different heating rates and temperatures via thermogravimetric analysis and nonlinear least square algorithm (Orfão et al. 1999; Grønli et al. 2002; Manya et al. 2003; Mészáros et al. 2004; Branca et al. 2005). Multistep reaction model was proposed by Branca and Di Blasi discovering the mechanism of isothermal wood degradation of cellulose, hemicellulose, lignin, and some extractives in different reaction zones (Branca and Di Blasi 2003). Figure 5 shows the multicomponent mechanism in this model whereas; A represents wood, B and D described as intermediate solid-phase reaction products. VM1, VM2, and VM3 represent volatiles generated in the three stages and final residue of char represents by C.

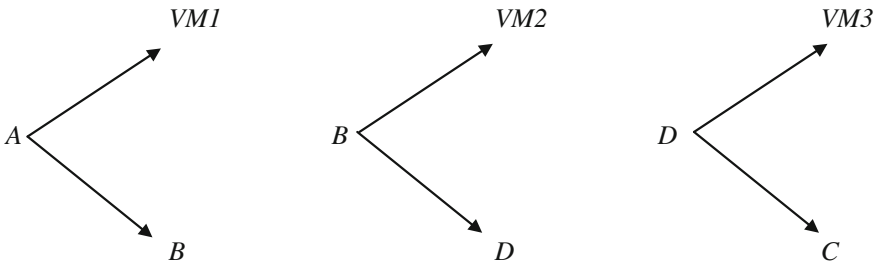

Fig. 5 Biomass decomposition mechanism; A—wood, B, D—intermediate solid-phase reaction products, VM1, VM2, VM3—volatiles generated in the three stages and C—the final residue of char (Branca and Di Blasi 2003)

Radmanesh et al. have proposed a three-independent parallel reaction model for biomass components devolatilization by using gas chromatograph and thermogravimetric analysis (Radmanesh et al. 2006). They also analyzed yield of tars and gases based on the heating rates. Moreover, Liden et al. proposed a kinetic model for wood pyrolysis. In this model, flash pyrolysis of wood was assumed according to two-parallel reaction model and stated that the gas yield with char and liquid tar then experienced decomposition by secondary homogeneous reactions as shown in Fig. 6 (Liden et al. 1988).

A kinetic model by Koufopanos et al. proposed that the small particles (cellulose, hemicellulose, lignin) were pyrolyzed by using summation of their individual reaction rates considering primary and secondary reactions (Koufopanos et al. 1991). It was found that the volatiles, gases, and char were produced from decomposition of biomass whereas volatiles and gases could react with char afterward to produce different compositions of volatiles, gases, and char. The model is shown in Fig. 7. However, Srivastava et al. have modified the model by Koufopanos et al. by considering the reaction order of biomass pyrolysis (Srivastava et al. 1996). They concluded that zeroth order has faster pyrolysis and the first order has higher final pyrolysis temperature because linear relationship of time and temperature.

5.2 Distributed Model

One type of model in which its pyrolysis products are formed by unlimited independent parallel reactions is generally called distributed model. Those parallel reactions have different activation energies derived using Gaussian distribution

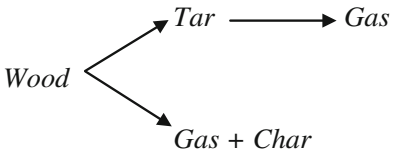

Fig. 6 Primary and secondary wood decomposition through pyrolysis (Liden et al. 1988)

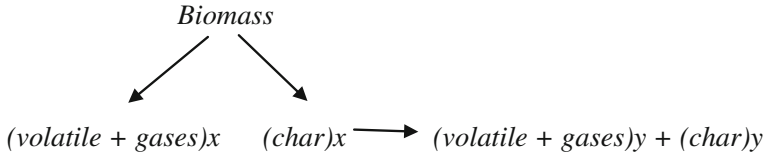

Fig. 7 Mechanism for primary and secondary biomass decomposition (Koufopanos et al. 1991)

function. A model proposed by Chen et al. about coal pyrolysis was discussed regarding the tar–char formation and gas evolution from pyrolysis of biomass. The model's name was FG-DVC that stands for Functional Group—Depolymerization, Vaporization, Cross-linking (Chen et al. 1998). Nevertheless, the model did not cover some of the following: secondary reactions effect, impact of mineral on kinetics of biomass, and heating rate. Niksa then came with a reaction model that can be used for coal devolatilization. This model was based on polymerization of chain macromolecules mixture into fragments that volatilize into tar, partially decompose into gases, or recombine with the narcent char matrix and the model was named as bio-FLASCHAIN (bio-FC) (Niksa 2000). However, the mechanism is very difficult to be implemented and validating it due to significant gaps in the supporting database. Figure 8 shows the reaction mechanism of distributed activation energy proposed by Niksa.

Moreover, there were some researches made on behavior of biomass components during transformation and their chemical structure under different mechanisms (Sheng and Azevedo 2002; Shen and Zhang 2001). The model is called Chemical Percolation Devolatilization (CPD) model. However, the secondary degradation of tar clusters that yields light gases were not considered. Thus, it leads to digression of results compared to experimental data. Nonetheless, Rostami et al. came with a new model by taking distributed activation energy mechanism (DAEM) for pyrolysis of biomass into account. The model made few changes in kinetic parameters obtained from experimental data could improvise the accuracy of

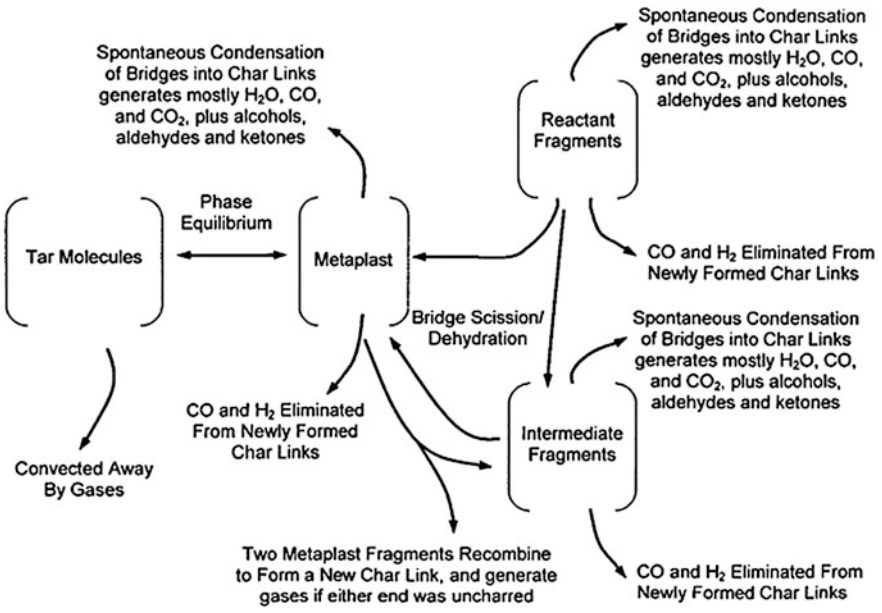

Fig. 8 Reaction mechanism of distributed activation energy (Niksa 2000)

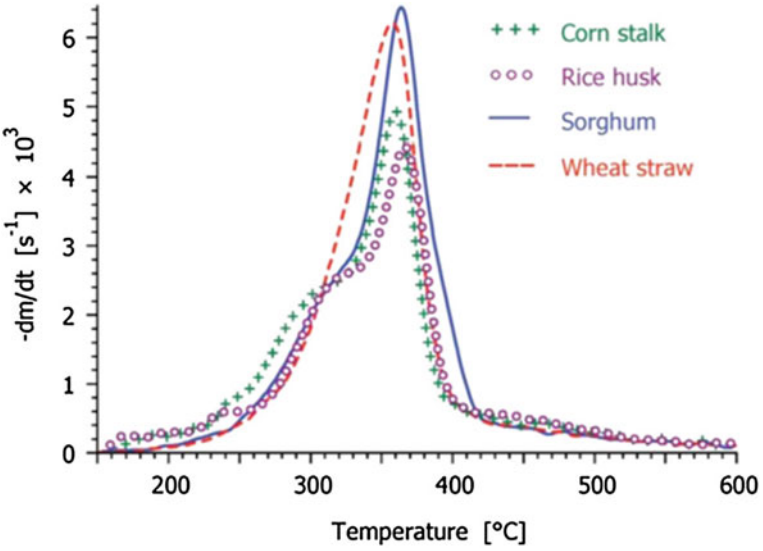

Fig. 9 Curves of mass loss rate using different biomass samples with eating rate of 40 K/min (Várhegyi et al. 2010)

the model (Rostami et al. 2004). Some other models were proposed by Becidan et al. (2007), Sonobe and Worasuwannarak (2008), Várhegyi et al. (2010) based on thermochemical decomposition that utilize the TG analysis technique for model validation with experimental data. In order to calculate model parameters, these models use the nonlinear least squares algorithm depending on mass loss rate of biomass samples with respect to temperature or time as shown in Fig. 9.

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Chapter 6
Selection and Utilization of Agro-industrial Waste for Biosynthesis and Hyper-Production of Pullulan: A Review

Bishwambhar Mishra, Deveeka Zamare and Akula Manikanta

Abstract The emergence of modern microbial technology and product biotechnology had significantly made over the way, scientists and researchers view differently the microbes and the product they biosynthesize. Pullulan is one of the most potent bio-compatible polymers which is basically synthesized by the *Aureobasidium pullulans*. This microbial pullulan acts as the promising biomaterial that is currently used for packaging of readily oxidized food materials, controlled drug delivery, tissue engineering and can also function as artificial molecular chaperones. Commercial pullulan is expensive. It was also estimated that the cost of pullulan is three times higher than the other polysaccharides. However, the cost of the raw materials required for pullulan production accounts for 30% of the total production costs. Therefore, it is essential to search out the cheapest substrate for the production of pullulan. From 1999 till date, different groups of researcher had found and reported various carbon source and nitrogen source from the waste products to be utilized for pullulan production. This review attempts to critically appraise the current literature on 'pullulan' considering its microbial sources and utilization of various agro-industrial wastes for pullulan production.

Keywords Pullulan · *Aureobasidium pullulans* · Agro-industrial waste Polysaccharides

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1 Introduction

Research studies in the field of carbohydrates have revealed that pullulan is one of the most emerging biopolymers, basically synthesized by *Aureobasidium pullulans*. This polymer is consisting of maltotriose units connected by α(1 → 4) glycosidic bond. The consecutive maltotriose units are inter-linked with α(1 → 6) glycosidic bond (Bender and Wallenfels 1961). This alternation of α(1 → 4) and α(1 → 6) bonds, is the main cause of its structural flexibility and hydrophilic nature. The daily intake of pullulan should be up to 10 g per day as per the FDA (Singh and Saini 2008). The major constraint prevailing on the use of pullulan is its cost that is three times more than the price of other polysaccharides. The general structure of pullulan has been illustrated in the Fig. 1.

Dry pullulan powders are white, crystalline or amorphous and non-hygroscopic and dissolve quickly in the cold or hot water. The pullulan shows its resistance to the mammalian amylases. Hence, it provides fewer calories and can be treated as dietary fibre. The employment and application of pullulan in biomedical and tissue engineering field are emerging owing to its biocompatible, non-toxic, non-immunogenic and inert nature. In comparison to dextran, the degradation rate of pullulan in blood serum is much quicker. It was found that the degradation index was 0.7 for pullulan with the intervals of 48 h of incubation while it was of 0.05 for the dextran in the same condition (Bruneel and Schacht 1995). Pullulan is suitable for all consumer groups because of its non-animal origin nature. It is a slow digesting macromolecule, which is colourless, odourless and hence it is used as very low calorie food additives. This is used as a texturizer and glazing agent in chewing gum and bubble gum. It is also used as foaming agent in milk-based desserts. Pullulan contributes major four areas of applications commercially like food, pharmaceutical, biomedical and other miscellaneous as described in Fig. 2.

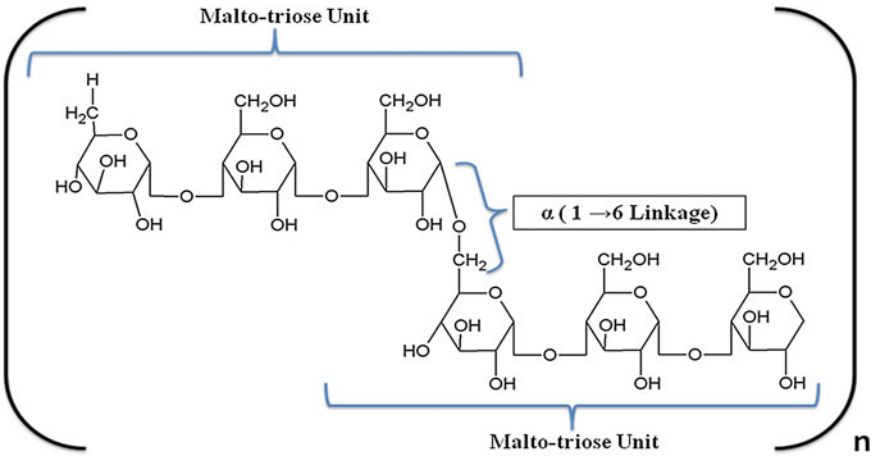

Fig. 1 General structure of the pullulan (Singh and Saini 2008)

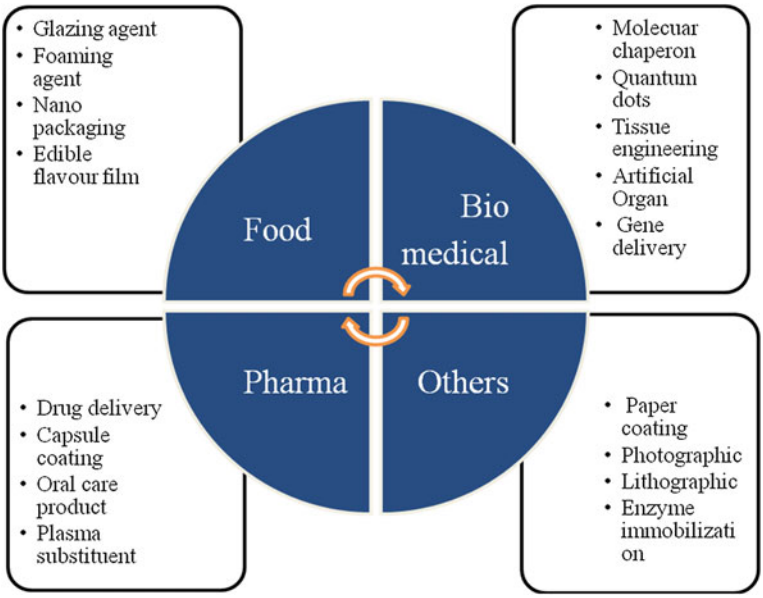

Fig. 2 Broad technological application of Pullulan

Table 1 List of reported microorganisms capable of producing pullulan

Serial no	Microorganisms	References
1	*Aureobasidium pullulans*	Leathers (2003)
2	*Tremella mesenterica*	Fraser and Jennings (1971)
3	*Cytaria harioti*	Waksman et al. (1977), Liva et al. (1986)
4	*Cytaria darwinii*	Waksman et al. (1977), Liva et al. (1986)
5	*Cryphonectria parasitica*	Forabosco et al. (2006), Delben et al. (2006)
6	*Teloschistes flavicans*	Reis et al. (2002)
7	*Rhodototula bacarum*	Chi and Zhao (2003)
8	*Aspergillus japonicus*	Mishra and Suneetha (2014a, b)

2 Microbial Sources of Pullulan

Although it is well-known fact that *A. pullulans* is a key producer of pullulan, but not all the strains of *A. pullulans* have the potential to synthesize pullulan (Ueda et al. 1963; Leathers et al. 1988).

Different types of microbial strains apart from the *A. pullulans* having the ability to produce pullulan have been summarized in Table 1.

3 Fermentative Production of Pullulan

In the industrial scale, pullulan is produced from the liquefied starch by using non-toxigenic and non-pathogenic strain under specific conditions. The upstream part of pullulan production by the fermentation deals with the initial step of the process like selection of the potential strain, inoculum preparation, medium development, growth kinetics, optimization etc., in order to get the product with maximum yield. In submerged fermentation, the nature of the liquid medium is changed drastically during production of pullulan. Initially, the liquid follows the Newtonian behaviour as the viscosity of the medium is very close to pure water. After sometimes, when the polymer is synthesized, the behaviour of the liquid medium changes to non-Newtonian due to increase in the apparent viscosity (Lazaridou et al. 2002).

Downstream processing is essential to meet the purity and quality of the final product from the fermentation broth. There are several steps which are mainly composed of separation of biomass from the culture broth, removal of melanin pigments, proteins and finally precipitation of pullulan with the appropriate organic solvent. Co-production of melanin pigment along with pullulan is a major problem. It was reported that the melanin is produced in the pentaketide pathway at the end of the fermentation (Siehr 1981; Dharmendra et al. 2003). However, pullulan is also synthesized during the late log phase or early stationary phase of the growth of the microorganism (Longfa et al. 2011). After the pigment separation, the main step is to do the precipitation of polymer with suitable organic solvent. Generally, two phases are developed after the precipitation. The deposition of the precipitated pullulan in the solvent system depends upon the molecular weight of the polymer. An appropriate and cost-effective downstream processing is the major requirement and necessary for the production of pullulan. The schematic representation of pullulan production has been illustrated in the Fig. 3.

All the major research in the process of pullulan production was carried out with *A. pullulans* as the yield is very high as comparison to other microbial sources. Pullulan production efficiency of different strains of *A. pullulans* has been summarized in Table 2.

4 Production of Pullulan from Agro-industrial Wastes

Production of pullulan from agro-industrial wastes is both feasible and ecologically sound. Therefore, it would be prudent to search for the available and suitable substrate and select the proper strain of the pullulan-producing organism to produce a valuable product, while protecting the environment from pollution. Pollution problems associated with accumulation of agro-industrial wastes and by-products increased the demand for bioconversion of the plant biomass to value-added compounds by economically feasible ways (Table 3).

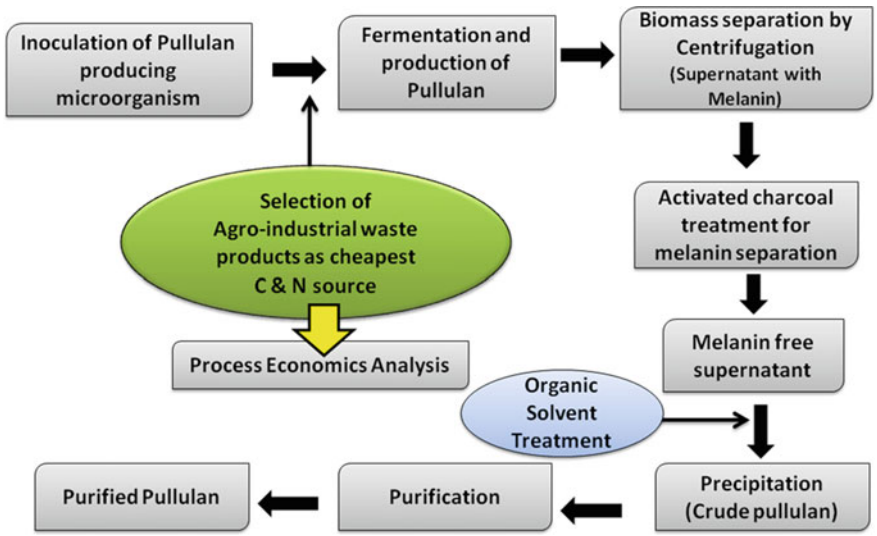

Fig. 3 Schematic representation of Pullulan production by fermentation

Table 2 List of various strains of *Aureobasidium pullulans* for pullulan production

Serial no	Microbial source	Pullulan concentration (g/L)	References
1	*Aureobasidium pullulans* HP-2001	5.5	Seo et al. (2004a, b)
2	*Aureobasidium pullulans* P 56	31.3	Youssef et al. (1999)
3	*Aureobasidium pullulans* NCIM 976	23.6	Dharmendra et al. (2003)
4	*Aureobasidium pullulans* HP-2001	11.49	Gao et al. (2011)
5	*Aureobasidium pullulans* ATCC201253	23.1	Cheng et al. (2011)
6	*Aureobasidium pullulans* SZU1001	25.6	Yu et al. (2012)
7	*Aureobasidium pullulans* LDT-1	31.25	Wu et al. (2012)
8	*Aureobasidium pullulans* SK 1002	30.28	Longfa (2010)
9	*Aureobasidium pullulans* CJ001	26.13	Chen et al. (2012)
10	*Aureobasidium pullulans* NPM2	25.1	Prasongsuk et al. (2007)
11	*Aureobasidium pullulans* FB-1	23.1 ± 0.02	Singh et al. (2008)
12	*Aureobasidium pullulans* RG-5	37.1 ± 1.0	Singh et al. (2012)

Table 3 Reported information for Production of Pullulan from the agro-industrial waste

Serial No	Agro-industrial alternate substrate	Type of fermentation	Microbial strain used	Pullulan yield(g/L)	References
1	Asian palm kernel	Solid state fermentation	*Aureobasidium pullulans* MTCC 2670	16.00	Sugumaran[a] et al. (2014)
2	Beet molasses	Submerged fermentation	*Aureobasidium pullulans* P56	49.00	Lazaridou et al. (2002)
3	Cassava starch	Solid state fermentation	*Aureobasidium pullulans* MTCC1991	27.5	Ray et al. (2007)
4	Cassava bagasse	Solid state fermentation	*Aureobasidium pullulans* MTCC 2670	30.28	Sugumaran[b] et al. (2013)
5	Coconut by-product (coconut water, coconut milk)	Submerged fermentation	*Aureobasidium pullulans* MTCC2195	38.3 (coconut water) 58.0 (coconut milk)	Thirumavalavan et al. (2009)
6	Corn steep liquor	Submerged fermentation	*Aureobasidium pullulans* RBF 4A3,	88.59	Sharma et al. (2013)
7	Jackfruit seed	Solid state fermentation	*Aureobasidium pullulans* NCIM 1049	34.22	Sugumaran[c] et al. (2013)
8	Jaggery	Submerged fermentation	*Aureobasidium pullulans* CFR-**77**	51.9	Vijayendra et al. (2001)
9	De-oiled jatropha seed cake	Submerged fermentation	*Aureobasidium pullulans* RBF 4A3,	83.98	Chaudhury et al. (2012)
10	Potatoes starch	Submerged fermentation	*Aureobasidium pullulans* P56	79.4	Yekta et al. (2011)
11	Rice hull hydrolysate	Submerged fermentation	*Aureobasidium pullulans* CCTCC M 2012259	22.2	Wang et al. (2014)
12	Soybean Oil	Submerged fermentation	*Aureobasidium pullulans* NRRLY-6220	17.4	Sena et al. (2006)
13	Soybean pomace	Submerged fermentation	*Aureobasidium pullulans* HP-2001	7.5	Seo et al. (2004a, b)
14	Sweet potato	Submerged fermentation	*Aureobasidium pullulans* AP329	29.43	Wu et al. (2009)
15	Brewery waste	Submerged fermentation	*Aureobasidium pullulans* P56	48.2	Roukas (1999)

The cost of pullulan production currently is relatively high as compared to other carbohydrate. Hence, it is very essential to search for inexpensive carbon and nitrogen sources, which are nutritionally rich enough to support the growth of the microorganism as well as the production of pullulan.

5 Sweet Potatoes

Sweet potato (*Ipomoea batatas*) is a cheap and easily available agriculture product. The major constituent of sweet potato is starch and little sugar. Sweet potato has been used as a suitable carbon source for various industrial fermentations. Wu et al. (2009) investigated the utilization of sweet potato waste by *A. pullulans* AP329 for pullulan production in submerged fermentation. Generally, starch should be hydrolysed to sugars before starting the fermentation of pullulan production. But, there is probably no need to add expensive β-amylase when sweet potato is used as carbon source in the fermentation. Because, it contains considerable amount of highly active β-amylase, which is the primary saccharifying enzyme in sweet potato.

6 Asian Palm Kernel

Research-based studies were done by Sugumaran et al. (2014a) in which four agro-wastes namely, wheat bran, rice bran, coconut kernel, and palm kernel were evaluated as a low carbon source the pullulan production by *A. pullulans* in solid state for fermentation at 50% moisture content. Palm kernel emerged to be the best carbon source among the four agro-wastes and yield 16 g/L pullulan. Later, the same group of researchers had studied the optimization of the process parameters for the pullulan production considering the carbon source as Asian Palm kernel with Response Surface Methodology (RSM). The pullulan yield was increased to 30.4 g/L. Thus, palm kernel appears to be a potential low-cost carbon source for the production of the pullulan.

7 Soybean by-Products

Soybean pomace is a major agro-industrial by-product from the soy sauce industry. It consists of carbohydrates and proteins as major component. Although soybean pomace has very essential utility, it is discarded as waste because of its high content of sodium chlorite (NaCl). This creates very serious environmental problems. Moreover, considering soybean pomace as a waste is a huge loss to the natural resources as it is a rich source of carbohydrates and proteins. A research-based

study was done by Seo et al. (2004a, b), in which yeast extract was replaced by soybean pomace as nitrogen source for production of pullulan by *A. pullulan* HP-2001.

8 Brewery Wastes

Spent grain liquor is a significant source of waste in the brewing industry. It is the liquor resulting from the final separation of the wort from spent grain in a brewing plant. It consists of water, proteins, mineral salts and total sugars as glucose. A number of brewery wastes were examined as substrates for fungal growth (Shannon and Stevenson 1975). Hang et al. (1977) reported that *Aspergillus niger* produces a significant amount of citric acid during the fermentation of brewery spent grain liquor. Pullulan production from brewery waste by *A. pullulans* has been investigated by Roukas (1999).

9 Coconut By-product

Coconut water is a naturally occurring clear liquid at the fruit's centre. It consists of easily digested carbohydrate in the form of simple sugars and electrolytes. Coconut milk is a liquid which is obtained from the grated meat of mature coconut. Especially, various factories producing desiccated coconut, copra and other coconut meat products (Coco sauce, coconut honey, coconut chips, roasted young coconut, coconut cream, coconut candy, coconut flour) generate the waste product in the form of coconut water and coconut milk. Due to higher Biological Oxygen Demand (BOD) of coconut by-product, it is considered as an active pollutant in the environment. This environmental problem has increased the interest of current researchers in coconut by-product and motivated its utilization in the production of such an industrially important product. Thirumavalavan et al. (2009) have studied and used both coconut water and coconut milk for the pullulan production. The coconut milk found to be more efficient for pullulan production as compared to coconut water as C/N ratio is higher in case of coconut milk.

10 Jackfruit Seed

Jackfruit (*Artocarpus heterophyllus*) tree is an evergreen tree that is mainly found in Florida, Central and Eastern African nations, India, Australia and most of the Pacific islands. Generally, only fleshy pulp of jackfruit is eaten and seeds are considered as the by-product and discarded as waste products. It was found that these seeds contributed 8–15% of the total fruit weight. These seeds contain both

proteins and carbohydrates content (Bobbio et al. 1978). The potential of jackfruit seeds as carbon and nitrogen source was studied by Sugumaran[c] et al. (2013) for pullulan production (34.22 g/L) in solid state fermentation. The optimal concentration of different factors for pullulan production was as follows: NaCl−1.69 g/L, KH_2PO_4 −4.16 g/L, $ZnSO_4{\cdot}5H_2O$−0.052 g/L and moisture content −47.9%, w/w.

11 Jaggery

Jaggery, a concentrated sugar cane juice with or without prior purification produced by cottage industries (also known as *gur*), containing 75–85% sucrose is widely used in India as a substitute of white and refined cane sugar. Vijayendra et al. (2001) and Ganduri et al. (2016) had used jaggery as carbon source for the production of pullulan with *A. pullulans* CFR-77 and by *A. pullulans* MTCC 2195 respectively.

12 Beet Molasses

Few researchers (Yekta et al. 2004; Lazaridou et al. 2002) had investigated about the production of pullulan by utilizing beet molasses as carbon source with a strain of *A. pullulans* P56 (a melanin-deficient strain). Maximum sugar utilization was found to be more than 90%. Overall, beet molasses were proven as an attractive fermentation medium for the production of pullulan by *A. pullulans*.

13 Cassava By-products

Cassava bagasse disposed from sago industry causes high pollution owing to its organic content and more biodegradability (Singh et al. 2008). As a result, biotransformation of these industrial solid wastes to value added products is helpful to our society to minimize the environmental pollution as well as cost of fermentation. Sugumaran[b] et al. (2014) have used Cassava bagasse for pullulan production in solid state fermentation with *A. pullulans* MTCC2670. Approximately about 600–650 tonnes per day of cassava bagasse are disposed from sago industry in India (Srivas and Anatharaman 2005). Ray et al. (2007) had investigated the production of pullulan by using cassava starch residues by *A. pullulans* MTCC1991. Cassava by-products a low-cost and easily available waste material from starch extraction from cassava could provide an economic advantage as a solid substrate as well as a carbon source for production of the emerging pullulan elaborated by the organism *A. pullulans*.

14 Corn Steep Liquor

Corn steep liquor is a high-protein (42%) feed ingredient, which is obtained from the soluble parts of the corn kernel in the steeping process. Sharma et al. (2013) have investigated about five different agricultural wastes (rice bran oil cake, soya bean oil cake, cotton seed oil cake, mustard seed oil cake and corn steep liquor) as nitrogen source for the production of pullulan by *A. pullulans*. Corn steep liquor was found to be best one, which can yield 77.92 g/L of pullulan. This fermentation process was also validated in a 7-L fermenter and a process economics was analysed with respect to cost of pullulan production. It was found that, corn steep liquor can make threefold reduction cost of raw materials for pullulan production in comparison to a conventional process. These observations can be useful in development of a cost-effective pullulan production.

15 De-Oiled Jatropha Seed Cake

Jatropha seed oil is used extensively for the bio-diesel production from last decades. Generally, after the bio-diesel production the seed cake has to be discarded as waste product. The de-oiled seed is very toxic to both human and animal health. Hence its use is restricted. In addition to that, the de-oiled jatropha seed cake comprises of 55–65% of biomass used for the production of bio-diesel. Hence it causes major environmental problem for its disposal. The potential of de-oiled jatropha seed cake used as a substrate for pullulan production was investigated by Chaudhury et al. (2012). It was found that, the yield of pullulan was 83.98 g/L in a 5L laboratory scale fermenter.

16 Potatoes Starch

The cells of root tuber of the potato contain starch grain. Large amount of waste residue has been released in the potato starch industry, which includes effluents and potato residues. This causes serious environmental problems. The major constituents of the potato starch waste consist of carbohydrates. These effluents are having COD (chemical oxygen demand) more than 30 g/L, hence it is rich with biodegradable components (starch, cellulose and proteins) and can be utilized by the microorganism. Yekta et al. (2011) had investigated the utility of potatoes starch waste for pullulan production by *A. pullulan* P56. The potato starch was liquefied by pullulanase and amyloglucosidase enzymes (Ca-alginate immobilized form) in a packed bed bioreactor. Maximum pullulan production was found to be 19.2 g/L and after optimization of various process parameters, the production was increased by 20% as compared to initial state.

The mixtures of potato starch hydrolysate and sucrose were also utilized for the production of pullulan (Chao et al. 2017). It was found that the mixtures of potato starch hydrolysate and sucrose could promote pullulan synthesis and possibly that a small amount of sucrose stimulated the enzyme responsible for pullulan synthesis and promoted effective potato starch hydrolysate conversion effectively. Thus, mixed sugars in potato starch hydrolysate and sucrose fermentation might be a promising alternative for the economical production of pullulan.

The utilization of raw potato starch hydrolysates for pullulan production was also investigated. The maximum pullulan production was found to be 36.17 g/L after 96 h of the fermentation. A comparative study was performed with glucose and sucrose as carbon source for pullulan production, which resulted 22.07 and 31.42 g/L of pullulan respectively (Shengjun et al. 2016). These results infer that raw potato starch hydrolysates can be utilized as carbon source for pullulan production in a cost-effective manner.

17 Rice Hull Hydrolysate

Rice hull is one of the most widely available agricultural by-products in many rice producing countries, which generates 120 million metric tonnes per year. The physic-chemical pre-treatment is required for efficient conversion of rice hull into the fermentable sugars. Hydrolysis process with dilute sulphuric acid has been used to recover usable sugars from the rice hull in efficient manner. A research based studies was done by Dahui et al. (2014) for the production of pullulan from rice hull hydrolysate by *A. pullulans* CCTCC M 201259. They found that acetic acid presents in the hydrolysate exert a negative effect on pullulan production. Hence, a mutagenic study was performed with the *A. pullulans* CCTCC M 201259 and a new mutated strain (designated as *A. pullulans* ARH-1) was isolated in a medium containing acetic acid. The maximum pullulan production was found to be 22.2 g/L by *A. pullulans* ARH-1 after 48 h, while that obtained by the parental strain (*A. pullulans* CCTCC M 201259) was 15.6 g/L after 60 h of fermentation.

18 Strain Improvement for Pullulan Production

Pollock et al. (1992) had investigated the mutagenic study with respect to *Aureobasidium* strain for the first time by using Ethidiumbromide as the chemical mutagen. The mutated strain produced higher molecular weight pullulan as compared to parent strain.

Gniewosz and Duszkiewicz-Reinhard (2008) had screened a mutant strain of *A. pullulans* A.p.-3 with reduced pigmentation by the physical mutagenesis for the higher production of pullulan. In one of the study, a pullulan-producing strain of *Aspergillus japonicus*-VITSB1 was mutated by UV rays and EMS mutagenesis.

The resulted mutant strain showed an increased molecular weight, less pigmentation with higher yield of pullulan (Mishra and Suneetha 2014a, b).

Periyasamy et al. (2015) had mutated a strain of *Aureobasidium mousonni* (NCIM 1226) through UV ray treatment for enhanced pullulan production. It was found that the mutated strain could produce 1.4 times more pullulan than that of parent strain. Most of the research in this orientation had carried out to find a melanin deficient strain for pullulan production.

Xiaoliu et al. (2012) had screened a mutant strain of *A. pullulans* SZU 1001 deficient in pigment production and was screened by complex UV and γ-ray mutagenesis studies. Medium composition optimization for increased pullulan molecular weight and production was conducted using this mutant.

19 Metabolic Engineering

There are very few reports available regarding the metabolic engineering for pullulan production. Wang et al. (2013) investigated the effect of ATP/ADP ratio for biosynthesis of pullulan by *A. pullulans*. They found that production of pullulan is directly proportional to the ATP concentration in the cell.

Ma et al. (2015) studied the about production of pullulan by a newly isolated marine yeast of *Aureobasidium melanogenum* P16 from inulin. They have integrated INU1 gene (from *Kluyveromyces maximum* KM) into the genomic DNA of *A. melanogenum* P16. As there was not any gene encoding inulinase in this yeast, so inulin can be directly converted to pullulan.

It was also found that homologous expression of ApUGPase in *A. pullulans* NRRLY-12974 can effectively improve the yield of extracellular pullulan. The yield of pullulan in engineering strain was improved to 1.3-fold time of parent strain (Haifeng et al. 2016). Recent study showed that knock-out of PKSIII gene in *A. pullulans* decreasing the production of melanin and pullulan and knock-in of gltP gene leads to increased production of heavy oil and pullulan (Jian et al. 2017).

20 Conclusion

Current research shows that production of pullulan from agro-industrial wastes is feasible and ecologically sound. Utilization of agro-industrial waste for the production of pullulan is a challenging. Enhancement of both purity and productivity of pullulan could be achieved by the employment of proper downstream processing and improving the C/N ratio of the substrate. Selection of proper substrate and microbial strain with optimized conditions can yield higher amount of pullulan.

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Chapter 7
Production, Characterization, and Applications of Microbial Poly-γ-Glutamic Acid

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Abstract Poly γ-glutamic acid is a promising biodegradable polymer from bacterial sources with intense applications in the field of medicine, wastewater treatment, food and cosmetics, etc. In the production process, the yield of polygamma glutamic acid varies with the various parameters like nutrient requirements, culture conditions, and the bacterial strains used for the production and the mode of fermentation. Industrial scale production of this high value-added product is not still feasible because of its high production cost. Due to its commercial interest, the cost-effective process for production, efficient recovery, and purification are the current area of interest. Here, main focus is on the production, purification, characterization, and applications of PGA from bacterial sources.

Keywords Poly γ-glutamic acid · Submerged fermentation · Solid state fermentation · Applications

Abbreviations

γ-PGA	Poly-γ-glutamic acid
DMSO	Dimethyl sulfoxide
TCA	Tricarboxylic acid
ICDH	Isocitrate dehydrogenase
GDH	Glutamate dehydrogenase
ODHC	2-oxoglutarate dehydrogenase complex
PDMS	Polydimethylsiloxane

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DSMR	Dry shiitake mushroom residue
SSF	Solid state fermentation
FDAA	1-fluoro-2,4-dinitrophenyl-5-L-alanine amide
GPC	Gel permeation chromatography
KD	Kilodalton
RI	Refractive index
NMR	Nuclear magnetic resonance spectroscopy
FT-IR	Fourier transform infrared spectroscopy
WSC	Water-soluble carbodiimide
PVA	Poly vinyl alcohol
NP	Nanoparticle

1 Introduction

The naturally occurring poly amino acids are poly-γ-glutamic acid, poly-ε-lysine, and cyanophycin (Obst and Steinbüchel 2004). Poly-γ-glutamic acid is an extracellular viscous material produced by several bacteria belonging to *Bacillus* genus. It is an anionic, water-soluble, biodegradable polyamide which is nontoxic to human and environment. Poly gamma glutamic acid is a polymer of glutamic acid units and is linked by γ-amide linkages (α-amino and γ-carboxylic acid units). It is produced in a ribosome-independent manner and polymerization by the enzyme system present on the membrane of the organism (Hajdu et al. 2008). It is free from protease attack because of the γ-amide linkages. Usually proteins and other peptides are made up of α-amino and α-carboxylic acid units which are susceptible to protease attack. It is an optically active polymer with chiral center in every glutamate residues. Stereochemically, three different forms of PGA have been found: a homopolymer composed of D-glutamate units, a homopolymer of L-glutamate units and a copolymer of D- and L-glutamate units.

PGA was first discovered by Ivanovics and Bruckner as a component of capsules of *Bacillus anthracis* and they found that the polymer was released into the medium on autoclaving, or on aging and autolysis of the cells. It was also present in the mucilage of "natto" (fermented soybeans, a traditional food in Japan) and later found that γ-PGA was freely secreted into the growth medium of *Bacillus subtilis* as a product on fermentation. Several *Bacillus* species have now been shown to produce γ-PGA extracellularly (Kunioka 1997). These include *B. licheniformis, B. subtilis, B. megaterium, B. pumilus, B. mojavensis*, and *B. amyloliquefaciens.* A halophilic archaebacterium, *Natrialba aegyptiaca* sp. is also known to produce PGA, but its difficulty in cultivation makes it unsuitable for fermentative production of PGA. *Bacillus licheniformis* and *Bacillus subtilis* are the most commonly used strains for fermentative production of PGA.

PGA has various physiological functions that differ according to the species synthesizing it and its environment. Soil bacteria mainly from the genus *Bacillus* except *B. anthracis* use the released PGA for sequestration of metal ions, thus increasing their resistance to adverse environments. PGA produced extracellularly by *Bacillus* also acts as the source of glutamate during starvation in the late stationary phase. Two highly pathogenic bacteria *B. anthracis* and *Staphylococcus epidermidis* synthesize surface-associated PGA, which helps them to escape phagocytosis and thus acts as a virulence factor. *B. anthracis* capsule is composed exclusively of the D-enantiomer, making it nonimmunogenic and prevents antibodies from gaining access to the bacterium. It protects *B. anthracis* from phage infections and *S. epidermidis* against antimicrobial peptides. Thus, anchored PGA protects the pathogen from the immune system and acts as a virulence factor, whereas released PGA may be a persistence factor that protects the bacterium from its environment. It is highly hygroscopic in nature. It can exist either in water-insoluble free acid form or as its salt with a variety of cations (Na^+, Mg^{2+}, K^+, NH_4^+ or Ca^{2+}), which is completely soluble (Ogunleye et al. 2015).

Here, the production aspects of PGA, purification, and its characterization by different techniques are mainly discussed. It also explains the applications of PGA in various fields, on which much attention has been recently focused.

2 Fermentative Production of PGA

2.1 Submerged Fermentation

2.1.1 Effects of Nutrients on PGA Production

PGA production by submerged fermentation is influenced by various media components. The yield, molecular weight, and stereochemical composition of γ-PGA are mainly influenced by nutrient composition. Researchers have focused on the nutritional requirements for the improved production of PGA and found that the nutrient requirements varied according to the strain used. Based on the glutamate obligation, PGA-producing strains are classified into two types: glutamic acid dependent and glutamic acid independent strains for PGA production. Exogenous glutamic acid dependent strains include *B. licheniformis* ATCC 9945 (Troy 1973). *B. subtilis* IFO 3335 (Kunioka 1995), and *B. subtilis* F-2-01 (Kubota et al. 1993). The exogenous glutamic acid independent strains include *Bacillus licheniformis* A35 (Chang et al. 2009), *Bacillus subtilis* TAM-4 (Ito et al. 1996), *Bacillus* sp. SAB-26 (Soliman et al. 2005), and *Bacillus amyloliquefaciens* LL3 (Cao et al. 2011). Glutamic acid dependent strains cannot produce PGA without the addition of glutamate in the medium. The amount of glutamate added to the medium depends on the strain used and also the other medium components. Glutamate showed positive interactions with other medium components during PGA

production. As the L-glutamate concentration increases, an increase in PGA production was observed with *Bacillus licheniformis* ATCC9945 but there was no effect on the molecular size of PGA produced (Kongklom et al. 2015). Glutamate-dependent strains and their yield are shown in Table 1.

The glutamate-independent strain *Bacillus subtilis* C10 utilizes citric acid and oxalic acid as the substrate for PGA production. It produces PGA by de novo pathway, and oxalic acid enhances the activity of pyruvate dehydrogenase essential for the synthesis of glutamate from glucose. The PGA yield of 27.7 g/l was obtained from this strain. High production of 33.84–35 g/l of PGA was derived from glutamate-independent strain *Bacillus methylotrophicus* SK19.001 using glycerol, sodium citrate, and peptone as the substrates (Peng et al. 2015). *Bacillus licheniformis* TISTR 1010 was found to be glutamate-independent strain, and it uses NH_4Cl as the main nitrogen source in a modified B medium. High yield of PGA was obtained by fed-batch fermentation, with the continuous supply of glucose and NH_4Cl (Kongklom et al. 2015). PGA yield by glutamate-independent strains is low compared to the glutamate-dependent strains. Therefore, the industrial production of PGA mainly focuses on glutamate-dependent strains.

Table 1 Glutamate dependent strains and their yield

Strains	Nutrients	Yield (g/l)	References
B. licheniformis ATCC 9945	Glutamic acid, glycerol, citric acid, NH_4Cl	23.00	Cromwick et al. (1996)
B. licheniformis ATCC 9945	Glutamic acid, glycerol, citric acid, NH_4Cl (Fed-batch)	35.00	Yoon et al. (2000)
B. subtilis IFO 3335	Glutamic acid, citric acid	20.00	Goto and Kunioka (1992), Kunioka and Goto (1994)
B. subtilis (chungkookjang)	Sucrose, $(NH_4)2SO_4$, glutamic acid	15.60	Ashiuchi et al. (2001)
B. licheniformis CCRC 12826	Glutamic acid, glycerol, citric acid, NH_4Cl	19.80	Shih et al. (2002)
B. subtilis NX-2	Glutamic acid, glucose yeast extract	30.20	Xu et al. (2005)
B. subtilis NX-2	Glutamic acid, glycerol	32.7	Wu et al. (2008)
B. subtilis (natto) MR-141	Maltose, soy sauce, sodium glutamate	35.00	Ogawa et al. (1997)
B. subtilis CGMCC 0833	Glutamic acid, $(NH_4)2SO_4$, DMSO, Tween-80, glycerol	34.40	Wu et al. (2008)
B. subtilis R 23 25.38	Glucose, citric acid, (NH4)Cl, NaCl glutamic acid, α-ketoglutaric acid	25.38	Bajaj and Singhal (2009)
B. liceniformis NCIM 2324	Glycerol, citric acid, (NH4)2SO_4, glutamic acid, glutamine, ketoglutaric acid	35.75	Bajaj and Singhal (2009)

The intracellular glutamate was produced by tricarboxylic acid cycle, and citrate acts as the main precursor for PGA production (Kunioka and Goto 1994). *Bacillus* uses extracellular ammonium to convert α-keto glutaric acid into glutamic acid. Thus, citrate acts as the main substrate for PGA production.

Bacillus methylotrophicus strain SK 19.001 was glutamate independent and found to produce 33.84 g/l of PGA produced within 36 h of fermentation in the medium containing glycerol, sodium citrate, and peptone as substrate. Intracellular L-glutamate was synthesized in this strain by the increased activity of L-aspartate amino transferase, glutamate synthase, and L-glutaminase enzymes. Organic nitrogen sources were used for the synthesis of intracellular glutamate (Peng et al. 2015). *Bacillus subtilis* MJ80 was found to produce PGA with or without the addition of glutamate and found to be facultative glutamic acid metabolizing bacterium. It could produce PGA from glutamate and also from soybean powder. *Bacillus subtilis* MJ80 was found to be glutamate-dependent and glutamate-independent strain. It produced 75.5 and 68.7 g/l of PGA from glutamate and soybean powder, respectively (Ju et al. 2014). *Bacillus licheniformis* NRC20 was found to be glutamate independent but enhanced the PGA production with the addition of glutamate (Tork et al. 2015).

Glycerol enhances the poly gamma glutamic acid production by increasing the metabolism of known precursors such as citrate and glutamate. It enhances the activity of poly glutamyl synthetase complex which involved in the racemization and polymerization of glutamic acid and thus increasing the PGA production (Bajaj et al. 2009). Addition of glycerol increased the secretion of PGA by changing the phospholipid composition on the cell wall and thus increases the permeability of the cell wall. Since PGA polymerization happens intracellularly and secreted extracellularly, the increase in permeability of the cell membrane increases the secretion of PGA (Du et al. 2005). In *Bacillus subtilis* CGMCC 0833, the carbon metabolic pathway was found to be regulated by glycerol, tween 80, and dimethyl sulfoxide. By the addition of glycerol, the activity of 2-oxoglutarate dehydrogenase depressed and carbon flux distribution for the synthesis of glutamate was enhanced. Tween 80 and DMSO stimulate the isocitrate dehydrogenase and the carbon flux enhances from 2-oxoglutarate to glutamate. Hence, addition of glycerol, tween 80, and DMSO enhanced the synthesis of intracellular glutamate. In addition, DMSO and tween 80 increase the cell permeability and thus enhances the intracellular uptake of substrate and secretion of PGA (Wu et al. 2008).

In *Bacillus licheniformis* NCIM 2324, L-glutamine and α-keto glutaric acid are found to be the metabolic precursor for the production of PGA. By the addition of these precursors, the yield and molecular weight of PGA increased (Bajaj and Singhal 2009). The addition of TCA cycle intermediates like glutamine and α-keto glutaric acid to the fermentation media leads to increase in PGA production by enhancing the activity of the enzyme, poly glutamyl synthetase complex. The addition of these precursors enhances the utilization of glutamic acid by the organism for PGA production (Bajaj et al. 2009).

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activity of poly glutamyl synthetase complex which involved in the racemization and polymerization of glutamic acid and thus increasing the PGA production. The addition of TCA cycle intermediates like glutamine and α-keto glutaric acid to the fermentation media leads to increase in PGA production by enhancing the activity of the enzyme, poly glutamyl synthetase complex (Bajaj et al. 2009). Addition of glycerol increased the secretion of PGA by changing the phospholipid composition on the cell wall and thus increases the permeability of the cell wall. Glycerol addition leads to decrease in C 16:1 and C 18:1 fatty acids and increase in C 10:0 and C 12:0 fatty acids which may improve the fluidity of the cell membrane. Since PGA polymerization happens intracellularly and secreted extracellularly, the increase in fluidity of the cell membrane increases the secretion of PGA (Du et al. 2005). In *Bacillus subtilis* CGMCC 0833, the carbon metabolic pathway was found to be regulated by glycerol, tween 80, and dimethyl sulfoxide. The addition of glycerol leads to depression of the activity of 2-oxoglutarate dehydrogenase and carbon flux distribution will be directed for the synthesis of glutamate. Tween 80 and DMSO stimulate the isocitrate dehydrogenase and the carbon flux enhances from 2-oxoglutarate to glutamate. Hence, addition of glycerol, tween 80, and DMSO enhances the synthesis of intracellular glutamate. In addition, DMSO and tween 80 increase the cell membrane permeability and thus enhance the intracellular uptake of substrates and secretion of PGA.

The stereochemical composition of PGA mainly affected by the Mn^{2+} supply to the production media. In *B. licheniformis,* Mn^{2+} enhances both biomass and PGA production by increasing the carbon source utilization such as citrate and glycerol. In *B. subtilis,* Mn^{2+} influences the glutamate racemase activity and thus increases the D-glutamate percentage in PGA.

PGA yield, molecular weight, stereochemistry, and the viscosity of the fermentation broth etc mainly influenced by the ions in the PGA production media. The KCl addition to the fermentation medium plays a vital role in controlling PGA production because K^+ induces its production. Addition of KCl to the fermentation medium effectively reduces broth viscosity and increases the productivity and molecular weight. K^+ regulates the D/L-glutamate ratio by reducing the L-Glutamate percentage in *B. subtilis* GXA-28 (Zeng et al. 2016) which showed a similar effect with Mn^{2+} in *B. licheniformis* ATCC9945A. Mn^{2+} influences the glutamate racemase activity and thus increases the D-glutamate percentage in PGA (Wu et al. 2006). The yield and the molecular weight of PGA produced can be controlled by verifying the NaCl concentration in the medium, but it mainly depends on the strain used. The high yield of PGA obtained when the NaCl concentration was 8%, but as the NaCl concentration increases in modified E medium, there is a decrease in γ-PGA molecular size (Wei et al. 2010).

High ferric ion (Fe^{3+}) concentrations lead to low-molecular-weight γ-PGA with enhanced production. High $FeCl_3$ concentrations could enhance the expression of γ-PGA synthetase genes (pgsA, pgsB, and pgsC), which leads to the improved production of γ-PGA. In addition, γ-PGA degradation genes contribute to the low-molecular-weight γ-PGA (Feng et al. 2017). PGA could be produced from inorganic nitrogen sources which are cost-effective than glutamate. Inorganic

nitrogen sources and α-keto glutaric acid metabolized to form glutamate which further involved in polymerization of PGA in *Bacillus subtilis* HSF1410 (Ren et al. 2015). And also nitrate enhances the PGA production in *Bacillus licheniformis* WX-02 by increasing the glutamate assimilation and also by increasing the biosynthesis of glutamic acid inside the cell. Nitrate reduction leads to accumulation of ammonium and thus increases the glutamate synthesis inside the cell. Nitrate acts as a positive inducer of PGA production (Li et al. 2014).

Addition of $CaCl_2$ in fermentation medium leads to decrease in viscosity of the media and enhancing the consumption of extracellular glutamic acid and thus helps in increased production of PGA. It also enhances the activity of three enzymes such as isocitrate dehydrogenase (ICDH), glutamate dehydrogenase (GDH) and 2-oxoglutarate dehydrogenase complex (ODHC), which are involved in conversion of 2-oxoglutarate to glutamate (Meng et al. 2016).

Due to its extensive industrial applications, the cost-effective production of PGA using cheap nutritional sources is one of the prime requirements. Several researchers tried to produce PGA from cheaply available carbon sources. Zhang et al. (2012) used cane molasses and monosodium glutamate waste liquor (contain 1–2% glutamic acid) by *Bacillus subtilis* NX-2 for the production of PGA and observed 52.1 ± 0.52 g/l of PGA under fed-batch fermentation. Instead of glucose, citrate, sucrose, fructose, and glycerol, the C_5 sugar xylose was used as substrate (Zhang et al. 2012). The production of 23.62 g/l PGA was obtained with xylose by *Bacillus subtilis* HB-1. Based on this, the multiple sugars containing substrate such as corncob fibers hydrolysate were used as an alternative carbon source for the production of PGA. The PGA obtained was high (24.92 g/l) compared to xylose, so that this low cost lignocellulosic biomass can be used for the industrial production of PGA (Zhu et al. 2014).

2.1.2 Effect of Culture Conditions on PGA Production

The PGA is produced as a capsular component or accumulated as highly viscous material in the fermentation medium. The increased viscosity of the fermentation broth leads to decrease in volumetric oxygen transfer and thus oxygen limitation. This affects the cell growth and PGA production (Kongklom et al. 2015). Oxygen plays an important role in the highly aerobic process like the production of PGA. Aeration of the fermentation broth can be increased by increasing the agitation speed. Increased aeration and agitation leads to increased glutamate utilization and thus increased the PGA production (Cromwick et al. 1996). Increased agitation-speed enhances the substrate utilization by increasing the activity of enzymes involved in metabolism and results in enhanced PGA production. Oxygen vectors such as n-hexane, n-heptane, n-hexadecane increases the solubility of oxygen in the fermentation broth and thus enhanced the PGA concentration and its molecular weight. However, n-dodecane addition leads to rapid cell growth, which results in loss of nutrients in the fermentation broth and thus decreased the PGA production and its molecular weight. Polydimethylsiloxane (PDMS) is an oxygen carrier that

increases the production of PGA in *Bacillus subtilis* BL53 along with the addition of metabolic precursors such as glutamine and α-keto glutamic acid. By the addition of polydimethylsiloxane to the fermentation medium, there is an increase in oxygen mass transfer rate and thus increases PGA yield.

Physiochemical stress strategy could achieve enhanced γ-PGA production in *Bacillus licheniformis* WX-2. There was an increase in PGA production by induced osmotic stress by 3% KCl, induced heat stress by 50 °C, and induced alkali stress by pH 8.5. The stress mediated strategy leads to the upregulation of the PGA synthetase genes pgsB and pgsC. This strategy could be used as an efficient method for the production of PGA in future. The growth of microorganism and PGA production are influenced by pH of the fermentation medium. Citrate acts as the main substrate for the production of PGA by *Bacillus* species and at pH 6.5, there is an enhanced citrate utilization and improved production of PGA (Cromwick et al. 1996). Two-stage pH control strategy was employed in *Bacillus subtilis* CGMCC 0833 as it is an important parameter for cell growth and for the utilization of extracellular glutamate. During first 24 h, the medium pH was controlled at 7, to obtain maximum cell growth and then to obtain improved production of PGA, pH was maintained at 6.5. The pH shift control strategy leads to increased utilization of glutamate and thus increased the PGA production (Wu et al. 2010).

The molecular weight of PGA depends on the strain used for PGA production and culture conditions. As there is increase in fermentation time, molecular weight of PGA decreases due to the action of the enzyme depolymerase. The PGA acts as a glutamate source for the microorganism during the late stationary phase. High molecular weight PGA is desirable for various applications such as viscosity adding agent and flocculent. The flocculating activity of PGA increases with the increase in molecular weight. Low-molecular-weight PGA required for the drug delivery and the antifreeze activity of PGA increases with the decrease in its molecular weight (Tork et al. 2015).

2.1.3 Modes of Submerged Fermentation

For industrial scale production of poly gamma glutamic acid, various fermentation modes have been tested such as batch, fed-batch and continuous culture, cell recycling and immobilization. Batch and fed-batch fermentation are most common fermentation strategies for PGA production.

To improve PGA production, the aerobic plant fibrous bed bioreactor was constructed and *Bacillus subtilis* NX-2 was immobilized in the bioreactor. Fed-batch fermentation was done to improve production of highly viscous PGA. Sugarcane bagasse, the cheap lignocellulosic biomass acts as the supporting material for immobilization of cells and to further increase the yield of PGA (Xu et al. 2014a). The cost-effective production of PGA was obtained with lower concentrations of yeast extract (40 g/l) and L-glutamate (30 g/l) in the formulated medium. It was done by fed-batch fermentation by maintaining the glucose concentration to 2–10 g/l. The PGA yield of 101.1 g/l was obtained by this method

which was higher than the batch fermentation (Huang et al. 2011). Due to low-cost media formulation, this approach can be used for the large-scale PGA production.

2.1.4 Recovery and Purification of Poly Gamma Glutamic Acid

The recovery and purification of PGA is mainly done by solvent precipitation. The addition of miscible solvents to water reduces the dielectric constant of water, which may cause the PGA in the solution to precipitate. Various solvents such as acetone, methanol, ethanol, 1-propanol, etc., could precipitate of PGA. Among these solvents, maximum recovery is obtained by the addition of ethanol. Cold ethanol provides better recovery than ethanol at room temperature. Recovery of PGA from the fermentation broth involves the following steps. First, remove the biomass from the fermentation broth by centrifugation or filtration. Then add four volumes of the particular solvent to the culture supernatant, and allow to precipitate for 12 h at 4 °C and centrifuge to recover PGA. Repeated precipitation will help to further purification of PGA. Finally, the pellet can be dialyzed to remove the salts and then lyophilized.

The drawback of ethanol precipitation involves the precipitation of proteins and other unwanted products from the fermentation broth and also the need of high amount of ethanol required for precipitation. Ultracentrifugation can be done to concentrate the cell-free broth. During ultracentrifugation, low-molecular-weight particles are rejected and usually retain macromolecules and colloids in a system. PGA with <100 KDa rejected and retain PGA with >100 KDa, and it helps to separate and recover PGA with desired molecular weight. Ultracentrifugation contributes to reduce the amount of ethanol required for precipitation of PGA.

Compared to ethanol precipitation, $CuSO_4$ precipitation is most efficient for poly gamma glutamic acid recovery. By comparing the efficiency and recovery of PGA obtained by $CuSO_4$ precipitation and ethanol precipitation, 85% of the PGA obtained by $CuSO_4$ induced precipitation whereas 82% recovery by ethanol precipitation. $CuSO_4$ precipitation offers better selectivity than ethanol precipitation because 48% of proteins get precipitated by ethanol precipitation but in case of $CuSO_4$ precipitation only 3% of proteins gets precipitated.

2.2 Solid State Fermentation

Solid state fermentation is defined as the fermentation of solids in the absence (or near absence) of free water in which substrate possesses the moisture to maintain the growth and metabolism of microorganism. Agro-industrial waste residues can be used as a solid substrate so that it acts as an environmentally friendly approach for the production of value-added products. Solid state fermentation offers a cheap strategy for the manufacture of poly gamma glutamic acid. Soybean cake powder,

wheat bran, dairy manure compost, swine manure, sweet potato, monosodium glutamate production residues, corn flour, etc., alone or in combination have been used as solid substrate for the production (Bajaj and Singhal 2011b; Yong et al. 2011a). SSF acts as an alternative to submerged fermentation. During submerged fermentation, the viscosity of culture broth increases as PGA production increases and thus decreases the oxygen mass transfer. The oxygen limitation affects the growth of microorganism and yield of PGA. Also SmF could be an expensive process, due to the high coast of media components. SSF offers a cheap strategy for the production of industrially important compound and also limits the problems encountered during SmF. The cost-effective production of poly gamma glutamic acid could be achieved by solid state fermentation.

For the production of PGA under SSF by *Bacillus amyloliquefaciens* C1, dairy manure compost rapeseed cake, corn flour, and monosodium glutamate production residues were used as the solid substrate. Nutrient requirements found to vary with the strain used. In this strain, the 43.7 mg gds^{-1} PGA was produced with the addition of citrate, $MnSO_4$ and $MgSO_4$ (Yong et al. 2011b). The average of 60 mg gds^{-1} was reported on *Bacillus subtilis* using swine manure as the solid substrate (Chen et al. 2005). The PGA yield of 83.61 mg gds^{-1} was obtained by the strain *Bacillus subtilis* CCTCC202048 with the mixed substrates of soybean cake powder and wheat bran supplemented with glutamate, citric acid, and NH_4NO_3 with initial moisture content of 65%, at 40 °C incubated for 42 h (Jian et al. 2005). From *Bacillus licheniformis* NCIM 2324 98.64 ± 1.61 mg gds^{-1} was obtained by using the soybean meal as substrate (Bajaj et al. 2008). Solid state fermentation was carried out with *Bacillus subtilis* NX-2 by using the dry mushroom residues and monosodium glutamate production residues (as the substitute of glutamate). Among dry mushroom residues, the Dry Shiitake Mushroom Residue (DSMR) was found to be the most suitable solid substrate. DSMR and monosodium glutamate production residues ratio was optimized to 12:8 and addition of industrial waste glycerol increased the PGA production under SSF to 107.7 mg gds^{-1}, which was the highest production till date under SSF (Tang et al. 2015).

2.2.1 Recovery After SSF

The recovery of poly gamma glutamic acid from the fermented substrate can be done by the following method. Treat the fresh fermented substrate with distilled water (1:10 w/v, based on initial dry weight of the substrate) in flasks, and mixed at room temperature on a rotary shaker 200 rpm for 2 h and then filtered through two-layer muslin cloth. The filtrate so obtained was centrifuged at 10,000 rpm for 15 min. Clarified supernatants were used for γ-PGA purification (Bajaj et al. 2008). Purification of PGA can be done by the addition of 4 volumes of alcohols such as methanol, ethanol, or other solvents for PGA precipitation. The precipitate was centrifuged at 12000 rpm for 20 min. The other impurities were removed by dialysis and finally lyophilized (Bajaj et al. 2008).

3 Characterization of Poly Gamma Glutamic Acid

The stereochemical composition of PGA can be done by circular dichroism (CD) and also by RT-HPLC. By circular dichroism determination of % of D/L-glutamate unit in pure PGA can be estimated from the standard graph of the same. By RT-HPLC, the D/L amino acids can be measured by Marfey's reagent, 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA). FDAA reagent reacts specifically to D and L amino acid to produce stable derivative and can be determined by RT-HPLC. The poly gamma glutamic acid obtained by fermentation was purified and hydrolyzed with 6 N HCl at 110 °C in a sealed and evacuated tube and hydrolysate neutralized and allowed to react with FDAA. RT-HPLC analyzed samples by using Eclipse XDB-18 column. Commercial D and L-glutamic acid derivatized with FDAA and stereochemical composition of the sample can be determined from the corresponding peaks. For amino acid analysis, hydrolysis of PGA can be done by the method mentioned above. Then thin layer chromatography can be done on a cellulose plate with solvent systems of butanol–acetic acid–water (3:1:1, w/w) and 96% ethanol–water (63:37, w/w) (Stewart and Young 1984; Yokoi et al. 1995). The purity of PGA can be determined by the presence of only of glutamic acid. Amino acids were detected by spraying with 0.2% ninhydrin in acetone. The total carbohydrate content can be determined by phenol sulfuric acid method montreuil (Montreuil et al. 1986). Bradford assay can determine the protein content by keeping bovine serum albumin as standard (Bradford 1976). The viscosity of the culture broth, after cell removal, can be measured using a conventional Ostwald viscometer at 30 °C and ratio between polymer viscosity and solvent viscosity gives the relative viscosity (Tork et al. 2015).

Gel permeation chromatography could be used to measure the number average molecular weight of the PGA. The number average molecular weight of PGA will be varied with the strain and the culture conditions. GPC can be done with Shodex KB800 series columns with a refractive index (RI) detector using deionized water with flow rate 1 ml/min as the mobile phase. Dextran standards with varying molecular weight can be used to construct a calibration curve (Shih et al. 2005). Molecular weight can also be determined by the measuring the diffusion distance of the concentric zone on neutral red plates. By this method, the molecular weight of PGA produced by *Bacillus licheniformis* NRC20 is determined as 1266 KD (Tork et al. 2015).

Structural elucidation of PGA can be done by H^1 NMR and C^{13} NMR spectroscopy. The peak shift of PGA has been reported by many researchers. Samples for NMR spectroscopy have to be dissolved in D_2O. Liquid chromatography can be done to determine the D-/L-glutamate composition of PGA with Crownpak CR (+) column (Goto and Kunioka 1992). Functional groups in PGA can be analyzed by Fourier Transform Infrared Spectroscopy (FT-IR).

4 Applications of PGA

Microbial PGA is produced by ribosome independent manner and has attractive properties such as water soluble, biodegradable, edible, and anionic in nature. It is nontoxic to human and environment. Hence, it could be used for extensive applications. Applications of γ-PGA are shown in Fig. 1.

4.1 Flocculation

It has extensive properties such as solubility in water, edible, biodegradable, and nontoxic to human and environment. These properties lead to the wide applications of PGA. It can be used as a bio-flocculent in wastewater treatment, downstream processing of food industries, and fermentation industries. In wastewater treatment, PGA is used for the flocculation of solid waste and metals (Deng et al. 2003). As the molecular weight increases, the flocculation efficiency increases. Ultra-high molecular weight PGA produced by the bacterial strains such as *Bacillus subtilis* P-104 shows high flocculating activity (Zhao et al. 2013). Cations, pH, and temperature are the main factors influencing the flocculation efficiency of PGA. Cations stimulate the flocculating activity of PGA by neutralizing and stabilizing the negative charge on the functional groups of the bio-flocculent by forming bridging between particles. Synergistic effects on flocculation occurred at different pH based on the cations and the extent of synergistic effect on flocculation decreased in the order, trivalent (Al^{3+} and Fe^{3+}), bivalent ($Ca^{2+}$and Mg^{2+}), and monovalent ions (Na^{+} and K^{+}). pH between 6 and 7 and temperature of 30 °C was found to be

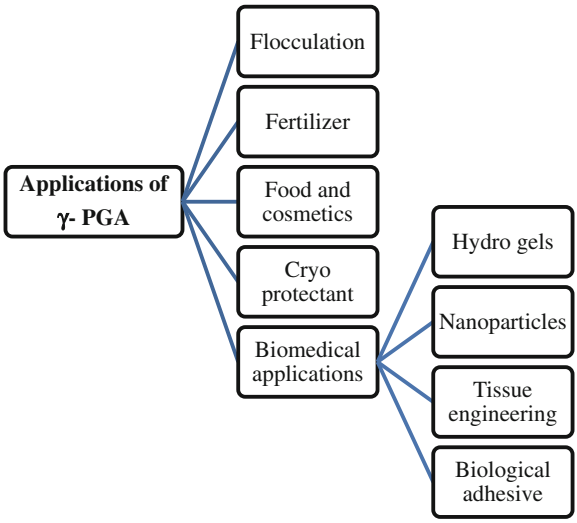

Fig. 1 Applications of γ-PGA

effective for flocculation by PGA. The flocculation activity of PGA decreased with increasing the incubation temperature and completely lost by heating up to 120 °C due to the destruction of polyamide structure of PGA.

PGA could efficiently remove basic dyes from aqueous solution. The electrostatic interaction of PGA and dyes leads to its sorption at pH above 5 and the removal of dyes from PGA takes place at pH 1, which helps to reuse the spent γ-PGA (Inbaraj et al. 2006). During wastewater treatment PGA could be used as a good adsorbent for the removal of mercury (II). The adsorption of mercury by PGA takes place at pH above 3 and reached maximum at pH 6. Desorption of mercury from PGA can be possible by treating it with the distilled water with pH 2 (pH adjusted to 2 by the addition of HCl) to reuse the spend PGA (Inbaraj et al. 2009). Presence of lead in soil inhibits plant growth. Lead removal from the soil can be made possible by PGA. Studies were carried out on *Brassica chinensis* L. seedlings in laboratory conditions and found that lead inhibited the growth of this plant. PGA acts as a good adsorbent for the lead at pH 5 (Chunhachart et al. 2014).

The synthetic flocculants can be replaced with PGA because it can be used as the flocculant in waste water treatment, downstream processing of food, pharmaceutical, and medicine industries. In food and fermentation industries, PGA can be used as a bio-flocculent to harvest microalgae. Lipid producing microalgae has commercial interest in biodiesel producing industries. Harvesting of the microalgae with PGA is cost-effective, and it avoids the loss of lipid due to the breakage of the algal cell during centrifugation and other harvesting techniques (Zheng et al. 2012). The γ-PGA produced from *Bacillus licheniformis* CGMCC 2876 was found to be an efficient bio-flocculent in the sugar cane industry. The color and turbidity of the sugarcane juice was IU 1877.36 and IU 341.41 with 0.8 ppm of γ-PGA, respectively. This was good as the sugar cane juice obtained by the most widely is used chemically synthesized flocculent in the sugarcane industry such as poly acrylamide with 1 ppm. Thus, PGA could be used as a potential substitute for polyacrylamide in the sugar refinery process (Yan et al. 2015).

4.2 Fertilizer

Plant growth and development are improved by the addition of fertilizers to the soil. PGA can be used as a synergist to chemical fertilizers to avoid the environmental pollution. PGA helps to improve growth by increasing the nutrient utilization even in depleted nutrient condition. The activity of soil enzymes such as urease, sucrose, and catalase increased and the total nitrogen accumulation in soil increased by the nitrogen immobilized microbes after the supplementation of PGA (Xu et al. 2013). PGA promoted the growth of Chinese cabbage and increased the content of total nitrogen, soluble protein, and soluble amino acids in leaves. The activity of enzymes involved in nitrogen metabolism and assimilation are increased by the addition of PGA. PGA facilitates the influx of Ca in the cytoplasm and Ca acts as a positive signal for nitrogen metabolism and thus promoted the growth of plants (Xu

et al. 2014b). The protease-producing bacteria *Bacillus subtilis* strain NX-2 is used for the fermentative production of water-soluble fertilizer by using soybean meal as a substrate under SSF. The protease will degrade the proteins present in the soybean meal and *Bacillus subtilis* strain NX-2 used these amino acids for the fermentative production of water-soluble fertilizer. The fermented product can be directly used as a fertilizer and showed improved effect on rapeseed growth (Wang et al. 2014).

4.3 Cryoprotectant

PGA is having antifreeze activity because of its high anionic amino acid composition (Shih et al. 2003). Polymers with acidic amino acids have high antifreeze activity compared to other polymers. PGA with molecular weight below 20,000 had antifreeze activity greater than that of glucose and the antifreeze activity decreased in the order Na salt = K salt > Ca salt > acidic form (Shih et al. 2003). PGA produced from *Bacillus subtilis* natto has the ability to protect *Lactobacillus paracasei* during freeze drying. PGA was found to protect the probiotic bacteria *Lactobacillus paracasei* significantly better than sucrose. The probiotic *Bifidobacteria* strains (*Bifidobacteria longum, Bifidobacteria breve*) were necessary for proper functioning of the gastrointestinal tract (Bhat et al. 2015). PGA protects these cells in fruit juices and helps to survive the cells in the harsh environments of the digestive tract.

4.4 Applications in Food and Cosmetics

PGA is a promising food ingredient because of its physiological and physic-chemical characteristics. Ca supplementation along with PGA, increases Ca absorption by increasing the solubility of Ca in the intestinal tract and also PGA is not susceptible to intestinal enzymes (Tanimoto et al. 2007). PGA has an antidiabetic effect because it reduces the rate of intestinal absorption of sugar. The absorption of insulin-mimetic inorganic salt vanadyl sulfate increases by conjugating with PGA. γ-PGA vanadyl complex having high insulin-mimetic activity than free vanadyl sulfate and thus reduces the intestinal absorption of glucose. Food supplement with K-γ-PGA composition prevents the increase of blood pressure by reducing sodium absorption and thus helps in controlling hypertension (Kishimoto et al. 2008).

Supplementation of PGA can prevent osteoporosis of bones because it increases the solubility of Ca in invitro and also in vivo. Thus, it helps in increasing the absorption of Ca in rats and postmenopausal women and thus increases Ca absorption in bones (Tanimoto et al. 2001, 2007). PGA is having antifreeze activity so that it acts as a cryoprotectant for frozen foods. PGA also used as a thickener in foods/beverages. And it prevents aging and improves the texture of foods. During

deep frying of foods, PGA can be added, and it reduces the oil uptake and moisture loss. Due to its water retention capacity PGA helps to control water evaporation and produce a dense matrix with improved integrity. Thus, PGA can be used as a functional oil reducing agent in deep-fat fried foods (Lim et al. 2012).

PGA is having a vital role in cosmetics because PGA-vitamin C complex increases the solubility of Vitamin C. Vitamin C is important for collagen formation and collagen helps in skin repair. Vitamin C also having antioxidation activity leads to antiaging. The properties of PGA such as hygroscopicity and skin compatibility make it an active ingredient in cosmetic compositions. In cosmetic compositions, PGA helps in maintaining the skin moisturization and elasticity by inhibiting the hyaluronidase enzyme that degrades the hyaluronic acid present in the skin dermis. And it helps in relieving allergic symptoms by inhibiting the permeability of inflammatory cells. The cosmetic ingredient with PGA-vitamin complex shows increased stability, improved absorption, and sustained release of vitamins from the complex (Sung et al. 2008, 2014).

4.5 Biomedical Applications

4.5.1 As Hydrogels

Hydrogel is a biocompatible material that can swell in water and have the capacity to hold water inside its structure. It has extensive applications in the field of drug delivery and as a scaffold in tissue engineering. Hydrogels can be prepared by γ-irradiation, chemical, or physical cross-linking. Biodegradable hydrogels were prepared by cross-linking of microbial PGA and L-lysine by amide bond in the presence of DMT-MM in water and form ester bond in the presence of WSC in DMSO. The use of chemical cross-linkers may be unfavorable for biological applications. The noncovalent interactions (physical cross-links) avoid the need for irradiation and toxic chemical cross-linkers (Murakami et al. 2011).

PGA reacted with PVA in aqueous solution to form hydrogel without any chemical treatment. Increasing the PGA concentration increases the water retention capacity and elongation of hydrogel but decreases the tensile strength. Adsorption of proteins and platelet adhesion on hydrogel decreases with the rise of PGA concentration and thus improved the blood compatibility of the hydrogel. Due to water resistance, blood compatibility, and mechanical properties, PGA-PVA hydrogel became good biomaterial for blood containing medical devices (Lin et al. 2006).

Chitosan-PGA PEC hydrogels are prepared by simple ionic gelation method in which amino group of chitosan and the carboxylic group of PGA interact to form hydrogels. The ionic interaction was confirmed by FT-IR. Due to the presence of PGA, the hydrophilic nature of hydrogel increased and thus having high affinity to cell and attracts serum proteins essential for cell attachment and proliferation.

It shows antibacterial activity against *E. coli* and *S. aureus* and was biocompatible for cell culture and proliferation (Tsao et al. 2010).

4.5.2 Nanoparticles

Drug and gene delivery are made possible by nanoparticles. Due to the small size of the nanoparticle, it could escape from the recticulo endothelium system and thus the circulation time in blood increases. PGA is hydrophilic and water soluble and used to deliver anticancer drugs. Nanoparticles formed from γ-PGA and chitosan have been used for the oral delivery of hydrophobic drugs and proteins. PGA-chitosan nanoparticles were found to be the efficient system for the delivery of insulin for the treatment of diabetics (Lin et al. 2005, 2007; Mukhopadhyay et al. 2012). The ζ-potential and the particle size can be modified by varying the composition of the reaction mixture. Particles of a mean diameter of 218 nm and ζ-potential of +21 mV could able to penetrate a monolayer of Caco-2 cells, which are an in vitro model system for the small intestine. It also functions as an effective carrier for DNA and siRNA delivery because of its preparation by simple ionic gelation method in aqueous solution without any solvents (Lee et al. 2008). The heavy metal such as lead and cadmium can be removed with superparamagnetic iron oxide nanoparticle coated with PGA. This could be prepared by coprecipitation method (Rajan et al. 2014). In vitro removal of lead and cadmium was done in simulated gastrointestinal fluid and metal solution and found that SPIONs-PGA nanoparticle could be used as a metal chelator for the clinical treatment of metal poisoning (Inbaraj and Chen 2012). PGA can be modified with phenylalanine ethyl ester to make the material amphiphilic in nature. It acts as a good carrier for the poorly water-soluble drugs (Akagi et al. 2005) and also acts as a carrier for protein delivery. γ-PGA-L-phenylalanine esters were non-cytotoxic material and shown not to release proteins entrapped inside the particle for 10 days under physiological conditions.

Targeted drug delivery helps to deliver the chemotherapeutic agents and other drugs to the specific sites without any side effects. Active targeting enhances the interaction between NP and the target site and thus increased internalization of drugs through receptor-mediated endocytosis. The targeting ligands mainly used are antibodies and antibody fragments, aptamers, peptides, sugars, and small molecules. Asialoglycoprotein receptors are overexpressed in hepatoma cells and galactosamine used as a ligand to target hepatoma cells. The anticancer drug, doxorubicin, and galactosamine as a targeting moiety were conjugated to the carrier γ-PGA. Conjugation of DOX to γ-PGA decreased its cytotoxicity on hepatoma cells, but the conjugation of galactosamine to γ-PGA-DOX restored the cytotoxicity of the conjugate to hepatoma cells. Low-intensity ultrasound treatment increased the cytotoxicity of γ-PGA-DOX conjugated.

4.5.3 Tissue Engineering

Tissue engineering is the process of developing biological substitutes to restore and maintain the functions of tissue. In tissue, engineering cells are cultured on a scaffold to form tissue and then implanted in patients body or cells with scaffold implanted directly into patients and the tissue will develop inside the body. This helps to overcome immune response between donors and acceptors. The anionic nature of poly glutamic acid is highly hydrophilic, this reduces its use as scaffold but the esterification of –COO– to γ-PGA ethyl, γ-PGA-propyl and γ-PGA-benzyl enhances its water resistance and thus become the versatile material for tissue engineering. γ-PGA-Bn showed better cell adhesion and viability compared to others and the attachment of integrin-binding RGD peptide to unmodified –COO– groups making the scaffold target integrin-binding mechanisms in cells. Thus, the electrospun γ-PGA-Bn scaffolds were developed as a good biomaterial for in situ human mesenchymal stem cell differentiation. For bone tissue engineering, PGA/hydroxyapatite monolith (PGA/HAp) was prepared by biomineralization and used as a scaffold. PGA/HAp monolith was found to be nontoxic to cells, and it absorbed bone morphogenetic protein-2 (BMP-2) and released into the medium during cell culture. PGA/HAp monolith enhances the BMP-induced alkaline phosphatase activity (which is an early stage marker for osteogenic differentiation) compared to PGA monolith itself. PGA/HAp acts as a good biomaterial for bone regeneration. Poly γ-glutamic acid graft chondroitin sulfate/polycaprolactone scaffolds could be used for cartilage tissue engineering. The extracellular matrix gets replaced by this scaffold because it provides the structure for the cells to be attached and proliferate. Polycaprolactone provides the mechanical strength and γ-PGA provides high water binding capacity and signal polysaccharides from chitosan. Thus, it helps to repair and regenerate articulate cartilage. The scaffold provides an ideal environment for cellular adhesion and the induction of differentiation.

Tubular scaffolds of gelatin and poly ε-(caprolactone) block poly(γ-glutamic acid) blending hydrogel act as the good biomaterial for cell proliferation. It has properties such as biocompatible, high elasticity, and the cell proliferation was in time-dependent manner. This scaffold was suitable for the proliferation of smooth intestinal muscle cells of rats. The in vitro degradation of the scaffold was fast but no degradation occurred when cells are present.

Human mesenchymal stem cells have great potential for tissue engineering and cell-based therapies. These stem cells have many roles in tissue remodeling and regeneration by differentiation into multiple lineages and immune suppression. Chitosan/PGA has good biocompatibility, and it assembled into polyelectrolyte multilayer films by layer by layer method. Chemokine stromal derived factor-1 into these complexes and are released periodically. CS/PGA PEMS with SDF-1 recruit hMSCs and thus promote the cell proliferation.

4.5.4 Biological Adhesive

Wound healing involves blood coagulation, inflammation, fibroplasia, collagen deposition, and wound contraction. PGA along with other components such as gelatin or collagen acts as an alternative to conventional surgical glue. Conventional surgical glue may lead to the low rate of degradation and chronic inflammation. The most common poly gamma glutamic acid based biological adhesive was water-soluble carbodiimide cross-linked gelatin and poly gamma glutamic acid hydrogel. This hydrogel glue was found to be effective in sealing the lung air leakage (Bajaj and Singhal 2011a). It acts as a potential replacement to the widely used surgical glue, fibrin. In diabetic rat models, wound healing was found to be improved by a novel layered hydrogel composed of alginate, chitosan, poly gamma glutamic acid. The swelling behavior of the hydrogel increases with the increase of the concentration of PGA and water vapor evaporation rate maintained at the ideal level for wound healing in this hydrogel. Ca release rate is high and Ca helps in releasing coagulation factors. Thus, AL-CS-PGA hydrogel acts as effective glue for wound healing in rat models (Lee et al. 2012).

5 Future Perspectives

PGA is an interesting polymer because of its wide applications in the field of medicine, agriculture, wastewater treatment and food industries, etc. Extensive research has been carried out on the economical production of PGA by many researchers. Culture conditions and the nutrient requirements vary with the strains used for PGA production. Different fermentation strategies are carried out for the effective production of PGA. The high production cost is the main drawback for the industrial scale production of PGA. The productions of PGA from economically feasible materials are the extensive area of research for the cost-effective production of PGA. The extensive studies are carried out on the economical production of the eco-friendly material.

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Chapter 8
Bioprocesses for the Production of 2,5-Furandicarboxylic Acid

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Abstract Recently, major industries focus on the chemical processes for the production of "green" alternative substance 2,5-furandicarboxylic acid (FDCA) for polymers, from glucose, fructose, and even from biomass through HMF as intermediate, although biological processes have greater significance nowadays as a less toxic method for the production of FDCA. Until now, only laboratory-scale production has been reported as biological approaches like whole cell using microorganisms and enzymatic approaches from many countries. According to the reports, for microbial production, a genetically engineered strain *P. putida* S12 with co-expressed genes showed the highest production of 150 g/L FDCA from HMF. For enzyme-assisted biocatalysis galactose oxidase M3–5, periplasmic aldehyde oxidase (PaoABC) and a catalase have been used as one-pot experiment for sequential oxidations higher substrate concentration HMF (100 mM) and obtained 74% of FDCA production. Acid precipitation (pH 0.5–3), cooling, and centrifugation followed by solvent extraction is normally used for the purification of FDCA from microbial broth and enzyme reaction mixture. From enzyme reaction media, a maximum of 15 mg has been obtained in pure form from a 40 mg (FDCA) production media. Further experiments are needed for the efficient biological production and purification of FDCA for industries from the current laboratory scale.

Keywords Biological production · HMF · FDCA · Biopolymer

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1 Introduction

2,5-Furandicarboxylic acid, an oxidized hydroxymethyl furan derivative is a valuable building block, has been reported as one of the 12 priority "Green chemicals" for the future by the US Department of Energy (Bozell and Petersen 2010). Since 2,5-furandicarboxylic acid has no polymerizing action, it cannot be used as such as a polymer unless it is combined with ethane-1,2 diol (mono-ethylene glycol) through esterification. This esterified or combined polymer known as polyethylene furanoate (PEF) can be used as analogs of petroleum-based polymers, and polyethylene terephthalate (PET) and polybutylene terephthalate (PBT) can reduce this nonrenewable energy use by 45–55% (Gandini et al. 2009).

Polymers like polyesters, polyamides, and polyurethanes from FDCA can be used for bottles, containers, nylons, coating resins, engineering plastics, and films. Apart from this, it can be used as an ingredient of fire foams, a drug to the kidney stone and artificial veins for transplantation. These derivatives of the FDCA could address the current estimated production of 4 metric ton a year with revenue of USD 10 million and an estimated production of almost 9 billion lb/yr, with product values between $0.85 and 2.20/lb (Werpy et al. 2004). This market is expected to reach 498.15 kilotons with a revenue of USD 498.2 million by 2020 at a CAGR of 36.70% (Grand view Research Inc. 2015).

Since 2,5-furandicarboxylic acid is a monomer that is not commonly found in nature, it is synthetically produced by the dehydration of fructose to 5-hydroxymethyl furfuraldehyde and its subsequent oxidations through chemical processes (Wang et al. 2015). The current FDCA manufacturers Avantium, Synbias, Carbone Scientific Tokyo Chemical Industry, V & V Pharma industries, and Chemsky produce it through chemical processes either through fructose or 5-Hydroxymethyl furfuraldehyde as substrates. So biobased FDCA could replace a large number of petrol-based chemicals, solvents, and greenhouse gas emissions. So as a leading producer, Avantium collaborated with BASF to develop a YXY process to produce biobased FDCA from plant-based sugars (Renewable energy sources) and other chemicals. It will be marketed by 2018 with the help of Coca-Cola. The FDCA production involves high temperature, high pressure, and the use of metal salts, organic solvents, and toxic chemicals in purification which is non-eco-friendly and expensive. As an alternative to chemical processes, bioconversion methods have to be developed for the production of FDCA from renewable sources.

2 Properties of FDCA and Its Polymer PEF

FDCA, also known as dehydromucic acid, has a chemical structure of $C_6H_4O_5$, which is a white solid insoluble in water and completely dissolves only in DMSO. It is a stable compound with melting point 342 °C and a boiling point of 420 °C.

It has a molecular mass of 156.09 g mol^{-1} and density of 1.604 g/cm^3. This substance has two pK_a values 2 and 28, and these properties make FDCA as a valuable basic substance for the new polyester industries. FDCA monomers cannot be used as directly as polymer unless it is combined with mono-ethylene glycol. The polymer made from FDCA and ethylene glycol is known as PEF, which gives existing polyester structure used nowadays. PET is the major polymer made from benzene-1,4-dicarboxylic acid with ethylene glycol, a widely used polymer used nowadays for packaging and other industries as plastic (Fig. 1). It is not recyclable and made from nonrenewable sources also. Now, Avantium has made biobased mono-ethylene glycol (MEG). If that bio-MEG combines with bio-FDCA, then 100% bioplastic can be made in nearby future.

3 Mechanism of Biotransformation of HMF into FDCA and Its Degradation in Microorganisms

HMF or 5-hydroxymethyl furfuraldehyde ($C_6H_4O_3$) is a toxic aldehyde to microorganisms. Six-electron transfer from HMF alcohol or five-electron transfer (from HMF) is needed for the successive transformation of HMF into FDCA for chemical and biological route transformation. This electron transfers system and

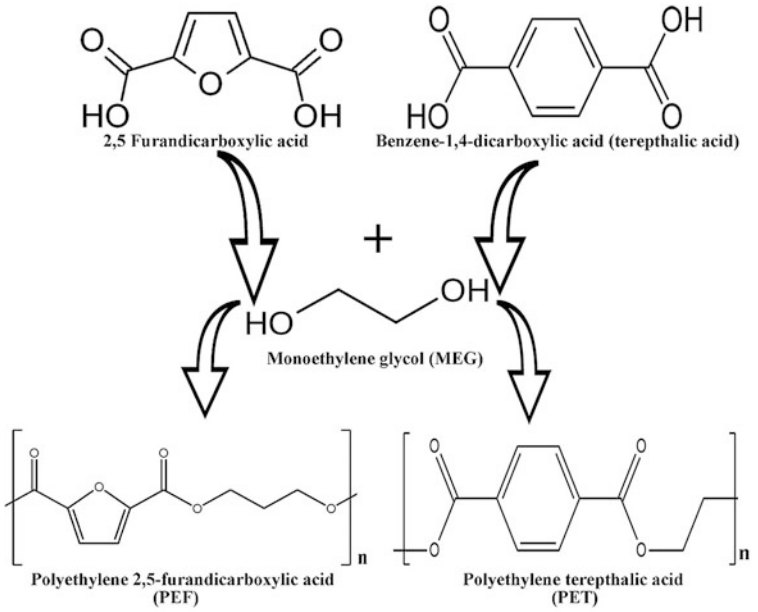

Fig. 1 Synthesis of PEF polymer from 2, 5-furandicarboxylic acid structure comparing with PET from a nonrenewable substance, benzene -1, 4-dicarboxylic acid

subsequently, gets oxidized (at least three oxidations on the appropriate place) by the oxygen, and either alcohol or aldehyde forms of HMF are formed. Since HMF is a toxic aldehyde, in the case of biotransformations, this electron transfer system is catalyzed by either peroxidases or internal and external dehydrogenases present in the microorganisms. This pathway was first reported in the microorganism *Cupravidus basilensis* HMF 14 completely. Since aldehyde moiety present in the HMF is toxic for most of the microorganisms and they convert that HMF into HMF alcohol or HMF acid, which is (alcohol or acid) less toxic normally to them. Selective oxidation of HMF will be converted into furanic chemicals such as HMF alcohol, maleic anhydride (MA), 5-hydroxymethyl-2-furancarboxylic acid (HFCA) (HMF acid), 2,5-diformylfuran (DFF), 5-formyl furoic acid (FFA), and 2,5-furandicarboxylicacid (FDCA).

Among them, most of the microorganisms produce HMF acid as the final product and lower the medium pH and thereby survive or grow very well in the medium. The furfuraldehyde (another toxic furan derivative) and HMF degradation by microorganisms are actually through 2-furoic acid and 5-hydroxymethyl-2-furoic acid (HMF acid) degradation routes (Kakinuma and Yamatodani 1964; Trudgill 1969). The microorganisms which produce HMF acid or other its related derivatives as end products are *Aceto bacterrancens* and *Serratia liquefaciens* (Mitsukura et al. 2004), *Fusarium bulbicola* (Shimada et al. 2010), and *Ureibacillus thermosphaericus* (Okuda et al. 2008). Microorganisms which produce any of the furfural or HMF derivatives after fermentation are *Amorphotheca resinae ZN1* (Zhang et al. 2010), *Cupriavidus basilensis* HMF14 (Koopman et al. 2010a, b), *Arthrobacter nicotiana*, *Telluriamixta* (Wierckx et al. 2010), Burkholderiales, Rhizobiales (Koopman et al. 2010a, b), Coniochaetales, Rhizobiales, Flavobacteriales, and Xanthomonadales (López et al. 2004). Some of the microorganisms producing 2-furoic acid formation from furfural are enterobacteriales (Abdulrashid and Clark 1987) and pseudomonadales (Trudgill 1969; Kakinuma and Yamatodani 1964).

2-Furoic acid degradation was first reported by the scientists Kakinuma and Yamatodani in (1964) and Trudgill in (1969). According to their pathway, toxic furfural degraded into 2-furoic acid which is biotransformed into 2-oxoglutaric acid by its internal enzymes. This 2-oxoglutaric acid gets involved into TCA cycle which is used as a metabolic supplement of the microorganisms. So authors concluded that the microorganisms with furfural addition showed good growth than other carbon or nitrogen media supplements. This is mainly known as furfural degradation pathway (Fig. 2b). After many years, authors (Koopman et al. 2010a, b) discovered that HMF alcohol degraded into HMF, HMF acid, FFA, FDCA, and 2-furoic acid by the microorganism *Cupriavidus basilensis* HMF14 by the action of HMF oxidase and nonspecific dehydrogenases (Fig. 2b). This bacterium showed high metabolic growth during the addition of HMF and furfural as the carbon source. So they connected HMF degradation into furfural degradation pathway already generated. These pathways opened really into a new way for the production of FDCA from microorganisms using HMF as a carbon source.

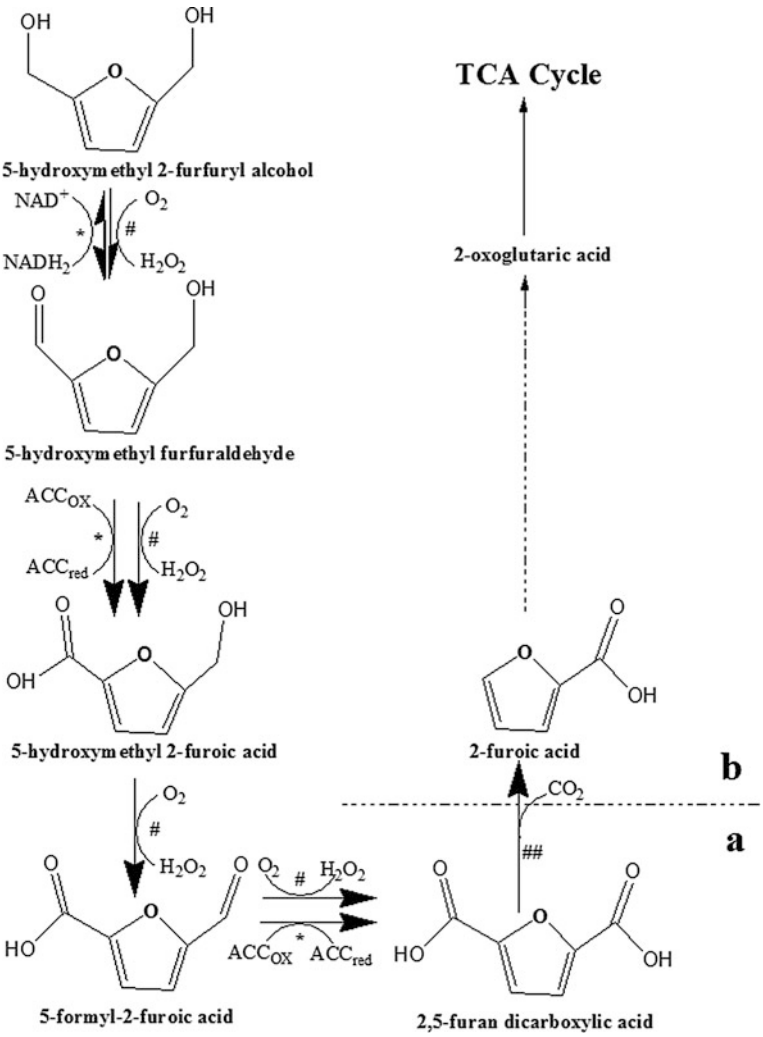

Fig. 2 Representation of the HMF metabolic pathway in a microorganism connected to furfural degradation pathway in which, * marking can be catalyzed by the enzyme HMF oxidase or by nonspecific dehydrogenases, and # can be represented by nonspecific aldehyde oxidases (ACC; acceptor) and ## is for 2,5-furandicarboxylic acid decarboxylase

4 Biological Production of 2,5-Furandicarboxylic Acid

A few approaches have been evolved for the bioconversion of substrates like biomass, C6 sugars glucose or fructose (Renewable energy sources), and 5-hydroxymethyl furfuraldehyde to FDCA using nontoxic biocatalysts. Lack of suitable enzymes for these substrates was considered as the major technical barrier

for an efficient technology (Werpy et al. 2004). So some of the biochemical approaches evolved slowly which mainly involve the conversion of the listed substrates either through microorganisms or its associated enzymes.

4.1 Microbial Production of FDCA

Reports convey that production of FDCA with microorganisms needed the substrate HMF in pure form. At least three subsequent oxidations of HMF, in which two oxidations are on an aldehyde moiety, eventually lead into FDCA. Generally, aldehydes make extensive damage to the organism's proteins, nucleic acids, and other cell organelles through the formation of reactive oxygen species (Zaldivar et al. 1999). HMF is a type of furan aldehyde which inhibits the activity of primary metabolic enzymes of microorganisms and they slowly face its death phase without further multiplication.

To overcome this, certain microorganisms have naturally evolved aldehyde dehydrogenases or oxidases which convert these furan aldehydes into their alcohol or acid product which are less toxic normally. The first report for the biological FDCA production using a microorganism was reported by the organism *Cupriavidus basilensis* HMF14 isolated from 5-(hydroxymethyl) furfural and furfural containing lignocellulosic hydrolysate. The authors characterized the HMF and furfural degradation pathways of this organism both at the biochemical and the genetic level. The formation of FDCA from HMF required an FAD-dependent oxidoreductase, encoded by a *hmfH* gene from hmfFGH'H gene cluster. This HmfH oxidase is an alternative to the nonspecific dehydrogenases (Koopman et al. 2010a, b).

The same authors identified another strain *Pseudomonas putida* S12 that was capable of utilizing HMF and furfural as sole carbon sources because of the presence of nonspecific "furanic dehydrogenases". They cloned the *hmfH* gene from *C. basilensis* HMF14 into *P. putida* S12 and catalyzed the oxidation of monocarboxylic acid HMF into 2,5-furandicarboxylic acid form by expressing HmfH oxidase (Koopman et al. 2010a, b). Some of the factors like initial HMF concentration, metabolic status, substrate, and product transport across the cell membrane affected the growth of *P. putida* S12-HmfH in the medium for efficient biotransformation. 15 (g CDW) l^{-1} of *P. putida* transported a maximum of 40 mM glycerol and 25 mM HMF from the medium, and the initial specific FDCA production rate (qFDCA) was 125.5 ± 1.5 μmol (g CDW)$^{-1}$ h^{-1}(Koopman et al. 2010a, b).

5-HMF biotransformation bacteria named *Burkholderia cepacia* H-2 was isolated from 5-HMF enriched campus surface soil from the university (National Yunlin University of Science and Technology campus). The 5-HMF biotransformation ability of this isolate was investigated by inoculating a pure *Burkholderia cepacia* H-2 into 10 mL MSM with 200 mg/L 5-HMF and observed the turbidity increase and FDCA production using HPLC. They optimized a medium with

2000 mg/L substrate concentration (HMF) with a pH of 7 and temperature 28 °C. During the process, they maintained neutral pH with buffer (Yang and Huang 2016) and obtained 1276 mg/L FDCA production.

As an available cheaper biomass, same authors selected marine macroalgae *Gracilaria verrucosa* and *G. amansii* as substrates for the production of HMF after thermal acid treatment and successfully transformed this algal hydrolysate containing HMF into FDCA. *Burkholderia cepacia* H-2 was inoculated in this zero fold and two fold diluted acid hydrolysate and 989.5 and 1031 mg/L FDCA production was reported after 24 h at pH of 7 and temperature 28 °C. It is the first report for the production of FDCA from a biomass containing HMF (Yang and Huang 2016).

4.1.1 Production Methods

Normally, FDCA from microorganisms is produced by fermentation method. Fermentation is a metabolic process that occurs in microorganisms like bacteria, fungus, and yeast for the conversion of useful nutrients like sugars (carbohydrates) for the production of acids, gases, and alcohols for their metabolic growth or survival by their enzymes. Industrial fermentation involves large-scale production of micro organisms in a fermenter for the production of biomass or useful products (metabolites) in the presence or absence of oxygen. Microorganisms convert the toxic aldehyde HMF into some other acids for surviving in the medium, which is a part of furfural degradation pathway. Most of the microorganisms convert HMF into HMF acid which is not toxic to them and very few microorganisms are capable of producing FDCA after a minimum three or four oxidations by its own oxidases.

4.1.2 Upstream Operations

A batch fermentation is a closed system (fermenter) with sterilized nutrient solution which is inoculated with the required amount of desired microorganisms (inoculum size), suitable media composition, agitation, temperature, and pH in the presence or absence of oxygen. The composition of the culture medium, concentration of biomass, and metabolite may change constantly due to the metabolism of the cells during fermentation and antifoam agent, and acid or base (for pH control) should be supplied continuously to maintain these conditions. Metabolites get accumulated during the growth phase of microorganisms that may be toxic to the cells and production of desired product may be reduced. Because of less expensive setup, batch process is widely acceptable for the industries. In the fed-batch fermentation, media components (substrates) are added in increments as the normal fermentation progresses. The major media elements are added at the beginning of the fermentation in small concentrations and these components continue to be added in small doses during the microorganism's production phase.

Batch Process for the Production FDCA

HMF oxidase gene was isolated from *C. basilensis* HMF14 and it engineered into *P. putida* S12, and it was inoculated into one-liter minimal media or Luria broth containing available carbon sources along with 100 mg ampicillin (*E. coli*) and 30 mg gentamicin as antibiotic agents. Precultures were incubated in one-liter flasks containing 150 ml mineral media. This was supplemented with 40 mM glycerol, 2 mM glucose, and gentamicin. The cells were harvested again and concentrated into 15 gl^{-1} CDW (Cell Dry Weight) in 10 ml mineral media containing 40 mM glycerol, 2 gl^{-1} $(NH_4)_2$ SO_4, 25 mM HMF, and gentamicin in 100 ml shake flasks. Diacids formed during the production of FDCA when they used glucose as carbon source and more than five fold increased buffer concentration was used to adjust the acidic pH. That is why authors used glycerol as a carbon source and resulted in 276 ± 89 μmol $(g\ CDW)^{-1}\ h^{-1}$ of FDCA (Koopman et al. 2010a, b).

For the biotransformation of HMF into FDCA, *Burkholderia cepacia* H-2 was inoculated into 125 ml batch reactors containing 40 mL MSM at a cell concentration of 0.05 ± 0.01 g/L. The medium was with 2000 mg/L substrate concentration (HMF) and kept for an incubation period of 48 h. After 24 h 1276 mg/L FDCA produced at pH of 7 and temperature 28 °C (Yang and Huang 2016).

Algal acid hydrolysate of *Gracilaria verrucosa* was selected in the presence of HMF for confirming the biotransformation effect of strain *B. cepacia* H-2 in a batch reactor. This algal acid hydrolysate is added with additional HMF (2000 mg/mL) also. Algal acid hydrolysate is taken in three forms like zero fold, two fold, and four fold diluted hydrolysate forms. After inoculation, the media is incubated at a temperature of 30 °C and pH 7. After 24 h 989.5, 1031, and 923.2 mg/L of FDCA were produced in the medium, in which two fold acid hydrolysate showed the highest production (Yang and Huang 2016).

Fed-Batch Process for the Production of FDCA

For the fed-batch, preculture of genetic engineered organism *P. putida* S12 (0.2 g l^{-1} CDW) is inoculated into the one-liter fermenter with modified mineral media containing 4 M glycerol, 0.1 M MgCl2, and 1 M HMF. HMF acid was increased little after and in order to avoid that, the rate of HMF feed reduced to 0.09 mM $(g\ CDW)^{-1}\ h^{-1}$ after 72.8 h and stopped after 117.4 h. During this time, FDCA production was also reduced to 0.096 ± 0.004 mM $(g\ CDW)^{-1}\ h^{-1}$. To increase the rate of oxidation and product formation from HMF, the feed rate of glycerol was increased to 12 mM $l^{-1}\ h^{-1}$. Oxygen was supplied at 11 ml min^{-1} for the first 25 h after that it changed into 200 ml min^{-1}. The pH of the media is maintained to 7 by the addition of 7% NH_4OH until its accumulation (4 g l^{-1}), after that it is replaced with 10 N NaOH at 30 °C. At the end of the cultivation (144 h), 182 ± 1 mM of FDCA obtained at 97% conversion and concentration of 30.1 ± 0.7 g l^{-1} (Koopman et al. 2010a, b).

Authors engineered HmfH and HmfT1 into *P. putida* S12 B-38 and HmfH, and HmfT1 and aldehyde dehydrogenase into *P. putida* S12 B-51 for a co-expression of these genes for the production of FDCA from HMF. Fed-batch fermentation medium is prepared in 2x MM medium supplemented with glycerol (40 g/L), yeast extract (10 g/L), glycerol (8 M) in 100 mM magnesium chloride, sodium salicylate (1 mM), HMF (4 M) at pH 7, and temperature 30 °C. The fermenter used here is Labfors 4 Bioreactor system (Infors Benelux BV) with two-liter capacity which was sparged with oxygen. The feed rate of HMF is 4 M soln/h. From this the organism, *P. putida* S12 B-38, successfully produced approximately 150 g/L FDCA with a cell dry weight of 28 g/L after 90 h (Wierckx et al. 2012).

4.1.3 Downstream Operations

FDCA should be in purified form for its day-by-day increased applications. For any activity, purity is an important matter according to its application. For FDCA, impure form has no sense and used at all either it is from chemical or biological production. For the batch, fed-batch, continuous, and enzymatic approaches also seeking a better downstream processing technology to purify FDCA from the fermentation broth or reaction mixture.

Whole-cell biocatalyst *P. putida* S12 is removed from the broth (250 ml) by centrifugation at 9500 g for 10 min. Collected supernatant is boiled for three minutes to remove the unwanted proteins followed by acidification with 96% H_2SO_4 at 4 °C and lowered the pH to 0.5. Obtained precipitate is centrifuged and the pellet contained FDCA. This pellet is washed with distilled water and centrifuged again. Now pellet is dried in air and dissolved in 675 ml of tetrahydrofuran and kept for shaking for 30 min at room temperature. This THF is vacuum dried at 50 °C until a clear dry powder is obtained. After elemental analysis, authors confirmed off-white powder consisted of 99.4 ± 0.28% of pure FDCA (Koopman et al. 2010a, b).

After fermentation of *P. putida* S12 B-38 and *P. putida* S12 B-51, the obtained FDCA was recovered from the broth by acid precipitation (for low pH), cooling, crystallization, and separation in crystallized form (Wierckx et al. 2012).

HPLC analysis of a reaction mixture of HMF to FDCA by chloroperoxidase from *Caldariomyces fumago* after 4 h showed 2 g/kg FDCA. Since it is a 20 ml of the reaction mixture, authors calculated approximately 40 mg of FDCA in the solution. After the process with the enzyme mixture, pH is adjusted to 3 and a precipitate obtained. This precipitate is resuspended in water and again pH adjusted to 3. The obtained precipitate is kept in a refrigerator for 2 days and air dried. It is spun down and again air dried. About 15 mg of FDCA was obtained from this reaction and confirmed the purity by TLC (Hanke 2009).

Authors used an enzymatic cascade reaction with galactose oxidase M3–5, periplasmic aldehyde oxidase (PaoABC), and a catalase for sequential oxidations of HMF. After the enzyme process reaction mixture is heated to 80 °C for 5 min and allowed to cool. After 17 h, reaction is stopped. For purification, its supernatant was

cooled to 0 °C after centrifugation and concentrated HCl was added dropwise until a precipitation is formed. The solution was then again centrifuged and the obtained pellet washed with 1 M HCl. The pellet is dissolved in acetone followed by concentration in vacuo three times and FDCA obtained as pale yellow solid in 74% yield (35 mg, 0.22 mmol) (McKenna et al. 2015).

4.2 Enzymes in the Production of FDCA

Chloroperoxidase from *Caldariomyces fumago* was the first enzyme used as the catalyst for the conversion of HMF into its desired products. It is an extracellular heme peroxidase, containing a prosthetic group iron (III) protoporphyrin (IX), intermediates in the catalysis of oxidation of different substrates along with hydrogen peroxide. In an optimized condition, 1000 units (118 pl) of chloroperoxidase were added into a reaction mixture containing 157.5 mg of HMF, 25 mL of distilled water, 2.5 mL of 1 M $KHPO_4$ buffer (pH 7.2), and 3% hydrogen peroxide. After HPLC analysis, production was calculated as 2 g/kg FDCA after the reaction. Since there were about 20 ml of the reaction remaining and low solubility of FDCA in the media, author predicted a maximum concentration of 40 mg of FDCA in the solution from this HPLC result. Medium pH was adjusted to 3 for precipitation followed by centrifugation, and the precipitate was resuspended in water. Again pH was changed to 3 to precipitate pure FDCA and kept in a refrigerator for 2 days. This was dried and 15 mg of FDCA was recovered in pure form which was confirmed by TLC (Hanke 2009).

The flavoprotein oxidase hyroxymethylfurfuraldehyde oxidase (HMFO), a FAD-dependent glucose—methanol—choline oxidoreductase family enzyme, is the first enzyme described to produce FDCA from both [(5-(hydroxymethyl) furan-2-yl] methanol (HMF alcohol) and HMF. This HMF oxidase was actually expressed in *E. coli* BL21 (DE3) and purified using affinity chromatography. Since they use molecular oxygen as electron acceptor, the enzyme does not need any other expensive cofactors for the oxidation of these substrates and its intermediates. Inserted oxygen in these intermediates actually originates from the hydrated aldehyde, and HMFO subsequently oxidizes that hydrated product to the carboxylic product and produces a byproduct H_2O_2. Oxidation of HMF alcohol and HMF needs four and three oxidations, respectively, for the production of desired product FDCA. The optimized reaction was carried out with 4 mM HMF, 20 mM HMFO, and FAD at a temperature of 25 °C and pressure 0.1 Mpa for 24 h yielded 95% FDCA. It was found to be substrates [5-(hydroxymethyl) furan-2-yl] methanol (HMF alcohol) and HMF had highest k_{cat} values as 22 and 9.9 s^{-1}, respectively. The reaction was stopped by providing a temperature of 70 °C to denature the HMF oxidase and analyzed by HPLC for the production of FDCA (Dijkman et al. 2014a, b).

In this enzymatic cascade (one-pot experiment), recombinant aryl alcohol oxidase (AAO) from *Pleurotus eryngii* (AAO cDNA expressed in *E. coli*) along with

an unspecific peroxygenase (UPO) from *Agro cybeaegerita* was used for the complete oxidation of HMF into FDCA. Aryl alcohol oxidase (flavoprotein) from glucose–methanol–choline oxidoreductase superfamily has the ability to oxidize aromatic primary alcohols and related aldehydes. The unspecific oeroxidase (UPO) from haemothiolate superfamily is capable of combining peroxide-borne oxygen into a variety of substrates. It has the capability to transform aliphatic and aromatic alcohols into its corresponding aldehydes by H_2O_2-dependent hydroxylation, which finally leads to the formation of carboxylic acids. In this work, aryl alcohol oxidase efficiently converted (two successive oxidations) the HMF into FFCA (Formyl Furan Carboxylic acid) in the presence of O_2. Even though low amount of FDCA was formed, high amount of H_2O_2 (approximately, two molecules in one reaction) gets liberated as a byproduct. The addition of UPO resulted in the faster oxidation of the FFCA into FDCA using excess H_2O_2 released by AAO. The optimized reaction mixture in 50 mM sodium phosphate (pH 7) buffer contained 3 μM HMF, 10 mM H_2O_2, aryl alcohol oxidase (5 μM, 54 units), and peroxidase (0.65 μM, 20 units) at 25 °C. This one-pot reaction mixture efficiently produced 91% of FDCA (Carro et al. 2014).

Hydroxymethylfurfural oxidase gene from Methylovorus sp. MP688 heterologously expressed in *E. coli* and this enzyme was characterized. This HMFO was similar to HmfH (another HMFO) from *C. basilensis.* Since it lacks a 35 amino acid, it had only 45% similarity with the normal HMF oxidase. The enzyme converted substrate 2 mM HMF into 92% FFA and 8% FDCA at a pH 8 and temperature 55 °C. The K_{cat} (s^{-1}) of this HMF oxidase for the substrates HMF, HMF acid, DFF, HMF-OH, and Furfuryl alcohol were found to be 9.9, 9.5, 1.6, 21.5, and 11.9, respectively. This data infers that this HMF oxidase is also suitable for the HMF oxidation pathway (Dijkman et al. 2014a, b).

Enzyme galactose oxidase used in this study was isolated from a fungus *Fusarium austro americanum* variant mutB and Peroxygenase used was isolated from a mushroom called *Agro cybeaegeritae* (AaP). Oxidations of HMF were carried out at 35 °C for 125 min in open glass tubes with 1 mM HMF, 0.005 mg/mL of galactose oxidase, and phosphate buffer (50 mM) of pH 6.5. Peroxidase was added 0.04 mg ep/mL as an initial dose (single) and additional 0.04 mg ep/mL dose was added after 60 min (multidose). This reaction mixture was bubbled with oxygen and was in stirred condition during entire reaction. After 5 min, aqueous hydrogen peroxide (20 or 40 mM) was added and total reaction mixture made up to 4 ml with aqueous solvent. These samples were inactivated by heating up to 75 °C for 5 min followed by centrifugation. It was quantified by HPLC analysis. In a single-dose reaction in the presence of H_2O_2 (2 mM), HMF was converted into 2,5-diformylfuran (DFF), formyl furan carboxylic acid (FFCA), and FDCA with a percentage of 1, 76, and 23, respectively. In a multi dose reaction in the presence of H_2O_2 (4 mM), HMF was converted into FFCA (54%) and FDCA (46%) with other same conditions (Kalum et al. 2014).

In an enzymatic cascade reaction, authors used galactose oxidase M3–5 and periplasmic aldehyde oxidase (PaoABC) and a catalase for sequential oxidations of HMF as a one-pot experiment. Galactose oxidase such as GOase M3–5 was isolated

from variants of *F. graminearum* galactose oxidase and periplasmic aldehyde oxidase (PaoABC) from *E. coli*. Galactose oxidase has the capability to oxidize primary and secondary benzylic alcohols with H_2O_2 as the product and aldehyde oxidase PaoABC is usually used for detoxification of aromatic aldehydes through aldehyde oxidation. Catalase used in the study converts H_2O_2 into oxygen and protecting the other two enzymes from oxidative damage. In the preliminary, cascade reaction using galactose oxidase and aldehyde oxidase converted 10 mM HMF into FDCA with the formation of normal intermediates in the pathway. After initial substrate concentration was increased to 20 mM, 44% FDCA was formed along with 50% 5-hydroxymethyl furoic acid (HMF acid). Substrate concentration at 50 mM with these enzymes produced 56% of FDCA after 17 h at ambient temperature and neutral pH. The whole reaction was completed after 8 h. From this reaction, authors confirmed that HMF acid is a poor substrate for galactose oxidase, and aldehyde oxidase is highly responsible for the formation of HMF acid and other intermediates at higher substrate concentration. Initial substrate concentration was increased to 100 mM along with the addition of catalase and aldehyde oxidase and optimized other conditions like pH (because of pH decline) for the whole oxidation process. After purification, 74% of FDCA obtained as pale yellow solid. This is the first report in which highest substrate (HMF) concentration was successfully used for the production of FDCA and the enzymes without cofactors (McKenna et al. 2015).

5 Scale up and Economics

Even though microbial and enzyme-assisted production of FDCA has been reported, there is no report obtained until now for the biological production of FDCA from industries.

6 Future Perspectives

Lab-scale biological productions of FDCA from HMF have been widely reported until now. No industries have started yet to produce it in commercial level, because one reason may be of current HMF and FDCA market price per kilogram amount. Actually, HMF (substrate) has equal or higher price than FDCA. Making purified substrate from cheaper source will be another challenge, especially using biomass or C6 sugars. The expense of downstream processing will be another challenge for the industries. Because if the product is impure it cannot be used for the applications. Also, right now there is no well-established or accurate downstream processing is reported from any of biological production works till now. Some authors have explained about THF extraction for purification which is not at all possible

because of the low solubility of FDCA in THF. So other cheaper, efficient, and nontoxic solvents have to be used for purification.

So large-scale production of pure substrate and product is a question to industries, why they go for its production? But the world is behind this "sleeping giant" HMF and FDCA because of its application and market value. For microorganisms genetically engineered strains with high copy number, higher substrate conversion (HMF) with low toxicity and efficient oxidase with subsequent oxidation can make a significant increase in the production of the product in recent years. In the case of enzymatic conversions reported, enzymes face inhibitions from intermediate like H_2O_2. But this can be overcome by catalase. Still, purity of substrate, low substrate affinity, low K_{cat}, and stability are problems for enzymes. It is essential to make some developments to replace oil-based polymers into renewable, reliable, cheaper, nontoxic, and recyclable plastic. Still research has to be progressed to develop for the efficient production of biobased FDCA.

Acknowledgements The authors would like to thank University Grants Commission, New Delhi for the financial assistance and support of this work.

Glossary

DFF 2,5-Diformylfuran

FDCA 2,5-Furandicarboxylic acid

FFA 5-Formyl furoic acid

HMF alcohol 5-Hydroxymethyl furfural alcohol

HMF 5-Hydroxymethylfurfuraldehyde

HMFCA (HMF acid) 5-Hydroxymethyl-2-furancarboxylic acid

PBT Polybutylene terephthalate

PEF Polyethylene furanoate

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Chapter 9
Biosynthesis of 1,3-Propanediol: Genetics and Applications

Narisetty Vivek and Parameswaran Binod

Abstract Increased demand for renewable fuels and remarkable growth of biodiesel industry resulted in the accumulation of huge quantities of crude glycerol as the by-product. In the context of utilizing this by-product as the raw material, various fine and bulk chemicals were synthesized, among them 1,3-propanediol gained more importance due to its flexible properties as a monomer. Understanding the microbial physiology and genetics with integrated knowledge on molecular tools and techniques helps in developing engineered biocatalysts for the synthesis of 1,3-propanediol in higher titers and productivity. The microbial production of 1,3-propanediol was reviewed in brief regarding, the chemical process and its limitations, glycerol dissimilation, genetics of dha regulon in various microorganisms, metabolic engineering approaches to improve glycerol utilization for 1,3-PDO production, new strategies in modes of fermentation along with the future prospective was outlined.

Keywords 1,3-Propanediol · Crude glycerol · Biodiesel · Polytrimethylene terephthalate · Dha regulan · Biorefinery · Mutagenesis · Co-substrate Homologous · Heterologous

1 Introduction

Biological production of 1,3-propanediol (1,3-PDO) from glycerol using *Clostridium pasteurianum* was reported by August Freund, an Austrian chemist. Recent developments in strain improvement and process development for improved

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biological production of 1,3-propanediol (1,3-PDO), increased its availability and market potential. Initially, 1,3-PDO-based polymers did not attain commercial interest as the process is technically and economically not feasible due to low availability and the high cost of the monomeric 1,3-propanediol. Over the past few decades, production of 1,3-PDO was developed along with the global market potential. A new polyester called polytrimethylene terephthalate (PTT) was synthesized using 1,3-PDO and terephthalic acid through polycondensation reaction, in 1995 Shell Chemicals announced a product named "Corterra™ PTT" and later in 2003 DuPont commercialized a bio-based PTT called "Sorona", in which 1,3-PDO is biologically derived from corn glucose (Bhatia and Kurian 2008; Kaur et al. 2012, 2013).

Glycerol, three-carbon compound with molecular formulae $C_3H_8O_3$, is highly reduced and reactive in nature due to three hydroxyl functional groups on three carbon atoms. Glycerol can be utilized as a cost-effective carbon source as well as substrate. The glycerol dissimilation is a coupled oxido-reductive process, yielding more reducing equivalents and energy molecules like ATP for growth and secondary metabolite production. In current scenario of research and development activities in biorefineries, glycerol is used in production of various chemosynthetic value-added products like 1,3-propanediol, 2,3-butanediol, dihydroxyacetone, butanol, propionic acid, succinic acid, ethanol, citric acid, acetic acid, mannitol, fumaric acid, arabitol, and vitamin B_{12} (Vivek et al. 2017b; Anand et al. 2010; Bhatia and Kurian 2008; Celińska 2012; Da Silva et al. 2009).

Polymers are the heavy molecular weight molecules composed of repeated monomeric units formed either through polycondensation or polyesterification reactions. These polymers are either natural or synthetic. The synthetic polymers are divided into two categories petroleum-derived and bio-based polymers. The man-made synthetic polymers have shown superior physical properties and tensile strength than the natural polymers such as PVC, PU, PE, and PP have been used in various industrial purposes like packaging, electronic components, toys, and construction materials (Liu et al. 2010a, b). The bio-based polymers like PGA, PHB, and PHBV have applications in the bioremediation and synthesis of biomedical materials. The synthetic polymers derived from petrochemical sources were mostly bio-resistant that is a major environmental concern and the risk of waste management has been a global issue (Umare et al. 2007). The development of bio-derived polymers offers an attractive and promising group of polymers that are biodegradable.

In this chapter, a detailed recent progress in employing advanced genetic engineering tools and process improving strategies to improve the yield and productivity of 1,3-PDO was discussed along with properties and traditional synthesis approaches for 1,3-PDO. A special illustration on dha regulan in different native microorganisms reported for 1,3-PDO production and constraints during the production process was elaborated. Later a concept of biorefinery approach, regarding utilization of additional carbon source for microorganism growth and energy production, i.e., co-substrate fermentations to improve the 1,3-PDO titers along with a short description about the synthesis of 1,3-PDO-based polymers and polyesters was provided.

2 Properties and Applications of 1,3-PDO

1,3-propanediol, an inflammable viscous organic compound with the molecular formula $CH_2(CH_2OH)_2$ with two hydroxyl groups at C1 and C3 carbon that makes it highly reactive for polycondensation reactions to make biodegradable polyesters, polyurethanes, polyethers and shows the behavior of good solubility in common polar solvents like water, alcohols, esters, and ethers. As a monomer, 1,3-propanediol is used as building blocks, chain extender, to increase thermal resistance and mechanical properties. The monomer can improve the physical nature of resins, adhesives, laminates, mouldings, detergents, cosmetics, deodorants, and powdered coatings. Due to its properties like less toxicity and heat stability, it was used in engine coolant formulations (Anand et al. 2010; Da Silva et al. 2009; Kaur et al. 2012). A major application of 1,3-PDO as the monomer is the production of polyester, polytrimethylene terephthalate (PTT). PTT is a semi-crystalline polymer synthesized by polycondensation reaction between the monomer 1,3-propanediol and a dicarboxylic acid either terephthalic acid or dimethyl terephthalate. The characterization of PTT has resulted in excellent physical and chemical properties like dimensional stability, low moisture absorption, weather resistance, better resilience, stress/recovery properties, high surface smoothness, length uniformity, and mechanical strength. These features are better compared to commercially available polyesters like polyethylene terephthalate and polybutylene terephthalate. The PTT has its opportunities in carpet and textile industries as well as in film preparations and packaging technology (Liu et al. 2010b; Bhatia and Kurian 2008; Umare et al. 2007). Recently, a novel application of 1,3-PDO as a plasticizer in preparation of composite films was reported where the monomer provides an additional property of elasticity and mechanical strength to the film (González et al. 2017).

3 Chemical Synthesis of 1,3-Propanediol

In a conventional approach, petrochemical derivatives were used for the synthesis of value-added chemicals and fuels. Similarly, petrochemically derived substrates like ethylene oxide and acrolein were used as raw materials for the production of 1,3-PDO on a commercial scale by two independent industries: Shell Chemicals (Shell process) and DuPont (Degussa process).

In the 1990s, Shell Chemicals developed a new catalyst for synthesis of 1,3-PDO from ethylene oxide. It is a two-stage process, in the first step ethylene oxide reacts with carbon monoxide and organometallic catalyst resulting in a hydroxyl aldehyde, later in the second stage aldehyde is reduced to 1,3-PDO by hydrogenation. An effective catalyst that was screened was homogenous cobalt that mediates the ring opening of ethylene oxide to react with carbon monoxide to form the three carbon hydroxyl aldehydes (Fig. 1). In the reduction reaction, synthetic hydrogen

gas in the presence of copper chromite catalyst was used as a hydrogen source. In the meantime, Shell Chemicals developed an improved one-pot synthesis with coupled reaction where ethylene oxide was reduced in the presence of a homogenous bimetallic catalyst formed from cobalt and ruthenium in 1:1 ratio with 1,2-diphospholanoethaneligand. Reaction tends to occur at elevated temperature and pressure in the presence of synthetic gas in methyl tert-butyl ether resulting in approximately 90% yield. With these technologies, Shell started a commercial polymer named Corterra™, the manufacturing unit was established at Geismar, Louisiana with a production capacity of 83,000 tons per annum.

Acrolein starting material in the Degussa process is known as the third major feedstock for 1,3-PDO production. In this two-step process, the first step is a hydration reaction in which 3-hydroxy propionaldehyde (3-HPA) was formed by the addition of water molecule to acrolein, later the intermediate 3-HPA was hydrogenated to PDO through catalyst-mediated reaction (Fig. 1). Acrolein is produced from propylene, but recent research has established a process that converts glycerol to acrolein (Liu et al. 2010a, b).

Although an efficient and high yielding synthetic chemical process was known and commercially established, there are disadvantages like high temperatures, pressures throughout the processes mediated by cost-effective catalysts like nickel, cobalt, ruthenium, etc. As the market demand of 1,3-PDO was emerging

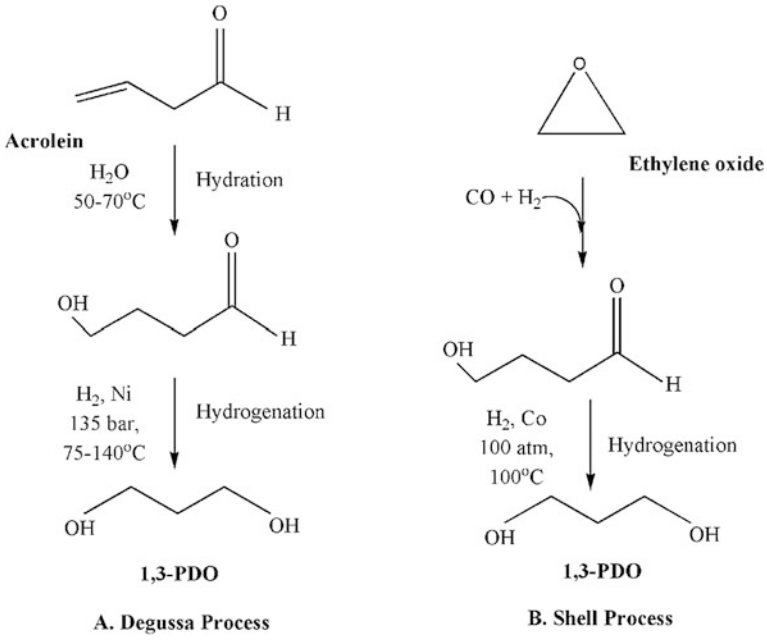

Fig. 1 Schematic representation of two chemical synthesis processes for 1,3-propanediol production A. Degussa process developed by DuPont and B. Shell process developed by Shell Chemicals

tremendously, as per the report the global demand for 1,3-PDO was 60.2 kt in 2012 and estimated to be 150 kt by 2019, there falls short of an environmental-friendly and economical process. Indeed, biotechnological processes were surveyed to be eco-sustainable using renewable sources as substrates and microorganisms as biocatalysts in optimum temperature conditions without liberating toxic intermediates or byproducts (Chemicals 2015; Markets 2015a, b).

4 Biotechnological Production of 1,3-Propanediol

4.1 Native and Nonnative 1,3-PDO Producers

It has been a decade since the first report on the microbial production of 1,3-propanediol was published. Later in 1914 a Bacillus sps., and an Enterobacteriaceae member in 1928 but a final biotechnological route was recognized in late 1990. Since then several microorganisms were isolated and screened for 1,3-PDO production from glycerol as sole carbon source. The natural producers like *Klebsiella pneumoniae* (Vivek et al. 2017a; Ashok et al. 2011; Avci et al. 2014; Cui et al. 2014; Hong et al. 2013; Jin et al. 2011a, b; Li et al. 2014; Liu et al. 2007; Oh et al. 2012; Petrov and Stoyanov 2012; Zhou et al. 2015), *Klebsiella oxytoca* (Galdeano et al.), *Clostridium butyricum* (Biebl 1991; Chatzifragkou et al. 2010, 2011, 2012, 2014; Ferreiraa et al. 2014), *Clostridium diolis* (Kaur et al. 2012), *Clostridium pasteurianum* (Dabrock et al. 1992; Jensen et al. 2012a, b),*Clostridium acetobutylicum* (Forsberg 1987), *Lactobacillus brevis* (Vivek et al. 2016), *Lactobacillus reuteri* (Baeza-Jiménez et al. 2011), *Lactobacillus diolivorans* (Pflügl et al. 2012, 2013, 2014), *Lactobacillus panis* (Kang et al. 2014a; Grahame 2013), *Citrobacterfreundii* (Anand et al. 2010; Celińska et al. 2015; Drożdżyńska et al. 2014; Ferreira et al. 2012), *Citrobacter werkmanii* (Maervoet et al. 2012), *Citrobacteramalonaticus* (Ainala et al. 2013) *Enterobacteraerogenes* (Anggraini 2014) and a newly isolated thermophilic strain AT1 (Wittlich et al. 2001) can utilize crude and pure form of glycerol as substrate to produce 1,3-PDO. In this vast pool of natural 1,3-PDO producers, *Klebsiella* sp., and *Clostridium* sp., are extensively considered and found to be the efficient producers, but these microorganisms are reported to be opportunistic pathogens, henceforth handling these microbes on an industrial scale will pose a risk (Celińska 2010; Kaur et al. 2012).

Overwhelming genetic engineering strategies and molecular techniques designed genetically modified microorganism by adopting the genes specific for 1,3-propanediol from natives. The GMOs like *E. coli* (Tong et al. 1991; Zhang et al. 2006; Wang et al. 2007; Jin and Lee 2008; Dabrowski et al. 2012) and *S. cerevisiae* (Rao et al. 2008; Ma et al. 2010, 2013) are heterologous hosts constructed by harboring specific genes from *K. pneumoniae*. Recent research perspective of isolating and engineering nonpathogenic and probiotic *Lactobacilli* strains for 1,3-PDO production was observed to be effective in producing similar titers to

Klebsiella and *Clostridium* sps. Initially, it was well known that 1,3-PDO production was observed in anaerobic conditions strains like *Klebsiella oxytoca, Enterobacter aerogenes, Lactobacillus* sp., were observed to produce in oxygen-limiting and aerobic conditions.

4.2 Metabolic Dissimilation of Glycerol

Organization of genes in a specific order, expression, and regulation of these genes in different environments under specific conditions would be supporting the organisms in a pattern of evolution. Metabolism of glycerol in these microbes is a coupled oxido-reductive process, in which glycerol acts as the sole carbon source for oxidative as well as a reductive pathway. In the oxidative pathway, NAD^+-dependent glycerol dehydrogenase enzyme encoded by *dhaD* gene converts glycerol to dihydroxyacetone (DHA), which is phosphorylated to dihydroxyacetone phosphate (DHAP) by transfer of phosphate group from ATP in the presence of kinase enzyme encoded by *dhaK* gene. This DHAP is further metabolized to phosphoenolpyruvate and pyruvate, synthesizing reducing equivalents and energy, for the growth and development of microorganism. To maintain equilibrium concentrations of $NAD^+/NADH^+H^+$ inside the microcompartments and cytoplasm, the reductive pathway was observed which depends on reducing equivalents generated by oxidative pathway (Celińska 2010; Kaur et al. 2012; Nakamura and Whited 2003).

In reductive pathway, glycerol is metabolized in two consecutive enzyme-catalyzed reactions to produce 1,3-propanediol as the end product. In the first step, glycerol dehydratase encoded by *dhaB* gene converts glycerol to 3-hydroxypropanaldehyde (3-HPA), the reactive intermediate reduces to 1,3-propanediol catalyzed by 1,3-PDO oxidoreductase (1,3-PDOR). Glycerol dehydratase, the rate-limiting enzyme in 1,3-PDO production, is cofactor (vitamin B_{12}) dependent in *Klebsiella* sp., *Citrobacter* sp., and cofactor-independent glycerol dehydratase was found in *Clostridium* sp. The enzyme glycerol dehydratase has three subunits dhaB, dhaC, and dhaE in genus *Citrobacter*, *dhaB1, dhaB2,* and *dhaB3* in *Klebsiella* sp., and as only one subunit *dhaB1* in *Clostridium* strains. Though enzyme glycerol dehydratase metabolizes the glycerol toward reductive pathway, glycerol in higher concentrations than the tolerance range is a suicidal reaction for the enzyme which gets inactivated. The reactivation of glycerol dehydratase is catalyzed by the enzyme glycerol dehydratase reactivase encoded by a globular complex polypeptide comprising $\alpha_2\beta_2$ subunits encoded by *dhaF* and *dhaG* genes. This mechanism of reactivation occurs in two steps ADP-dependent cobalamin release and ATP-dependent dissociation of apoenzyme factor complex, where ATP hydrolysis renders the conformational change in inactive form of the enzyme to ADP-bound active form of the enzyme (Németh and Sevella 2008; Kaur et al. 2012; Celińska 2012).

The intermediate produced in the first step is toxic to growth and metabolic activity of microorganism upon accumulation, which can be overcome by the action of 1,3-PDO oxidoreductase enzyme which metabolizes 3-hydroxypropionaldehyde to 1,3-propanediol (Barbirato et al. 1996). 1,3-PDOR gene is encoded by *dhaT* gene. NADH is the cofactor for 1,3-PDOR which is produced in the oxidative pathway (Fig. 2). In general reduction of NAD^+ to $NADH^+H^+$ occurs in electron transport mechanism in oxygen sufficient conditions, but in anaerobic and micro-anaerobic conditions the metabolism should favor reductive pathway for regeneration of these reducing equivalents or should produce more reduced compounds like ethanol, lactate, etc., via oxidative pathway. Hence in the metabolic pathway of glycerol, anaerobic conditions will be suitable for organisms to depend on the reductive pathway for regenerating reducing equivalents, in turn producing 1,3-propanediol. An isoenzyme of 1,3-propanediol oxidoreductase and NADP-dependent dehydrogenase transcribed and translated using YqhD gene was found in *E. coli* strains showing increased activity than 1,3-PDO oxidoreductase in native producers (Cao et al. 2006; Chen et al. 2011).

4.3 Genetics of dha Operon

The genes responsible for dissimilation of glycerol and assimilation of 1,3-propanediol anaerobically in the bacteria are clustered into the dha operon. Organization, sequence of genes, and homology of the enzymes have been investigated in *Clostridium butyricum*, *Clostridium pasteurianum*, *Clostridium acetobutylicum*, *Klebsiella pneumoniae*, *Citrobacter freundii*, *Citrobacter werkmanii,* and *Citrobacter amalonaticus*. In *Clostridium butyricum*, nucleotide sequence analysis revealed six open-reading frames *dhaS, dhaA, dhaB$_1$, dhaB$_2$, dhaT*, ORF 6, and a truncated ORF 7, which are transcribed in the same direction. The genes *dhaB$_1$* and *dhaB$_2$* are found upstream to *dhaT*. The transcriptional terminator is found downstream of *dhaT*, which is the master regulator of the operon. The ORF *dhaB$_1$, dhaB$_2$* and *dhaT* comprise of 2364, 915 and 1158 base pairs, respectively, which translate into enzymatic subunits with a molecular mass of 88,074, 34,149, and 41,558 Da respectively. Each ORF can be distinguished with gap bases after a stop codon and start codon of adjacent ORF and has a ribosome-binding site upstream to start codon. The two genes *dhaS* and *dhaA* act as signal transduction system in *Clostridium* sp., where *dhaS* acts as sensor molecule and *dhaA* as a regulatory unit. The structural arrangements of these genes in an open reading frame are represented in Fig. 3. Enzyme glycerol dehydratase encoded by *dhaB1* in *Clostridium* sp. was found to be vitamin B12 independent and homology studies added supportive information showing homology with the native producers of 1,3-propanediol with vitamin B_{12}-dependent glycerol dehydratases, gene *dhaT* encodes 1,3-propanediol dehydrogenase having 76–85% identical to 1,3-PDO dehydrogenase produced by *Clostridium pasteurianum*, *Citrobacter freundii,* and *Klebsiella pneumoniae* (Raynaud et al. 2003, 2011; Nakamura and Whited 2003).

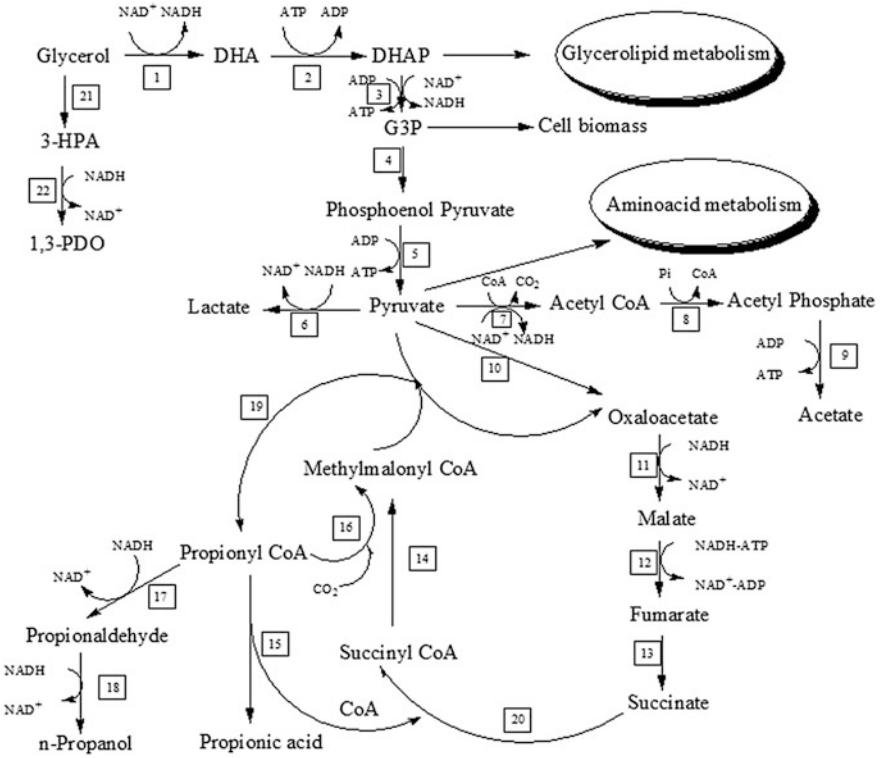

Fig. 2 Glycerol dissimilation in oxidative and reductive pathways. The numbers represent the enzyme-mediated reactions *1* Glycerol dehydrogenase, *2* Dihydroxyacetone (DHA) kinase, *3* Isomerase, *4* G3P dehydrogenase, *5* Pyruvate kinase, *6* Pyruvate kinase, *7* Pyruvate dehydrogenase, *8* Phosphotransacetylase, *9* Acetate kinase, *10* Pyruvate carboxylase, *11* Malate dehydrogenase, *12* Fumarase, *13* Succinic dehydrogenase, *14* Methylmalonyl CoA Isomerase, *15* Acyl CoA synthase, *16* Methylmalonyl CoA carboxylase, *17* Propionaldehyde dehydrogenase, *18* Propanol dehydrogenase, *19* Propionyl CoA carboxylase, *20* Succinyl CoA synthetase, *21* Glycerol dehydratase, and *22* 1,3-PDO dehydrogenase

The dha operon mediating 1,3-propanediol production in *Klebsiella pneumoniae* was sequenced and characterized. The availability of genomic data will be helpful for construction of engineered strains. The *K. pneumoniae* gene carrying dhaB and dhaT genes is sequenced along with the *dhaB* subunits *dhaBα, dhaBβ, dhaBγ* and observed the tandem arrangement, along with the transcribing behavior in different directions, under the control of different promoters (Skraly et al. 1998; Tao et al. 2012; Celińska 2012).

Conserved sequences with the identity of 96% were revealed in *Citrobacter werkmanii* strains compared to *Citrobacter freundii* and 87.5% identical to *Klebsiella pneumoniae* dha cluster. Nucleotide sequencing and chromosome walking revealed a 12,911 nucleotide long dha cluster similar to other native producers. Oxidative pathway of glycerol initiated by glycerol dehydrogenase

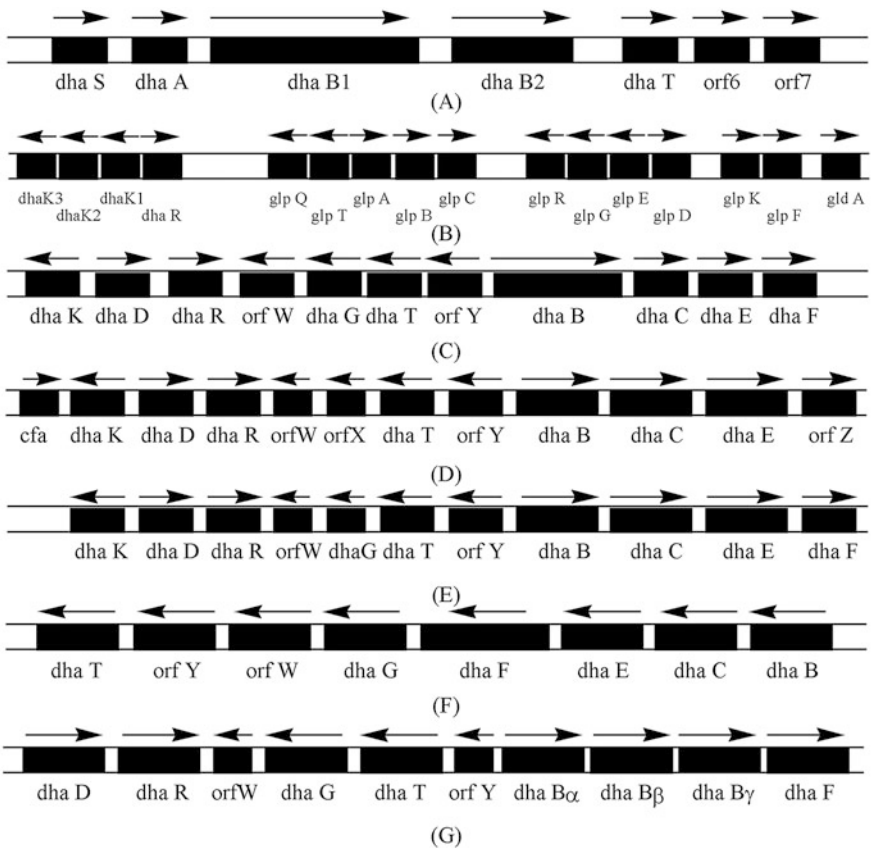

Fig. 3 Schematic representation of dha regulon genes involved in the oxidative and reductive pathway of glycerol in various organisms. **a** *Clostridium butyricum* VPI1718 **b** *Citrobacter amalonaticus* Y19 **c** *Citrobacter freundii* **d** *Citrobacter freundii* **e** *Citrobacter werkmanii* DSM 17579 **f** *Clostridium pasteurianum* DSM 525 **g** *Klebsiella pneumoniae* ATCC 25655

coded by *dha D* gene is a hexamer in *Citrobacter freundii* comprising of 365 amino acids in each subunit and in *Citrobacter werkmanii* gene transcribes into 365 amino acid long peptide chain. Genetic makeup and sequence of amino acids in the peptide reveals a 99% homology between *Citrobacter freundii* and *Citrobacter werkmanii.* The second important enzyme is dihydroxyacetone kinase for oxidizing DHA obtained from glycerol to DHAP. This kinase enzyme is encoded by *dhaK*. It is the first gene in the dha cluster in *C. freundii*, *K. pneumoniae,* and *C. werkmanii*, whereas *C. butyricum* has two dha K genes in the gene cluster. The transcription of both genes *dhaD* and *dhaK*—is in opposite direction in *C. freundii*, *K. pneumoniae,* and *C. werkmanii*, but it is in the same direction in *C. butyricum*, which shows the homology between the *enterobacteriaceae* members and organization difference between different genera. In all of these native 1,3-PDO producers, the genes

responsible for the reductive pathway *dhaBCE* and *dhaT* are found downstream to dhaD and dhaK. The expression of the dha cluster is regulated by dha R in *Citrobacter* sp., and *Klebsiella* sp., similar todhaS and dhaA in *Clostridium* sp. Two unannotated orf sequences, Orf W and OrfY are found in *C.werkmanii*. Apparently, their functionality is activating glycerol dehydratase by converting the inactive cobalamine cofactor to the active form of adenosylcobalamin (Maervoet et al. 2014).

A new candidate *Citrobacter amalonaticus* was reported for 1,3-propanediol production lacking a dha cluster. However, sequencing analysis revealed glycerol and diol-utilizing genes on a pdu-like an operon as in *Lactobacillus* species. Oxidative assimilation of glycerol is carried out by a series of genes—*glpK, glp D, glp ABC* found scattered all over the chromosome. *Citrobacter amalonaticus* shares homology with *E. coli* in gene sequence and arrangement. Both lack the genes *dhaB* and *dhaT* required for reductive utilization of glycerol and production of 1,3-PDO. *Citrobacter amalonaticus* was reported as efficient glycerol assimilator and has a vitamin B_{12} synthesis operon, the major cofactor for glycerol dehydratase. The unknown *YdhD* or any other oxidoreductase isoenzyme is found to convert 3-HPA to 1,3-propanediol. The fermentative pathway in *C. amalonaticus* was found active when exogenous electron acceptors like formate were supplemented into the fermentation media (Ainala et al. 2013).

In *Lactobacillus* species like *L. reuteri*, *L. buchneri,* and *L. diolivorans*, the propanediol-utilizing Pdu gene cluster plays a role in glycerol dissimilation and 3-HPA synthesis when glycerol and glucose are co-fermented. *Lactobacillus* species lack the enzymes for an oxidative metabolism of glycerol (Stevens et al. 2011; Vaidyanathan et al. 2011) so glycerol can only be used as a co-substrate for fermentation. 1,3-propanediol oxidoreductase, an NAD^+-dependent enzyme having similar properties like the dhaT gene of *K. pneumoniae* and *L. reuteri*, was found in *L. brevis* and *L. buchneri* (Veiga-Da-Cunha and Foster 1992). In *L. panis* PM1 *Pdu CDE* codes for glycerol dehydratase of 9 gene operon *Pdu ABCDEGHKJ* genes. A transcriptional repressor *PocR* with 359 amino acid length was found upstream to *pdu* operon coding in opposite direction. The repressor upon overexpression showed a deleterious effect on diol dehydratases enzyme activity and 1,3-propanediol production, experimentally 22% decrease in activity and 40% decrease in production. The 1,3-propanediol oxidoreductase gene *Pdu Q* and a putative alcohol dehydrogenase were found in locus, not of *Pdu C* gene locus (Kang et al. 2014b).

The genes responsible for glycerol dissimilation, generation, and regeneration of reducing equivalents and energy sources by yielding value-added products and by-products though differ in each organism will find similarities in one or other extent and few are totally identical and homologous. The gene organization of dha cluster and Pdu cluster in *Enterobacteriaceae*, *Clostridiaceae*, and *Lactobacillus* members are represented in the figure.

4.4 Different Modes of Fermentation for 1,3-PDO Production

The biotechnological process was observed as environmental-friendly process compared to chemical synthesis, various limitations like low product yield, inhibition of process mediated by substrate, products, or intermediates are bothering the process. In case of 1,3-PDO production, the substrate glycerol is inhibitory at specific concentrations to different strains, the similarly concentration of 1,3-PDO and other by-products like organic acids and alcohols. To overcome these limitations several approaches of fermentation were carried out to improve the yields of 1,3-PDO and to design an economical process.

These are the following strategies:

4.4.1 Fed-Batch Fermentation

The strain *Clostridium butyricum* DSM 10702 was observed to be tolerant to glycerol concentrations up to 70 g/l and in fed-batch fermentation under the controlled conditions the strain could produce 36.1 g/l using crude glycerol as the substrate. Later, the author pretreated the crude glycerol with various ion-exchange resins and has observed increased yields and titers op 1,3-PDO to 41.4 g/l (Loureiro-Pinto et al. 2016a). A high 1,3-PDO producing *Clostridium butyricum* strain AKR102a was reported with titers of 93.7 and 76.2 g/l with pure and crude glycerol respectively (Wilkens et al. 2012). A scale up of the process with the same strain at 200 L capacity resulted in 61.5 g/l 1,3-propanediol (Wilkens et al. 2012). Another strain Clostridium butyricum VPI 1718 was observed to produce 1,3-PDO at a concentration of 70.8 g/l with 0.55 g/g yield (Chatzifragkou et al. 2011). A high yielding species of *Clostridium, C. diolis* DSM 15410 was reported to produce 67.8 g/l 1,3-PDO with pure glycerol as the sole carbon source (Kaur et al. 2012) also a similar concentration of 68.1 g/l was observed with Citrobacter freundii FMCC-B 294. The author has observed the strain has tolerance to different concentrations of crude and pure glycerol (Metsoviti et al. 2013). *Klebsiella pneumoniae* DSM 4799 strain growing in raw glycerol with feed inlet concentration of 35–40 g/l observed to produce 80.2 g/l 1,3-PDO (Jun et al. 2010). A fast conversion of pure glycerol using K. pneumoniae XJ-Li strain with optimized physical and media parameters was observed to produce 38.1 g/l 1,3-PDO with 0.7 molar yield (Zhang et al. 2007).

4.4.2 Biorefinery Approach: Concept of Co-substrate Fermentation

The dismutation process of glycerol is to regenerate reducing equivalents for metabolic equilibrium between oxidative and reductive pathway. The theoretical maximum of 1,3-propanediol yield from glycerol is approximately 0.72 $g_{1,3\text{-PDO}}/g_{glycerol}$

(Biebl and Marten 1995). If cost-effective raw materials are supplemented with glycerol as carbon sources in a co-fermentation strategy, there could be a chance in increasing the yield of 1,3-PDO. In this aspect of co-substrate fermentation external carbon sources are used for biomass growth and generation of reducing equivalents whereas glycerol was recognized only as a substrate for 1,3-PDO production. The oligomeric sugars obtained after enzymatic hydrolysis of lignocellulosic biomass was used as co-substrates in glycerol fermentation. With mixed substrates like corn stover hydrolysate and glycerol, an increase in 31% yield was observed with *C. diolis* DSM 15410 strain in fed-batch fermentation (Xin et al. 2016). Similarly higher titers about 71.58 g/l 1,3-PDO were obtained *K. pneumoniae* ME-303 strain using corn straw enzymatic hydrolysate as co-substrate. Co-fermentation using hydrolysates was also reported by Pflugl et al., where *Lactobacillus diolivorans* DSM14421 strain was observed to produce 73.7 g/l in fed-batch fermentation with glucose to glycerol molar ratio of 1:1 (Pflügl et al. 2014). During hydrolysis of lignocellulosic biomass a liquid stream obtained after acid pretreatment has major pentose sugars like xylose and arabinose, a *K. pneumoniae* ME-303 strain was observed to utilize the xylose as a carbon source and increased 1,3-PDO yield from 0.52 to 0.63 $g_{1,3\text{-PDO}}/g_{glycerol}$ (Jin et al. 2011b).

4.4.3 Immobilized Cells

In 1,3-PDO fermentation, the concentration of biomass plays a very crucial role in the metabolism of glycerol. In the literature perspective, the rate of 1,3-PDO formation depends on the rate of biomass. The biomass concentration in the fermentation media can be improved by various methods, in which immobilization is one of the strategies. In immobilization, the cells are either entrapped or adhered in an inert matrix, which allow the transfer of substrate and media components across the matrix allowing cells to grow in number and increase the biomass concentration. As the biomass concentration is increasing, the rate of substrate utilization and product yield increases from batch to batch. In a study, *Clostridium beijerinckii* B-593 cells were immobilized on to glass beads and glass rushing rings and carried out continuous fermentation resulting in a maximum volumetric rate of 7 g/l/h (Gonen et al. 2013). In the immobilized bioreactors productivity and operational stability can be maintained with lowering the operational costs. In a recent report, *Clostridium butyricum* DSMZ 10702 entrapped in calcium alginate beads were used in fluidized bed bioreactor. The maximum production observed was 20.5 g/l with 2.8 g/l/h productivity with a feed concentration of 50 g/l glycerol at 0.05 h^{-1} dilution rate (Loureiro-Pinto et al. 2016b).

4.4.4 Mixed Culture Fermentation

1,3-PDO producers prevail in methanogenic, facultative aerobic, and obligate anaerobic environments like sewage sludge and sediments (Gallarado et al. 2014),

compost, decaying straw, anaerobic distillery wastewater, and soil samples (Selembo et al. 2009). In the production of bulk chemicals, the mixed cultures have add on potential in economic and production stability. In 2013, Dietz and Zeng reported a mixed culture from municipal wastewater treatment facility with a molar yield of 0.56–0.76 and production titers of 70 g/l with 2.6 g/l/h productivity in fed-batch fermentation. In the fermentation broth, acetic and butyric acids were observed as by-products that describe presence of methanogens and other acid-producing bacteria (Dietz and Zeng 2013). A syntrophic growth of 1,3-PDO producer and acid-utilizing microorganism was investigated with *Clostridium butyricum* and methanogenic archaea *Methanosarcina mazei*, in this process removal 70% of acid by-products like acetic and formic acids were observed when methanol was used as co-substrate (Bizukojc et al. 2010).

4.5 Classical Random Mutagenesis to Improve 1,3-PDO Production

In the biotechnological 1,3-PDO production, feedback inhibition was observed where the accumulation of 1,3-PDO is toxic to glycerol dehydrogenase enzyme resulting in reduced glycerol consumption. Hence with increased 1,3-PDO concentrations, the selective osmotic pressure was induced in *Clostridium butyricum* DSM 5431 strain with proton suicide method resulting in two mutant strains. These mutants were evaluated to have 44% increase in glycerol consumption and 50% increase in 1,3-PDO production compared to wild-type strain. The scenario was replicated in decreased acid concentrations that increase the metabolic flux of NADH toward reductive pathway for regeneration of NAD^+ and increased 1,3-PDO dehydrogenase activity (Abbad-Andaloussi et al. 1995). Another strain *Clostridium pasteurianum* was chemically mutated using ethyl methane sulphonate (EMS) to tolerate the higher concentrations of glycerol. The resulting mutants have six times increase in cell dry weight compared to wild type and 1,3-propanediol production up to 33% and also there was 46% increase in butanol production (Jensen et al. 2012b; Kubiak et al. 2012). In the fermentation, each strain has specific ORP values which favor the growth rate and end product synthesis, for *Klebsiella pneumoniae* M5aL wild-type strain value, lies between −160 and −190 mV. Hence, the mutants are selected in a way that it tolerates a wide range of reduced environments, YMU1 mutant of the parent strain can tolerate −280 mV and has enhanced 1,3-propanediol production of 63.1% compared to parent strain (Du et al. 2007). In 2009 Otte et al., worked on genome shuffling of chemically (NTG) mutated strains of *Clostridium diolis* DSM 15410. The strain was improved in such a way to tolerate glycerol concentration up to 138.15 g/L (1.5 M) and 91 g/L (1.2 M) 1,3-propanediol concentration compared to wild-type strain tolerance level of 80% was increased, in fed-batch fermentation the strain produced 80 g/L 1,3-propanediol with 80% increase in titers compared to parent strain (Otte et al. 2009).

4.6 Homologous and Heterologous Expression for Improved 1,3-PDO Production

In metabolic dissimilation of glycerol, the oxidative pathway results in end products like organic acids that alter the pH of the medium to acidic affecting the growth and metabolic activity of the microorganism resulting in decreased yields of desired products like 1,3-propanediol (Zeng et al. 1993). To overcome these limitations, *Klebsiella oxytoca* M5aL *ldhA* mutant was created by knocking out lactate dehydrogenase gene as in reduced environments the lactic acid produced utilize NADH in excess. These mutants produced 83.56 g/L 1,3-propanediol and 60.11 g/L 2,3-butanediol when sucrose was used as co-substrate along with glycerol (Yang et al. 2007). Later Zhang et al. knocked out acetoin reductase gene specific for 2,3-butanediol production in *Klebsiella oxytoca* resulting in increased production of 42 and 62% conversion efficiency of glycerol to 1,3-propanediol and 46% increase in cell dry weight compared to the wild-type strain (Zhang et al. 2012). Another research group observed inactivation of acetoin reductase gene and expression of formate dehydrogenase gene in *Klebsiella pneumoniae* strains. The strains showed increased 1,3-propanediol production about 15.9% in batch and 21.7% in the fed-batch process with a final concentration of 72.2 g/L (Wu et al. 2013). Heterologous expression of pyruvate decarboxylase (*pdc*) and aldehyde dehydrogenase (*aldB*) from *Zymomonas mobilis* into acetolactate (*als*)-deficient *Klebsiella pneumoniae*, to enhance the generation of reducing equivalents and conversion of 3-HPA to 1,3-PDO, resulted in increased production of 68.2 g/L, 0.63 g/g yield and 1.42 g/L/h productivity (Lee et al. 2014).

The heterologous expression of 1,3-propanediol oxidoreductase (YqhD gene) from *E. coli* was expressed in *Lactobacillus reuteri* ATCC 55730. The analysis of post-fermentation media resulted in 34 and 13% increase in productivity and molar yield of 1,3-PDO (Vaidyanathan et al. 2011).

Overexpression of homologous or heterologous genes coding for rate-limiting enzymes alter the production levels, if a specific gene expression rate is higher at the similar conditions in another host compared to the native strain. In *Shimwellia blatte,* the expression levels of 1,3-PDO oxidoreductase (*dha T*) gene was higher compared to dha T gene of host *C. freundii* AD970. Hence, the author heterologously expressed dha T gene from *S. blatte*, in *C. freundii* AD970 resulted in the twofold higher concentration of 1,3-PDO production than the wild-type strain. A final concentration of 49.9 g/L 1,3-PDO with a yield of 0.49 $g_{PDO}/g_{glycerol}$ and 0.32 g/L/h productivity was observed (Celińska et al. 2015).

The heterologous expression of dha operon from *Klebsiella pneumoniae* ATCC 25955 into *E. coli* AG1/pTC1 strain was carried out and resulted in the yield of 0.46 $g_{PDO}/g_{glycerol}$ (Tong et al. 1991). Later, a new construct was engineered using *dha B* genes from *Citrobacter freundii* and *yqhD* from wild-type *E. coli* strain into *E.coli* JM109 and resulting in recombinant *E. coli* JM 109 (*pHsh-dha B-yqhD*) strain (Zhang et al. 2006). The genes *dha B, dha T,* and *yqhD* along with Shine-dalgarno sequence are PCR amplified and ligated with PLPR promoter. The recombinant strain

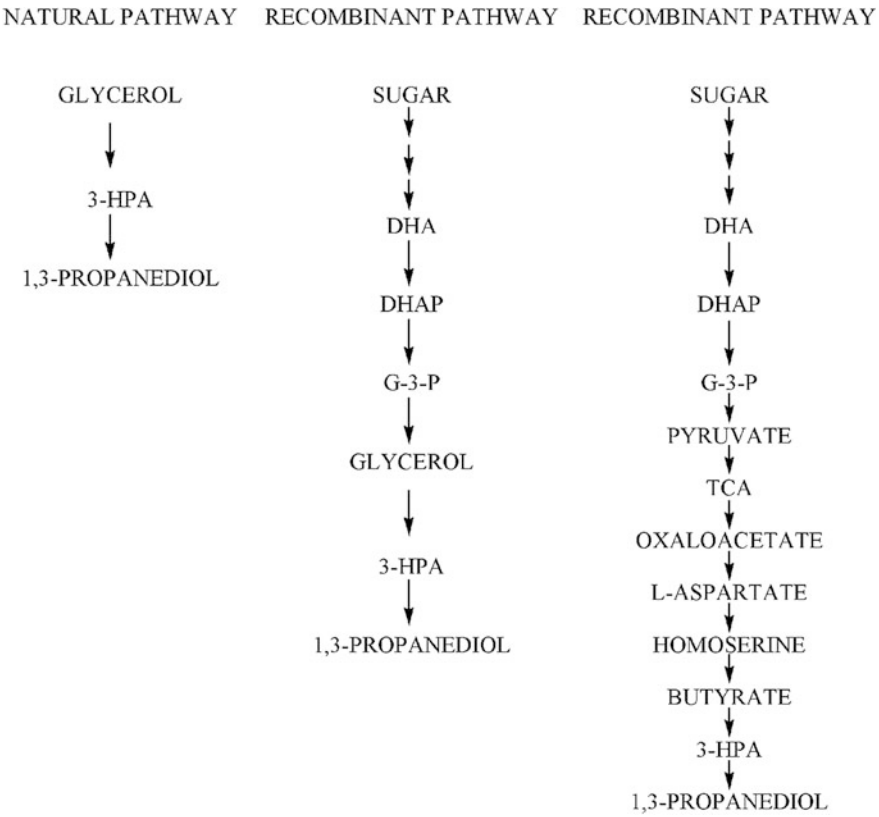

Fig. 4 Metabolic illustration of native glycerol and recombinant glucose to 1,3-propanediol producing pathways. Abbreviations: *3-HPA*—3-hydroxypropionaldehyde; *DHA*—Dihydroxyacetone; *DHAP*—Dihydroxyacetone phosphate; *TCA*—Tricarboxylic acid cycle; *G-3-P*—Glycerol 3-phosphate

expressing the dha operon is validated using high cell density fermentation under initial aerobic and continued anaerobic conditions resulting in 104.4 g/L 1,3-PDO, 2.61 g/L/h productivity and with a conversion rate of 90.2% g PDO/g glucose (Tang et al. 2009). The comparative difference between the natural 1,3-PDO production pathway and the genetically engineered pathways was illustrated in Fig. 4.

5 Downstream Processing

The value of a product synthesized either through conventional chemical method or biological method the market potential and commercial value depends on the purity and cost effective downstream process. Attention toward downstream process of

1,3-PDO is required as the purity of the chemical matters in the synthesis of polymers. Separation of 1,3-PDO from the fermented broth depends on various factors, like by-products, residual substrates, macromolecules, and physical characters like high boiling point (214–219 °C) and hydrophyilicity render purification difficult and the various process has been investigated. The first method was devised by Malinowski using liquid–liquid extraction (Malinowski 1999), where organic solvents are used to create a soluble and insoluble phase where the product being hydrophilic, transits into the hydrophilic solvent and later it was extracted by evaporating the solvent phase. The later reactive extraction method was devised using acetaldehyde to react with 1,3-propanediol to form 2-methyl,1,3-dioxane (2-MD). The dioxane formed reacts with organic solvents to purify 1,3-PDO, xylene, ethyl benzene, and toluene showed a recovery of 69, 71, and 74% (Malinowski 2000; Hao et al. 2005). In solvents, ethanol and 2-propanol could form single phases where extraction will be difficult, but acetone formed two phases. In ATPS system two combinations of inorganic salts and solvents like $(NH_4)_2SO_4$/acetone and K_2HPO_4/acetone were found to be efficient in extraction of 1,3-propanediol from the fermented broth. The highest purity of 98% was obtained with ethyl acetate as the solvent for extraction and later silica resin, purification resulted in a final yield of 70% (Cho et al. 2006). Based on sorption and desorption of 1,3-propanediol various resins are synthesized and modified to increase the adsorption efficiency of the resin specifically toward 1,3-PDO, 001X7H-pretreated cation-exchange resin with efficiency of 360 mg/g PDO (Wang et al. 2014), H^+ ion-exchange resin (Rukowicz et al. 2014), the XAD-7 and XAD-16 (Luerruk et al. 2009) where adsorption behavior of XAD-7 is higher than XAD-16, but desorption is greater in XAD-16 (Luerruk et al. 2009). Another little efficient but economical and continuous downstream processing technique like pervaporation using Na-ZSM-5 zeolite (Li et al. 2001c) and X-type zeolite membranes (Li et al. 2001a, b) was explained. In the fermented broth, as an initial step biomass and soluble proteins should be removed to make the downstream process easier. Two different approaches are put forward for this and step one is flocculation (Hao et al. 2006) using polyacrylamide and chitosan, another is microfiltration using hollow fiber cartridge (Anand et al. 2011; Avci et al. 2014). In flocculation, the absorbance of spent medium decreased to 0.02 and reduced the protein concentration to half. But in the second strategy, after microfiltration by using activated charcoal 96% of soluble proteins are removed along with decolorizing the broth. Notably, 15% loss in the concentration of 1,3-PDO was observed when treated with activated charcoal. After flocculation, the resultant supernatant was flown through four countercurrent reactors with butyraldehyde and supernatant in opposite directions. The reaction resulted in the formation of acetals of 1,3-PDO, 2,3-butanediol and glycerol. The mixture contains 98% of 1,3-PD and 2,3-BD acetals and only 10% of glycerol acetals. The recovery of glycerol acetals increased with increase in butyraldehyde volume. Later obtained reaction mixture was hydrolyzed to get a concentration of 407 g/L 1,3-PDO, 252 g/L 2,3-BD, 277 g/L glycerol, and 146 g/L glycerol acetal while the feed volume consists of 15 g/L 1,3-PDO. Anand et al., used vacuum distillation to concentrate the sample and remove the minimum concentrations of

ethanol and then purified using silica gel chromatography with chloroform and methanol as mobile phase resulting in 75.47 overall yields in the purified manner (Hao et al. 2006; Anand et al. 2011).

6 Conclusion and Future Outlook

It is reliable to depend on the sustainable and renewable raw materials than the diminishing natural fossil fuel sources, conserving the nature, addressing the global climatic and environmental concerns. The outbreak of various biotechnological techniques and tools, availability of knowledge regarding physiology and genetics of microorganisms, resulted in the development of biological processes for the production of various fine and bulk chemicals like 1,3-propanediol. The technical barriers for the greener approach will be the competitive market by the chemical methods, cost of the raw materials, yield, and productivity of the end product, production of undesirable by-products and cost-effective downstream process. With the current tailor-made approaches the genetically engineered microorganisms can be developed for utilization of multiple substrates producing desired end product without any additional byproducts making the downstream process simple and whole process could be costeffective. The involvement of scientific knowledge, biofuels and biorefinery industries in future can be the replacement of whole chemical market with bio-derived bulk and fine chemicals.

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Chapter 10
Biosynthesis and Technological Advancements of Biosurfactants

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Abstract The growing industrial development and concomitant efforts to protect mother Earth from alleviating pollution levels has greatly demanded the use and disposal of milder chemicals and xenobiotics. Of the widely used xenobiotics, surfactants have an increasing market demand due to its prevalent role in pharmaceutical preparations, food industry as well as almost all foaming products. The use of biosurfactants in place of chemical surfactants has gained momentum owing to the low toxicity, higher biodegradability and better environmental compatibility of biosurfactants. The current review outlays the various biosurfactants produced and their significance in various industries. An outline of the various biosynthetic pathways, challenges and advancements in the synthesis of the two mostly used biologically synthesised biosurfactants—rhamnolipids and sophorolipids has been discussed.

Keywords Biosurfactant · Microbes · Rhamnolipids · Sophorolipids Xenobiotics

1 Introduction

Surfactants find a myriad of applications in human life extending its role in household products to a vast array of industrial processes. The realm of surfactant activity is widespread to different industries including food (Kralova and Sjoblom 2009), environmental remediation (Cheng et al. 2017), textiles (Jing-xin 2004), fuel extraction (Torres et al. 2003; Chistyakov 2001), biotechnology (Singh et al. 2007), antimicrobials (Ginkel 1989) and many more. Chemical modification of renewable or non-renewable substrates is currently done to meet this great demand of surfactants

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(Behler et al. 2001). However, it leads to various environmental problems and health concerns owing to the indiscriminate use of chemical surfactants (Rebello et al. 2014).

Life cycle assessment of surfactant synthesis and disposal undoubtedly proves the levels of its toxicity at different ecosystems. The prevalence of some fluoridated surfactants such as PFOA commonly used in nonsticky utensils in human blood (Calafat et al. 2007), marine animals and birds (Groffen et al. 2017) are some instances to substantiate it. The LCA analysis of PFOA alone indicates that maximum exposure comes from emission on the application (117.0 t), followed by manufacture (3.9 t), consumer exposure (1.2 t), wastewater treatment plants (10.6 t) and environmental emission of 12.8 t (Meng et al. 2017). Surfactant synthesis, in turn, causes increased emission of greenhouse gases, which contribute to the increased global warming, ozone damage, and climatic variations. Surfactant toxicity is evident in almost all sectors of the ecosystem including both biotic (Meng et al. 2017) and abiotic elements of the atmosphere and water bodies (Pittinger et al. 1993).

1.1 Advantages of Biosurfactants

The choice of eco-friendly biosurfactants against chemical counterparts has attained relevance owing to its biodegradability, low toxicity, ecological acceptability, superior foaming ability, enhanced selectivity and increased specific activity (Cameotra et al. 2010). Since its first use in the 1980s, the field of biosurfactant research has gained great impetus and ecological significance due to increasing rates of pollutions associated with chemical surfactants. Figure 1 schematically represents the damage caused by surfactant chemical synthesis. Studies indicate that biosurfactants even in very small concentrations give stable emulsions at different environmental conditions compared to chemical surfactants requiring large concentrations (McClements and Gumus 2016).

Additionally, biosurfactants are found to be antibacterial (Mani et al. 2016; Ndlovu et al. 2017), antifungal (Chen et al. 2017), antiviral (Pang et al. 2017; Vollenbroich et al. 1997) and immunologic in nature, which adds an extra advantage to be used in medicine and therapeutics (Rodrigues et al. 2006). The antifungal role of surfactants in preventing various mycotic infections of plants is also evident by inhibitory action of surfactin and fengycin against *Mycosphaerella fijiensis* (Gonzalez-Jaramillo et al. 2017). Though economically biosurfactants are not a wise choice for synthesis than chemical surfactants, the growing interest for greener and biodegradable commodities has expected the biosurfactant market to rise in 2018–2020 approximately to $25 billion (http://www.transparencymarketresearch.com/specialty-and-biosurfactants-market.html; http://www.grandviewresearch.com/press-release/global-biosurfactants-market).

The current review targets to outlay the economised production of various biosurfactants focusing on the predominantly biosynthesised biosurfactants—rhamnolipids and sophorolipids. The review also outlays the biosurfactant utility in various sectors and biosynthetic pathways involved in their generation. The

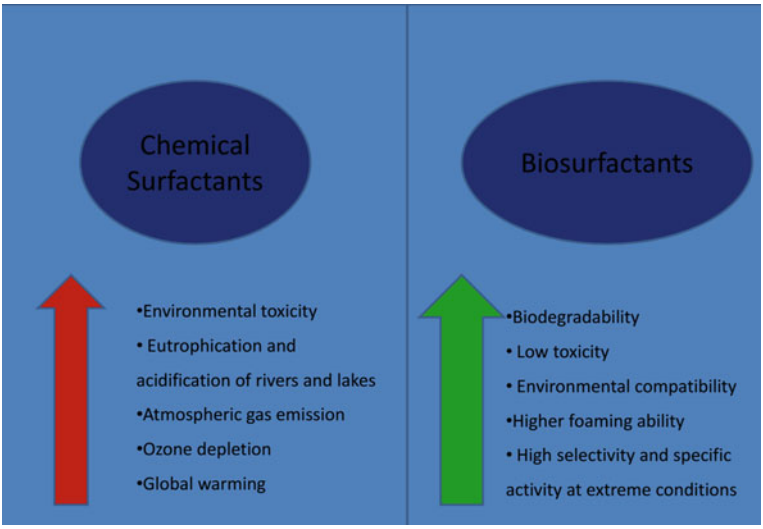

Fig. 1 Significance of biosurfactants over chemical surfactants a schematic representation

challenges faced by the biosurfactant industry are also described along with the progress developed by productive research.

1.2 Types of Biosurfactants

The term biosurfactant refers to amphipathic molecules derived from biological origin (plants or microbial) capable of reducing the surface tension of liquids. Though the term biosurfactant is used for microbially derived surfactants, any surfactant formed with any of the hydrophilic or hydrophobic part of biological origin is also included as a biosurfactant. Biosurfactants based on the mode of synthesis can be divided to first-generation biosurfactants usually termed green surfactants (involves chemical synthesis from renewable resources) and the second-generation biosurfactants or true biosurfactants (involves biological synthesis from renewable resources. The former category includes members such as alkyl polyglucosides, sucrose esters, etc., whose hydrophobic part alone is derived from a renewable resource. Such biosurfactants are produced using either plant- or animal-derived oils with biobased carbohydrates (Tmakova et al. 2017).

The second generation of biosurfactants are mostly produced biologically by fermentation using microbes, for example; rhamnolipids, sophorolipids, surfactin, etc. Chemical classification of biosurfactants greatly depends on the combinatorial linking of three different biomolecules, viz carbohydrates, lipids and proteins or their components to generate glycolipids, lipopeptides, oligopeptides, fatty acids/neutral lipids, phospholipids, polymeric molecules and particulate derivatives (Muthusamy et al. 2008). The glycolipids are of diverse kinds including

sophorolipids (produced by *Candida bombicola, Candida tropicalis*), rhamnolipids (*Pseudomonas aeruginosa, Burkholderia* sp.), cellobioselipids (by *Cryptococcus humicola*, *Ustilago maydis, Pseudozyma flocculosa)*, mannosylerythritol lipids (by *Pseudozyma* sp.) and trehalose lipids (*Arthrobacter* sp.). The major lipopeptides and oligopeptides include surfactins and subtilisin (*Bacillus subtilis)* and viscosin and syringomysin (*Pseudomonas fluorescens)*. Table 1 lists the different types of biosurfactants produced by microbes and their industrial utility in various fields. Studies indicate that yet new types of biosurfactants are reported from new isolates as in the case of *Wickerhamomyces anomalus* CCMA 0358 (Souza et al. 2017).

Biosurfactants play diverse functions in the bacterial physiology, viz (a) as components of cell membrane (Cortes-Sanchez et al. 2013), (b) biomolecules enabling xenobiotic solubilisation and utilisation in hydrocarbon-loaded environment (Kaczorek et al. 2008), (c) as antibiotics (Magalhaes and Nitschke 2013), (d) hemolytic activity in human pathogenesis (Ashdown and Koehler 1990), (e) quorum signalling (Daniels et al. 2006), (f) regulating swarming (Wang et al.

Table 1 Microbial sources of biosurfactants

Type of biosurfactant	Microorganism
Glycolipids	
Trehalose lipids	*Rhodococcus erithropolis* Arthobacter sp., *Tsukamurella* sp. and *Arthrobacter* sp.
Rhamnolipids	*Pseudomonas aeruginosa, Pseudomonas putida, P. chlororaphis, Bacillus subtilis, Renibacterium salmoninarum*
Sophorolipids	*Candida bombicola, C. apicola*
Mannosylerythritol lipids	*C. antartica, Kurtzmanomyces* sp.
Phospholipids	*Acinetobacter* sp., *Corynebacterium lepus*
Lipopeptides	
Viscosin	*Pseudomonas fluorescens*
Serrawettin	*Serratia marcenscens*
Surfactin	*Bacillus subtilis*
Subtilisin	*Bacillus subtilis*
Gramicidin	*Bacillus brevis*
Polymyxin	*Bacillus polymyxia*
Lichenysin	*B. licheniformis*
Fatty acids	*Corynebacterium insidibasseosum*
Particulate surfactin	*A. calcoaceticus*
Polymeric	
Emulsan	*Acinetobacter calcoaceticus*
Biodispersan	*Acinetobacter calcoaceticus*
Liposan	*Candida lipolytica*
Carbohydrate–lipid–protein	*Pseudomonas fluorescens*
Mannan–lipid–protein	*Candida tropicalis*

2014), (g) biofilm formation (Bonnichsen et al. 2015), etc. They are produced either externally or intracellularly in response to various environmental factors, carbon sources and sometimes as factors to overcome stress.

1.3 Industrial Synthesis—Current Scenario

The effective utilisation and commercialization of a fermentation protocol is economic effectiveness, substrate access and high yield. The recycling of agro-industrial residuals for biosurfactants is a cost-effective strategy for economised production (Makkar et al. 2011). Figure 2 represents the structure of various commercially produced biosurfactants including both chemically modified and biosynthesized biosurfactants. According to biosurfactant market evaluation published in 2015, of the various biosurfactants produced industrially methyl ester ketone accounted for the highest biosurfactant market followed by alkyl polyglucosides and rhamnolipid (http://www.grandviewresearch.com/industry-analysis/biosurfactants-industry).

Biosurfactants are produced in various countries including companies such as BASF Cognis (Alkylpolyglucosides), Jeneil Biotech (rhamnolipids), Evonik (sophorolipids), etc. Of the commercially produced biosurfactants, methyl ester ketones and alkyl polyglucosides are derived from renewable resources like waste

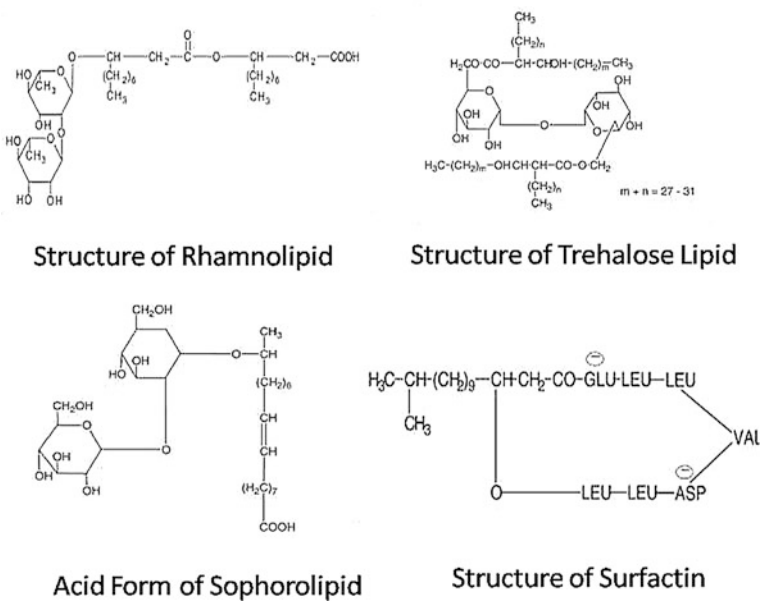

Fig. 2 Chemical structure of commercially produced biosurfactants

oil which further undergoes chemical modifications to yield the final biosurfactant product. However, rhamnolipids and sophorolipids are microbially derived, biologically synthesised rather than mere modification of biologically derived resources and thus are considered for study in this review as biosynthesized biosurfactants.

Various factors such as microbial type, carbon source, stirring, oxygen availability, physical factors (pH, temperature), aeration, nutrient level, bioprocess conditions, etc., greatly influence the production rate of biosurfactants. The biosynthesis and commercialization of biosurfactants is effective only to a small extent owing to poor yields and elevated post-production expenditure. Strategies adopted to overcome this crisis include the use of cheaper raw materials, high yielding fermentation protocols and use of novel or mutant hyperproducing strains (Muthusamy et al. 2008). Biosurfactants are produced using large number of carbon sources including vegetable oil, mineral oil (Stoimenova et al. 2009), glycerol (Putri and Hertadi 2015), palm oil (Radzuan et al. 2017), vineyard pruning (Vecino et al. 2017), SDS (Rebello et al. 2013), etc. Currently, biosurfactant research is advancing at a higher rate yielding new possibilities of replacing synthetic surfactants with eco-friendly candidates to a great extent in the near future.

1.4 Rhamnolipids

1.4.1 Chemistry and Biosynthesis

Rhamnolipids are microbial surface-active glycolipids containing one or more rhamnose moieties attached to a different type of fatty acids. The wide variety of rhamnolipids produced are critically analysed for their biopotential and applications (Chrzanowski et al. 2011). Chemically rhamnolipids contains dimers of 3-hydroxy fatty acids of different carbon lengths linked to a mono- or di-rhamnose moiety through a beta glycosidic bond. Naturally, they are released as mixtures of mono-rhamnolpids or di-rhamnolipids, for example rhamnolipid congeners Rha-C10-C10, Rha-C10-C12 and Rha-Rha-C10 were produced by *Pseudomonas aeruginosa* on growth on SDS as a carbon source (Rebello et al. 2013). The ratio of mono-rhamnolipid and di-rhamnolipid ratio generated is greatly influenced by carbon sources (Nicolo et al. 2017) and environmental factors.

Three main proteins, viz *RhlA*, *RhlB* and *RhlC* are involved in the biogenesis of rhamnolipids. *RhlA* utilises the b-hydroxydecanoyl–ACP precursors from the bacterial fatty acid synthetic pathway and convert them to hydroxyl-alkanoic acid (HAA), which serves the building blocks for mono- and di-rhamnolipids (Timmis 2002). Rhamnolipid formation utilises hydroxyl-alkanoic acid (HAA) to which two different rhamnose molecules are sequentially added to form mono-rhamnolipid and di-rhamnolipid respectively. The initial transfer of rhamnose to HAA is catalysed by *RhlA*. The transfer of dTDP-L-rhamnose to either HAA, or a previously generated mono-rhamnolipid is then catalysed by *RhlB* and *RhlC* (Deziel et al. 2003).

1.4.2 Challenges in Rhamnolipid Production

Rhamnolipids are most explored biosurfactants and are produced mainly by *Pseudomonas aeruginosa* sp. utilising various carbon sources such as soybean (Giani et al. 1997), corn (Linhardt et al. 1989), olive (Robert et al. 1989), hexadecane (Tuleva et al. 2002), detergents such as SDS (Rebello et al. 2016), etc. In majority of the countries, high production costs hinders or slows down rhamnolipid industrial production to some extent. Problems related to costly raw materials, foam generation during production, low yield and increased post- production recovery charges are some factors contributing to such a scenario (Muthusamy et al. 2008).

The major challenges faced in its industrial production are (1) use of opportunistic pathogen *Pseudomonas aeruginosa* for biosurfactant production requires extra care in handling (Van Bogaert et al. 2007) (2) generation of excessive foam during large-scale production (3) formation of mixture of different congeners requires further purification thereby increasing cost (4) laborious and expensive downstream processing accounts for 70–80% of production cost (5) low yield (6) sophisticated fermentation, time-consuming production.

1.4.3 Bioreactors to Optimise Production

Rhamnolipids are produced both by shake flask or reactors as batch, continuous or fed-batch cultures. The latter technique proved superior to mere batch cultures, with approximately 1.3-fold increase in rhamnolipid yield and high substrate–product conversion ratios (Lee et al. 2004). Similarly fed-batch mode of recycling stationary phase *P.aeruginosa* into new culture media accounted for 100% increase in rhamnolipid yield in another study (dos Santos et al. 2016). Further advancements of fed-batch culture with a fill and take strategy yielded better rhamnolipid yields than conventional fed-batch modes (He et al. 2017). The use of semi-solid state fermentation technique using glycerol and wheat bran substrates for rhamnolipid production effectively reduced the foam generation associated with rhamnolipid synthesis (Wu et al. 2017). The excessive foam generated during rhamnolipid synthesis could be effectively overcome by methods of simple foam fractionating techniques reaching enrichment factors up to 200 (Beuker et al. 2016) or by use of stop valves as a foam breakers (Long et al. 2016).

Renewable resources such as agro wastes could help to reduce the cost of rhamnolipid synthesis to a factor of 10–30% compared to chemical surfactants (Satpute et al. 2017). With the increasing levels of glycerol generated as a by-product of biodiesel production, methods utilising glycerol for biosurfactant synthesis has also gained much interest (Randhawa and Rahman 2014). The continuous mode foam fractionation of biosurfactants from bioreactor has been found to be effective in controlling the foam generated during production as well as increasing the rate of mono-rhamnolipid production to a fivefold (Diaz De Rienzo et al. 2016). The use of hollow fibre reactors containing immobilised cells of

Pseudomonas aeruginosa along with nitrate reduction instead of oxygen showed no problems associated with foam generation as observed in aerated systems (Pinzon et al. 2013).Thus, the compilation of information of different research around the world could surely help to economise rhamnolipid yields.

1.4.4 Genetic Engineering to Optimised Production

Attempts to resolve the first challenge of *Pseudomonas aeruginosa* pathogenicity has already resulted in the expression of its biosynthetic genes in *E.coli*, but the yields were less (Ochsner et al. 1994). The various recombinant DNA-based attempts to improve rhamnolipid production ranged from attempts to introduce LacZY genes of *E.coli* in *Pseudomonas* (Koch et al. 1988), transposome-mediated integration of rhlAB gene in *E.coli* (Wang et al. 2007) as well techniques to induce mutations in wild strains. Synthesis and expression of rhlAB gene and rhaBDAC gene cluster in recombinant *E.coli* under the influence of various synthetic promoters gave good rhamnolipid yields and they were further optimised by media engineering strategies (Gong et al. 2015). The recombinant expression of *P. aeruginosa* and *Burkholderia rhlAB* and *rhlC* genes in *E.coli* yielded di-rhamnolipid congeners to a greater extent (Du et al. 2017). Studies utilising mutation induced optimisation of rhamnolipids are also evident via transposon Tn5-GM-induced mutations of *Pseudomonas* (Koch et al. 1991), gamma ray mutations (Iqbal et al. 1995) and *N*-methyl-*N*-nitro-*N*-nitrosoguanidine-induced random mutagenesis (Tahzibi et al. 2004) yielding some successful reports on rhamnolipid yield.

The heterologous rhamnolipid production in *Pseudomonas putida KT2440* by the introduction of *rhlAB*-genes helped to overcome pathogenicity of *P.aeruginosa* strains, quorum-sensing regulation and made possible biomass free production of the biosurfactant (Wittgens et al. 2011). The use of biofilms of the above recombinant *P.putida KT2440* was also found to be a good source to produce mono-rhamnolipids (Wigneswaran et al. 2016). The carbon sources such as fatty acids transcriptionally delay the expression of *rhlC* gene and this could be used to control the ratio of monorhamnolipid to dirhamnolipids produced in the fermentation (Nicolo et al. 2017).

1.4.5 Application Potential

Rhamnolipids aid the solubilisation and easy uptake of hydrophobic xenobiotic compounds, thus becoming useful in soil remediation (Chebbi et al. 2017) and enhanced microbial oil uptake (Safdel et al. 2017). Rhamnolipids also extend its activity in the field of medicine by differentiating fibroblasts and keratinocytes to help in early wound healing (Stipcevic et al. 2006), targeted killing of myofibroblasts as therapy of scars (Shen et al. 2016). The antimicrobial role of this biosurfactant facilitates its use against plant pathogens (Sha et al. 2012) as well as

human pathogenic microbes (De Rienzo et al. 2016; Magalhaes and Nitschke 2013). Apart from the antimicrobial properties of rhamnolipids, the high emulsifying property makes it a cleansing agent in detergents and other cleansers. The biopesticidal properties of rhamnolipids enable the control of various insects also (Kim et al. 2010).

1.5 Sophorolipids

1.5.1 Chemistry and Biosynthesis

Sophorolipids are glycolipids produced extracellularly by various non-pathogenic yeasts such as *Candida bombicola, Candida apicola, Candida bogoriensis* (Tulloch et al. 1968) and *Wickerhamiella domericqiae* (Chen et al. 2006). Chemically, they contain a glucose disaccharide sugar sophorose, formed by a glucosyl-β-(1-2)glucosyl linkage between the sugar moieties. The complete sophorolipid is generated by joining the fatty acid with the sophorose moiety. In the majority of the sophorolipids, fatty acids of 22 carbon chain length are found, with the remaining 10% containing 24 carbon atom-based fatty acids. The non-pathogenicity of the host strains makes sophorolipids more advantageous than rhamnolipids derived from *Pseudomonas aeruginosa,* which are opportunistic pathogens. Sophorolipids are found as mixtures of free acidic, lactonised form and acetylated derivatives; with the lactonised form having better foaming and solubility agents (Elshafie et al. 2015). The greater degree of acetylation of sophorolipids makes them more water insoluble enabling its easy recovery. The acetylated derivatives of this biosurfactant are also a good antiviral agent and have cytokine stimulatory effect.

Biosynthesis of sophorolipids works in combination with fatty acid synthesis which yields the building blocks for sophorolipids, catalysed by the enzyme cytochrome P450 monooxygenases a member of CYP52 family. The first step in the sophorolipid synthesis is the formation of hydroxylated fatty acids from fatty acids (Van Bogaert et al. 2013). The formation of sophorolipids is further catalysed by two sequential glycosylation reactions to yield a β-D glucosyloxy fatty acid (Esders and Light 1972) which further gets acetylated on the breakdown of acetyl CoA as per the metaCyC sophorolipid biosynthetic pathway shown in Fig. 3 (Caspi et al. 2014). The acidic sophorolipids get lactonised by specific proteins secreted by *S. bombicola* (Ciesielska et al. 2016).

1.5.2 Challenges in Sophorolipid Production

The key hold-up to its economised synthesis is the increased expenditure associated with production. However, the use of low-cost renewable agro wastes, waste oil (Makkar et al. 2011), molasses and coconut oil (Hoa et al. 2017), etc., could reduce the cost of production to a large extent. The major companies manufacturing

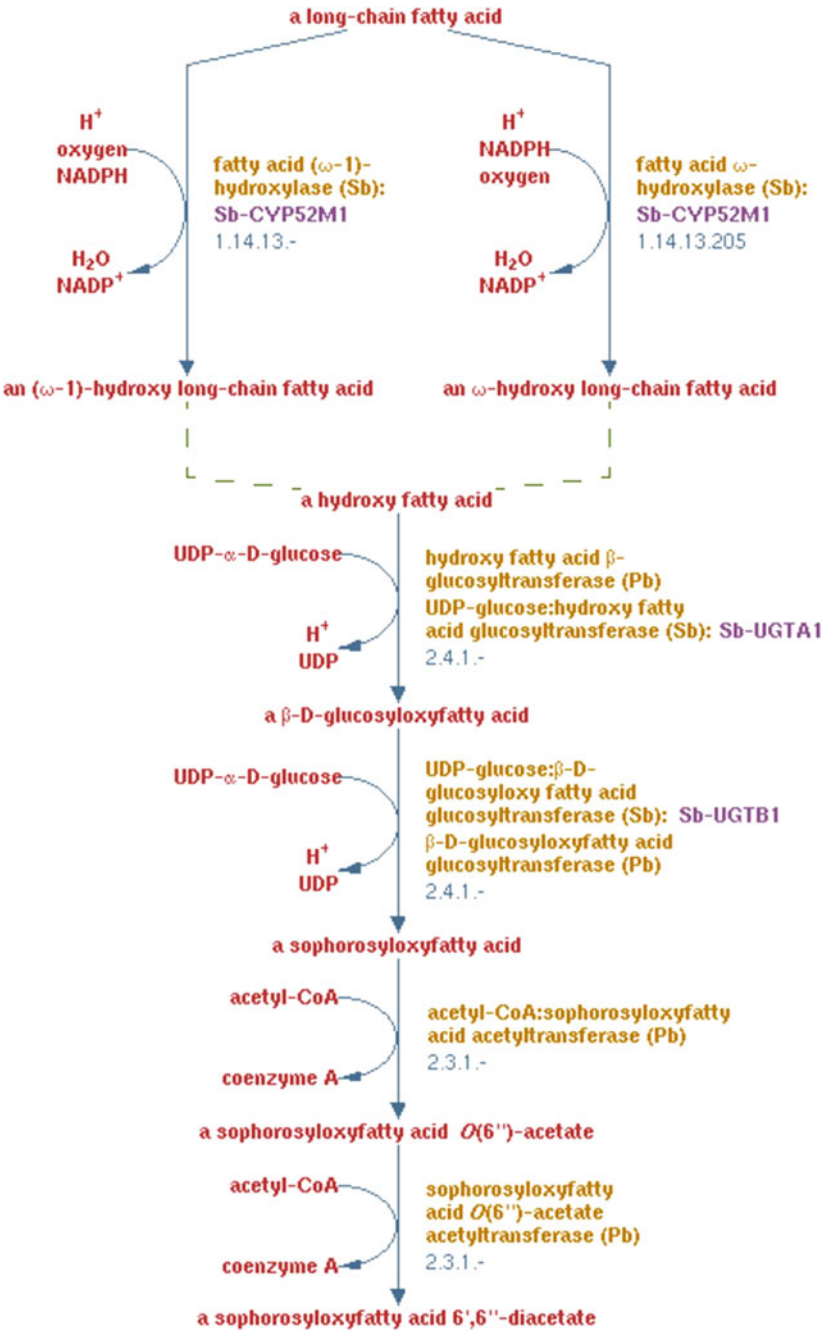

Fig. 3 Biosynthetic pathway of sophorolipids (Caspi et al. 2014)

sophorolipids include Evonik, Japan-based Saraya, South Korea-based MG Intobio, Japan-based Allied Carbon Solutions and US-based Synthezyme. Most predominantly sophorolipids are used for the production of cosmetics, toiletries and in medical field. The ecofriendliness of sophorolipids as well as its high yield of around 400 g/L has aroused much industrial interest of these biosurfactants (Van Bogaert et al. 2011).

1.5.3 Bioreactors to Optimised Production

Sophorolipids are produced by *Candida bombicola* from fat- or oil-contaminated wastewater both in batch and continuous reactions (Daverey and Pakshirajan 2015). Solid-state fermentation of *Starmerella bombicola* using agro wastes such as molasses, oil cake and straw yielded 0.179 g of sophorolipid per gram dry matter (Jimenez-Penalver et al. 2016). The combinatorial use of fed-batch method along with ultrasounds greatly increased the sophorolipid yield from waste vegetable oil (Maddikeri et al. 2015). Pilot-scale optimisation of *Starmerella bombicola* lactone esterase overexpression strain was done to get a highly pure diacetylated sophorolipid, which was suitable for chemical modification to generate sophorolipid amine oxides (Delbeke et al. 2016). High cell density fermentation with increased nutrients and optimised physical parameters using *Candida bombicola* was done in a fermentor to achieve productivity of 200 g/L/day (Gao et al. 2013). This was found to be of much relevance for industrial synthesis.

1.5.4 Genetic Engineering

Most of the recombinant work on sophorolipids is related to the identification of genes involved in sophorolipid synthesis. The proteins encoded by YP52M1 gene cluster catalyse the hydroxylated fatty acids synthesis—an essential step for sophorolipid synthesis (Van Bogaert et al. 2013), while glucosyltransferase gene UGTA1 catalyses first addition of glucose to the fattyacid derivative generated in the former step (Saerens et al. 2011). The cloning and recombinant expression of the glucosyltransferase gene from *C. bombicola* in *Sacharomyces cerevesiae* was done to study the broad spectrum glycosylating activity of this enzyme gtf-1 on sterols and fatty acids (Solaiman et al. 2014). The further lactonization of sophorolipids is found to be catalysed by an esterase, entitled *S. bombicola* lactone esterase which was characterised to prove that the above protein generates the lactonised form of sophorlipids by a serine hydrolase mechanism (Ciesielska et al. 2016). Most of the scientific developments related to sophorolipids are mostly patented such as the generation of sophorolipid transport protein (Soetaert and Van Bogaert 2012) which regulates the secretion of sophorolipids.

1.5.5 Application Potential

The anticancerous role of sophorolipid and its derivatives was effective in the treatment of pancreatic cancer and oesophageal cancer (Fu et al. 2008; Shao et al. 2012). Though the spermicidal and anti-HIV properties of this biosurfactant makes it a good topical contraceptive, the higher rates of cytotoxicity discourages its long-term use as a microbicidal contraceptive (Shah et al. 2005). They are used as surfactants, emulsifiers, antimicrobials, in environmental remediation of heavy metals, insoluble aromatic compounds and also in microbial-enhanced oil recovery (Elshafie et al. 2015). Moreover, it also is used in the generation of ω and ω-1hydroxy fatty acids, an essential component of perfumes (Van Bogaert et al. 2007).

2 Conclusions

Biosurfactant industrialised production is the need of the hour for the better ecological sustenance of the environment as well as the well-being of the human health. The cost factor of the biosurfactants could be overcome by using renewable resources as feedstock, response surface methodology-based optimisation, hyper-producing strains, better extraction protocols and cost-effective production protocols. The use of different expression systems using recombinant technology, in combination with media engineering and better extraction methods such as foam fractionation would help to achieve better yields. With successful attempts in the directions of rhamnolipids, sophorolipids and other biosurfactants the vision to replace chemical surfactants by biosurfactants would be possible in the near future.

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Chapter 11
Recovery of Nutraceuticals from Agri-Food Industry Waste by Lactic Acid Fermentation

Lyned D. Lasrado and Amit Kumar Rai

Abstract The enormous amount of by-products produced in the food and agricultural sector and the current attention towards sustainability is attracting researchers to look into possibilities of its utilization in the recovery of nutraceuticals. Nutraceuticals are food or food products that confer health and medical benefits, and are instrumental in the prevention and treatment of diseases. The agri-food industry by-products are an excellent source of proteins, lipids, fibre and other bioactive compounds. Among the different methods used for the extraction of these bioactive compounds (nutraceuticals), fermentation by lactic acid bacteria is one of the economical and eco-friendly approaches. During fermentation, chemical changes induced in the organic substrate by the action of microorganisms aids in the formation of bioactive compounds, either by a process of hydrolysis of large polymers to simple molecules, or transformation of substrates. This book chapter discusses the role of lactic acid fermentation in transformation/hydrolysis of by-products for the efficient recovery of nutraceuticals. The bioprocess of recovery of nutraceuticals from waste by lactic acid fermentation with better efficiency adds value to the food waste, reduces environmental pollution and has a positive impact on the economy.

Keywords By-products · Fermentation · Lactic acid bacteria · Nutraceuticals Health benefits

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1 Introduction

Agri-food industry waste or by-products produced during agricultural production, industrial processing, manufacturing, distribution and food service sector pose a major waste handling dilemma. While some by-products are deemed suitable for manufacturing value-added products especially fodder, the majority of waste is deemed to be disposed. The disposal of waste into the environment is inconvenient to the ecosystem (Kumar et al. 2017; Rai et al. 2012). The high-water activity and nutritional content of waste facilitate microbial decomposition which may cause undesirable effect on human health due to the large amount of methane and carbon dioxide emissions, and sanitation issues (Helkar et al. 2016). Thus, the utilization of the waste generated by food and agricultural sector is imperative as well as challenging. In the recent years, food and agricultural waste are purported as a valuable source of products (dietary supplements/food additives) that provide additional health benefits, beyond basic nutrition. These products are bracketed under the term nutraceuticals (Galanakis 2013).

The by-products resulting from several agri-food processing wastes are rich in numerous useful biomolecules and precursors of bioactive components (Mao et al. 2013; Rai et al. 2012b, 2013; Rustad 2003). The routinely used methods for extraction of bioactive compounds include the liquid–liquid or solid–liquid extraction, subcritical and supercritical extractions, pressurized liquid extraction, microwave and ultrasound extraction, fermentation technology and enzymatic hydrolysis (Joana Gil-Chávez et al. 2013; Rai et al. 2012a). Among them, fermentation of by-products using specific enzyme-producing bacteria is gaining more popularity as it helps in recovery of biomolecules as well as the transformation of recovered products into biomolecule with higher bioactivity (Bijinu et al. 2011; Pihlanto et al. 2012; Rai et al. 2011). Recently, several protein-rich by-products have been used for the production of bioactive peptides using specific protease producing microorganisms (Rai et al. 2011). Among the microorganisms used for bioconversion of waste, lactic acid bacteria (LAB) has garnered great attention owing to its potential to effectively convert inexpensive agri-food industrial residues, to valuable bifunctional products, including bioactive protein hydrolysate, oligosaccharides, phenolic compounds (Aranday-Garcíaa et al. 2017; Martins et al. 2011). The advantage of using LAB is that they have generally recognized as safe (GRAS) status, which makes the process eco-friendly and prevents the usage of hazardous chemical agents. Apart from that, LAB also produces bioactive metabolites such as bacteriocins, γ-aminobutyric acid (GABA), lipopeptides, oligosaccharides, vitamins and compounds having probiotic effect in the products (Leroy and De Vuyst 2004). In this chapter, the viability and practicality of utilizing agri-food industry waste for the recovery of nutraceuticals waste by lactic acid fermentation will be discussed. Lactic acid bacterial fermentation-driven release of functional bioactive compounds from discards of agri, fruit, dairy, bakery, brewery, meat and fishery sectors will be deliberated.

2 Biotechnological Approaches for the Recovery of Bioactive Compounds

In recent years, biotechnological approaches are gaining popularity, because chemical and physical methods result in some undesirable effects on the quality of the product for food application. Particularly in case of lipids, extreme temperature and pH may have an undesirable impact on *cis* n-3 PUFA by oxidation resulting in a product having a negative health impact. Mineral acids may help in the hydrolysis of carbohydrate and proteins in by-products but they do not have GRAS status and it is very difficult to remove these acids from the hydrolyzed product. Researchers have used organic acids for the production of bioactive protein hydrolysate but that has not proven to be much effective in comparison to fermentation techniques (Rai et al. 2009a, b). The different types of biotechnological approaches for the treatment of agri-food processing by-products are discussed briefly.

2.1 Fermentation Technology

Fermentation technology has been employed for effective recovery and transformation of agri-food processing by-products into valuable products. Apart from the recovery of valuable compounds solid-state fermentation (SSF) of agri-food industrial residues has garnered a lot of attention over decades for the production of enzymes, organic acids and other biotechnological products. Solid-state fermentation can be described as microbial growth and subsequent formation of product on a solid substrate in the absence/or near absence of moisture/water (Pandey 2003). Bioconversion of agri-food industry wastes for bioactive mining relies to a great extent on SSF, as it is an economical process resulting in wide range of industrially important products. To develop an efficient bioprocess under SSF a number of critical factors must be considered, which include the knowledge of optimal operation conditions, the substrates and microorganisms used (Martins et al. 2011). Fungi and yeast are favoured as starter cultures in SSF owing to their ability to adapt to the low moisture content of the fermentation media, however, bacteria-based SSF processes have also been widely reported (Nigam and Pandey 2009).

Among different bacteria used for fermentation, LAB carries an advantage over others due to their GRAS status and health-promoting properties (Leroy and De Vuyst 2004). This bioprocess involving LAB has been the subject of several studies directed towards utilisation of waste and recovery of bioactive compounds (Tables 1 and 2). Some examples include recovery of bioactive peptides from protein-rich bioactive hydrolysate (Ahn et al. 2009; Rai et al. 2010a; Sanjukta and Rai 2016) and release and biotransformation of polyphenols (Kaur et al. 2013; Pathak et al. 2014). Yeast and fungi are other potential candidates for bioconversion of by-products to bioactive molecules. Antioxidant and anticancer agents produced by SSF of pineapple waste with *Kluyveromyces marxianus* NRRL Y-8281 (Rashad

Table 1 Application of lactic acid bacteria for the recovery of nutraceuticals from animal-based by-products

Industry waste	LAB strain	Recovery of nutraceuticals	References
Whey	*Lactobacillus helvelticus*	Bioactive peptides	Ahn et al. (2009)
Freshwater fish viscera	*E. faeium* HAB01, *P. acidilactici* K7, *P. acidilactici* NCIM5368	PUFA and bioactive protein hydrolysate	Rai et al. (2010a, 2011)
Chicken liver	*Pediococcus acidilactici* NCIM5368	Bioactive protein hydrolysate	Chakka et al. (2015)
Shrimp waste (head and thorax)	*Lactobaccilus* spp. strain B2	Chitin recovery	Cira et al. (2002)
Shrimp industry waste	*Lactobaccilus* sp., *Pediococcus* sp.	Chitin and astaxanthin	Bhaskar et al. (2007b)
Shrimp waste	*Lactobacillus plantarum* 541	Protein hydrolysate and chitin	Rao et al. (2000), Rao and Stevens (2006)
Mixed marine processing waste	*Lactobacillus* sp. B2	Protein hydrolysate	Ramírez-Ramírez et al. (2008)
Sphyraena enis waste	*Lactobaccilus* sp. B2		
Shrimp head	*Streptococcus thermophilus*	Carotenoid	Mao et al. (2013)
Shrimp waste	*Lactobacillus plantarum* PTTC1058	Recovery of chitin and chitosan	Khanafari et al. (2008)
Crayfish	*Lactobacillus pentosus* 4023	Recovery of chitin	Bautista et al. (2001)
Shrimp waste (*Penaeus indicus*)	*Lactobacillus platarum* B4496	Recovery of carotenoid	Sachindra et al. (2007)

PUFA polyunsaturated fatty acids

et al. 2015), release of phenolic aglycones from cranberry pomace by SSF using food grade fungus *Lentinus edodes* (Vattem and Shetty 2003), and production of antioxidant nutraceuticals by SSF of pomegranate peel and creosote bush leaves using *Aspergillus niger* GH1 (Aguilar et al. 2008).

2.2 Enzymatic Hydrolysis

Enzyme preparations either alone or in mixtures have been successfully used for the recovery of bioactive compounds from plant- and animal-based raw materials (Baiano 2014). Enzymes such as cellulases, hemicellulases, pectinases, etc., hydrolyze cell wall components thereby increasing the cell wall permeability by hydrolysing the cell wall components resulting in the release of polysaccharides,

Table 2 Application of lactic acid bacteria for the recovery of nutraceuticals from plant based by-products

Waste material	LAB strain	Bioactive component	Bioactivity	References
Rice bran	*Lactobacillus plantarum*	–	COX-1 antagonistic function	Kim et al. (2015)
Rapeseed and flaxseed meals	*Lactobacillus helvelticus*	Peptides	Lipid peroxidation and angiotensin 1-converting enzyme inhibition	Pihlanto et al. (2012)
Radish brine	*Lactobacillus plantarum*	Anthocyanins and phenolic compounds	Antioxidant	Jing et al. (2014)
Litchi pericarp	*S. thermophilus* *Lactobacillus casei*	Phenolic compounds	Antioxidant	Li et al. (2013)
Soy whey	*Lactobacillus plantarum* B1-6	Phenolics, and isoflavone aglycone contents	Antioxidant	Xiao et al. (2015)
Okara/ soymilk	*Lactobacillus delbruekii* subsp. *delbruekii* SNC33	Alkali-stable polysaccharides	Bile acid-adsorptive capacity	Takagi et al. (2008)
Grape Marc	*Lactobacillus paracasei* 14A, *Lactobacillus plantarum* 12A and PU1, and *Bifidobacterium breve* 15A	–	Antioxidant	Campanella et al. (2017)

antioxidants and other bioactive compounds (Puri et al. 2012). Enzyme-assisted extraction processes has been successfully used for the extraction of a variety of bioactive products from agri-food industry waste, including the extraction of lycopene from tomato peel (Zuorro et al. 2011), anthocyanin from grape skin (Munoz et al. 2004), carotenoids and soluble fibre from carrot pomace (Stoll et al. 2003; Yoon et al. 2005), phenolics from citrus peel (Li et al. 2006), vanillin from vanilla green pods (Ruiz-Terán et al. 2001), and lignans from flaxseed hull (Renouard et al. 2010). The application of enzymes for complete extraction of bioactive compounds is proven to be a promising and effective alternative for the conventional solvent-based extraction methods. Enzyme pre-treatment of raw material contributes to reducing the extraction time, and thus provides increased yield and better quality products (Meyer 2010). Enzymes treatment has also been studied for recovering nutraceuticals from fish-processing waste (Swapna et al. 2011). Enzymes have been applied for the recovery of protein hydrolysate (Slizyte et al. 2005; Swapna et al. 2010a), lipids rich in PUFA (Swapna et al. 2011; Rustad 2003) astaxanthin and chitin as a potential nutraceutical (Auerswald and Gade 2008).

2.3 *Metabolic Engineering of Lactic Acid Bacteria*

Metabolic engineering (ME) uses recombinant DNA technology to modify specific biochemical reactions or to introduce new pathways for the production of new metabolites/products (Demain and Adrio 2008). Metabolic engineering of LAB includes strategies that have been aimed at driving the sugar metabolism towards end products other than lactic acid such as sugars of low calorific value, oligosaccharides or L-alanine. Another approach in ME is directed towards escalating the flux through complex biosynthetic pathways, resulting in the formation of the B-vitamins folate and riboflavin (Hugenholtz et al. 2002). Production of exopolysaccharide by LAB such as Lactococcus and Streptococcus is an upcoming concept in metabolic engineering, since naturally LAB produces very low amount of food grade EPS compared to that produced by non-dairy bacteria (Papagianni 2012). Even though there has been remarkable progress in ME of LAB, the focus on engineering LAB for bioconversion agri-food industry waste is scanty.

3 Lactic Acid Bacteria Associated with Agri-Food Industry Waste

LAB comprise a wide group of Gram-positive, low-GC, acid-tolerant bacteria which are in general non-sporulating and non-motile. LAB have been traditionally and industrially used as starter cultures for food fermentation and also as probiotics (Mazzoli et al. 2014). LAB are one among the most promising candidates for application in bioconversion of plant- and animal-derived biomass into various value-added products. Several LAB have been isolated from several agri-food processing by-products with the idea of selecting natural isolated for recovering nutraceuticals from respective sources (Rai et al. 2009a, 2011; Jini et al. 2011). They have a different requirement for nutrition and thus grow abundantly and specifically in regions were the specific nutrition is available, e.g. they are often associated with animal intestines and oral cavities (e.g. Lactobacillus, *Enterococcus faecalis*), plant leaves (Leuconostoc and Lactobacillus) as well as decaying animal and plant matter (Ashe and Paul 2010). LAB belonging to the genus; Lactobacillus, Streptococcus, Leuconostoc and Lactococcus were isolated from dairy effluent samples (Ashe and Paul 2010).

Most of the studies on isolation of LAB have been reported from the intestine of fish and poultry (Jini et al. 2011; Ozyurt et al. 2017). Recently, Ashayerizadeh et al (2017) isolated *Lactobacillus plantarum, L. ramnosus* and *L. fermentum* from poultry slaughterhouse waste and suggested their potential application in the fermentation of poultry waste. Recently, eight LAB strains (*Enterococcus gallinarum, Streptococcus* spp., *Lactobacillus lactis* subsp. *lactis*, *Lactobacillus brevis*, *Lactobacillus plantarum, Pediococcus acidilactici, Leuconostoc mesenteroides* subsp. cremoris and *Lactobacillus delbrueckii* subsp. *lactis*) were isolated from the

natural fish flora (skin, muscle and gut of sea bream, mullet, sea bass, carp and European catfish), were used for fermentation of seafood processing waste (Ozyurt et al. 2017). In their study, Jini et al (2011) have isolated proteolytic LAB strains belonging to *P. acidilactici*, and *E. faecalis* from freshwater fish visceral waste for fermentation of fish processing by-products.

4 Nutraceuticals from Agri-Food Industry Waste by Lactic Acid Fermentation

Fermentation using LAB is an eco-friendly approach wherein specific enzyme-producing LAB generates organic acid in situ, which also helps in the preservation of waste apart from the recovery of useful biomolecules (Rai et al. 2009a, 2011). In addition to organic acid, some of the potential LAB produces proteinaceous antimicrobial compounds (bacteriocins), which acts as a wide range of bacterial pathogens (Jini et al. 2011; Rai et al. 2009a). Several authors have also suggested that application of LAB for fermentation of by-products may also result in the product having a probiotic effect (Leroy and De Vuyst 2004). Lactic acid fermentation has been used for production and recovery of nutraceuticals from different types of agri-food industry waste, which is briefly discussed in this section.

4.1 Fish and Shellfish Processing Waste

Fish-processing industries across the globe generate considerable quantities of inedible as well as edible by-products. If we consider that nearly 45% weight of a fish turns into processing by-products, 63 MMT of fish-processing wastes (Total fish production—140 million metric tones) is the annual result of fish-processing industries across the globe (Rai et al. 2012b, 2013). Lactic acid fermentation has been considered as an emerging technology for an eco-friendly, effective and economical approach for recovery of nutraceuticals from fish processing (Rai et al. 2010a, b, 2011; Bhaskar et al. 2007a). Lactic acid fermentation of fish-processing by-products has been employed to recover bioactive protein hydrolysate (Rai et al. 2011; Rutu et al. 2014), PUFA rich lipids (Rai et al. 2010a, 2011) carotenoids having antioxidant potential (Sachindra et al. 2007; Bhaskar et al. 2007b) and chitin (Rao and Stevens 2006). A detail of application of different LAB species reported for recovery of nutraceuticals from fish-processing waste is shown in Table 1.

Fermentation using potential microorganisms allows simultaneous recovery of different types of nutraceuticals depending on the enzyme-producing ability of the starter culture (Rai et al. 2011). Recently, fermentation of fish visceral waste using *P. acidilactici* has resulted in the simultaneous recovery of PUFA rich lipids (yield >90%) and bioactive protein hydrolysate (Rai et al. 2010a, 2011). Further

studies showed that protein hydrolysate resulted after fermentation exhibited antibacterial activity against bacterial pathogens like *E. coli* MTCC118, *Salmonella typhi* FB231, *S. itridicus* FB256, and *Micrococcus luteus* and high antioxidant activities (radical scavenging activity and total antioxidant activity) (Rai et al. 2011). Bioactive protein hydrolysates are rich in bioactive peptides, which depending on amino acid composition, peptide size and sequence of amino acids exhibit several functional properties (Sanjukta and Rai 2016). Lactic acid bacterial fermentation results in products having antibacterial activity, which can be either due to the peptides produced by the bacteria or due to peptides produced on hydrolysis of the proteinaceous substrate (Rai et al. 2009a). This fraction having bioactive protein hydrolysate has a potential application in functional feed/ingredient in aquaculture.

Lipid content in fish-processing wastes are comparable to meat portion and has been reported to be in the range of 4–43.8% with higher content of unsaturated fatty acids, particularly linolenic acid, eicosapentaenoic acid and docosahexaenoic acid (Rai et al. 2012a; Swapna et al. 2010b). Recently, lactic acid fermentation with different LAB isolated from fish visceral waste has resulted in the recovery of oil rich in PUFA (Rai et al. 2011). The oil recovered by lactic acid fermentation using *P. acidilactici* NCIM5368 has shown similar effect to cod liver oil in reducing cholesterol and triglyceride levels in serum and liver; and HMG-CoA reductase in liver microsomes (Rai et al. 2012b, 2013). Further, oil recovered from fish-processing waste by fermentation enhanced the activity of antioxidant enzymes in tissues (serum, liver, brain and heart), modulated the activities of membrane-bound enzymes (Na^+K^+ ATPase, Ca^+ATPase and acetylcholine esterase) and improved the fatty acid composition in microsomes of tissues similar to group fed with cod liver oil (Rai et al. 2015). In a recent study, feeding fish oil recovered from fish waste increased EPA and DHA concentrations in liver and meat of broiler chicks in comparison to control groups (Muhammed et al. 2017). Recovery of fish oil rich in EPA and DHA will not only offset the high demand of fish oil but will also minimize problems associated with their disposal. Lactic acid fermentation using *P. acidilactici* NCIM5368, *Enterococcus faecium* NCIM5335 and *P. acidilactici* FD3 have also resulted in the recovery of functional lipids and bioactive protein hydrolysate from freshwater fish heads (Rutu et al. 2014).

4.2 Shrimp Processing Wastes as Sources of Chitin and Carotenoid

A large amount of waste produced on shrimp processing industries is rich in nutraceuticals such as carotenoid (astaxanthin) and chitin (Prameela et al. 2017). Fermentation of shrimp industry waste using potential LAB has been done for (i) preservation of shrimp waste (Cira et al. 2002; Fagbenro and Bello-Olusoji 1997; Shirai et al. 2001), (ii) production of chitin (Rao et al. 2000; Bautista et al. 2001;

Healy et al. 2003; Rao and Stevens 2006) and (iii) recovery of carotenoids (astaxanthin) (Sachindra et al. 2007). Shrimp waste fermentation was initially carried out with the idea of preservation of waste for further application. Such studies included analyzing the effect of different starter cultures, fermentation time and carbohydrate sources on quality of fermented silage (Cira et al. 2002; Rai et al. 2012a; Shirai et al. 2001).

Protein hydrolysate, carotenoids, chitin and its derivatives recovered from shrimp processing by-products have been reported to possess nutraceutical potential in food and feed industry (Aranday-García et al. 2017; Mao et al. 2013; Sachindra and Bhaskar 2008). In a recent study, *L. brevis* and *Rhizopus oligosporus* was used for shrimp processing waste fermentation and the product in the form of protein hydrolysates and astaxanthin exhibited very strong radical scavenging activity (Aranday-García et al. 2017). Astaxanthin is a powerful antioxidant in comparison to other naturally available antioxidants and it exhibits beneficial effects against cardiovascular disease, inflammation and oxidative damage (Zhang et al. 2015). Previously, *Pedicoccus acidolactici* CFR2182 was found to be the best lactic acid bacteria among different cultures evaluated for their efficiency in the fermentation of shrimp processing waste (Bhaskar et al. 2007b). Later, the fermented lyophilized powder was found to be rich in low molecular weight peptides exhibiting strong antioxidant activity (Sachindra and Bhaskar 2008). Fermented shrimp waste using LAB has also been reported to possess essential amino acids and balanced amino acid composition (Lopez-Cervantes et al. 2006). Shrimp head fermentation with *Streptococcus thermophilus* resulted in high antioxidant activity, which was attributed to the increase in astaxanthin the fermented and hydrolysed shrimp head (Mao et al. 2013). Carotenoids recovered from crustacean wastes can be used to reduce oxidative stress and also as a source of pigmentation in aquaculture. Astaxanthin recovered from shrimp waste has been used for pigmentation of cultured fishes through dietary manipulation (Weeratunge and Perera 2016).

Chitosan is a bioactive derivative of chitin formed after chitin deacetylation to varying degrees. Research on chitin and chitosan oligomers for their nutraceutical potential has attracted researchers involved in the development of functional foods due to their versatile immuno-enhancing and protective effects against some infectious pathogens and antitumour activity (Shahidi and Abuzaytoun 2005). Chitin is traditionally recovered from crustacean wastes by chemical treatment of two-step process namely deproteinisation and demineralization, making this process ecologically aggressive resulting in a source of pollution (Rao and Stevens 2006; Rai et al. 2012a). The first step helps in removal of proteins (using alkali—e.g. 4% sodium hydroxide) followed by removal of ash (using acid—e.g. 4% hydrochloric acid). Fermentation using LAB having GRAS status is an eco-friendly alternative to the ecologically aggressive chemical process for the production of chitin (Rao et al. 2000; Bautista et al. 2001; Healy et al. 2003; Rao and Stevens 2006). The *L. plantarum* NRRL-B 14768 strain was reported to be used for fermenting shrimp processing waste resulting in the efficient recovery of chitin and carotenoid (Prameela et al. 2010). Previously, Healy et al (2003) has also shown that lactic acid fermentation along with proteolytic bacteria of waste prawn shell is an effective

approach for recovery of valuable biomolecules such as chitin and protein hydrlysate from shell waste. Fermentation method using microorganism having GRAS status is more eco-friendly and has advantages over chemical methods, as it results in an enhancement in the recovery of astaxanthin along with chitin and chitosan without the usage of chemical agents (Prameela and Hemalatha 2015).

4.3 Poultry Processing Waste

Most of the studies on utilization of poultry processing by-products have been carried out using commercial enzymes and very few reports are available on lactic acid fermentation.

Poultry processing industries produce both edible (liver, gizzard, meat scraps and blood) and inedible (feather, intestine, feet, bones and head) wastes (Chakka et al. 2015). In a recent study chicken liver protein hydrolysate was prepared by fermentation using *P. acidilactici* NCIM5368 (Chakka et al. 2015). The fermented protein hydrolysate exhibited good antioxidant activity and strong antibacterial activity against *Listeria monocytogenes* and *Bacillus cereus* and moderate activity against *M. luteus* and *Yersinia enterocolitica*.

Functional and bioactive protein hydrolysates were prepared by lactic acid fermentation of chicken eggshell membrane using the *L. plantarum* (Jain and Anal 2017). The hydrolysate exhibited reducing power potential, DPPH radical activity and angiotensin I converting enzyme inhibitory activity apart from being effective against various foodborne pathogens. In another study, feeding fermented boiler by-products with *L. plantarum* as a starter culture to pigs results in the improved feed: gain ratio and lower count of pathogenic bacteria in the intestine (Urlings et al. 1993). In a recent study, Ashayerizadeh et al. (2017) suggested that fermentation using LAB is an advisable method for the utilisation of nutrients present in poultry slaughterhouse waste, which can also result in the production of probiotic supplement.

4.4 Bran

Rice bran, a major by-product of the rice-polishing process predominately contains phytosterols enriched with ferulic acid (FA). FA and its derivatives are popular active ingredients used in the development of therapeutics for the prevention and treatment of cancer, owing to their antioxidant potential. The fermentation of rice bran by LAB has been reported to cause the release and bioconversion of FA. Two *P. acidilactici* isolates have shown great potential in the release of ferulic acid from rice bran and its biotransformation to bio-vanillin (Kaur et al. 2013). Vanillin which is used as a flavour and aromatic compound is also reported to possess antioxidant and anticancer properties (Pedroso et al. 2013; Bezerra et al. 2016). Rice bran water

extract fermented with *L. plantarum* is reported to demonstrate a direct COX-1 antagonistic function for platelet aggregation mediated diseases like myocardial infarction and thrombosis. Analysis of the phenolic acid compositions revealed ferulic acid as the predominant phenolic acid in the rice bran water extract (Kim et al. 2015). Cellulolytic, lignolytic and pectinolytic enzymes released by the LAB are responsible for the change in the polyphenol profile during the fermentation process. These enzymes break the chemical components of plant cell walls loosening the cell wall matrix. The ester bonds that link the phenolic acids to the cell wall matrix are cleaved by enzymes like ferulic acid esterase and ferulic acid decarboxylase consequently releasing ferulic acid (Huynh et al. 2014).

Pre-fermentation of bran with LAB can be instrumental in improving the flavour and textural qualities and hence the prebiotic potency of bran-rich products. Investigation of dextran production in wheat and rye bran by fermentation using two *Weissella confusa* strains, revealed that both, particularly the latter, are favourable matrices for the production of dextran in technologically significant amounts (Kajala et al. 2015). Dextran, is known to support the growth of probiotic bacteria and possess other desirable attributes like resistant to hydrolysis by simulated gastric juice and α-amylase (Tingirikari et al. 2014).

4.5 Whey a By-product of Dairy Industry

Whey, the yellowish liquid remaining after milk has been curdled and strained, is the largest by-product of the dairy industry. Milk protein acts as a precursor of bioactive peptides, which are produced during lactic acid fermentation and exhibit several bioactive properties such as antioxidant, antihypertensive, antimicrobial, cholesterol reducing and anticancer activity (Rai et al. 2016, 2017b). Fermentation of whey by *Lactobacillus helvelticus* is reported to release anti-hypersensitive peptides IPAVF. AQSAP, AHKAL, and APLRV which show potent angiotensin 1-converting enzyme (ACE) inhibition. Whey fermented with *L. brevis* also contained a strong ACE inhibitory peptide identified as AEKTK (Ahn et al. 2009). LAB possess extracellular proteinase which hydrolyses milk proteins specifically and liberates peptides.

The efficacy of three LAB strains; *S. thermophilus* CRL 804, *L. delbrueckii* subsp. *bulgaricus* CRL 454 and *Lactobacillus acidophilus* CRL 636 as a starter culture for developing functional whey-based drinks was evaluated. *S. thermophilus* CRL 804 produced the highest amount of lactic acid and *L. delbrueckii* subsp. *bulgaricus* CRL 454 proteolytically released the branched chain amino acids Leu and Val. In conclusion, the starter culture containing a combination of *S. thermophilus* CRL 804 and *L. delbrueckii* subsp. *bulgaricus* CRL 454 showed high potential to be used for developing fermented whey-based beverages (Pescuma et al. 2008). In another study, reconstituted whey powder (10 and 16%. w/v) was fermented with 64 different strains of the LAB at 37 °C for 24 h to develop an effective starter culture for whey-based beverage formulations. Maximum

degradation of allergenic whey protein, β-lactoglobulin, resulted on fermentation using *L. acidophilus* CRL 636 and *L. delbrueckii* subsp. *bulgaricus* CRL 656 and a number of peptides and amino acids were released in the first 6 h of incubation (Pescuma et al. 2012).

4.6 Meal

Meal, a by-product obtained during the processing of seeds to produce dietary oils has been exploited for the extraction of nutraceuticals through lactic acid fermentation. Zeng et al. (2013) developed a cheap and simple method using a combination of LAB fermentation and protease hydrolysis to increase the production of bioactive ACE inhibitory peptides from peanut meal. Rapeseed and flaxseed meals fermented with Lactobacillus were also able to produce compounds (mainly peptides) that inhibited lipid peroxidation and angiotensin 1-converting enzyme (Pihlanto et al. 2012). Sesame meal contains two major groups of lignans, namely oil-soluble lignans and glycosylated water-soluble lignans, among which, sesaminol triglucoside is the major lignan that displays biological activities. Though the antioxidative potential of sesaminol triglucoside is weak in vitro, hydrolysis of the glycosides attached to the lignin salleviate oxidative stress. Owing to their high β-glucosidase activity LAB are suitable candidates for the bioconversion of sesame meals (Pathak et al. 2014). KuoChing et al. (2010) reported a marked increase in the antioxidative and anti-inflammatory activities of the sesame meals fermented with LAB.

Soybean meal is a by-product obtained during extraction of soybean oil and is a rich source of high-quality protein and isoflavones in bound form (Wongputtisin et al. 2015). Fermentation of soybean-based substrate using protease and β-glucosidase-producing bacteria can enhance the biological property by the production of bioactive peptides and free isoflavone, respectively (Rai et al. 2017a; Sanjukta et al. 2017). Soybean meal fermented with *L. plantarum* Lp6 has shown to enhance antioxidant and angiotensin-converting enzyme inhibitory activities due to the production of bioactive peptides during fermentation (Amadou et al. 2010; Wang et al. 2014).

4.7 Radish Brine

Fermentation of Radish brine with *L. plantarum* resulted in the release of antioxidative anthocyanins and phenolic compounds (Jing et al. 2014). Four different anthocyanins were identified in the brine, which were characterized as pelargonidin-3-diglucoside-5-glucoside derivatives acylated with one coumaric acid, one ferulic acid, or both. Eight different free phenolic acids were detected in radish brine, and fermentation facilitated free phenolic acid release from conjugated or insoluble forms (Jing et al. 2014). Radish brines generated as one of the major

waste from industrial radish pickle production are therefore valuable to produce antioxidative nutraceuticals.

4.8 Litchi Pericarp

The pericarp of *Litchi chinensis* is a good source of A-type procyanidins (flavonoids), which are being considered as natural dietary supplements due to their high biological activity in vivo. *S. thermophilus* is reported to be able to metabolize procyanidin A2 to its isomer, and *Lactobacillus casei* can decompose flavan-3-ols into 4-hydroxyphenylpropionic acid, 3,4-hydroxyphenylacetic acid, m-coumaric acid, and p-coumaric acid. This suggests that bioconversion by LAB is an efficient method to convert litchi pericarp procyanidins to a more effective antioxidant agent (Li et al. 2013).

4.9 Soybean Whey

Soybean whey is a liquid waste generated during production of tofu (soy curd), which has serious disposal problems (Monajjemi et al. 2012). Soy whey fermented using *L. plantarum* and *S. thermophilus* showed high antioxidant activity suggesting its nutraceutical potential (Monajjemi et al. 2012). Soy whey extract fermented with *L. plantarum* B1-6 possessed elevated total phenolics, and isoflavone aglycone contents, and displayed higher ferric reducing antioxidant power, reducing power, ABTS, superoxide and hydroxyl radical scavenging activity compared to unfermented soy whey extract. Additionally, fermented soy whey extract exhibited greater protection against Fenton's reagent-induced oxidative DNA damage (Xiao et al. 2015).

4.10 Okara

Raw okara is a yellowish-white material consisting of the insoluble parts of soybean seeds, which are retained in the filter during the production of soymilk (Jiménez-Escrig et al. 2008). Soymilk containing okara (okara/soymilk) fermented by *Lactobacillus delbruekii* subsp. *delbruekii* SNC33 showed higher in vitro bile acid-adsorptive capacity compared to unfermented okara/soymilk. Alkali-stable polysaccharides were generated by lactic fermentation which increased the short-chain fatty acids (propionic and butyric acid) in rat intestines and faecal bile acids ultimately exerting hypocholesterolemic effect (Takagi et al. 2008). Studies have also suggested that the effect on the microbial production of cecal short-chain

fatty acids as well as polyamines in LAB fermented *okara*/soymilk is depended on the intake of additional dietary fibre (Takagi et al. 2009).

4.11 Grape Marc

Grape marc or pomace is the solid remains (skins, pulp, seeds and stems) of grapes. Grape marc contains large amounts of natural antioxidants, hemicellulose sugars and fatty acids making it suitable for fermentation for the recovery of bioactives (Rivera et al. 2007). The ability of selected LAB and bifidobacteria to grow on grape marc rich in polyphenols leading to the valorisation of grape marc through both the increase in probiotic biomass and supply of antioxidant compounds has been reported. *Lactobacillus paracasei* 14A, *L. plantarum* 12A and PU1, and *Bifidobacterium breve* 15A showed highest cell density (>9.0 CFU/g) in grape marc (GM)-based media supplemented with 1% of glucose (GMG). In comparison with the un-inoculated GMG, cell-free supernatant (CFS) of fermented GMG displayed an evident rise in antioxidant activity in vitro. The protective effect of the fermented grape marc samples towards induced oxidative stress Caco-2 cells was ascertained by the determination of the intracellular ROS production and detoxification by DCFH-DA assay. Fermented GMG exhibited markedly higher antioxidant activity on Caco-2 cells compared to the un-inoculated control. Confirming these results, the SOD-2 gene expression also showed lower prooxidant effect induced by four grape marc preparations when compared to the control (Campanella et al. 2017).

5 Conclusions

Agri-food industry wastes offer a huge wealth of nutraceuticals that have potential application in functional food and feed industry. Use of eco-friendly processes like lactic acid fermentation would also minimize the application of hazardous chemicals (acid/alkali) by the processing industry. Lactic acid fermentation can be an efficient and economical approach integrated with respective bio-processing operations to reclaim nutraceuticals associated with waste, which can create an environmental hazard. Further, the recovered nutraceuticals like bioactive protein hydrolysates, peptides, chitin, lipids, polyphenols and carotenoids can be effectively utilized in aquaculture feeds and nutraceutical industry. Future studies on characterization of nutraceuticals derived during lactic acid fermentation will result in the discovery of novel bioactive molecules with specific health benefits.

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Chapter 12
Manno-oligosaccharides as Prebiotic-Valued Products from Agro-waste

Shweta Singh, Arabinda Ghosh and Arun Goyal

Abstract Agriculture is the backbone of Indian economy, is carried out for primary products where a significant amount of agro-waste is produced. About 72% of fruits and vegetable production in India goes waste, due to their processing variability which meets about 1.4% of the total global trade. Therefore, the agro-waste utilization for human benefit is the major concern for environmental sustainability. Such waste can be effectively used for the production of useful compounds for animals and human beings. Lignocellulosic agro-waste has been explored for the production of energy as biofuels. Such biomass can also be considered to be useful for the production of prebiotic oligosaccharides. Different agro-wastes such as copra meal, potato peel, rice straw, wheat straw, corncob, palm kernel meal, etc., can be used for the production of different oligosaccharides. Oligosaccharides are short chains of monosaccharides of the size ranging from two to ten units of monosaccharides or simple sugars. The oligosaccharides are highly stable and are used as dietary supplement possessing prebiotic, antioxidant activity and potential immune-modulating properties. Manno-oligosaccharides can be produced from pretreated agro-waste by hydrolysis with endo-β-(1 → 4)-mannanase. MOSs has significant stability against α-amylase, intestinal juice, and simulated gastric juice which is an essential parameter for oligosaccharides to serve as a prebiotic. Nevertheless, MOSs also supports higher growth rate of probiotic bacteria *Bifidobacterium infantis and Lactobacillus acidophilus* as compared to commercial prebiotic. In addition to prebiotic properties, MOS significantly reduces the growth

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of colon cancer cells that depicts their antagonistic properties without any cytotoxic effect against the normal cell culture. This chapter will describe the roles of manno-oligosaccharides recovered from agro-waste which have applications as prebiotics, acting antagonistic agent against cancer cells and as anti-allergic agents.

Keywords Agro-waste · Non-digestible oligosaccharides (NDO) Manno-oligosaccharide (MOS) · Prebiotics · Galactomannan

1 Introduction

The agricultural practices, environment and human health are intrinsically linked. Human life and environment quality critically depends on agricultural production. The rapid industrialization and sustained urban growth results in the buildup of waste and cause immense deterioration in the general quality of urban life. Agricultural practices on earth surface leave considerable amount of agricultural waste, some of it is recycled for the production of organic manure, while large amount of it remains unused and therefore, poses a disposal problem (Raval 2014). Uncontrolled burning of agricultural waste in fields is not only a hazardous disposable practice but, is also wastage of large amount of useful bio-resource for energy (Raval 2014). In Asia, around 350 million tons of agro-waste is generated annually such as sugarcane waste (bagasse and leaf top), paddy straw, wheat straw, coconut meal, coconut husk, vegetables waste, food waste, jute fibre, cotton stalk, groundnut shell, wooden mill waste, palm kernel meal, spent coffee ground, etc. (Pappu et al. 2007; Madurwar et al. 2013). Around 72% of fruits and vegetable production in India goes waste due to their processing variability which meets about 1.4% of the total global trade (Ramanathan and Parthasarathy 2014). The increasing level of agro-waste has become a major concern for both developed and developing countries. Therefore, the proper management of agricultural waste for its conversion into value-added product is required (Tilman et al. 2002).

Agro-industrial wastes like fruit and vegetable wastes can be recycled and reused for the production of prebiotics and valued-added functional food product such as non-digestible oligosaccharides (Patel and Goyal 2011; Shaharuddin et al. 2014; Rahmani et al. 2017). Non-digestible oligosaccharides (NDO) are short chain of monosaccharides (two to ten) and configuration of anomeric carbon atom (C1 or C2) makes glycosidic bond of NDO non-susceptible (Roberfroid and Slavin 2000; Weijers et al. 2008). Its physico-chemical properties shown to act as prebiotic are beneficial to human health, and therefore used as an ingredient for functional food (Qiang et al. 2009). Prebiotics term was first given by Gibson and Roberfroid who had written “pre” in place of “pro,” which means “before” or “for” (Gibson and Roberfroid 1995). They are non-digestible food ingredients that improve gut micro-ecology, i.e. it selectively stimulates the growth of beneficial microflora (*Bifidobacteria*) in colon (Mussatto and Mancilha 2007; Patel and Goyal 2011), etc.

The prebiotics have low calorific value, possess antitumour, cholesterol-lowering and antioxidative properties (Liu et al. 2010). They act as potential immune modulator and they increase production of short-chain fatty acids in colon by decreasing the pH of gastrointestinal tract (Mussatto and Mancilha 2007) and also increase the absorption of minerals in the colon and vitamin B production in gastrointestinal tract (Mussatto et al. 2007).

Manno-oligosaccharides are NDOs can be produced from pretreated agro-waste by hydrolysis with endo-β-(1 → 4)-mannanase (E.C. 3.2.1.78). This enzyme catalyzes the random hydrolysis of β-1,4 mannosidic linkage present in the backbones of mannan, glucomannan, galactomannan and galactoglucomannan (Titapoka et al. 2008) and releases oligosaccharides of various degree of polymerization. The abundance of MOS is higher as compared with mannose monosaccharide (Titapoka et al. 2008). MOSs hydrolysate has significant stability against ά-amylase, intestinal juice and simulated gastric juice which are essential attributes of a prebiotic (Titapoka et al. 2008; Ghosh et al. 2015). Nevertheless, MOS supports higher growth rate of probiotic bacteria *Bifidobacterium infantis and Lactobacillus acidophilus* as compared to a commercial prebiotic agent such as inulin (Ghosh et al. 2015). In addition to prebiotic properties, MOS significantly reduces the growth of colon cancer cells that depicts their antagonistic properties without showing any cytotoxicity against normal cell culture (Ghosh et al. 2015) and also acts as anti-allergic agent (Ozaki et al. 2007).

2 Manno-oligosaccharides

MOSs are mainly of two types α-MOS and β-MOS oligosaccharides. They consist of a linear chain of mannose sugar, possess prebiotic properties and are used as a feed additive to prevent pathogen colonization in the digestive tract of human and animals (Srivastava et al. 2017). β-MOS can be produced by random hydrolysis of mannans by endo-β-1,4-mannanase (EC 3.2.1.78) and show variability with respect to their degree of polymerization and presence/position of substituents (Srivastava et al. 2017). In the 1980s, it was discovered that mannose and methyl-α-D mannoside inhibits *Salmonella* infections in broilers chicken. In bacteria, lectin receptor (contain D-mannose) is blocked by mannose and thus inhibiting the bacterial binding (Freter and Jones 1976; Oyofo et al. 1989). Different researchers published that *Salmonella* binds mannose by the help of Type 1 fimbriae and reduce its colonization in broilers chicken intestine by 90% (Oyofo et al. 1989; Rezaei et al. 2015).

3 Substrate for Manno-oligosaccharides Production

Manno-oligosaccharides are produced from commercial substrates (Fig. 1) and from agro-waste, i.e. from lignocellulosic biomass, which are rich in mannans (Blibech et al. 2011; Otieno and Ahring 2012). Mannans are the important

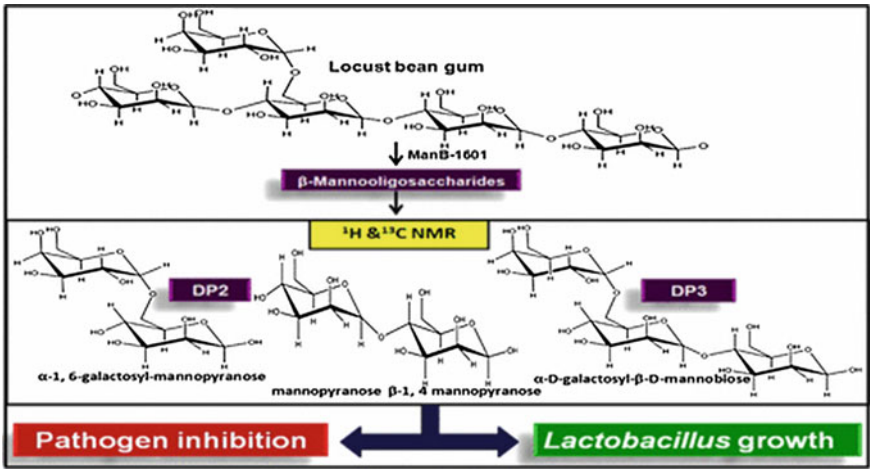

Fig. 1 Manno-oligosaccharides from Locust bean gum and its beneficial effects (Srivastava et al. 2017)

constituent of hemicellulose in the secondary cell wall of softwood of lignocellulosic biomass. It is the second largest group in hemicelluloses after xylan (Ojima 2013). Mannans are the heteropolymers and contain β-(1→4)-D-mannopyranose backbone and often substituted with galactose or glucose moieties through β-(1→6) and β-(1→4) linkages (Blibech et al. 2011; Otieno and Ahring 2012). It is a storage polysaccharide, e.g. Ivory nut is a source of mannan, also present in cell wall polysaccharides in yeasts (Bartnicki-Garcia 1968; Barfod et al. 1990).

3.1 Types of Mannans Present in Hemicellulose Content of Agro-waste

On the basis of their backbone, glucose and galactose side chains mannans were classified into the following groups: galactomannans, glucomannans, galactoglucomannans (Moreira 2008; Schröder et al. 2009).

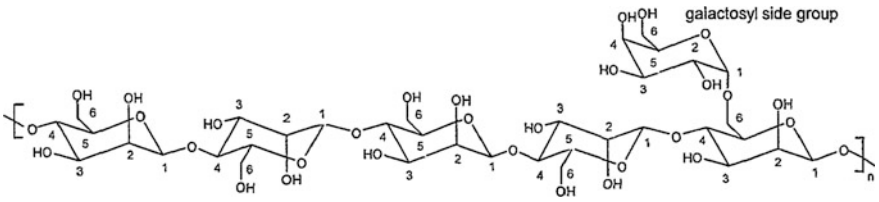

Fig. 2 Chemical structure of galactomannan (Cui et al. 2009)

3.1.1 Galactomannans

They are natural water-soluble polysaccharide consisting of β-(1 → 4)-linked D-mannose backbone, randomly substituted with a single unit of α-(1 → 6)-linked D-galactose residues as displayed in Fig. 2 (Moreira 2008; Jian et al. 2013).

3.1.2 Glucomannans

They are natural water-soluble polysaccharide consisting of randomly arranged β-(1 → 4)-linked D-mannose and β-(1 → 4)-linked D-glucose backbone. The molar ratio of glucose and mannose is 1:1.6 (Fig. 3). They are generally found in the secondary walls of softwood (Katsuraya et al. 2003).

-G-G-M-M-M-M-M-G-M- (G: Glucose & M: Mannose).

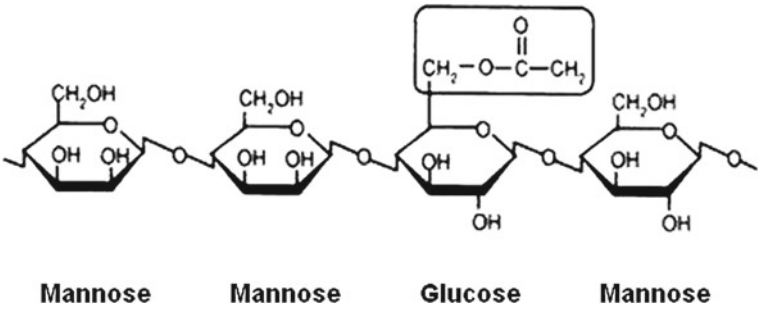

Fig. 3 Chemical structure of glucomannan (Maeda et al. 1980)

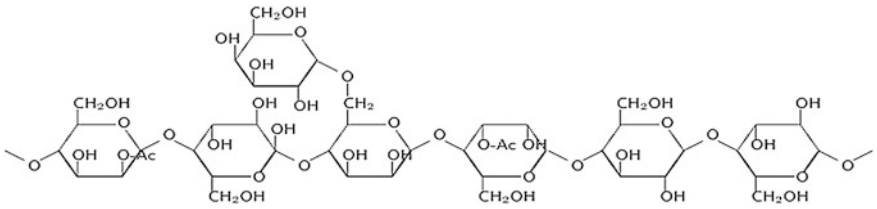

Fig. 4 Chemical structure of galactoglucomannans (Willför et al. 2008)

3.1.3 Galactoglucomannan

They are natural water-soluble polysaccharides consisting of backbone of randomly distributed β (1 → 4)-linked D-mannose and D-glucose units with the branching of α (1 → 6)-linked D-galactose units attached to mannose units (Fig. 4) (Willför et al. 2008). C2 and C3 hydroxyl group in mannose are partially substituted by acetyl groups (Willför et al. 2008).

3.2 Commercial Mannans

The commercially available polysaccharides, viz. carob galactomannan from *Ceratonia siliqua*, locust bean galactomannan (carob gum) and guar galactomannan from *Cyamopsis tetragonolobous* (Linn), *Caesalpinia pulcherrima* are rich sources of galactomannan (Maeda et al. 1980; Srivastava and Kapoor 2005; Cui and Wang 2009; Ghosh et al. 2013; Srivastava et al. 2017). Production of manno-oligosaccharides from commercial substrates poses a major drawback because of their high costs, therefore it is not economically viable.

3.3 Agro-waste

In current scenario, the production of manno-oligosaccharides from mannan-rich agro-waste (biomass) is economical and therefore of great interest. In India, around 72% of fruits and vegetables go waste due to their processing variability, which is about 1.4% of the total global trade (Ramanathan and Parthasarathy 2014). Therefore, the agro-waste utilization for human benefit is the major concern for environmental sustainability (Tilman et al. 2002). Different types of agro-waste such as copra meal, palm kernel meal, spent coffee ground, potato peel, corn stover, etc., can be used for the production of different types of oligosaccharides (Table 1). Among agro-waste copra meal, palm kernel meal, spent coffee cake were the main substrates which possess high percentage of mannan and used can be for manno-oligosaccharides production (Utami et al. 2014; Campos-Vega et al. 2015; Ghosh et al. 2015).

Table 1 Different agro-wastes with their hemicelluloses content (% dry weight)

Feedstock	Mannan	Xylan	Arabinan	Galactan	References
Palm kernel meal	57	3.7	–	–	Ong et al. (2004)
Spent coffee ground	39.3	0.3	0.4	2.5	Jooste et al. (2013)
Copra meal	21	–	–	–	Gosh et al. (2015)
White Pine	15.8	7	–	–	Eslyn et al. (1975)
Radiata pine	11.3	5.9	1.6	2.5	Otieno and Ahring (2012)
Poplar	3.9	14.49	0.6	1	Wyman et al. (2009)
White Oak	2.9	18	2.4	0.4	Otieno and Ahring (2012)
Maple	2.9	17.3	2.8	–	Otieno and Ahring (2012)
Hard maple	2.9	16.8	1.7	–	Otieno and Ahring (2012)
Buckeye	2.8	14.9	2.1	–	Otieno and Ahring (2012)
Basswood	2.8	17.5	2.1	1.1	Otieno and Ahring (2012)
Walnut	2.6	16.5	1.8	–	Otieno and Ahring (2012)
Sycamore	2.7	17.1	1.7	–	Otieno and Ahring (2012)
Legume shrub	2.2	11.6	1.4	1.5	Otieno and Ahring (2012)
Cottonwood	2.2	13.3	0.6	0.6	Otieno and Ahring (2012)
Cherry	2.1	19.8	1.8	–	Otieno and Ahring (2012)
Corn stover	1.8	21.4	3.5	2.5	Öhgren et al. (2007)
Black locust	1.8	13.1	13.1	0.9	Otieno and Ahring (2012)
Wheat straw	1.1	18	3.4	0.5	Salvachúa et al. (2011)
Potato peel waste	0.75	3.52	3.53	6.91	Liang and McDonald (2014)
Hybrid poplar	0.5	14.6	0.3	0.3	Otieno and Ahring (2012)
Beech	0.9	20.8	1.5	–	Otieno and Ahring (2012)
Switchgrass	0.8	21.1	2.8	1	Suryawati et al. 2009
Hickory	0.6	19.9	1.2	–	Otieno and Ahring (2012)
Bagasse	0.48	22.5	2	1.4	Alves et al. 2010
Tall fescue	0.3	13.8	2.9	1.1	Otieno and Ahring (2012)
Big blue stem	0.3	21.2	2.7	0.8	Otieno and Ahring (2012)

3.3.1 Copra Meal

Copra meal is the dry coconut flesh which is produced as the by-product in coconut oil industry has high economic value and generally regarded as agricultural waste without any nutritional value (Saittagaroon et al. 1983; Ghosh et al. 2015). The copra meal production is up to 1.7–2.1 million tons annually (Saittagaroon et al. 1983; Hossain et al. 1996). Copra meal is left after oil extraction and water decantation from copra. Around 30–40% weight of coconut is used in oil production and the remaining coconut after oil extraction was used as copra meal (Saittagaroon et al. 1983). The abundance of galactomannan (65%) and mannan (21%) in copra meal thrives its way as a suitable source of manno-oligosaccharides (MOS) production (Hossain et al. 1996; Ghosh et al. 2015). A process was developed where galactomannan was recovered from copra powder after pretreatment (Saittagaroon et al. 1983). Effective

pretreatment of defatted copra meal yields 48% carbohydrate and 5% lignin in this 72% mannose, 25% glucose, 3% galactose and traces of xylose and arabinose were present (Saittagaroon et al. 1983).

3.3.2 Palm Kernel Meal

Palm kernel meal is the by-product of palm kernel generally regarded as agricultural waste produced during palm oil processing. It contains approximately, 16.4% crude fibre and 15.4% crude protein (Adrizal et al. 2011). The abundance of β-mannans in fibre composition of palm kernel meal was approximately 20–40% (Utami et al. 2014). So because of its high hemicellulose content, it was used in the production of MOSs (Utami et al. 2014).

3.3.3 Spent Coffee Ground (SCG)

Spent coffee ground (SCG) is the by-product after processing of coffee beans and fruits. Coffee is the second largest trade commodity after petroleum and coffee-producing industries generate residue from coffee fruit during processing almost greater than 50% of the total mass of coffee fruit (Campos-Vega et al. 2015). Approximately, 6 million tons per year SCG is produced worldwide during processing (Tokimoto et al. 2005). The abundance of mannan in SCG was approximately, 20–30% of dry weight of SCG, that thrives it as a suitable source of MOSs production. SCG has potential ingredients for the food industry, so it can be used for an overall increase in the sustainability of coffee agro-industry. It mainly contains (by dry weight) 47% mannoses, 30% galactose, 19% glucose, 4% arabinose with mannan as a major polysaccharide (Mussatto et al. 2011).

3.3.4 Potato Peel Waste

The potato peel waste is the leftover component after the use of potato. It contains (w/w), 25% starch, 30% non-starch polysaccharide, 20% acid insoluble and acid soluble lignin, 18% protein, 1% lipids and 6% ash (Camire et al. 1997). As analyzed by Liang and McDonald (2014) potato peel waste contains fermentable carbohydrate, viz. (% dry mass) starch (16.83), galactan (6.91), xylan (3.52), arabinan (3.53) and mannan (0.75).

4 Synthesis of Manno-oligosaccharides

Manno-oligosaccharides are produced by depolymerization of mannan through various methods, viz. physical method, chemical method and enzymatic hydrolysis. Both physical and chemical methods are effective in this process but the residual

content leads to the environmental safety issue. Enzymatic hydrolysis process is comparatively slower than the earlier methods, but more effective and precise where enzymes are selective and kinetically lead to the hydrolysis of glycosidic linkages between mannose moieties from mannan polysaccharide to release manno-oligosaccharides.

4.1 Physical Method

Polysaccharide can be depolymerized by autohydrolysis at 190 °C (Carvalheiro et al. 2004), by irradiation with ionizing radiation such as γ-rays, microwaves (Al-Assaf et al. 2006; Prawitwon et al. 2007; Singh and Tiwari 2009) and by hydrothermal processing (Rose and Inglett 2010).

4.2 Chemical Methods

Galactomannan polysaccharides from seeds of *Mimosa scabrella* and *Stryphnodendron barbatiman* was hydrolyzed to manno-oligosaccharides by using mild acid hydrolysis (H_2SO_4) (Ganter et al. 1995). Mannan polysaccharide of plant cell wall can be depolymerized by hydrolysis using strong alkali (NaOH) and the reaction is further neutralized with strong acid (HCl) for the production of manno-oligosaccharides (Maru et al. 2015).

4.3 Enzymatic Hydrolysis

Enzymatic hydrolysis is the most common method for hydrolysis of mannan that can be kinetically controlled to produce desired end products. For the depolymerization of mannan, endo-β-mannanase is used. It catalyzes the random cleavage of β-(1 → 4)-mannosidic linkages within the backbones of mannan, glucomannan, galactomannan and galactoglucomannan resulting in the production of various manno-oligosaccharides (Ghosh et al. 2015). Kurakake et al. (2006) isolated endo β-mannanase from *Penicillium oxalicum* SO and employed to hydrolyse the guar galactomannan that resulted in the production of various manno-oligosaccharides of the degree of polymerization (dp) ranging from 2 to 8. An optimized process of enzymatic hydrolysis of *Gleditsia sinensis* gum (galactomannan) for production of dp 1-5 manno-oligosaccharides was described by Jian et al. (2013). The high contentment of mannan polysaccharide in agro-waste such as copra meal showed to a major reservoir of manno-oligosaccharides. A controlled time-dependent hydrolysis of copra meal mannan by using recombinant endo-β-(1 → 4)-

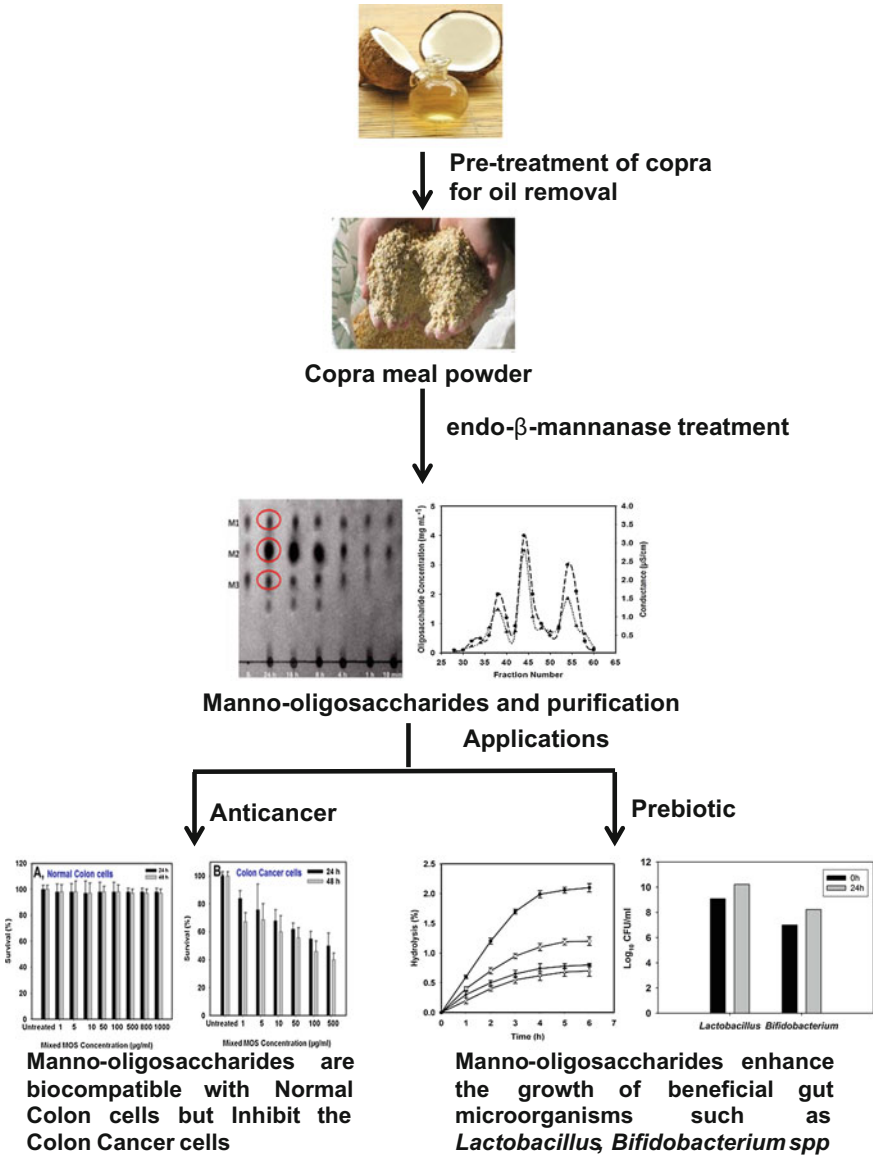

Fig. 5 Schematic presentation of manno-oligosaccharides production from copra meal and their applications

mannanase of family 26 glycoside hydrolase (*Ct*Manf) from *Clostridium thermocellum* ATCC 27405 yielded mannobiose (dp2), mannotriose (dp3) and mannotetraose (dp4) (Fig. 5) (Ghosh et al. 2015).

5 Structural Analysis and Quantification of Manno-oligosaccharides

Physico-chemical properties are linked to the function of oligosaccharides. To understand this relationship, the structural characterization of oligosaccharides is important. In structural analysis, the sequence, molecular weight, type of linkage, branching and anomeric configuration are determined in oligosaccharides. The techniques such as FTIR, Thin Layer Chromatography (TLC), Mass Spectrometry (MS) and Nuclear Magnetic Resonance Spectroscopy (NMR) are used in the structural characterization of oligosaccharides. Among these techniques, MS and NMR are the most prevalent analytical methods for structural characterization because of their high sensitivity, precise results and versatility (Patel and Goyal 2011). Isolation of functional manno-oligosaccharides is done by using high-performance liquid chromatography and carbon-Celite column chromatography. Functional manno-oligosaccharides in a reaction mixture are quantify by using different techniques such as UV spectrophotometry combined with Artificial Neural Networks (ANN), mass spectrometry TLC, high-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD), high-performance chromatography with a refractive index detector, capillary electrophoresis with a UV-diode array detector (Dias et al. 2009; Patel and Goyal 2011; Ghosh et al. 2015).

6 Applications of Manno-oligosaccharides

6.1 Manno-oligosaccharides as Prebiotic

Manno-oligosaccharides alter the gut microbiota by stimulating the growth of selective microflora such as *Lactobacillus* sp. and *Bifidobacterium* sp., which inhibit the growth of pathogenic bacteria in the human colon and gastrointestinal tract of other animals (Gibson et al. 2000). Gut microbiota in human is formed by more than 500 different bacterial species; this showed that the total number of bacterial cells present in colon is far exceeding the total number of eukaryotic cells forming/present in the body (Conway 1995; Gibson and Beaumont 1996; Tannock 1999), therefore it plays an essential role in host biochemistry, immunology, physiology and resistance in gut infections (Walter 2008). Gut microflora in humans has great importance in metabolic processes such as energy extraction from food that controls obesity, cardiovascular disease, diabetes, depression (Rolfe 2000; Tremaroli and Bäckhed 2012). *Lactobacillus* sp. and *Bifidobacterium* maintain gut homeostasis and human health by inhibiting the growth of pathogenic microorganism such as *Escherichia coli* sp., *Salmonella* sp. and *Listeria monocytogenes* in the gastrointestinal tract, in the urinary tract, etc. (Klaenhammer 1988, 1993; Rivière et al. 2016). The antagonistic nature of *Lactobacillus* sp. and

Table 2 Effect of mixed manno-oligosaccharides and inulin on growth of probiotic and non-probiotic bacteria (Ghosh et al. 2015)

Bacteria	Mixed manno-oligosaccharides ($\times 10^7$ cells/ml)		Inulin oligosaccharides ($\times 10^7$ cells/ml)	
	0 h	34 h	0 h	34 h
Lactobacillus acidophilus 4495	12 ± 4.0	280 ± 6.0	12 ± 4.0	160 ± 4.0
Bifidobacterium infantis 41661	1.0 ± 0.2	30 ± 0.8	1.0 ± 0.1	17 ± 0.7
E. coli DH5α	0.2 ± 0.02	ND	0.2 ± 0.02	ND
E. aerogenes 3030	0.7 ± 0.02	ND	0.7 ± 0.2	ND

Bifidobacterium sp. is because of the production of inhibitory compounds such as hydrogen peroxide, organic acids, bacteriocins and broad-range antibiotic-like substances (Denev 2006). Organic acids produced by *Lactobacillus* sp. and *Bifidobacterium* sp. reduce the pH of human colon and therefore decrease the solubility of bile salts, increase mineral absorption, decrease ammonia absorption, inhibit the growth of harmful microorganism and had anti-inflammatory properties (Rivière et al. 2016). *Lactobacillus* sp. and *Bifidobacterium* also produce vitamin B and lowers the blood cholesterol level in the gastrointestinal tract of human (Gomes and Malcata 1999). Therefore, the growth of beneficial microflora in the gut has great value.

Recent studies showed that manno-oligosaccharides from copra meal possessed prebiotic properties (Ghosh et al. 2015). They showed that mixed manno-oligosaccharides from copra meal have application in prebiotic. He had reported that manno-oligosaccharides increase the number of prebiotic bacteria such as *Bifidobacterium infantis* and *Lactobacillus acidophilus* from 1.0×10^7 to 30×10^7 cells/mL and 12×10^7 to 280×10^7 cells/mL, respectively, within 34 h as compared to standard prebiotic inulin and on the other hand it does not support the growth of pathogenic enteric bacteria (*Escherichia coli* and *Enterobacter aerogenes*) (Table 2). Thus MOS can be used as prebiotic agents.

6.2 *Manno-oligosaccharides as Antitumor Agent*

In Western industrialized countries, the second most frequent type of cancer is colon cancer in humans (Wollowski et al. 2001; Fernández et al. 2015). Use of prebiotic and probiotic is favourable over commercial anti-anticancer agents in cancer treatment. These prebiotic and probiotic agents possess anticancer properties and detoxify the genotoxins in the gastrointestinal tract (Wollowski et al. 2001). Recent studies showed that manno-oligosaccharides from copra meal possessed antitumour properties (Ghosh et al. 2015). They showed that mixed manno-oligosaccharides from copra meal possessed antitumour activity against colon cancer HT29 cell line. It was reported that MOS at 500 μg/ml concentration

reduce the percentage survival of HT29 cells to 50% after 24 h of incubation and 40% after 48 h of incubation, however normal colon cancer cells remain unaffected by MOS treatment. These results indicated that manno-oligosaccharides significantly, affect human colon carcinoma cell HT29 line by inhibiting its proliferation and thus possessed the antitumour activity (Fig. 5). Thus, MOS can be used as chemotherapeutic agents for colon cancer treatment.

6.3 Manno-oligosaccharides as Anti-allergic Agent or Immune Modulator

In the past few years, atopic allergies have increased continuously, mainly because of the change in food habits and environment. Allergic reactions are immunological reactions associated with an antigen present in food and environment (Ozaki et al. 2007). For the suppression of allergic reactions prebiotic and probiotic agents were used rather than commercial anti-allergic agents (Fukasawa et al. 2007). The increase in serum IgE antibody, infiltration of acidophil cells and cytokine production (IL-8 and TNF-α) indicate allergic inflammation in the body (Ozaki et al. 2007). Recent studies showed that manno-oligosaccharides from coffee mannan suppress allergic reactions (Ozaki et al. 2007). In vitro studies of manno-oligosaccharides from coffee mannan on ovalbumin-sensitized mice showed that there was a decrease in serum IgE titre from 56.2 to 36.2. There was a significant decrease in cytokine production from Peyer's Patch i.e. cytokine TNF-α level decrease from 348 pg/ml to 142 pg/ml and cytokine IL-8 level decrease from 287 pg/ml to 21.7 pg/ml (Ozaki et al. 2007). Thus manno-oligosaccharides possess anti-allergic properties and have a potential to be used as anti-allergic agent.

7 Conclusion

The agro-waste rich in hemicellulose content can be used to recover mannan and manno-oligosaccharides. The utilization of agro-waste as a source of mannan will not only overcome the problem of its disposal but also lead to the synthesis of the value-added products. Manno-oligosaccharides can be used as prebiotics to improve the growth of beneficial microflora in the gastrointestinal tract and therefore, can be used for functional food applications. They also impart antitumour and anti-allergic properties to the host, so it can be used as a potential chemotherapeutic agent and as an immune modulator. The clinical trials of manno-oligosaccharides on the animal model or human may further confirm their health benefits.

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Chapter 13
Computational Modelling and Prediction of Microalgae Growth Focused Towards Improved Lipid Production

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Abstract In response to compelling demands worldwide for sources of renewable and eco-friendly energy feedstock, research and development in microalgae as a sustainable alternative has garnered interest. In order to make microalgae-derived fuel more competitive than fossil fuels in terms of cost, bottlenecks like scalability, better biomass production and enhanced lipid production without nutritional stress need to be resolved. In this chapter, the various computational modelling methods applied to microalgae growth in various environmental conditions have been reviewed. The possibility and potential of employing these models for better lipid production have also been highlighted, as better predictability of models can lead to better transgenic algal platform. Moreover, the upcoming models integrating omics data with flux analysis have also been discussed that has resulted in updated simulation due to the incorporation of data about novel genes. Lastly, the need for close collaboration between biochemical engineers, molecular biologists and modellers

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have been emphasised to validate the models on natural environment apart from laboratory conditions.

Keywords Computational modelling · Flux balance analysis · Product optimisation · Microalgae · Lipid and biomass production

1 Introduction

Microalgae-derived biofuel has gained a lot of interest in the past decade as an efficient carbon dioxide mitigator and a producer of energy-dense reduced carbon as storage molecules called triglycerides that can be converted into transport grade biodiesel. The carbon removal efficiency, photosynthetic rate and oil content of microalgae are several times better than land plants, making them highly beneficial environment-friendly microbes in an attempt to alleviate energy crisis and global warming (Gonçalves et al. 2013; Georgianna and Mayfield 2012). The limitations of microalgal biofuels are primarily economic as they need to be competitively priced against fossil fuels. The costs associated with cultivation under operating conditions are one of the significant among others, as these parameters affect the biomass and lipid productions directly. Microalgae have been proven to accumulate high amount of TAG under stress like light, salinity, nitrogen depletion, phosphorus and iron depletion, etc., but at the same time biomass productivity reduces along with reduction in photosynthetic efficiency due to the cessation of growth. Thus, under stress total lipid production is affected adversely resulting in lower oil yield which is undesirable economically. The more understanding of the metabolic intricacies of lipid metabolism like cross-talk among different sub-compartments of lipid production, function of regulatory proteins, rate-limiting steps, supporting metabolic pathways that provide the carbon flux, partitioning of carbon pool and lastly influence of environmental factors would help us understand the principles of TAG production (Banerjee et al. 2017; Ho et al. 2014; De Bhowmick et al. 2015).

With the advent of incoming omics approaches, i.e., large amount of data sets used for ‘quantification as a whole’ studying the cellular behaviour during various levels of regulation such as genomic, transcriptomic, proteomic and metabolomic levels (Merchant et al. 2007; Park et al. 2015; Nguyen et al. 2011; Valledor et al. 2014; Radakovits et al. 2012). Based on these molecular biology data, metabolic network reconstruction can be made from the computational models and bioinformatics (Boyle and Morgan 2009; May et al. 2009). These integrative modelling techniques enable us to build and improve the existing genome-scale reconstruction models having more predictive capabilities that can be utilised towards the ultimate goal of rational metabolic engineering for high lipid yield through genome engineering tools. Here, we discuss the ongoing attempts of computational modelling needed for pathway prediction and data analysis with focus on increased lipid production (Lenka et al. 2016; De Bhowmick et al. 2015).

2 Computational Modelling and Objective Response Prediction

With the advancement in computational capabilities along with advanced softwares, modelling has been employed to many microalgae for pathway prediction, data analysis and optimisation of bioproducts (Packer et al. 2011; Jiang et al. 2014; Yang et al. 2010; Tevatia et al. 2012). Several modelling techniques have emerged, each having their distinct quantitative and qualitative specialities with pros and cons, which are applied either individually or together (Lenka et al. 2016; Nikoloski et al. 2015) (Fig. 1). Till now, *Chlamydomonas reinhardtii* being the first among the microalga to be sequenced and extensively researched (Terashima et al. 2011; Choi et al. 2013; Merchant et al. 2007; Wang et al. 2011; Moellering and Benning 2010), models are primarily based on *C. reinhardtii* data to predict its growth against several environmental conditions (Dal'Molin et al. 2011). Computational models help reducing the cost and time of wet laboratory work by letting us predict the relationship of algal cell growth with the changing environment and the bio-products they produce as end product of metabolism (Chang et al. 2011). For predictions, genome-scale reconstructions (GEM) are made based on the database about metabolic pathways and enzymes from various organisms like KEGG (Kyoto encyclopaedia of genes and genomes) and MetaCyc. Such complete GEMs were built by Chang et al. (2011) and Dal'Molin et al. (2011). The models generally used to predict biochemical or metabolic processes are graph or topology based, flux balance analysis, kinetic modelling, dynamic reduction of unbalanced modelling (DRUM) and constraint-based modelling using energetics. One central theme that all the approaches revolve around is how the metabolites flow through a pathway. Among them, FBA and kinetic modelling make the most quantitative approach, so has been observed to yield good results in the prediction of TAG enhancement. The following equations are provided based on which flux and kinetic models are formulated (Baroukh et al. 2015a; Kauffman et al. 2003; Edwards et al. 2002):

$$A + 2B + 3C = 5D \tag{1}$$

$$\frac{\mathrm{d}}{\mathrm{d}t}\begin{pmatrix} A \\ B \\ C \\ D \end{pmatrix} = Nv = N\begin{pmatrix} VA \\ VB \\ VC \\ VD \end{pmatrix} = 0 \tag{2}$$

$$\text{Reaction rate} = -\frac{\mathrm{d}[A]}{\mathrm{d}t} = -\frac{\mathrm{d}[B]}{2\mathrm{d}t} = -\frac{\mathrm{d}[C]}{3\mathrm{d}t} = \frac{\mathrm{d}[D]}{5\mathrm{d}t} \tag{3}$$

$$= -vA = -\frac{vB}{2} = -\frac{vC}{3} = \frac{vD}{5} \tag{4}$$

Equation 1 describes that one molecule of A, two molecules of B and three molecules of C react to produce five molecules of D. Equation 2 forms the basis for FBA where loss or gain of metabolite molecules with time depends on the

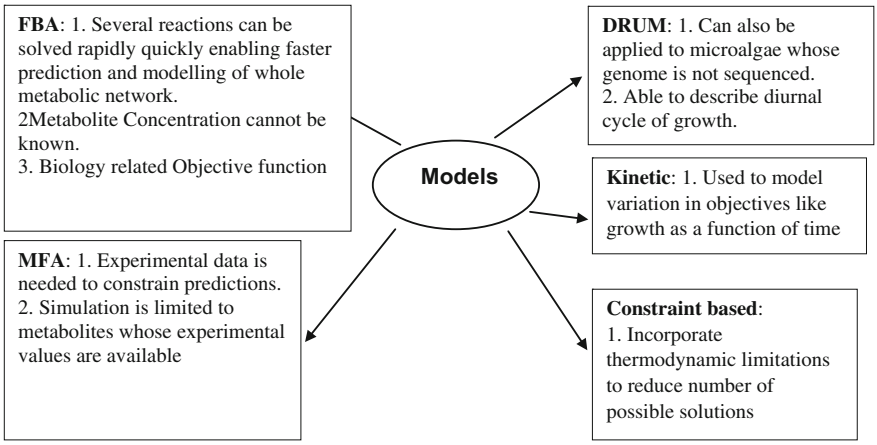

Fig. 1 Precise comparison of different techniques that are used to model microalgae growth—advantages and limitations

stoichiometric matrix called 'N', formed by reactants *A*, *B*, *C* and product *D*, and flux called '*v*' for each of the metabolites in the reaction concerned. This equation is set in quasi-steady state, where incoming flux of *A*, *B*, *C* is equal to outgoing flux of *D*, and the sum equals zero. Equations 3 and 4 which form the basis of kinetic model signify the relationship between the rate of change of concentration of *A*, *B*, *C* and *D* with their fluxes, where flux associated with consumption of reactant *A* (i.e., rate of loss of *A*) equals half the flux of *B* and one-third the flux of *C* and one-fifth the flux of product *D* (i.e., rate of formation of *D*). Recently, computational modelling has been explored where different algal growth types (Baroukh et al. 2015a) and different phases of metabolism along with biomass productivity are described (Baroukh et al. 2015b).

3 Flux Balance Analysis

This approach examines the flow of metabolites through the system that can be extended with the obtained data to predict through entire metabolic network. FBA applies to steady-state conditions, where the incoming flux of metabolite must be equal to the outgoing flux. FBA is best explained by Eq. 2 where the experiments are not conducted to determine the metabolite fluxes unlike MFA, a variant of FBA (Orth et al. 2010). Ideally, studies have shown that FBA does not comply with unique steady-state flux distribution and FVA (flux variance analysis) needs to be carried out to know the range of fluxes that agrees with the objective function (Nikoloski et al. 2015). In this approach, multiple levels of limits or bounds can be set such as reaction conditions and objective functions that restrict the flux within a certain range. For example, objective functions like maximisation of biomass or

TAG and minimisation of nutrient use have been attempted in FBA (Wu et al. 2015). Integration of FBA and MFA approaches has also been attempted for making better models by using the time course omics data in FBA analysis (Kleessen et al. 2015). FBA has been used in lipid synthesis pathways where the importance of particular enzyme has been predicted. It has been done by sensitivity analysis of a pathway by changing the constraints of the individual reactions and observing the resulting effect on lipid flux (Chang et al. 2011; Dal'Molin et al. 2011). In addition to solving the small fluxes in individual reactions, the gene knockouts (InDels) can also be studied in these reactions by FBA. A modification of FBA, minimisation of metabolic adjustment (MOMA), is more suited to calculate the change in internal fluxes caused by the mutants (Bhowmick et al. 2015).

GEMs are used in FBA that incorporates genomic, transcriptomic and proteomic data (Dal'Molin et al. 2011). AlgaGEM has been employed for maximisation of hydrogen production under various growth conditions while minimal utilisation of carbon and energy (Dal'Molin et al. 2011). Another GEM, iRC1080, was developed by Chang et al. (2011) that maximised biomass production by modelling the effect of light utilisation. This model also identified the optical spectral range for light absorption and simulated various phototrophic, mixotrophic and heterotrophic growths under various light regimes and environmental changes. An updated model, iCre1135, was introduced by Imam et al. (2015) that considered novel genes and improvised reactions (e.g., pyruvate carboxylase reaction was treated as reversible) to simulate TAG production during autotrophic growth. FBA models have been used in several studies related to lipid production to better understand the (i) interconnection between energy and carbon metabolism in different light regimes (light/dark) in *Chlorella sp.* (Muthuraj et al. 2013) and *Chlorella pyrenoidosa* (Feist et al. 2009), (ii) dynamics of the carbon sharing between lipid and carbohydrate/starch in light regime in *Ostreococcus tauri*, (iii) flow of carbon flux towards lipid formation under nitrogen stress in *Phaeodactylum tricornutum* (Levitan et al. 2015) and (iv) primary metabolism by calculating the intracellular flux in *C. reinhardtii* (Boyle and Morgan 2009)

Thus, more updated models with crucial modifications can generate better simulated genetic engineering strategies having enhanced TAG yield.

4 Metabolic Flux Analysis

MFB modelling approach is constructed on experimental determination of flux through metabolite concentration using mass spectrometry and then fitting them in numerical data. Wu et al. (2015) used non-stationary C-13 based INST-MFA rather than steady-state MFA, as intracellular flux can be measured through INST-MFA. The metabolite levels and intracellular flux were experimentally validated by LCMS and kinetic isotope tracing. This data was fed to FBA to maximise objective function like biomass concentration and corresponding specific growth rates were predicted. A significant finding from this study was that the acetyl-CoA utilisation

during heterotrophic growth was 33-fold higher than autotrophic growth, thereby showing a possible way of augmenting TAG accumulation during heterotrophic growth, which is by enhancing the acetyl-CoA pool. They proposed that this phenomenon could be exploited by inhibiting citrate synthase, an enzyme of Kreb's cycle, which can help flow the acetyl-CoA flux towards lipid synthesis (Wu et al. 2015). One important application of MFA is to predict the genetic changes that can produce desired traits at optimum level, by in silico creation of mutation that leads to desired products. Kliphuis et al. (2012) conducted experiments on *C. reinhardtii* under red light illumination to record the biomass, lipid, carbohydrate and protein content to infer that a balance must be reached between light intensity growth rate in order to maximise productivity of both bioproducts and biomass.

5 Kinetic Modelling

A kinetic model ideally deals with rate equation of a chemical reaction of interest that is featured by a reaction rate, order of reaction, concentrations of reactants and products. Kinetic models can also be used to track a variable like biomass, lipid content or pigment concentration with change in time. The rate equation will define the rate of change of that variable against change in time, i.e. it can be the entire time course of cultivation for a particular microalga (Ullah et al. 2006). From Eq. 2, the rate equation can be derived where 'k' is the rate constant; [A], [B] and [C] are initial concentrations of the reactants and 'x', 'y' and 'z' are the order of the metabolites generated through experiments provided in Eq. 5:

$$\text{Rate} \ = k_1[A]^X[B]^Y[C]^Z \tag{5}$$

When several equations are considered, each rate equation becomes simple ordinary differential equation (ODE) coupled with one another. Differential equation solvers like ode15s are employed to obtain (solution space) a set of solutions to that equation. In ODE solvers of a kinetic model, the inputs provided can be initial concentrations of metabolites or rate equations itself while the output being the concentrations of product with change in time say, biomass or lipid content (Ullah et al. 2006).

Several models are existing that describe the kinetics of microalgae growth with variation of culture parameters. Kumar et al (2014) described the growth kinetics considering light intensity, temperature and other process parameters. The kinetic model equation is provided below:

$$C = \frac{C_{\max}}{1 + \left(\frac{C_{\max}}{C_0} - 1\right)e^{-k_c t}}, \tag{6}$$

where C is the biomass concentration (gl^{-1}), t is the time, Kc is the growth rate (day^{-1}), C_{max} is the maximum biomass (gl^{-1}) and C_0 is the initial biomass (gl^{-1}). Kc was experimentally determined for each alga under variable CO_2 concentrations

in that study. The MAE (mean absolute error) is determined by Eq. 7 to test the fitness of the model:

$$\mathrm{MAE} = \frac{1}{n}\sum_{i=1}^{n}\left|\frac{C_{\mathrm{predicted}} - C_{\mathrm{experimental}}}{C_{\mathrm{experimental}}}\right| \times 100\%, \tag{7}$$

where $C_{\mathrm{experimental}}$ and $C_{\mathrm{predicted}}$ are the observed and model-derived values of biomass concentration, respectively.

It has been established that lipid accumulation increased in nitrogen depletion with simultaneous decrease in biomass growth resulting in lowering total lipid content. Now models are being constructed to model the objective functions of growth and lipid under varied concentrations of ammonium under carbon dioxide sparging (Tevatia et al. 2012). Experimental results about biomass and lipid were fitted in differential equations of Baranyi–Roberts and Luedeking–Piret, respectively. Empirical model generated equations that predicted with reasonable accuracy about the correlation between specific growth rate and lipid content which may help a decision-maker in maximisation of either of the conflicting objectives (biomass or lipid) at varying levels of ammonium (Tevatia et al. 2012). An interesting observation was found from kinetic modelling of algal growth rate, lipid yield and glycerine consumption during photoheterotrophic condition in *Chlorella minutissima* that lipid and biomass production was at its best in heterotrophic than autotrophic conditions (about 26–36 times) and initial glycerine concentration helped increase in both lipid and biomass productivities (Yang et al. 2010). This analysis was possible by carrying out a sensitivity analysis of the experimental results to create a model using Universal Inverse Modeling Code (UCODE) to estimate the parameter values. Similarly, algal growth rate and neutral lipid accumulation were modelled by Packer et al. (2011) in *Pseudochlorococcum* sp. The model established a relationship between the effect of light and nutrients like nitrogen, iron, phosphorus on growth and lipid production. Prediction models have been built by response surface methodology (RSM) optimisation tool involving oleaginous algae like *Nannochloropsis* sp. and *Dunaliella tertiolecta* to maximise lipid content. The models demonstrated that individual stress factors like salinity and light intensity along with their synergistic effects are the most potent inducers of lipid yield. In addition to that, higher biomass and carbon availability can add to the total lipid content (Banerjee et al. 2016a, b; Kumar et al. 2015). Thawechai et al. (2016) obtained similar results using *Nannochloropsis* sp. for maximisation of maximise biomass and lipid productivity. High light intensity and elevated CO_2 level were predicted to be important for lipid production. Very recently, a kinetic model was used called Verhulst logistic that simulated the relationship between inorganic carbon availability and light with maximum biomass growth and specific growth rate based on preliminary experimental results. This updated model can be readily employed to model objective function like lipid production against carbon source as carbon supply is an important limiting factor (Packer et al. 2011). These updated models along with the genetic information already obtained from detailed systems biology and other studies (Table 1) can lead to more accurate simulation of lipid

Table 1 Genes and proteins responsible for lipid production that can be utilised in metabolic modelling (Lenka et al. 2016; Banerjee et al. 2017)

Genes	Utility
1. Transcription factors like ABI4, AP215, WRI1, NRR1, MLDP, DOF, bZIP	Regulation of lipid metabolic pathways and enzymes like DGAT1
2. Enzymes like DGAT1, AAE, ACCase, KASII, KAS, GPAT	Responsible in lipid synthesis and fatty acid synthesis
3. Lipase, PDAT1, PNPLA3	Helps in lipid remodelling during stress

production that can help in rational metabolic engineering and transgenics (Banerjee et al. 2016a, b) reducing the number of experiments and cost associated with it.

6 Conclusion

Coordinated efforts have been made at physiological, molecular and cellular levels to understand the lipid regulatory network through high throughput analysis, transcriptomics and proteomics data. These data sets sometimes at whole genome level reveal the information about rate-limiting steps in a pathway, enzymes, regulatory molecules and novel genes that lead to common objective of lipid production. Several biochemical engineering experiments have also been performed that described the effect of extrinsic factors like light intensity, salinity, temperature, nutrients, carbon source and photobioreactor have on various microalgal cultivation modes like autotrophic, heterotrophic and mixotrophic. These set of studies have provided a wealth of information for the modellers to update and modify their models about lipid production and biomass growth and come up with model that predicts or simulates the metabolic processes with improved accuracy. Efforts must be initiated to validate the models those predicted in laboratory, in actual natural conditions through gradual scale-up and repeated cycles of simulation and testing must be carried out. As there is limited research outside *Chlamydomonas reinhardtii*, model building must be attempted in oleaginous strains of *Nannochloropsis*, *Dunaliella* and *Botryococcus braunii* with a focus on more lipid production. Serious efforts and close collaborations are needed between biologists in wet laboratory and modellers to design better-engineered algae that are committed towards lipid production.

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Chapter 14
Perennial Energy Crops on Drained Peatlands in Finland

Narasinha J. Shurpali

Abstract Finland has a large area under peatlands (more than 10 M ha). Under pristine conditions, peatlands are a sink for atmospheric CO_2 and a source of methane to the atmosphere. Since the 1950s, peatlands in Finland have been drained for forestry, agriculture, and peat extraction for energy. Once drained, peatlands transform into large sources of CO_2 to the atmosphere and weak sinks for methane. Finding a suitable after-use option for drained peatlands is complicated by the specific nature of the drained peatland type. As an after-use option on a cutover peatland, the cultivation of a perennial bioenergy crop on a drained peatland in eastern Finland was explored during 2004–2011. The long-term measurements of greenhouse gas exchange from this study site showed that the benefits from bioenergy crop cultivation vary strongly depending on the climatic conditions during the crop cultivation phase.

Keywords Bioenergy · Greenhouse gas exchange · Climate change

1 Introduction

Peatlands occupy a small part of the Earth's land area (Turetsky et al. 2015). However, they are globally important because of their high carbon content. Pristine peatlands are currently a weak carbon sink and a moderate source of methane (Frolking et al. 2011; Nilsson et al. 2008). Peat is a decaying organic matter that has accumulated under-saturated conditions. Formation of peat occurs in areas with surplus soil moisture (Camill 2000). Peatlands are common in regions with high precipitation excess (e.g., the temperate and boreal zones). The northern peatlands account for about a third of the world soil carbon (Gorham 1991). They sequester

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more carbon dioxide from the atmosphere than they emit. This biogeochemical C cycle is greatly perturbed when peatlands are subjected to drainage and land use. Since the 1950s, nearly 60% of the original peatland area has been drained for several purposes such as forestry, agriculture and peat extraction for energy (Minkkinen et al. 2002). About six million ha of peatlands have been drained in Finland (Laiho and Laine 1997). The drainage of peatlands affects the regional carbon cycle in a way that the originally carbon accumulating ecosystems are transformed into strong sources of CO_2 to the atmosphere.

Drainage of peatlands has an important role in regulating the regional and thus, the global climate (Limpens et al. 2008). A lowering of the water table triggers drying (Artz et al. 2008) so that the soil decomposition processes tend to be not limited by the lack of oxygen (Fenner and Freeman 2011). With more oxygen available, the soil organic matter (SOM) decomposes more rapidly leading to more carbon dioxide being released into the atmosphere. Simultaneously, methane emissions from drained peatlands decline due to a decrease in methane production and increased oxidation. Moreover, several studies hinted that the drained peatlands may even act as small-scale methane sinks (Hyvönen et al. 2009).

While the use of peatlands as an economic resource was deemed necessary, the fact that such land use (drainage) leads to environmental problems is increasingly being realized the world over since the 1990s. Hence, continued attempts are being made in Finland and elsewhere to find suitable land use options for drained peatlands after their intended land use (Vasander et al. 2003). Simply abandoning these lands is considered environmentally untenable. Therefore, several options have been suggested as after-use options: intensive forestry, rewetting, restoration to a functional peatland, creation of artificial wetlands, use of cutover peatlands for agriculture and cultivation of energy crops, etc. Decision on which option to use at a given drained peatland is complex. In view of the peatland formation processes and origin, every peatland is different in its physiography, hydrology, the physical and chemical characteristics of the remaining peat, etc. In addition, the physical layout of the drainage ditches varies from one location to the other. Thus, peatland restoration and reclamation criteria are specific to the peatland site under investigation (Vasander et al. 2003).

2 Ecosystem Greenhouse Gas (GHG) Exchange

Quantifying GHG (CO_2, CH_4, and N_2O) exchange from a given ecosystem and understanding the processes that describe the measured exchange rates are necessary for identifying sinks and sources of GHGs. Since the GHGs are radiatively important, their increasing levels in the atmosphere are a cause for concern. Increasing GHG concentrations in the atmosphere is directly linked to increasing global surface temperature and thus to climate change (Oreskes 2004). Currently, the two most widely used techniques for measuring GHG exchange are the eddy

covariance (EC) and the closed chamber (manual or automated) methods (Riederer et al. 2014). The eddy covariance method measures turbulent GHG fluxes for monitoring trace gas flux dynamics at a landscape level (Baldocchi 2014). EC towers have become a common approach for monitoring direct and continuous (year-round) GHG fluxes from different ecosystems. Nowadays, the EC fluxes of CO_2 and water vapor are routinely made across all continents. A global network of EC stations called "FLUXNET" links across a range of regional networks in different parts of the world (http://fluxnet.fluxdata.org). This network has registered more than eight hundred active and historic flux measurement sites distributed in diverse climatic and vegetation zones.

EC fluxes are measured by estimating the covariance between the fluctuations in vertical velocity and target scalar mixing ratios (water vapor, all GHGs and biological volatile organic compounds, etc.) at a given measurement site (Baldocchi 2014). With the eddy covariance method, one can measure fluxes directly over a large area with no disturbance to the microenvironment under investigation. High-frequency EC measurements are collected for the purpose, capturing diurnal and seasonal dynamics. EC method is the first choice if the research objective is to characterize the site average GHG sink/source strength. However, as the EC method presents a large area integrated flux rate, chamber-based methods (either manual or automated) assume importance if the research interest is in understanding the inherent spatial heterogeneity of a site in terms of the distribution of cold and hot spots of GHG dynamics. EC method is resource-intensive, as this method requires expensive, fast response instruments for detecting small changes in the atmospheric GHG concentrations. Large datasets collected through EC techniques require personnel trained in instrumentation and micrometeorology. On the other hand, manual chamber methods are inexpensive, easy to use, and are suited for developing process-level understanding of soil, plant, and microbial interactions.

3 Annual Cropping Systems on Drained Peat Soils

Finland is the world's northernmost agricultural country. Finnish farmlands reach from the 60th latitude to north of the Arctic Circle. The major crops include cereals (wheat, rye, barley, and oats), turnip, rape, potato, sugar beet, and grasses (Natural Resources Institute Finland 2017). These soils have been reported to trigger increased annual emissions of CO_2 and N_2O (Statistics Finland 2015). The GHG emissions from agricultural peat soils in the Nordic countries (Finland, Sweden and Norway) have been compiled (Maljanen et al. 2010). This compilation of all available data on GHG emissions as of the time of this publication included 16 perennial grass sites, 6 sites cultivated with barley, and 1 site each with potato and carrot. Agriculture plays a minor role in the Nordic economy that is dominated primarily by the forestry sector. Nevertheless, considering the fact that the Nordic region is among global regions that are most vulnerable to climate change, the

available data on GHG emissions from Nordic agriculture are far too limited for developing proper mitigation strategies. The GHG emissions from agricultural peatlands vary in magnitude owing to differences in cultivation methods, crops, and climatic conditions. Based on the available data, it can be inferred that the peat soils used for crop cultivation are significant sources of CO_2 and N_2O to the atmosphere (Maljanen et al. 2010).

Most of the measured GHG emissions from agricultural peat soils are based on chamber methods with a few studies employing the EC method to monitor the net ecosystem CO_2 exchange (NEE) (Maljanen et al. 2010). Depending on the water table level, croplands on peat soils are small sinks or sources of CH_4 (Hyvönen et al. 2010). Annual crops on peat soils (cereals) are larger sources of CO_2 and N_2O than perennial grasslands owing to increased pore space resulting from regular tillage. The cereal crop cultivated on a peat soil in Finland has been shown to be a minor sink for CH_4 and a source of CO_2 (17.7 t CO_2 ha^{-1}) and N_2O (17.1 kg N_2O ha^{-1}). Annual N_2O emission from a potato field has been estimated to be 15.7 kg ha^{-1} (Regina et al. 2004). Compared to mineral soils, annual crops on drained peat fields have been shown to be a net source of CO_2 owing to a high degree of decomposition of the soil organic matter. In addition, these soils have been known to emit high amount of N_2O to the atmosphere in comparison to mineral soils as they contain a large N-pool. While CO_2 accounts for 80% of the global warming potential of an agricultural peat soil, N_2O emitted from these soils accounts for the remaining potential. The methane sink strength of the drained peat soils is too low to have any cooling impact unless the water table level is artificially elevated to reintroduce methanogenic microbes in these peat soils.

4 Perennial Agriculture on Drained Peat Soils

It is apparent from the above discussion that converting agricultural drained peat soils into croplands or grasslands for forage has not succeeded as a sustainable after-use option for drained peatlands in Finland. To circumvent this problem, policy suggestions were made to employ perennial energy crop cultivation on such problematic soils, a potential sustainable solution in the Nordic region, Germany, and Estonia where peat extraction for energy is a common practice (Lewandowski et al. 2003). Indeed, the idea of growing reed canary grass (RCG—*Phalaris arundinaceae*, L.) as a perennial bioenergy crop on cutover peatlands in Finland gained momentum since the mid-1990s. This crop was the obvious choice at the time as several studies reported that RCG is well suited for cultivation under low temperatures and humus rich, waterlogged, nutrient-poor soils of the Nordic countries (Lewandowski et al. 2013). An additional reason for selecting this crop as a source of renewable energy was that the atmospheric carbon taken up by the crop during the process of photosynthesis would compensate the emissions resulting from the combustion of harvested RCG biomass. Thus, the RCG cultivation was considered to be a carbon-neutral energy source (Lind et al. 2016). However, the

basis for such assumptions was a few studies wherein the aboveground biomass or productivity was viewed as an indicator of the ability of this crop to fix the atmospheric carbon. Most of the studies conducted on reed canary grass cultivation on organic soils in Finland focused on measurement of rates of biomass accumulation under different agronomic practices (Sahramaa and Jauhiainen 2003). The objective of these studies was to generate biomass data for peat energy industries to assess the role of RCG as a fuel supplement to peat-derived energy. Such studies addressing the agronomic traits of the RCG crop recommended the use of RCG as a perennial bioenergy crop on marginal soils of cutover peatlands in the Nordic countries (Sahramaa and Jauhiainen 2003). However, some studies suggested that the bioenergy crops should be banned from cultivation on organic soils owing to their high CO_2 and N_2O emissions (Beringer et al. 2011; Crutzen et al. 2008). In view of these conflicting reports, the Biogeochemistry Research Group at the University of Eastern Finland undertook a field experimental study with the broad aim of investigating the environmental impact of the cultivation of RCG on the carbon balance and N_2O emissions from a cutover peatland. The results of this study are summarized in the following.

The field experiment was conducted on a 15-ha cutover peatland site with reed canary grass cultivation in the Linnansuo peat harvesting area (62° 30′N latitude and 30° 30′E longitude) in the Tuupovaara village in eastern Finland. Based on the 1961–1990 climatic averages, the region experiences a mean temperature of −11.9 °C in January and 15.8 °C in July. The annual mean temperature of the region is 2.0 °C and annual precipitation amounts to 600 mm. The mean annual duration of snow cover in the region is 183 days with a mean annual snow depth 63 cm. An aerial view of the study site with the location of the EC instrument tower marked in red is shown in Fig. 1 (left panel).

The instruments were mounted on a tower (Fig. 1: Middle panel) at a height of 3.7 m above the ground and aligned at an angle of 225°, in the direction of the predominant winds in the region. The EC system consisted of a 3-D sonic anemometer (CSAT-3, Campbell Sci.) and an open-path infrared CO_2/H_2O

Fig. 1 Left panel: aerial view of the drained peatland site in Eastern Finland where the reed canary grass (RCG) was cultivated as a perennial bioenergy crop (the red mark shows the location of the Eddy Covarinace (EC) instrument tower at the site); middle panel: the EC instrument tower with various instruments mounted at 3.7 m above the soil surface; right panel: a close-up of the fast response infrared gas analyzer (IRGA) and three-dimensional sonic anemometer mounted on the EC tower (*Photo credits* Alpo Hassinen and Jari Huttunen)

analyzer (IRGA, Li-7500, LI-COR) for measuring high-frequency (10 Hz) vertical wind velocity and CO_2 and water vapor mixing ratios, respectively (Fig. 1: Right panel). Supporting measurements included air temperature, air relative humidity and pressure, net radiation and its components, photosynthetically active radiation wind speed and direction, soil temperature profile, precipitation, soil water potential, soil moisture, water table level, and snow depth. The measurements began in early 2004 and continued until the end of 2011. Flux calculations were performed employing the standard procedures (Papale et al. 2006) to obtain 30 min averaged flux estimates. Further details on the data processing procedures are available in our published work on the topic (Shurpali et al. 2009, 2010, 2013). In addition to the EC-based estimation of net ecosystem CO_2 exchange, chamber-based emissions of soil CO_2, CH_4, and N_2O emissions were carried out at the study site (Hyvönen et al. 2009; Shurpali et al. 2008).

The various crop-growing seasons during the study period exhibited contrasting climatic conditions. Based on several years of continuous CO_2 balance measurements, the RCG cultivation system was found to be a strong sink for atmospheric C on organic soils (Shurpali et al. 2009, 2010, 2013). In response to varying rainfall patterns, the net ecosystem CO_2 exchange (NEE) from the perennial bioenergy crop showed a wide interannual variation. Carbon cycling in this ecosystem was found to be sensitive to the amount and timing of precipitation. The crop fixed large amounts of atmospheric CO_2 during wet years (annual rainfall above the 30-year average), while the photosynthetic potential of the crop was drastically reduced during dry years owing to the climatic stress (Shurpali et al. 2009).

Some studies have reported that biofuels may not bring the intended atmospheric benefit owing to high emissions of N_2O during crop cultivation (Crutzen et al. 2008). For example, the amount of N_2O emitted from bioenergy crops such as rapeseed and corn have been found to be twice the amount of N_2O than previously thought (Crutzen et al. 2008). Such high N_2O emissions override any benefits of avoiding fossil fuels and potentially contributing to global warming (Crutzen et al. 2008). However, the bioenergy crop at our site emitted consistently low amounts of nitrous oxide and methane each year (Hyvönen et al. 2009). The low N_2O emissions resulted from the perennial nature of the crop cultivation system (requiring no tillage on an annual basis), low NPK fertilizer requirement of the crop, and poor nutrient status of the organic soil (C:N ratio of greater than 40). Methane emissions in this drained organic soil were low due to reduced methane production and high methane oxidation (Kettunen et al. 1999).

Having characterized the carbon balance and soil GHG emissions, a proper accounting of all energy inputs and outputs of the RCG crop was done to perform a life-cycle assessment (LCA) (Shurpali et al. 2010). For the purpose, the various crop management practices, costs associated with fertilizer manufacturing, and distances (about 70 km) involved in transporting the biomass from the field to nearby combustion plants were considered. Owing to low nutrient requirement of the crop and biomass yields, the estimated management-related emissions were a minor part of the bioenergy LCA in this ecosystem. Chemical inputs (fertilizers and lime) and transport of harvested biomass were the major components in the annual

total CO_2 emissions related to the management of the RCG cultivation system at this study site (Shurpali et al. 2010). Net GHG emissions from the bioenergy crop were compared with those from coal. Except during exceptionally dry years, net GHG emissions from bioenergy were found to be consistently lower than emissions from coal (Shurpali et al. 2010). This study showed that bioenergy crops could be cultivated on such marginal organic soils as the one under investigation in this study. This study also refuted the gross generalization that bioenergy crops are carbon-neutral. Net GHG emissions from the bioenergy crop were compared with those of coal. The long-term field experiment described here showed that the bioenergy benefits are strictly dependent on climate. Favorable climatic conditions (well-distributed rainfall and moderate temperatures) favor atmospheric C fixation in crop biomass and in the soil (Shurpali et al. 2009, 2010). However, adverse climatic conditions leading to climate stress affect the photosynthetic potential of the bioenergy crops and thus reducing the bioenergy benefits. The RCG crop cultivation on this soil was found to be equivalent to using coal in terms of the net GHG emissions per unit of energy during dry growing seasons. The bioenergy crop performed better than coal during years when the climatic conditions favored high CO_2 sequestration by the plants and soil.

From an environmental friendliness point of view, bioenergy crops hold promise. The long-term field data from this experiment show that environmentally sustainable bioenergy production is possible even on some organic soil types. More long-term studies on different soil types and climatic conditions are needed for developing better bioenergy strategies. With this in view, RCG as a perennial crop on a mineral soil in Eastern Finland was cultivated (Lind et al 2016). The RCG crop had higher capacity to take up CO_2 from the atmosphere on a mineral soil than on the drained peatland site. However, while the N_2O emissions from the drained organic soil were negligible, they were high [2.8 kg N_2O ha^{-1} (Shurpali et al. 2016)] on the mineral soil. Therefore, for developing sound bioenergy policies, complete life-cycle analyses need to be performed. These analyses should take into account GHG dynamics during the crop cultivation phase and all other input and output costs in terms of CO_2 equivalents. In conclusion, whether a bioenergy crop is sustainable in all aspects depends not only on the environmental friendliness but also on the suitability of the biomass type for combustion in incineration plants. Burning certain biomass types may have detrimental effects on the furnaces used for combustion and the emanating flue gas may contain chemicals that are not conducive to human health (Sippula et al. 2017).

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Part III
Environmental Assessment and Waste Management

Chapter 15
Bioenergy Conversion from Aquatic Weed Water Hyacinth into Agronomically Valuable Vermicompost

Ankaram Snehalata and Kothur R. Rao

Abstract Increasing amount of waste due to urbanization, lifestyle and shift of huge masses from rural to urban largely contributes to the need for waste management. This developed a need of categorizing the waste into municipal waste, industrial waste, agricultural waste, hospital waste, etc., which will make it easy for the proper disposal, reuse and recycling of waste so determined effort should be taken for the management of waste. For this purpose, proper canalization of segregation, distribution, disposal, recycling, reuse of waste without deteriorating the environment is a matter of great concern. To succeed in these all the stakeholders like users creating waste, environmentalist, scientist, municipal stakeholders and doctors should take an initiative and need to participate in the same. For proper management of solid waste, continuous efforts have been made by recycling and reusing the plant weed. Vermicomposting is the biocomposting process of organic waste by earthworms and bacterial action directing the stabilization of organic matter. The final product produced in this process is known as vermicompost and it assists in the enrichment of soil as well as useful for sustainable agriculture. This technology is a boon for recycling of the solid waste generated from various sources including aquatic weeds. The prime objective of the present investigation is to convert waste into wealth. Its application to the agricultural field is also retrieving the waste garbage into gold. The present investigation is mainly focused on vermicompost production from water hyacinth (*Eichhornia crassipes*) collected from local perennial Sambhaji tank from Solapur and its application to a commercial crop groundnut (*Arachis hypogaea*) for total yield and biomass production. The significant output of the commercial crop groundnut displays the extended use of vermicompost in agriculture, providing an insight and approach for organic farming

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in future. Our results suggest that the vermibiotechnology is an eco-friendly and economically feasible technology and it is also helpful for converting waste into bioenergy.

Keywords Waste management · Vermicompost · Eichhornia crassipes Sambhaji tank · Arachis hypogaea · Bioenergy

1 Solid Waste: The Preamble

Solid waste is unwanted debris resulted from various residential, industrial and commercial activities. It may be categorized depending on their contents or the hazardous potential of the waste that causes an impact on humans and environment. Rise in large-scale industries, rapid urbanization, increase in population density and also changing lifestyle has challenged the balance between environment and man (Yadav and Garg 2011). Solid waste includes most of organic, domestic refuse, commercial and institutional wastes, municipal solid waste, ashes, sludge sewage, etc. Also agricultural waste from dairy, poultry and farmyard exhibits most of the organic contents. This article also highlights waste management through recycling of an aquatic weed by employing the earthworms, the final product of which is a boon to agriculture and organic farming.

1.1 Impact on Human and Environment

Discharging the waste products in the open land or releasing into the neighboring water bodies would be problematic for both land and water ecosystems. Man is responsible for polluting the major environment including air, water, soil, etc., as a result of illegal waste disposal and failure of definite scientific protocol for waste management. This results in breeding of infectious vectors in the society which may cause contagious diseases (http//:www.unescap.org).

1.2 Indian Context

Per capita waste generated in India is 0.498 kg/day and municipal solid waste generated ton per day (TPD) is 129,593 and 47,301,346 ton per year TPY (Annepu 2012). Reuse techniques of solid wastes: use the energy efficiently, preventing pollution and beneficial to society. Agricultural and animal waste may be converted into manure by composting and producing biofertilizer (i.e. vermicompost) and biofuel (Furedy and Choudhary 1996). It is highly essential and responsibility of one and all to collect, treat and dispose of the waste generated from various sources

in a sustainable manner. Dumping of these wastes leads to the emission of various dreadful gases which may pose serious threats to humans and overall environment, therefore the immediate attention has to be diverted to the management of municipal solid waste in a more scientific, sustainable and eco-friendly manner. The application of three concepts reuse, recycle and reduce is contributed towards depleting the environmental pollution which may lead to the healthier environment and cleaner surroundings.

2 Pollution of Aquatic Bodies by Weed

Water bodies are an important source of natural resources, on which almost all the activities (viz. agriculture, human consumption, industrial, etc.) of human are totally dependent. Water bodies like lakes, ponds, pool and wetlands form the major freshwater bodies whose ecosystem is enriched with biotic and abiotic interactions. When these freshwater bodies get overexploited by aquatic vegetation, it is said to be an infestation of aquatic weed. The main root cause of pollution of aquatic bodies from urban areas is mainly due to the release of domestic sewage and effluents directly into these precious resources. The waste enters into the aquatic bodies enriched by the nutrients induce enormous growth of aquatic weeds.

*2.1 Water Hyacinth (*Eichhornia crassipes*)*

Eichhornia crassipes is a perennial aquatic plant with long fibrous roots, rounded leaves, leafy stem with showy lilac-coloured flowers. A single plant has an ability to cover an area of 600 m^2 in few months. The factors responsible for the rapid proliferation of water hyacinth are their ability to grow on open water, rapid growth by means of stolons, low environmental demands and bladder-like petiole which makes the plant float, dissemination of seeds and reproduction by both sexual and asexual methods (The Wealth of India 1952).

Dense mats of water hyacinth reduce light to submerged plants hence decreasing oxygen levels in aquatic bodies (Ultsch 1976). These weeds make a negative impact on the water bodies, affecting biodiversity, fish production, nutrient loss of water, hindering oxygen interaction, causing insects and vectors, problems for navigation (Lancar and Krake 2002; Becker et al. 1987). Water hyacinth and alligator weed due to their overgrowth and dense mat block the route for boating and even ships (Lancar and Krake 2002). The application of water hyacinth raw material for manufacturing handmade papers and boards reported 1 ha. of infested pond area will yield 0.9–1.8 tons of dry matter basis per day (Thyagarajan 1983). The proliferation of water hyacinth rapidly is due to the availability of nutrients through anthropological activities such as the discharge of wastewater in water bodies, industrial effluents, run off from land, etc. (Ojeifo et al. 2000).

2.2 Worldwide Prevalence and Eradication of Water Hyacinth

Its prevalence around the world included Africa, Asia, Australia and North America (Téllez et al. 2008). Water hyacinth is introduced in India as an ornamental plant in botanical garden in Bengal (Biswas and Calder 1954). A field study done by (Narayanan et al. 2007) indicates that majority of states from India are infested with water hyacinth. These include in Kerala (from Ashtamudi wetland, Sasthankotta lake, Vembanad-Kol wetland), in West Bengal (from East Kolkata wetlands), in Madhya Pradesh (from Bhoj wetland), in Uttar Pradesh (from Upper Ganga river), in Himachal Pradesh (from Chanderertal wetland), in Punjab (from Harike lake, Kanjli wetland, Ropar wetland), in Andhra Pradesh (from Kolleru lake) and in Manipur (from Loktak lake) are greatly polluted with exorbitant infestation of this dreadful weed (Narayanan et al. 2007). Water hyacinth can also be used as compost and mulch, as it contains higher concentrations of nitrogen, phosphorous, potassium, calcium, magnesium (Mukhopadhyay and Hossain 1990). It is used as raw material in industries for paper, plastics, as a fodder for cattle, etc. (The Wealth of India 1952). Free-floating aquatic weeds are mainly water hyacinth (*Eichhornia crassipes*), water fern (*Salvinia molesta*), water lettuce (*Pista stratiotes*), duckweed (*Lemna minor*), rooted floating weeds like arrow head (*Sagittaria guayanensis*), nikalmi (*Ipomea hederacea*) (Lancar and Krake 2002).

Sambhaji tank is situated at 17° 38′ 52″N latitude and 75° 54′ 14″E longitude, 2 km from the centre of the city. Thousands of migratory birds visit every year and few of them are flamingoes, demoiselle cranes, etc. This tank is a recreational point for boating, bird watching center, also it is used for immersion of idols, other offerings of god like (nirmalya), fishing is done and is a beautiful place that attracts many tourist visitors. But now this tank is invaded by the water hyacinth aquatic weed which is creating problematic situations like blocking the routes for boating, fishing, affecting the biodiversity of tank and influencing the recreation. Various attempts have been made to remove manually from Sambhaji tank and subsequently heaped in huge quantities as dumps on the roadside in piles. This dumping makes the environment an invitation for many disease-causing insects and vectors. Water hyacinth weed interferes with the activities of birds, hinders the use of larvicides and mosquito repellents thus creating a menace (The Wealth of India 1952).Water hyacinth forms a dense mat over a water body surface, thereby obstructing the sunrays to penetrate through surface thus affecting the aquatic plant growth due to which plants decay and pollute the water body (The Wealth of India 1952). The tank is facing the problem of pollution due to domestic wastewater entering the lake from nearby residential and commercial establishments, washing of clothes, animal washing, etc. Due to an infestation of water hyacinth, Sambhaji tank is facing water pollution, which causes health hazards to public and municipal sewage waste management (Figs. 1, 2, 3, 4 and 5).

Fig. 1 Sambhaji Tank, Solapur (without infestation)

Fig. 2 Sambhaji Tank, Solapur overcrowded with water hyacinth

2.3 Bioconversion and Reuse Techniques by Energy Trapping

Non-conventional energy can be generated from different types of organic wastes like domestic, industrial, agricultural and aquatic weeds. Plant or animal waste is

Fig. 3 Boating in Sambhaji Tank, Solapur

Fig. 4 Obstruction for boating in Sambhaji Tank, Solapur due to overgrowth of water hyacinth

the best source of organic material for conversion into usable products or energy sources such as composting, liquid or gaseous fuel by biological processes or agents: such as certain microorganisms, some detritivores or enzymes (Wise et al. 1981). The bioenergy can be utilized for various purposes in the form of biogas, biofuel, biofertiliser, etc. The recent technologies like anaerobic digestion,

Fig. 5 Loading of water hyacinth weed from Sambhaji Tank, Solapur for disposal

gasification, incineration, pyrolysis and pelletization are implied in sustainable solid waste management (Zhu et al. 2008). Energy can be trapped, created and reutilized in the form of biogas, biofuel, thermal power, biofertilizers, etc., from various solid wastes.

3 Why Vermicomposting?

Huge amount of waste is generated in India which is sent for dumping and land-filling. Vermibiotechnology can be used to minimize this waste by converting it into nutrient-rich vermicompost. Worm castings also contain plant beneficial microbes and improve soil health. Vermicompost is superior as compared to chemical fertilizers because it not only enriches the soil but also provides organic matter needed for the soil. Vermicompost is superior to chemical fertilizers as it also decreases the use of pesticides for controlling plant pathogens. Decomposing and breakdown of organic wastes are time consuming when it is dumped and left as it is, but earthworms feed voraciously on decomposed waste, fragment the organic waste rapidly, resulting output which is nontoxic, has a potency of economic value. Decomposed organic wastes have a large population of pathogenic microbes which is harmful to humans and environment but vermicomposting reduces pathogenic microbial population (Kumar and Shweta 2011). It plays a leading role in minimizing environmental problems of soil, water and air pollution. Vermicompost technology can be practiced as cottage industry which lifts up the economic graph and provides employment and also supports a supplementary income.

Vermicomposting is a low-cost technology and potentially agronomical valuable, could be practiced near wetlands, encouraging farmers to imply and harvest the weed.

3.1 Role of Earthworms in Solid Waste Management

Earthworms improve soil aeration and porosity, rapidly decomposes the organic matter by process of feeding on it, fragmentation and excretion of nutrient-rich casts (Edwards and Bohlen 1996; Shuster et al. 2000). Various organic wastes such as coir pit, kitchen waste, agro residues, industrial wastes, sludge are being processed by earthworms into valuable bioenergy-rich vermicompost (Ramalingam et al. 2004; Ndegwa and Thompson 2001; Garg et al. 2006; Nair et al. 2006). Solid waste management of filter mud and sawdust by vermitechnology employing two exotic and one local earthworm species *Eisenia fetida* along with *Eudrilus eugeniae* and *Perionyx excavatus* in monoculture and polyculture vermireactor systems proved to be a potential conversion into value-added end product (Khwairakpam and Bhargava 2009).

3.2 Studies on Bioconversion of Water Hyacinth

Vermicomposting proved an alternate promising option for controlling the menace of water hyacinth waste. Studies on water hyacinth vermicompost have been emphasized by Gajalakshmi et al. (2002), employing different earthworm species. The annelid, *Eudrilus eugeniae* was found to be an effective candidate for composting of different combinations of water hyacinth. Vermicompost of pre-composted grass clipping and water hyacinth amended with cow dung proved to be a successful treatment for solid waste management of water hyacinth with minimum complexity and economic viability (Ansari 2011). Application of water hyacinth to marigold plants improved growth and yield by enhancing nutrient supply in the soil to plant (Paul and Bhattacharya 2012).

4 Experimental Protocol

4.1 Earthworm Culture and Rearing

Earthworm species, *Eudrilus eugeniae* was procured from Zonal Agricultural Research Station, Solapur. *Eudrilus eugeniae* is fast growing and converts organic waste rapidly (Dominguez et al. 2001). The stock culture of *Eudrilus eugeniae* was grown by raised bed method using partially decomposed farm yard manure and cow dung as a food source.

4.2 Fragmented and Decomposed Water Hyacinth and Cow Dung Collection Procedure

Water hyacinth was obtained by deweeding manually and collected and dumped on the edge of road at the site of Sambhaji tank, Solapur, Maharashtra. This weed was cut into small pieces by mechanical shredder and sundried for 1 week. Cow dung was collected locally from livestock Ahimsa Ghoshala, Karamba, Solapur. The main purpose of adding cow dung is that it enhances the speed of vermicomposting (Muthukumaravel 1996).

Composting was done in specially constructed pits. During experimentation, the treatment used was 50% water hyacinth + 50% cow dung. An inoculum of 500 mature earthworms was released in decomposed organic waste. During pre-composting period, to ensure proper circulation of air and to prevent odour, the pit mixture was regularly turned up and down. Vermicompost treatment (T1) was compared against decomposed water hyacinth + soil treatment (DT1). Average moisture was preserved by sprinkling desired amount of water. To prevent moisture loss and from predators, the pit was covered with gunny bags. The average duration of the experiment was 150 days. Initial 60 days for decomposing and 90 days was required for vermicompost production. For the experimentation, two independent pits were used:

Pit 1: The contents of pit 1 were decomposed water hyacinth (DT1) and soil (60 days)
Pit 2: 50% water hyacinth + 50% cow dung for 90 days after the release of earthworms (T1).

For determination of physico-chemical and microbial parameters, samples were drawn from both the units, i.e. decomposed stage and after 90 days of vermicomposting.

4.3 Estimation of Physico-chemical Parameters

The worm worked decomposed waste was subjected to nutrient analysis. The samples of decomposed treatment and vermicompost of experimental group were analyzed for chemical analysis: total nitrogen (N) by Kjeldahl method (Black et al. 1965), phosphorous by Olsen method (Olsen et al. 1954), potassium by flame photometry (flame photometer Elico made with C 1361 flame), pH of the samples were estimated by pH metre (Elico) with L1 612 pH analyzer, Electrical conductivity by EC bridge (Elico, CM-183EC-TDS) and moisture was determined by loss on drying method.

*4.4 Field Experiment Using Groundnut (*Arachis hypogaea*)*

A field experiment was conducted to study the effect of vermicompost on growth, yield and quality parameters of commercial crop groundnut during rabbi season in a private field at Karamba, which was 15 km from Solapur. The experimental site consisted of black-brown-textured soil and was neutral in reaction.

The physico-chemical properties of field soil were EC 0.21 (mS/cm), pH 7.90, Organic Carbon 0.73, Nitrogen 0.60%, Phosphorus 0.44%, Potassium 0.22%, C:N 27:1. The climate was hot and temperature ranged between 40 and 42 °C during experiment and irrigated by supply of bore water. In plot treatment, 10 plants were randomly selected and tagged with labels for reading of various morphological characteristics.

At village Karamba, Tal. North Solapur, medium deep well-drained soil was selected and two harrowing were given to the selected field for groundnut sowing. The well-decomposed farm yard manure (FYM) was well spread in the field at the rate of 250 kg/ha. as per recommendation. The gypsum application (250 kg/ha.) was also given in the selected field. Regarding chemical fertilizers, the dose was 25 kg nitrogen and 50 kg phosphorous/hectare was applied. The variety TG.26 was selected for the trial. The field layout as per the treatments was done and it was replicated by five times. The seeds were treated by Trichoderma at the rate of 5 g/kg of seed before sowing for better germination and healthy seedlings. The rhizobium seed treatment at the rate of 25 g/kg was also given to the seed before sowing. The seeds were sown by hand at the distance of 30 cm in between rows and 10 cm in between plants.

The plot size was of dimension 2.0 × 1.5 m and was of Randomised Block Design (RBD) method. Groundnut seeds applied were 100 kg/ha. The doses of vermicompost and chemical fertilizers were given to the plot at the time of sowing as per the treatments. One hawing and one weeding were given to the groundnut fields and plots were kept free from weeds. The protective irrigations were given to the plot as and when required at 15–20 days interval. The pest and disease incidence was recorded at 15 days interval till the harvest of the crop. An experimental plant was studied for morphological counts such as plant growth, height, number of leaves, branches and pods/tagged plants. Wet and dry pod along with fodder yield was recorded in kilogram/hectare and ton/hectare. The pest incidence of aphid, jassid, thrips and leaf minor was recorded on the crop. These were controlled by one spraying of dimethoate 30 EC at the rate of 500 ml in 500 L of water/hectare. The leaf minor pest was controlled by spraying quinalphos 25EC at the rate of 1000 ml in 500 L of water/hectare. There was no incidence of tikka or rust disease on the crop and hence no any fungicide sprays were given to the crop. The data obtained during experimentation was subjected to statistical analysis which included paired sample t-test and ANNOVA. p value significance was also calculated of decomposed treatment against vermicompost and comparing field output (Bailey 1965).

Randomized Block Design method layout for field experiment of Groundnut (*Arachis hypogaea* L.)

	R I	R II	R III	R 1V	R V
N ↓ S	T1	Control	T1	Control	T1
	Control	T1	Control	T1	Control
	T1	Control	T1	Control	T1
	Control	T1	Control	T1	Control
	T1	Control	T1	Control	T1

4.5 Selection of Groundnut for Field Experiments

Groundnut contributes 35% of world production by India. It is adaptable to all soil types and textures. The yield under irrigated conditions is double as those grown as dry crops. The yield in India is greater as compared to Africa and the USA. India cultivates groundnut in an average area of 6.41 million hectares. Groundnut is an important source of edible proteins and oil in India. India is on lead after China in groundnut production, contributing around 19% of the world production. Groundnut yield in India is 1.13 ton/ha. The major states in India involved in the production of groundnut are Andhra Pradesh, Tamil Nadu, Gujarat and Maharashtra contributing towards 90% of production (Mehrotra 2011).

Around 80% of groundnut in the country is cultivated during kharif season. Grown in tropical and subtropical areas between 25 and 28 °C and under 500 mm rainfall in the loamy and black soil. Botanical name of groundnut is *Arachis hypogaea* of leguminous family, measures 1–5 ft. in height, the plant is a hairy, tap-rooted which has many nodules. In Khandesh, Vidharba, Sangli and Satara in Maharashtra, groundnut harvest is done during the month of January–May, whereas in Ahmednagar, Pune and Solapur district, the crop is cultivated between March and August. In Marathwada crop rotation of groundnut and wheat is practiced. Solapur, Latur, Amravati and Jalna are four major districts in Maharashtra producing 0.7 million tons of edible oil (Mehrotra 2011). At present there are 98 oil mills including 170 expellers, 152 big and 18 small expellers in Solapur city. Groundnut production in Solapur district during the year 2007–2008 with an average yield was 4.80 ton/ha. (http://www.coin.fao.org/cms/world/india/en/Home.html). Groundnut has 25–33% protein, carbohydrate 10.2%, fat 40.5% and calorie value 500–600. Groundnuts are rich in vitamin, B-complex which includes thiamin, riboflavin and nicotinic acid. Groundnuts are good source of vitamin E (Wrenshall 1949). Oil extracted from groundnut seeds is an important food source. Groundnut cake used as cattle feed in India which has good amounts of protein. Groundnut stems with shoots and branches are fed to cattle in green and dry form. Groundnut shells can be bioconverted into manure to improve soil health as well can be used as a biofuel (Talwar 2004). Due to high fat and protein content, groundnut is a

valuable energy-giving food. It is used for oil, seeds, flour and butter, while the plants as fodder and husk for agricultural purpose. Groundnut cake is used for feeding farm animals.

4.6 Studies on Field Experiments

Organic farming has taken a rapid speed to meet the essential nutrient inputs to soil, because use of chemical fertilizers lead to deterioration of both soil and agricultural products and as well environmental pollution (Liu et al. 2009; Padel et al. 2009). Vermicompost is a stabilized, fine porous material loaded with plant beneficial microbes and nutrients readily to take, has increased water holding capacity (Dominguez 2004).

Growth and yield parameters influenced by vermicompost application were studied on crops like tomato (*Lycopersicum esculentum*), garlic (*Allium stivum* L), sweet corn hybrids (*Zea mays*), rice, cicer, pisum, etc. (Joshi and Vig 2010; Suthar 2009; Lazcano et al. 2011; Sudhakar et al. 2002; Sinha et al. 2010). Increased germination rate reported in crops like groundnut, cucumber, red amaranth, pak choi, Chinese mustard green (Hidalgo 1999; Lee et al. 2012). Favourable effects of vermicompost application on various crops in terms of yield, growth, etc., have been reported. Vermicompost besides having nutrients also shows the increased source of nitrogen useful for growth of plant beneficial microbes (Ravindran et al. 2008). Vermicompost affect positively on plant growth by better aeration to the plant roots, enhancing readily available water, triggering nitrogen, phosphorous and potassium exchange for better plant growth (Manivannan et al. 2009).

5 Aftereffect of Vermicompost Application on Field Experiment

5.1 Physico-chemical Parameters

Moisture was increased in vermicompost by 59.05 ± 2.94% compared to decomposed treatment (49.48 ± 0.79%), with percent variation of 19.34% significant at $p < 0.01$ (Figs. 6, 7 and 8). Electrical conductivity increased in T1 by 1.69 ± 0.17 mS/cm as compared to decomposed treatment 0.64 ± 0.35 mS/cm). The vermicompost result was significant at $p < 0.001$ with percent variation of 164%. pH content was 8.34 ± 0.85 in decomposed treatment and seen reduced in vermicompost by 6.00 ± 0.20 significant at $p < 0.05$ with a significant difference of 28.05%. Nitrogen percentage in T1 was enhanced by 1.50 ± 0.06% while it was 1.01 ± 0.01% in decomposed water hyacinth with significant difference of 50.00% at $p < 0.001$, Phosphorus content in vermicompost was 1.89 ± 0.03 compared to

decomposed treatment (0.89 ± 0.02) significant at $p < 0.001$ with percent variation of 112%. Potassium level was increased in vermicompost treatments after 90 days, in T1 the value was 1.15 ± 0.48 compared with control (0.23 ± 0.03) significant at $p < 0.001$ with percent variation of 400%. C:N ratio in decomposed water hyacinth was 19.63 ± 0.06, which was decreased after the release of earthworms in vermicompost treatment (11.00 ± 0.03) with percent variation of 43.00% significant at $p < 0.001$.

5.2 Pod Yield

The total yield of groundnut pods on a dry basis in control group was 0.546 tons/ha. It was increased in T1 group vermicompost applied field was 1.178 ton/ha. (115%) as compared to control. The results were subjected to ANOVA and F value was 78.96 significant at $p < 0.001$ (Figs. 9 and 10).

Parameters	Decomposed water hyacinth	Vermicompost treatment T1	Percent Variation %	P value
Moisture (%)	49.48±0.79	59.05±2.94	19.35	P<0.01
EC (mS cm^{-1})	0.64±0.35	1.69±0.17	164.00	P<0.001
pH	8.34±0.85	6.00±0.20	28.05	P<0.05
Nitrogen (%)	1.01±0.01	1.50±0.06	50.00	P<0.001
Phosphorous (%)	0.89±0.02	1.89±0.03	112.0	P<0.001
C:N ratio	19.63±0.06	11.00±0.08	43.00	P<0.001

T1- 50% water hyacinth+50% cow dung

Fig. 6 Analysis of physico-chemical characteristics of decomposed water hyacinth and vermicompost water hyacinth (*Eichhornia crassipes*) (Mean ± SD, $n = 3$)

Treatment	RI	RII	RIII	RIV	RV	Mean	kilogram/ Hectare	Ton/hectare (wet)	Ton/hectare (dry)
T1	0.388	0.300	0.350	0.360	0.370	0.353	1178.660	2.230	1.178
Control	0.164	0.160	0.170	0.150	0.175	0.163	546.000	1.601	0.546
F value: 78.96***									

Values are Significant at ***P<0.001 (Mean±S.D, n=3)

Fig. 7 Effect of vermicompost processed by *Eudrilus eugeniae* on groundnut (*Arachis hypogaea*) dry pod yield compared with control

Treatment	RI	RII	RIII	RIV	RV	Mean	kilogram/ Hectare	Ton/hectare (wet)	Ton/hectare (dry)
T1	0.445	0.450	0.460	0.465	0.459	0.456	1519.330	3.010	1.519
Control	0.214	0.220	0.222	0.230	0.240	0.225	750.660	2.149	0.750
F value: 151.9***									

Values are Significant at ***P<0.001 (Mean±S.D, n=3)

Fig. 8 Effect of vermicompost processed by *Eudrilus eugeniae* on Groundnut (*Arachis hypogaea*) dry fodder yield compared with control

Fig. 9 Result of vermicompost effect on plant and pods compared with control

5.3 Dry Fodder Yield

Dry fodder yield of groundnut crop *Arachis hypogaea* after harvesting was more from vermicompost applied field as compared to the soil as control applied field. The dry fodder yield in T1 treated plot was 1.519 ton/ha. (102%) compared to control treated plot (0.750 ton/ha.). The vermicompost result was significant at F value of 151.9 at the value of $p < 0.001$ (Figs. 11, 12, 13 and 14).

Fig. 10 Result of vermicompost effect on groundnut plant and pods compared with control

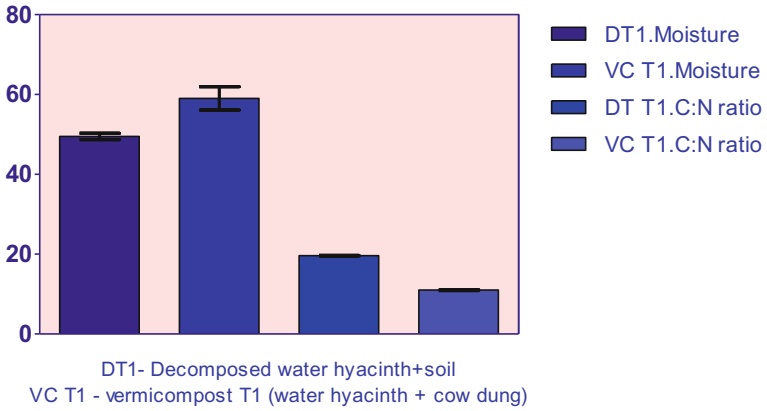

Fig. 11 Moisture content and C:N ratio in vermicompost treatment compared with decomposed water hyacinth

6 Discussion

Vermicompost is organic manure produced by the activity of earthworms in collaboration with gut microflora. It contains rich macro and micronutrients (organic carbon, nitrogen, phosphorous, potassium, calcium, magnesium, sulphur, copper, iron, manganese zinc, boron, sodium, chlorine). Besides these nutrients, physical parameters (moisture, electrical conductivity, pH, ash) have a greater role to play in soil structure. In the present investigation the experiments carried out for converting

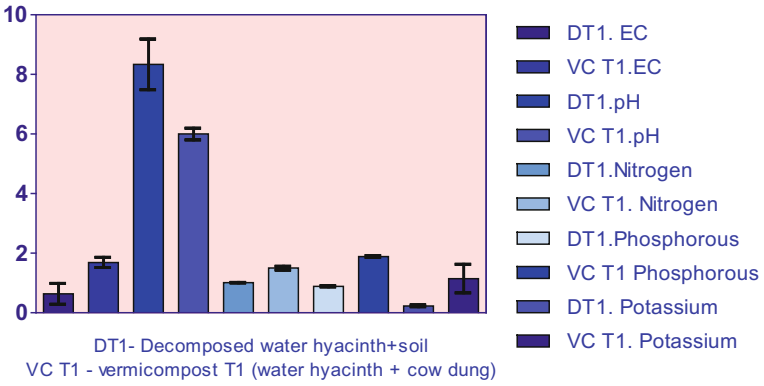

Fig. 12 Electrical conductivity, pH, nitrogen, phosphorous and potassium content in vermicompost treatment compared with decomposed water hyacinth

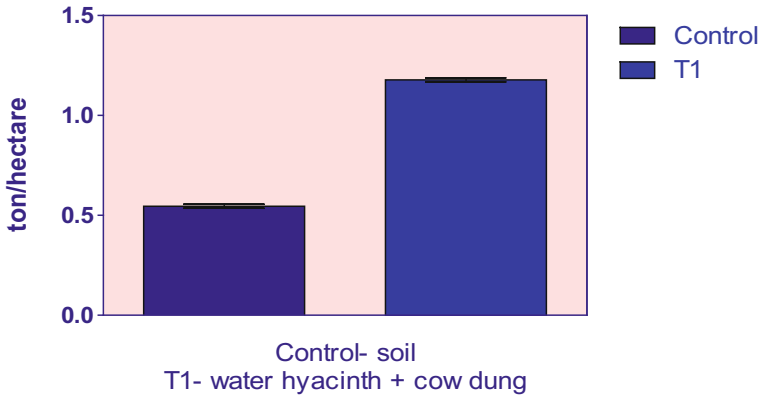

Fig. 13 Effect of vermicompost processed by *Eudrilus eugeniae* on groundnut (*Arachis hypogaea*) dry pod yield compared with control

water hyacinth, an aquatic weed into useful vermicompost with the association of earthworm species, *Eudrilus eugeniae* using pit method resulted in significant variations in micro, macro and physical parameters of the vermicompost produced from pit method. In the present investigation, the pit method noticed enhancement of the moisture in T1 treatment (Fig. 6). During the conversion of press mud sugarcane waste to vermicompost, it was observed that moisture level of vermicompost between 65 and 75% enhanced microbial population, biomass and cocoon production of earthworm (Parthasarathi 2007). From our results, it is clear that from all the pit method of vermicompost cow dung along with water hyacinth enhanced the moisture contents. In the present study, the electrical conductivity in millisiemens per cm (mS/cm) from vermicompost produced with the help of pit method observed increased values when compared with control decomposed water

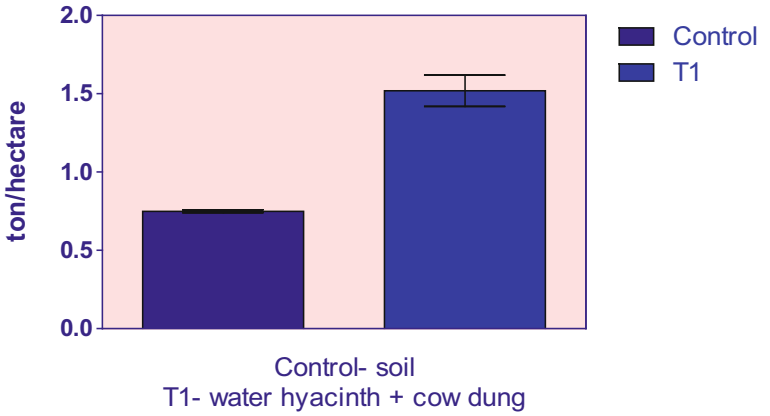

Fig. 14 Effect of vermicompost processed by *Eudrilus eugeniae* on groundnut (*Arachis hypogaea*) dry fodder yield compared with control

hyacinth. A significant increase in electrical conductivity levels from compost without worms to worm worked vermicompost was seen during the vermicomposting process, which may be boosted by earthworm activity, the soluble salt level increases in combined action of microorganisms present in the organic raw matter and in the digestive tract of earthworms (Karmegam and Daniel 2008). The variations in pH alter many macro- and micronutrients mobility in the plants. In the present study, pH content from vermicompost, produced with the help of pit method noticed a decrease in the values. While recycling the organic wastes of cow manure, poultry and food industry waste to produce vermicompost with the help of *Eisenia fetida* a decrease in pH at the end of the experiment was noticed, when compared to initial values (Yadav and Garg 2011). pH variations from different sources of waste may be credited to physic–chemical attributions of wastes used in experimentation. pH change may be due to the formation of organic acids from feeding substrates or due to the type of substrate used as raw organic matter (Suthar and Singh 2008). Earthworm casts contain twofold of both macro and micronutrients, reduced C:N ratio compared to that of garden compost (Saranraj and Stella 2012). While converting milk processing industrial sludge along with plant waste into vermicompost with the help of *Eisenia fetida* there was significant drop in C:N ratio as that of initial substrate waste which may be due to the loss of carbon during respiratory activities and microflora associated with a decrease in carbon: nitrogen ratio of the substrate (Suthar et al. 2012). According to (Gupta et al. 2005), the lowering of C:N ratio from final product vermicompost is an indication of the maturity of composting. While studying the conversion of organic material into composted final product there was a reduction in C:N to 8.13 for the vermicompost and 1.05 for the compost (Castillo et al. 2010).

Nitrogen is important for plant growth and overall to the food and fibre production of the livestock. In the present study, the nitrogen content from

vermicompost produced by pit method with the help of *Eudrilus eugeniae* after using organic raw material resulted in an increase in nitrogen content. During bioconversion of domestic waste into vermicomposting worked by two epigeic earthworm species *Perionyx excavates* and *Perionyx sansibaricus,* there was enhanced nitrogen content from the vermicompost which may be due to inoculation of earthworms in the waste material that significantly enhanced the amount of nitrogen due to earthworm-mediated nitrogen mineralisation of the waste (Suthar and Singh 2008). Earthworm contributes through mineralization of nitrogen, secreting its mucus and excreta providing the sites for chemical reactions, encourages the proliferation of nitrogen fixers (Singh and Suthar 2012; Dominguez 2004). Phosphorous is required in large quantity for routine growth in living organisms. The energy is derived from phosphorous-containing compounds ATP and ADP hence continuous phosphorous supply is highly essential for energy inputs. Rise in level of available phosphorus worked with earthworms from press mud might be due to break down and release of phosphorus by co-ordinated action of faecal phosphatases of these annelid worms and microbial activity in the casts (Vinotha et al. 2000) and due to microbial activities producing organic acids, solubilizing inorganic phosphorous, mineralization of organic phosphorous by phosphatase activity of earthworms (Scervino et al. 2010; Eivazi and Tatabai 1977). Potassium is required for plants attaining optimal growth. In the present study, the potassium content from the vermicompost produced through pit method enhanced the potassium content. Macronutrient Potassium level was reported to be increased in worm worked compost which may be initiated by the action of both *Perionyx sansibaricus* and microbes that boosted the rate of mineralization (Suthar 2007).

6.1 *Energy Recovery from Water Hyacinth Biomass Through Vermicomposting*

In the present investigation, the total dry yield in the form of pods enhanced to its maximum yield in T1 treatment when compared to control (0.546 ton/ha.) of the groundnut crop (Fig. 7). Similarly, the dry fodder biomass after harvesting the groundnut crop (120 days) vermicompost application produced maximum fodder in T1 treatment 1.519 ton/ha. when compared to control group (0.750 ton/ha.). Effect of 4% vermicompost application produced from municipal garbage on growth and productivity of canola an important oil crop, (*Brassica napus*) was displayed by increased growth, biomass and yield of canola under normal irrigation. There was 47.19% improved yield after 4% vermicompost application and hence vermicompost can be used for sustainable plant production because it is an efficient plant growth medium (Rashtbari et al. 2012). Vermicompost act as a chelating agent and further regulate the utilization of microminerals to the plant which finally influences the overall development of the plant (Giraddi 1993). A study on field application of vermicompost in combination with nitrogen fertilizer on wheat recorded an increase

in dry crop husk (16.2 g/plant) and wheat grain yield (3.6 ton/ha.) (Desai et al. 1999). The combined application of nitrogen, phosphorous and potassium fertilizers and vermicompost increased the yield of cabbage (58.67%) over control which may be attributed to increased availability of native and applied macro and micronutrients (Devi and Singh 2012). The application of vermicompost produced from the organic waste resulted in improved growth and yield of an ornamental plant *Amaranthus* species. An overall increase in nutrient contents especially nitrogen, phosphorous and potassium was seen which finally enhance the total yield attributed towards higher availability and uptake of plant nutrients and growth promoting substances during the entire duration and crop harvesting (Uma and Malathi 2009). Similar might be a case in the present investigation in which eco-friendly manner of converting aquatic weed into vermicompost was utilized and observed higher yield of the commercial groundnut crop. From our studies, it is clear that water hyacinth aquatic weed after converting it into useful vermicompost with the help of pit method by using *Eudrilus eugeniae* can be applied on the field trials using groundnut crop. In vermicompost treatment pod and biomass yield (fodder yield) increased significantly compared with control. The total dry matter yield in vermicompost treated plot was 1.519 ton/ha. as compared to control plot which yielded 0.750 ton/ha. (Fig. 8). This might be due to the substantial amount of nutrient supplying to the plant treated with vermicompost which leads to the increase in the number of branches, leaves, height of plant and finally pod number and groundnut yield for sustainable agriculture. Recycled vermicompost could be a boon for the farmers. The fresh fodder (2.62 ton/ha.) makes up 52% of total fresh biomass and dry fodder (1.88 ton/ha.) makes up 46% of total dry biomass (Bassam 2010). The studies also cleared the better impact of various combinations such as vermicompost produced from water hyacinth amended with cow dung on field experiments. The positive impact of vermicast to field soils is it makes it fertile enough to withstand same crop growth and yield in repetitive cultivations which later require smaller concentrations of vermicast, whereas chemical fertilizers have to be applied in increasing doses which have a negative impact on soil properties.

In our studies, the initial biomass we used for vermicomposting process reduced to its half, i.e. 35% of original waste after it was decomposed and ready to introduce earthworms. Thus, a huge amount of biomass can be reduced volumetrically by vermicomposting and can be prevented by dumping this large amount of solid waste through landfilling. The reduction of waste occurred during pre-vermicomposting period reduced the time required for vermicomposting and area of worm bed with waste stabilization. A study conducted on effects synthetic nutrients such as iron, manganese, zinc, copper, molybdenum and boron and their influence on earthworm growth during vermicomposting displayed a marked increase in worm growth rate with significant waste volume reduction (Palaniappan and Thiruganasambadam 2012). The volume of municipal solid waste reduced to 35% of total volume in the process of vermicomposting in the Ujjain city (Dalal 2012).

The vermicompost serves as an option for energy generation. Wastes such as sugarcane, spent tea and grated coconut meat turned into vermicompost proved to produce electrical voltage that light up the LED. Thus, it can be an alternative for

battery, reducing the negative impact of chemicals in environment (Karim et al. 2011). Rich organic sources such as rice husk, bagasse, groundnut shells and maize cobs are effective sources of electricity generation, heat and cooking fuel due to low concentrations of carbonaceous substances. These also contribute less to total greenhouse gas emissions. Sum total of crop including grain is used as bioenergy feedstock. The remnant to product ratio of groundnut was estimated to be around 0.5–1.2 for pods and 2.2–2.9 for straw; which explains that every ton of nut production yields 500–1200 kg of shells, 2.2–2.9 tons of straw residue are harvested; in total groundnut crop yields between 3.7 and 5.1 tons of biomass per hectare. These residues are an efficient source of solid biofuel, with a relatively high energy content of 16 Mj/kg for shells and 18 Mj/kg for straw—with advanced bioconversion technologies (cellulosic ethanol or pyrolysis) this 'waste' biomass can be turned into liquid fuels and bioproducts; alternatively, it could be densified and used in biomass (co-firing) power plants (http://news.mongabay.com/bioenergy/). Groundnut shell has great potential for domestic, industrial energy resource. Groundnut shell residue is a good source of energy for purposes like fuel, cattle feed, making boards, can replace cork, as a mulch, etc. Groundnut shells show woody characteristics with moisture 8.76%, Carbon 15.50%, ash 20.3 and volatile matter 54.96.

Combustible gases like H_2, CO, CH_4, CO_2 and N_2 production were successfully produced from groundnut shell (Sivakumar and Krishna 2010). Also, the amount of producer gas, i.e. hydrogen was seen in higher concentration than coconut shell and rice husk. Groundnut crop is termed as an energy crop because it not only yields food as peanuts but also net yield waste, compost from feedlots and dairies are left behind. All of these items can be used for fuel (http//:www.InfinitePower.org). Ethanol was extracted from groundnut shell biomass (Akpan et al. 2005). Agriculture residue such as groundnut shell biomass was used in the production of briquettes which is an alternative source of energy, since briquettes are used as fuel energy (Maninder and Kathuria 2012). Biomass briquettes are substitutes for electricity, heat and cooking fuel. It is found to generate around 61,000 MW energy and briquettes manufacturing industries may turn as employment hubs for around 15.52 million appointments. Farmers too are benefitted and can earn $6/ton by supplying their farm residues to these industries (Tripathi et al. 2000). The end use of briquettes is mainly for replacing coal substitution in industrial process heat applications (steam generation, melting metals, space heating, brick kilns, tea curing, etc.) and power generation through gasification of biomass briquettes (Tripathi et al. 2000). Groundnut shell was used as a biomass which was blended with coal to produce environmental-friendly biofuel briquette in an effective way (Onuegbu et al. 2012). It was also observed that ash contents of groundnut shell were less than that of coal, the lower the ash content, the better and the quality of the fuel. It was found that groundnut shell ash is equivalent to Portland cement concrete and can be used as a substitute for the later (Alabadan et al. 2006). The blending of cement with groundnut husk proved to be effective in soils containing chemical magnesium sulphate and sodium chloride (Adole et al. 2011). In a feeding trial on mature cows, they were fed with peanuts along with shells. The food intake

and digestibility data significantly proved that whole peanut along with shells as a source of energetic nutritional valued food (Myer et al. 2009).

7 Concluding Note

From our results, it is evident that vermicompost derived from water hyacinth an aquatic weed has enhanced nutritional value and showed effective outcome on growth and productivity of groundnut crop. Thus, the overall biomass is also increased which contributes towards economic agriculture practices and environmentally eco-friendly method. Our approach of vermicomposting has provided a scope for the solid waste utilization of water hyacinth in Sambhaji tank, Solapur, India in an eco-friendly manner. This could become a source of wealth for Solapur Municipal Corporation if subjected for mass bioconversion of solid waste into a biofertilizer. This will simultaneously control the environmental pollution caused by aquatic weed. Finally, it can be concluded that earthworms are good bioengineers for recycling organic waste produced from the aquatic weed, i.e. water hyacinth. They are the natural bioreactors that can degrade the organic matter biologically, enrich the soil fertility and have a prime role in the total yield of crop. Vermicomposting is the best practice; can be adapted as eco-friendly, economically feasible, pollution-free approach of converting the waste biomass and retrieving the same in the form of useful vermicompost. Excess use of chemical fertilizers can be avoided by using aerobically produced biofertilizers. This is the method of recycling, reuse and reutilizing waste by bioconversion of water hyacinth into a useful product and later such vermicompost effectively used for cultivation of agricultural crops. This technology can be used for sustainable agriculture practices which enrich the soil by addition of nutrients as well as enhancement of microbial population. Vermicompost considered as gold produced from garbage has a greater nutritive value which has combined effects in the overall growth and yield of agricultural crops.

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Chapter 16
Mitigation of Global Warming Potential for Cleaner Composting

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Abstract With the rapidly growing human population, urbanization, and the uplifting living standards in all over the world, huge amount of solid waste was generated. It is expected that the entire amount of word solid waste production would climb from more ~3.5 million to >6 million tons/day from 2010 to 2025, and the rate would continue to increase and reach a peak to over 11 million tons/day about by the year 2100. Thus, how to manage those increasing solid waste generation was becoming a great issue for the sustainable civil infrastructure. Organic waste, which includes food scraps, yard wastes, agricultural wastes, and process residues, took the largest proportion of the overall generated solid waste by 46%. But ~90% of the solid waste is directly disposed into the landfill that can produce a considerable proportion of flue gases (generally the CH_4 and N_2O gases) due to the anaerobic mineralization of bio-available organic matter. Composting is an eco-friendly alternative "Old & Gold" technology to landfilling for the management of organic waste. Its principal interest lies in its potential to recycle the organic nutrients through compost application. The life cycle measurement has been extensively used as a mean of evaluation for impact assessment, such as overall

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warming potential, along with different waste management technologies. The typical methods assumed for composting comprise transportation of organic waste, viable machinery, greenhouse gases (GHGs) emissions during the curing phase, as well as the end-product application. There is a divergence in the adopted operational methodology to determine its environmental effect. This chapter deliberates on the variations of life cycle computation of solid waste management that involved different global warming potentials of composting. It is also based on the GHGs mitigation approaches to minimize the global warming impact by aerobic composting. The element of the study is to examine the difference in the inventory investigation for composting, and its fundamental mechanism and the significant inventory for an additional assessment. This study establishes that the GHGs emissions which emit directly during the composting process supply additional global warming prospective rather than further emissions. The bulking agent is used for the mitigation of overall warming potential. The measurement of the composting and its impact on global warming prospective is widely dependent on many defined efficient components. The environmental impact should be examined based on the operational approach and the input feedstock to generate a basis with minimized discrepancies among studies. Consecutive exercise is compulsory to evaluate the everlasting assistance of composting on environment, health, and soil properties to further identify its effect as a cleaner technology. Demonstration of mitigation method of field-research is an essential step toward the acquiescence of organic farming with global GHGs emissions moderation object through composting. Therefore, in this chapter, provides a detailed account of the technological advancement and composting approaches in global prospects for mitigation of GHGs emission, environmental safety, and human health protection.

Keywords Inventory analysis · Cleaner composting · Greenhouse gas mitigation Global warming potential

1 Introduction

The global warming is an abnormality which identified by the continuous heating of the atmosphere, the earth's shallow, and oceans. The average earth's temperature has increased by 0.8 °C bygone the earlier century and probable that earth temperature is likely to increase by ~1.133–6.42 °C in the subsequent 100 years found by the Environmental Protection Agency (EPA). In addition, the majority of the climate scientists confirmed that the increasing trade of global warming on earth is now facing is not because of the natural occurrence but is basically from the consequence of anthropogenic activities. Global warming initiated with the greenhouse effect, which is occurred by the breakdown of the ozone layer, and synergy between planet climate and incoming sun radiation. The atmospheric gases for the most part responsible for the greenhouse effect are known as "greenhouse gases" and take account of water vapor, methane (CH_4), nitrous oxide

(N_2O), and carbon dioxide (CO_2). The CO_2 is the chief greenhouse gases (GHGs) found in the environment. The GHG has high transmittance of visible light which from the sun radiation has high absorbent for the long-wave radiation emitted from the earth. Consequently, strong absorption of infrared radiation on the ground was happened, causing the earth's temperature rise. The effect of global warming includes climate, glacier, ecology, agriculture, biology, and ocean. As is well recognized, environment change has earlier than now had recognizable effects on the environment: Escalation in average earth temperatures, liquefying ice glacier, increasing ocean water height, extra extreme weather events, ecosystem variations, worldwide depilation of agricultural and residential lands, disappearance of species, and so on. Ice cores melting of Antarctica, Greenland, and tropical-mountain iceberg indicates that the earth's environment transforms gradually with GHGs emission. Ancient testimony can also be observed in ocean sediments, coral reefs, tree rings, and course of obscure rocks. This ancient climate confirmed that recent warming rises approximately ten times rapid than the moderate rate of ice-age-resumption warming (National Research Council 2006).

Most of the climate experts reveal that the essential motive of the present global warming bias is anthropogenic inflation of the "greenhouse impact" warming that produced when the environment ambush heat radiating from earth against space (IPCC 2014; Naomi Oreskes 2004), which means that GHGs are the most contributor. By the way of some other routes in the environment, CO_2 makes its own way into the atmosphere. For example, fossil fuel combustion releases CO_2, which is the number one anthropogenic source of carbon dioxide. Over deforestation is also a major patron to higher CO_2 emission in the earth's atmosphere. The Global Forest Resources Assessment (2010) finding showed that the CO_2 produced nearly a billion tons per year due to the deforestation and mix in the entire surrounding atmosphere. On the other hand, the study of EPA also indicates that the GHGs inventory of the U.S. sinks from 1990 to 2012 and CH_4 can come from innumerable natural sources, but anthropogenic activities are responsible for $\sim$60% of CH_4 emissions. Except for fossil fuel combustion, one of the majorities of important sources of GHGs emitted comes from organic waste. The widespread use of organic waste management will produce an amount of GHGs during the process, such as landfill, incineration disposal, and compost. Composting is one of the management approaches for handling the organic waste, especially as alternatives to landfill (European Commission 2008), which is because the main advantage is the capability to recycle organic minerals through compost application for organic farming. In this background, composting devotes to significant amount of GHGs emissions as well as to reduce emissions. However, extremely a lesser amount of GHGs are produced in composting process due to aerobic mineralization of organic matter and rapid mixing/turning process of the mass amount of organic solid waste.

The aim of this chapter is to introduce the evidence, cause, and impact of climate change, discuss the relationship between climate change and GHGs from a global view, furthermore, summarize the organic waste generated and its proper

management based on the GHGs emission control, analyze the strategies for GHGs mitigation, and put forward the prospects toward the present circumstances of the cleaner composting.

2 Overview of Organic Waste Generation and Greenhouse Gases (GHGs) Emission Responsible for Climate Change: A Global and National Status

With the progressive industrialization and renovation of human operative standard, more and more organic waste was produced, which has lost its original value or not lost its value, but was abandoned during production, life, and other activities; the state of the organic substances may be organic, liquid, or gaseous. At present, the world's city annual output of organic waste is about 2 billion tons, and it is estimated to reach 3 billion tons in 2025 (Charles et al. 2009). The rising of organic waste accounts for about 40–70% of the full amount municipal solid waste in China, which mainly includes food waste, sewage sludge, livestock manure, septic tank, fecal sludge, garden waste, agricultural waste, and so on. With the urbanization, population growth, and industrialization process speeding up, the output of organic waste will increase rapidly, and the tendency of high water content will emerge (Zhang et al. 2010). Organic waste is classified into four categories: (1) primary production wastes (such as straw, litter waste, etc.), (2) secondary production (such as manure), (3) sideline organic fraction of solid wastes (such as agricultural solid wastes), and (4) waste of anthropogenic activities (such as biosolids and municipal solid waste) (Yang 2002). The United States divides organic waste into seven basic types: animal manure, crop residues, sewage sludge, food waste, industrial organic waste, wood processing waste, and domestic garbage. The complex biochemical reactions of organic waste will pollute water, soil, air, and even endanger human health. Therefore, to seek an economic and efficient method of organic waste treatment, achieve the goal of harmless, and reduction and reuse have attracted researchers' extensive attention.

However, the generation of waste and its composition varies between and also within countries, and waste management is closely relevant to development status, economic, and national politics. In Japan, the largest significant approach of management of waste is incinerated. The processing capacity accounts are, therefore, for >75% of the gross amount of waste provoked. The proportion of landfill is less than 3% of the total production. Landfills remain the main waste channel in the United States, and the composting was also the main form of waste. With the increasing attention to reuse of waste, waste composting process has been used as an important measure of waste recycling, especially in kitchen and garden waste. Besides the recycling of recyclable waste (including compost), the domestic waste treatment methods adopted in Germany are mainly incineration, mechanical, biological treatment, landfill, and several other methods.

The fact is that GHGs can be emitted from organic waste production to management. Consequently, the Intergovernmental Panel on Climate Change also found that there is a more than 95% anthropogenic action up to the past 50 years, which have warmed our atmosphere. Consequently, modern civilization and industrialization activities also have increased the planet CO_2 concentration from 280 to 400 ppm in the past 150 years (IPCC 2014). An extensive variety of eaves of potential GHGs emissions have been formed. Rogner et al. (2007a) revealed the technical information on GHGs emission and concluded that unless energy approach replaced essentially, the world would progressively lean on fossil fuels at the end of 2030. It is predictable in order to indicate that additional 80% of the overall energy will initiate from fossil fuels. This outcome was positioned on "numbers of testimony" and "tremendous compliance" in the article (Rogner et al. 2007b). It is estimated that yearly CO_2 emissions from energy-related activities in 2030 were 40–110% greater than in 2000, at the side of extensively higher in developing country (Rogner et al. 2007b). It was estimated that each year greenhouse gases emissions per capita in the developed country territory prevails significantly lesser (2.8–5.1 tons CO_2) compared to developed country locality (9.6–15.1 tons CO_2) (Rogner et al. 2007c). Prominence gradually indicated a rise in yearly world GHGs emissions (the "Kyoto" gases, consistent in CO_2-corresponding) of 25–90% by 2030, than in 2000 (Rogner et al. 2007b).

Annual per capita GHGs emissions in the developed countries like USA, UK, and Europe are commonly ten times higher than developing countries such as China and India. But due to China's rapid industrial development and population, their annual per capita GHGs generations are rapidly reaching among the equal of those in the *I group* of the Kyoto Protocol. Further, other nations with gradually rising GHGs discharge are India, Australia, South Korea, and Iran (which alone from the oil-rich Persian Gulf countries now have the principal per capita GHGs production estimate in the world). Apart from this, the EU-15 and USA GHGs emissions are significantly reduced per capita per year following the trends over the time.

3 Key Aspects of Waste Management and Climate Change Mitigation: A Global Trend

Based on the authenticity that an enormous extent of organic waste is generated in the world, effective waste management is an important means to reduce GHGs emissions by monitoring the environmental change. The anthropogenic and essential actions are required for the management of solid waste from its origination point to its ultimate management (United Nations Statistics Division 2017). This combined all the other approaches such as collection, transportation, management, and ultimate demolition of solid waste equally guideline monitoring. It is also ambient juridical and supervisory framework that relates to waste management practices, enveloping guidance for recycling, etc. Because of solid waste

management rules and regulation, they are not constant among all the developed and developing countries, both regional and provincial (Davidson 2011).

First phase is the waste collection; different countries and regions wastes vary greatly about collection methods. Subside assortment is the greater simple practices of collecting waste routinely through specialized trucks in most of the European countries, Canada, New Zealand, and USA. In rural region, solid waste may require to be taken to the storage station and then shifted to the relevant disposal aptitude center. In a lesser developed nations, there is no official system of waste collection. At present, the most extensively used disposal processes are incinerated, landfill, and composting, according to the country's economic situation and the basic national conditions. Organic waste recycling is a kind of resource recovery methods that indicate to the assortment and recycle of organic waste like barren beverage bottle. Decomposable organic components such as food scraps, plant debris, and paper waste can be recycled through composting and anaerobic digestion methods. As a result of solid waste energy recovery, the pyrolyzation, combustion, gasification, and landfill gas recovery are the technologies by which the non-recyclable materials thermally converted into usable electricity or fuel. Resource recycling is the accurate alternative of solid waste disposal, which was estimated for eco-friendly management and definitive next level application; on the other hand, the types of materials to be recycled varied from city to country.

The key point of waste disposal is to prevent waste being generated including the reuse of second-hand products to encourage consumers to avoid the use of disposable products. However, waste management in the developing and economic transition cities is facing constant challenges and many struggles because of weak institutions, shortage of resources, and the process of urbanization. Another important issue is the transboundary movement of waste. The "Basel Convention" approved by 172 countries abolished the transportation of hazardous wastes from developed to developing countries. The Basel assemblage has been assimilated into the EU solid waste transportation supervision. Although nuclear waste is studied dangerous, it does not fall under the authority of the Basel Congress. The impressive organic waste management will devote to the rebate of global GHGs emissions and the information of atmosphere change mitigation. Climate change moderation subsists of actions to reduce the magnitude long-term troposphere variation. Mitigation of GHGs is an environmentally good approach; climate change is a cabinet of the misfortune for commons in accordance with the IPCC's Assessment Statement (2014). But efficient climate change variation could not be accomplished if individual assistant (regional, provincial, or country) acts separately in its individual favor implying the order for joint processing. A number of transformation operations, on the other hand, have aspects of exclusive good as beneficial activities may require more in a horizontal line to each person, regions or countries that control them for the short term, although the expenditure of such anthropogenic activities remains another problem, especially for poor and developing countries people (IPCC 2014). In accord with the United Nations Framework Convention on climate change (UNFCCC), all the nations are in compliance with the regulation of the climate change. The main aim of the UNFCCC is to reduce the

atmospheric concentrations of flue gases at one level to reduce within the human limitation with the climate variation. The environmental quality will be improved with a new impersonal laying down "parties will do the best" to reduce warming potential below 1.5 °C as per the Paris Agreement of 2015 (Sutter and Berlinger 2015). The present scenario of global GHGs emission does not reflect to be persistent with reducing global warming to lower 1.5 or 2 °C. At the center of most regulation is the mitigation of GHGs production through decreasing waste quantity and switching the energy to low-carbon power sources (IPCC 2007). Global major amplitude to mitigate climate change through find supplementary energy paternity, fossil fuel stage-out, interest side executive (energy efficiency and conservation, demand part grid authority, demand part switching source, lifestyle and performance, and dietary modify), sinks and negative emissions (reforestation and afforestation, avoided desertification and carbon storage and capture), and geo-engineering (solar radiation management and CO_2 elimination). These aspects have attracted more and more attention all over the world and are being strictly implemented.

4 Scenario of GHGs Emission from Waste Management

In 2005, the waste dumping areas make an involvement in GHGs emissions, and expected to increase at ~3–5% of the total emissions amount by anthropogenic activities (UNEP 2010). The generations of GHGs are inferable from a range of stages of a waste process scheme such as collection, separation, transportation, dumping, and other activities of all waste types will have different levels of GHGs emissions. According to the characteristics of solid waste itself, and the main purpose of solid waste disposal is resource conservation and resource regeneration, the technical route of solid waste management should be also responsible. First, avert the generation of solid waste; second, make full use of solid waste rational; finally, the proper management of solid waste that cannot be further used temporarily (Ling 2010). For solid wastes which are temporarily unavailable and new solid wastes such as debris generated during the regeneration of solid waste resources, we choose to use risk-free final disposal. There are many types of solid waste treatment methods, of which the most common of three, namely, sanitary landfill, incineration, and composting. Every solid waste disposal practice produced GHGs, either direct or indirect process.

However, landfill is the basic technique of solid waste management in most countries of the world. Unscientific way landfilling of unsorted solid waste has become an important source of GHGs (Huang et al. 2015). Landfill gaseous emissions are the most important starting place of GHGs generation from the waste management region that emits ~700 Mt CO_2-e (estimate for 2009). The organic matter of the waste under the covered landfill mainly goes through anaerobic decomposition, and the components of gas product mainly include CH_4 and CO_2, more than 90%, meanwhile produce H_2S, NH_3, and other trace gases (Wei et al. 2009). In terms of CH_4 alone, as important GHGs, its 100-year global warming

potential value is 25 times that of CO_2, and the rate of contribution to the greenhouse effect is about 22% higher than CO_2 alone (Zhanyun et al. 2014). The volume concentration of CH_4 in landfill gas can reach 55–60%; the annual global emissions are about 40 Tg; the total amount of methane released from global landfill disposal is the world's third anthropogenic methane source, accounting for 13% of global CH_4 emissions; and the CO_2 greenhouse effect is equivalent to 840 million tons (Eggleston 2006). In addition to CH_4 and CO_2 as the main components of landfill gas, its volume concentration in landfill gas can reach 40–45%, CO_2 with landfill gas and CH_4 together into the atmosphere; because of its biogenic, it is not included in the total GHGs emissions but it is an important part of a landfill gas (Zhanyun et al. 2014).

In recent years, the incineration method to dispose waste is much attentional because of its high degree of harmless, huge reduced capacity, which can recover heat, timely processing, and a series of advantages. It has become the main way to deal with garbage in developing countries (Zhang et al. 2006). The waste biomass after the incineration volume can be considerably reduced by 90% and the weight can be decreased by 80%. Due to the complexity of the solid waste composition, the produced contaminant of waste incineration is more problematic and more complex and toxic (Dong 2008). Incineration of waste, in addition to producing SO_x, NO_x, and dust, also produces HCl and highly toxic organic pollutants PCDD/Fs and heavy metals (Pb and Hg) and dioxins. From a global warming point of view, pyrolysis or thermal transformation of waste is described by a near full oxidation of organic matter to CO_2. Some organic waste debris may not be completely mineralized and thus generated as CO and various hydrocarbons. Depending on the flue gas cleaning system, nitrous oxides (N_2O) may also be generated. All such emissions from the incinerator involved in global warming (Astrup 2009). Municipal solid waste in different countries because of their different component contents, GHGs emissions, is also different. In waste incineration process, the most important way of GHGs emissions is the CO_2 generated by burning of the fossil source of waste. In China, GHGs emissions (calculated as CO_2 equivalent) during the generation of municipal solid waste incineration are 166–212 kg/t. The contribution of waste fossil carbon combustion to GHGs emissions is 257 kg/t. GHGs are produced by other processes (transportation, etc.) that account for 11–18% (He 2011). As a cost-effective technology to make organic solid waste resource, harmless and stabilization, composting technology is widely used in livestock and poultry manure and sludge treatment (Zheng et al. 2011). Manure and sludge after composting not only can become harmless, stable, and its available nutrients are more conducive to plant absorption but also the humus produced can develop the soil properties and can be used as an exceptional organic fertilizer. During the composting process, a large amount of CH_4 and N_2O are produced due to local anoxia and anaerobic–aerobic alternation in some areas. The amount of N_2O produced is about 0.02–9.9% of the total nitrogen, the amount of CH_4 is 0.1–12.6% of the initial total carbon, and the NH_3 released during composting can also be partially converted to N_2O in the atmosphere, resulting in indirect greenhouse effect (Yang et al. 2012; Wang et al. 2016; Awasthi et al. 2017).

5 Conventional Waste Management Verses Cleaner Technology

At present, there are many kinds of solid waste treatment methods, of which the most traditional are the three, namely, landfill, incineration, and composting. The application of landfill is the most widely used, and also the proportion is highest with 81.8% of the full amount; incineration accounted for 14.5% of total proportion; the composting effect is good but only used in individual areas; and limitations of the composting are larger than landfill and incineration, accounted for not more than 3.7% of entirety proportion (Wang et al. 2010).

Landfill is a waste management, which stacks the waste in a facility that is impermeable and almost isolated from the surrounding environment. This facility is generally built on land, so it is also called "land disposal". Sanitary landfill is a kind of landfill technology that differs from traditional landfill. In addition to careful and rational site selection for landfill sites, the facility is also required to apply anti-leakage treatment and cover up compaction to eliminate groundwater and atmospheric secondary pollution. Sanitary landfill includes anaerobic landfill, semi-aerobic landfill, and aerobic landfill. The structure used in anaerobic landfill is the simplest and does not require construction cost too much. The biogas formed subsequent to landfill can be reused. As a result, it is extensively globally used. Aerobic and semi-aerobic landfills are demanding on site; the cost is higher than anaerobic landfill, but the decomposition of organic matter is extremely fast and significantly shorten the cycle of waste stabilization, and also a positive impact on exudates quality (Bogner et al. 2008). Though landfill is widely used in waste management, this technique has obvious flaws. Primarily, it is much more complicated to manage the pollution created by this process. For solid waste landfill, the most important forms of contaminated media and contaminants are leachate contaminated, and the main source of leachate is precipitation. As the landfill cannot do strict anti-rain operation, production of leachate is inevitable; meanwhile, characteristics of landfill leachate are complex, and it is difficult to control and handle it. Second, the landfill will occupy a lot of land. And the value of the land around landfill will also be significantly reduced. Third, it is an elongated expression. They not only require to be strictly managed at some stage in the process of the landfill, still after the closing of the landfill, but also still require such a long-term management operation. Domestic landfill needs to set up landfill gas drainage system to smooth the export of methane-based landfill gas to prevent the accumulation of landfill gas to avoid explosion, and the corresponding landfill gas and malodorous gas treatment systems.

Incineration is currently the most commonly used solid waste high-temperature disposal technology. Incineration is under high-temperature conditions, and the organic matter in solid waste is oxidized and decomposed, thus achieving the goal of destroying harmful organic materials and mitigating the sum of total waste dumped. Incineration is suitable for disposal of organic waste but can also be used for the inorganic waste high temperature to melt curing (Pardo et al. 2015).

Incineration is one of the solid waste treatment processes which has the following advantages. First, it can completely decompose and destroy harmful organic matter, thus maximizing the harmless degree which the waste being treated. Second, the final decomposition of organic matter is carbon dioxide and water, after purification can be directly discharged into the atmosphere. Therefore, high-temperature incineration can minimize the amount of solid waste residues, that is, to minimize the landfill land and land occupied by solid waste, it can efficiently defend land property. Third, solid waste incineration using a fully enclosed factory processing mode that can maximize the control of pollution of the material leakage which may cause the procedure of transport, storage room, and feeding. Fourth, the heat generated during the incineration process can be recycled, so it can enable the regeneration of the solid waste which cannot be recovered. Beside this, incineration also has numerous disadvantages. First, the complexity of incineration technology led to a high demand of personnel quality and technical management. Second, because of the high requirements for equipment and pollution control of incineration, incineration costs are much higher than other disposal techniques. Third, utilization of incineration has few limitations on the uniqueness of the waste properties. Furthermore, due to the complexity of solid waste and the instability of its composition, as well as the complexity of incineration products, it results in a difficult to control the pollutants.

Composting is the process of degrading biomass, organic matter in waste into humus by the reaction of microorganisms under aerobic or anaerobic conditions. During aerobic composting, aerobic bacteria absorb, oxidize, and decompose the waste. Through their own life cycle, microorganisms decomposed complex organic matter to simple inorganic substances; meanwhile, the energy required for the growth of microorganisms is produced, the another part of the organism is synthesized into new cytoplasm, and so the microbial growth and multiplication increased microbial diversity (Sánchez et al. 2015; Awasthi et al. 2016, 2017). Anaerobic composting is under an anaerobic environment; use anaerobic microorganisms to complete the decomposition and transformation of organic matter. Many developed and developing countries put into a habit for anaerobic digestion and composting of organic fraction of solid waste (restaurant wastes or kitchen, waste of garden, and sewage sludge). Collectively, approaches are the most excellent functions used to source divide waste into fractions. As soon as composting aerobically decomposes waste and forms CO_2, a humic portion, and water; carbon storage occurs in the remaining compost. Composting can be sustainable at realistic cost in developing countries. Nevertheless, with the rapid development of technology, composting technology is more popular and feasible in the field of organic solid waste management. While striving to improve landfill, incineration and composting technology, according to the different characteristics of solid waste composition, is used to promote the comprehensive processing technology research and application. Biological treatment technology in solid waste management has great potential and broad prospects. Among all processing methods, bio-treatment technology, which is represented by composting, has the advantages of low cost, low operating cost, and trouble-free approach (Wang et al. 2010). In China, the

main components of MSW are kitchen waste and organic matter, and nutrient content is high and can reach 60–70%, while the water content of general organic is 70% higher; from the point of view of reduction and resource utilization, anaerobic digestion is a very suitable technique (Bo et al. 2003). South Korea has done a lot of research work on anaerobic digestion about kitchen waste, and there are many successful experiences worth learning from (Han and Shin 2002). New cleaner technologies such as thermal plasma gasification technology (Youngchul et al. 2010), hydrothermal catalytic gasification technology (Onwudili and Williams 2007), and thermal cracking technology (Ji-Qing and Xiao-Jing 2004) have been gradually applied in the treatment of solid waste, and landfill bioreactor technology (Visvanthan et al. 2010) for co-disposal of electronic waste with municipal solid waste has also begun to be applied to solid waste treatment. Improve utilization rate of solid waste and expand the view of comprehensive utilization that is one of the main development directions of solid waste treatment technology. Realizing the recycling of solid waste is an effective and sustainable way that not only can reduce the pressure on solid waste treatment facilities but also can reduce the utilization of environmental property and GHGs emissions.

6 Potential Impact of GHGs Emission Through Waste Management

Each and every waste management activity generated huge quantities of GHGs both directly and indirectly. On the other hand, then taken as a whole, climate change impact or its emission settlement with the cleaner organic waste management process will chance on net GHGs, computing for equally GHGs generation and savings. Presently, it is estimated that approximately 34% of all municipal solid waste (MSW) is either recycled or leveraged for energy recovery; the rest greater part moves to the waste dumping spaces (Bogner et al. 2008). Hence, there is a remarkable opportunity to endorse diversion of waste from landfills at the municipal point with guidance on standards and recommended approaches/strategies at the countrywide level. Some strategies at the municipal level include post-consumer recycling to avoid waste generation and reduce GHGs emissions from the production process, post-consumer fluorinated gases managing (fluorinated gas reduction), wastewater, and treatment of sludge to mitigate the CH_4 emissions. Landfills cause methane generation, chief GHGs, with twelve time's negative effect than carbon dioxide over its lifetime. The GHGs footprint of incineration depends on whether the process is simple incineration, which only emits CO_2, or part of a waste to energy system. The latter also emits CO_2, but the energy produced reduces the overall demand for energy, most of which is produced by burning fossil fuels, which also emit CO_2. There is a broad variety of operational practices of landfill in

place, with remarkable changes in the developed and developing nations. As many developing countries lag behind in waste management techniques, most cities rely on dumping in open landfills and release untreated sewage into waterways. While the mean per capita waste generation remains under that of developed countries, rapid rural-to-urban migration and changing consumption patterns are causing speedy expansion situation of waste accumulation in growing nations. In addition to methane emissions, developing countries are facing serious public health impacts resulting from the lack of sustainable waste management. The methane from the landfill place is the by-product of the organic waste breakdown with active action of mix microbes. These are creating a number of undesired situations and cause negative impacts on the climate as well as human health. These harmful conditions may comprise asphyxiation and explosions, objectionable odor, and displacement of oxygen in the nearby soils which slow down the normal growth of ingenious plant. On the other hand, landfill gas instead of being a significant source of risk and pollution could be considered as a significant source of renewable energy (Karagiannidis et al. 2010).

The landfill gas used as an energy source can successfully mitigate the generation of harmful greenhouse gases, so the recovery of gas from landfill site with energy generation was found to be the most resourceful utilizable way in mitigating the gaseous negative impact from big landfill sites. Incineration of MSW was also an adequate technology to reduce GHGs emissions, especially if the energy produced can minimize the burning of fossil fuels. The gaseous emissions due to the transportation of waste are very little when compared with the landfill emissions. Even if the transportation mileage is two times higher due to increasing segregation and recycling the greenhouse influence of transportation could be only 3–4% than landfill waste (Pipatti and Wihersaari 1997), some potential strategies to address landfills are processes that reduce GHGs generation compared to landfilling. These processes can include thermal processes such as incineration and industrial co-combustion as well as mechanical biological treatment (MBT) with the landfill of residuals and anaerobic digestion. By improving landfill management to reduce GHGs emissions at any scale (Bong et al. 2016), by using the modern approaches of waste management, cities can keep away from the per annum generation of GHGs. The climate impact of composting is due to both direct process emissions and indirect upstream and downstream emissions. Direct emissions from composting facilities result from fuel combustion in equipment and from decomposition of the organic material. As composting produces CO_2 from biogenic carbon sources, it does not contribute to national GHGs inventories for the waste sector. When compost is applied to ground, additional, negligible emissions will be emitted as organic compounds are slowly mineralized to biogenic CO_2. Therefore, compost applied to soil has a medium or long-term potential to store carbon and also a good choice to reduce the major amount of GHGs emissions (Amlinger et al. 2008).

7 Need and Gap Analysis of GHGs Mitigation Strategies

The developed and growing nation's waste management unorganized and organized sectors are increasingly not aware of cleaner technologies and GHGs emissions health and environmental impact with their link to global climate changes. The conventional approaches to solid waste sector mainly focus on end-of-pipe solutions, and the focus is on collection and disposal and not on use again and reduction practices. "*We dump—They collect*" is the most common outlook that has been cultured among residents, institutions, as well as industries by this approach over a long period. Waste management is not their concern; it is a municipal liability (Santuccia et al. 2014). Moreover, they are not identifying the problems of human and the natural ecosystem caused by global warming as well as their part as GHGs emitters due to mismanagement of waste. Furthermore, utilities are forecasting the future limitations of having to operate in a carbon foreshadow business environment. They want to prepare this potential operational limitation and to act in an environmentally accountable, economically feasible, and politically strategic approach by mitigating their GHGs emissions. To make informed decisions, utilities need to be familiar with the characteristics of the climate change problem and the many variables, uncertainties, and prospective costs and profit on specific GHGs mitigation options. Valid data on the steps concerned with, and the resources obtainable for, the GHGs reduction improvement approach have not been synthesized into an arrangement to help out utility choices. It requires direction concerning the GHGs emission reduction activities that are most excellent operation option to their management.

It is a remnant complex for nations to identify exactly and precisely how and where greenhouse gas emissions are released into the environment, to what degree the nation's mitigation rules and legislations are operational, if they are doing well or not, and how. Often, most of the developed and developing nation's information are not significantly sufficient to make adjustments for the waste management sector purpose. The influence of the countries mitigation action plan is difficult to authenticate. Furthermore, country GHGs information are not directly compatible with the reporting requirements of global needs. The existing management strategy of generated waste in the rapid growing world is not environmentally feasible and sustainable, because it relies only on dumping of waste on landfills places. It is apparent that the strategies of waste management mainly focusing on landfills site will show the way to enhance high GHGs emissions. Faced with the outcome and costs of operations, governments have reached a concurrence internationally that world emissions need to be mitigating remarkable. The developed and developing nations are working toward an international framework for rapid emission mitigation action plan.

The vital needs of the GHGs emission mitigation action plan are as follows:

- Waste handling and management sectors are key emitters in the developed and developing nations;
- Plan for climate GHGs mitigation is relevant to sustainable development goals;

- Need to improve capacity to collect, analyze, and clean technologies activity data and GHGs statistics;
- International agency should report regularly for GHGs from waste sector;
- Fill data gaps and build capacity among developed and developing nations;
- Provide an internationally accepted and neutral data common platform for evaluation of national information;
- Build emission mitigation targets in a waste management plan;
- Monitor routine performance, responding to policy and requirements of local and global GHGs initiative's activities;
- Build and report GHGs information which are relevant with international and national standards; and
- Provide authentic evidence of GHGs developmental for carbon financing (Fig. 1).

The waste management is the important and vital sector in the minimization of the GHGs emission caused by the global unscientific practices, viz., collection, handling, and open dumping. A variety of factors are widely affecting the GHGs emission from the composting including temperature, aeration level, moisture, and sources of nutrients. The important factors during the organic waste management are affected, in turn, by organic waste type and its properties, storage and handling, and improper transportation. It is important to note that the proper organic waste management is a vital process for any waste management sector because improper and unscientific use of waste can lead to unenthusiastic and harmful impact on the each and every niche of the environment. There is very little documentation available on whether and how the country has modified waste management (or related) legislative policies for reducing the greenhouse gases for the improvement

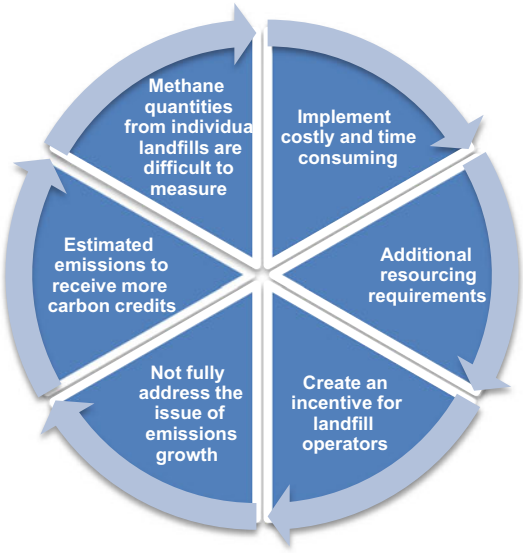

Fig. 1 Challenges and gap analysis of GHGs emission from waste management

Table 1 Gap analysis for the estimation GHGs emission from waste management sector

Sector	Activity	Gaps	Required effort to address the gaps
Waste management	• Collection and transportation	• Low maintenance • High fuel CONSUMPTION • Full fugitive emissions from vehicles	• Cleaner motor fuel • Obtain full fugitive emissions data from transportation activities
	• Treatment	• Unscientific approach	• Cleaner composting technology
	• Landfilling	• Facility-level • Emissions from waste dump activities	• Measure facility-level

of surrounding climate. To explore GHGs mitigation question in the context of cleaner technologies, the new approach to understanding how effectively governments respond to new challenges by analyzing the current policy regime and identifying potential gaps in the different steps of waste management and their carbon credit opportunities is shown in Table 1.

The GHGs emissions are common due to improper unscientific management (nonexistence of recovery and treatment options) of organic municipal solid waste, particularly organic waste. Hence, the reduction of GHGs emissions (CH_4 and CO_2) from municipal solid waste involves the following:

- Mitigation of the quantity of waste;
- Separation of organic wastes; and
- Management of waste to recover resources (compost–aerobic treatment) and energy (biomethanation).

Mitigation of waste generation is possible through least waste generation, proper segregation at source level, reuse, and recovery of waste. Composting is one of the most suitable treatment processes for organic waste in a sustainable manner, whereas recycling is frequently used for inorganic materials. Wastes that cannot be treated or recycled are ultimately disposed at disposal sites or landfills. Separation at the source level with treatment at ground levels plays an important role in mitigating organic fractions getting into disposal sites responsible for the emission of GHGs.

7.1 Approaches and Strategies for GHGs Mitigation

From the composting areas, the GHGs emissions need to be calculated and subtracted from the emissions avoided calculated beyond. Gaseous emissions may come from the composting activity and from the equipment used to control the

complete process. CO_2 free from the composting is measured biogenically, so does not calculate in GHGs calculations. Although it is hypothetically probable for methane to be released by a poorly managed compost facility, the Environment Protection Agency (EPA) has accomplished that there are a very few confirmations that this in fact happens, so considers any releases insignificant. On the other hand, the fuel and electricity used to operate the equipment and buildings result in anthropogenic releases. The EPA projected that diesel utilizes at compost facilities consequence in the release of 35 pounds of CO_2 per wet ton of processed feedstocks. Using this amount, the 14,800 tons of organic waste predominately diverted from Portland would as a result, in 259 tons of CO_2 being released. This tonnage would be cut down from the net profit of the avoided emissions. In actuality, the definite use would have to be standardized and confirmed. For instance, a investigation by the Recycled Organics Unit in Australia probes the release of about 30 lb of carbon dioxide per ton of garden residuals windrowed from fuel utilized, and only an supplementary 6 lb per ton of electricity.

7.2 Direct Mitigation of GHGs Generation

The waste generation is directly connected to urbanization, population, and affluence. The growing rate of waste generation can be coupled with the gross domestic product energy consumption per capita and classified final consumption per capita (Richards 1989; Bingemer and Crutzen 1987; Rathje et al. 1992; USEPA 1999; Mertins et al. 1999; IPCC 2000; OECD 2004; Bogner and Matthews 2003). In developed nations looking forward to mitigate waste generation, an existing objective is to decouple waste generation from economic dynamic forces such as GDP (EEA 2005; OECD 2003; Giegrich and Vogt 2005). In the majority of developed and developing nations with increasing urbanization, population, and prosperity its ultimately a major challenge for every municipal site to collect, recycle, treat, and dump of growing amounts of wastewater and solid waste.

7.3 Avoidance of GHGs Generation

The greenhouse gaseous emissions from the region of agriculture can be mitigated from side to side by the execution of better sustainable management practices. For instance, the options of manure storage space method should be mainly based on ecological assessment criteria, as well as production capacity. By the composting process, all the farmhouse animal dungs are used as slurry and stockpile and a decrease of 0.70 Tg CO_2 per year would be found. Likewise, by the methane generation collecting and then burning from obtainable slurry amenities, a refuse of 0.76 Tg CO_2 per year would be achieved. The latest CH_4 emission factors were projected based on these outcomes and incorporated into the IPCC methodology.

Under cool conditions of North America, the methane emission would be 45 kg methane per hectare per for dairy farm animals manure rather than 36 kg methane per hectare per, and 3 kg methane per hectare per for farm animals manure to a definite amount than 1 kg methane per hectare per (Pattey et al. 2005) contribution that dung treatment makes to entirety country farming emissions of N_2O and CH_4 differ; however, it can go higher than 50% in nations reporting to the UNFCCC (2009). On dairy farm management, decisions act together with ecological controls such as water availability and temperature of key microbial processes (that is, nitrification, denitrification, methanogenesis, and methane oxidation), troubling the quantity of emissions from each stage of the dung management level. The current considerate of manure management disturbed the direct and indirect N_2O emissions and CH_4 emissions, set up a new data comparing direct N_2O emissions following dispersion of a range of manure types of diverse methods, and emphasize some of the mitigations being considered by environmentalist and policy-makers (Chadwick et al. 2011).

The emission of GHGs could be mitigated by optimizing the compost heap dimension as larger heap amplifies methane and nitrous oxide emissions due to deprived aeration (Fukumoto et al. 2003). Unnatural exposures to air and rotating usually decrease methane emission (Lopez-Real and Baptista 1996), at the same time as rising compost pile porosity could decrease nitrous oxide release (Møller et al. 2000). Bedding matter used in farm animal's feedstock not only affects the ammonia emission in the feedlot pen, except GHGs emission during composting. On the other hand, composting in open windrow, woodchip, or straw bedding is completed with no differentiation to cattle feedlot manure GHGs emissions (Hao et al. 2004). The effects of going on a diet manipulation on manure values can also put back to affect GHGs release from manure composting. While an 85% of grain of barley diet was replaced with 60% dehydrated distilled grains and no more than 25% barley grain, nitrous oxide generation from composting farm animals dung was high but methane generation was not pretentious (Hao et al. 2011). The larger nitrous oxide release can be recognized to the higher nitrogen content in DDGS. Separation of municipal solid waste followed by recycling (for metals, paper, plastics, and textiles) and anaerobic digestion or composting gives the less net flux of GHGs, compared to other supplementary approaches for the MSW management. In relationship with landfilling unprocessed waste, composting or anaerobic digestion of wastes and recycling of paper generates the overall greatest reduction of net GHGs.

8 Policies and Guidelines for GHGs Mitigation Through Waste Management

Compost has extensively been used for the enhancement of soil fertility, increased water upholding, and mitigating interconnected understanding of drought. The compost application on unfertile lands has enhanced sequestration of carbon present

in the soil, stabilizing the carbon stage and an effective waste management approach for fall in the intensity of GHGs generation (Rathmann Challenge Guidelines 2017). Methane, carbon dioxide, and nitrous oxide emissions from the waste-related areas on wastewater, land, incineration, and other waste-related activities are reported. The solid waste and GHGs emissions are closely connected with each other directly, which means that reducing GHGs produce from MSW is chiefly affected by various policy and regulatory strategies that can usefully be grouped into those that encourage energy and other source recoveries from waste materials, restrict choice for ultimate waste disposal, promote waste recycling, and reuse and encourage waste minimization (Calster et al. 2015).

The GHGs account data can provide the information about GHGs emissions at a national and regional level. It includes the following:

- Quantity of GHGs emission and gases;
- Time sequence trends in GHGs emission from a different source;
- The approach used for the assessment of GHGs emission; and
- Information of parameters used for the assessment of GHGs emissions.

The combinations of the following information of GHGs are useful for the preparation of the policy plan for the GHGs mitigation. It includes the following:

- GHGs emission from different sources that require mitigation action;
- Potential reductions in GHGs and objective for future reductions to be met through mitigation activity;
- Prioritization of mitigation planning; and
- Development of monitoring approaches for evaluating the mitigation policy planning at each point.

The Framework Convention on Climate Change is an international platform formed from the integration of 150 nations in June 1992 by the United State (U.S.) in Rio de Janeiro. The union committed signatory nations to take a non-binding action to reduce GHGs emissions to 1990 levels by 2000. The release levels of GHGs for post-2000 were determined through subsequent negotiations subsequent to the Rio Convention. The first of these GHGs mitigation debates occurred in Berlin in April 1995. The first conference on the "Framework Convention on Climate Change" was mainly decided to launch a 2-year progression to define measures and advance commitments to mitigate the GHGs generation in the post-2000 periods. This agreement afterward became known as the "*Berlin Mandate*". The second conference was held in Geneva from July 8–19, 1996. The third conference is scheduled for December 1997 in Kyoto, Japan. The **Intergovernmental panel on Climate Change (IPCC)** has built international GHGs mitigation guidelines that provide the fundamental data on the estimation of GHGs. The approach of IPCC concluded that individual activities have caused an unfavorable effect on environment. The IPCC strategy also initiated the theory of "Tier" to represent methodological complexity levels. The new guidelines, the 2006 IPCC strategy for national greenhouse inventories (IPCC 2006), summarize

three-tier methodologies (Tier 1, 2, and 3) for each GHGs emission source. The methodology of tier 1 is composed of the fundamental approach, tier 2 includes the intermediary, and tier 3 is sometimes referred to as higher tier methods and is generally measured to be further accurate.

The methodologies of tier 2 and 3 are action data openly connected with GHGs producing activity. It can provide further correct GHGs emission information than a tier 1 method. Policy-makers use GHGs emission results in the preparation of policies for GHGs mitigation. If they have entrance to more accurate GHGs emission information, it can be taken more effectively mitigation actions. The methodologies of tiers 2 and 3 are widely used activity data that are directly linked with GHGs producing activities, and the development achieved as a consequence of each mitigation action will be with and without human intervention reflected in the GHGs inventory under these methods. Consequently, to make sure efficient mitigation policy plan, the developments of an inventory of GHGs based on tiers 2 and 3 methods are suggested (Kaneko and Kawanishi 2016).

8.1 Ministerial Declaration and Future Climate Change Activities

On the last day of the Geneva Convention, a ministerial declaration was adopted that calls for, or encourages, the development and establishment of a legally binding post-2000 limits on GHGs emissions. Provisions within this agreement, the "Geneva Declaration", encourage the development of a legally binding emissions goal for developing nations and emissions reduction commitments from developing countries; endorse the conclusions of the IPCC report; support a U.S. recommendation that legally binding medium-term emission targets that are pursued; and urge expedited development, application, dispersion, and transfer of climate-friendly procedure.

8.2 Indian Efforts on GHGs Mitigation

The number of policies has been formed in India that contributes to atmosphere gaseous emission mitigation by reducing or avoiding GHGs emissions. The first "*National Action Plan on Climate Change*" policy was formed in June 2008 which recognized eight central part "national missions" operations in the course of 2017. The first act in worldwide climate cooperation was to transform the environment agency "the Ministry of Environment, Forests and Climate Change" (Government of India 2013). The National Action Plan on Climate Change (NAPCC) is given that the current and outlook policies focused chiefly on the climate improvement and different programs. Used by governments for an extended phase, policies based

on market are formed to manage environmental contamination at a diversity of influence points. They are efforts by altering relative prices—increase the cost of emission-intensive actions and/or reduce the cost of minor-emitting alternatives—to supply producers and consumers with an economic incentive to adopt at the end. Policies that can be deliberated market-based comprise fees and taxes, subsidies, and the use of the pollution control trading process. Market-based policy provides financial incentive to elicit definite performance from entities liable for GHGs emissions, whether consumers or producers. These shorts provide and summarize policies based on market meant, at decreased GHGs emissions in a number of most important emerging economies: India, China, Brazil, South Korea, and South Africa. By implementing authoritarian and market-based policy transversely their economies, these countries are looking forward to endorse cleaner technologies and behavior transform while also promoting economic improvement and growth (Moarif and Rastogi 2012).

8.3 Cap-and-Trade Regulations for Cleaner Composting

Cap-and-trade regulations limit the quantities of pollutants (in this case GHGs) that entities can emit into the environment, and can supply financially feasible incentives for falling emissions even further (below the cap). The "cap" sets a restrict value on GHGs emissions, while the "trade" makes a marketplace for carbon allowances. Regulated entities (for instance, a coal fire power plant) can either reduce emissions from their own facilities, or can purchase "emission reductions" from other regulated entities that have to mitigate their emissions lower than their cap (and therefore have some left over). The most important components mixed up in a cap-and-trade plan are caps, reporting, and monitoring. Limit (cap) of GHGs release for any company is set up by international, federal, or local governing body. The government then decides on coverage, or the sectors and sources of carbon that must meet the terms with this limit. To make sure, observance with these cap systems must as well be present to examine origin point, checking and verifying each and every source's coverage of carbon production. Sources, on the other hand, may go further than their ranges, or in excess of the cap, if they had traded with an additional source.

Waste management (WM) activities practiced in various emergent nations release high quantities of GHGs in the environment. High GHGs generation is mostly due to landfilling; therefore, solid waste composting areas create significant opportunities for carbon reduction, which could eventually twist out to be carbon credits tradable. Emission mitigation can also be shaped freely by non-synchronized entities (such as a compost facility) to be used by synchronizing entities to offset their individual emissions. The "United Nation (UN) Framework Convention on Climate Change" in 1992 formed that the Kyoto Protocol was the basis for preliminary carbon cap-and-trade steps. There were 165 pieces of law formulation first

introduced in the 110th congress in the middle of 2007, of which at least were correlated with cap-and-trade programs (Kapoor and Ambrosi 2007).

8.4 Carbon Credit Through Composting

Carbon offsets or carbon credits commonly referred to as certified, tradable greenhouse gases emission mitigation, used inside a cap-and-trade program. The emissions of least gas do not regularly produce carbon credits consequences from a formal "protocol" or procedure that verifies, quantifies, and certifies qualifying reductions of emission from suitable projects. Credible carbon credits as a whole represent real, demonstrable, everlasting, scientific, and enforceable emission mitigation. Generally, the carbon credits are appropriately registered by a carbon "exchange or registry" to make possible market trading and ensure that the same credits are not selling more than one time. The quantified CO_2 equivalents (CO_2) in metric ton units usually used the carbon credits.

The zero emission of carbon dioxide equivalents per ton is measured as one credit and each carbon credit fetches the firm approximately $3–6. In Europe, it is €5 per tons of CO_2 but is expected to rise to €7.50 during the course of the year 2014. The value will potentially reach a new high of €180 billion by the year 2016. The carbon credit remuneration continues year after year. And the most excellent part is that it is quite easy to implement technologies known to reduce emissions provided the project meets certain criteria. The firms trading credits have two options to choose from depending on the life of the project—fixed crediting period of 10 years or first period of 7 years extendable twice for a total period of 21 years. The benefits of cleaner sustainable technologies are the capacity to gain funding, commencing a prototype carbon fund under the support of the World Bank. The fund is formed by contributions from many developed nations. Indian firms may well take the lead to use cleaner technologies to earn credits and secure funds. From 2005 to 2006, the importance of the total world market for carbon credits tripled. More than one billion tons of credits in 2006 were sold in the course of the European Trading scheme with a marketplace pace of about $20 billion (by the nations with Kyoto Protocol adopted) (Kapoor and Ambrosi 2007). Those countries which are not agreement in accordance with the Kyoto cap-and-trade process of the United State (U.S.), the market is very little but still considerable: in 2006, the carbon traded on the Chicago Climate Exchange (CCX) over 10 million tons with a value of over $40 million, in view of the fact that the 2003 Chicago Climate Exchange (CCX) prices have differed from a lower amount of than a dollar per metric ton to approximately $5. Due to international agreements and accomplishment of climate change, the carbon market is one of the highest increasing markets for financial commodities. The growing apprehension and increasing responsiveness are required for pollution control; the perception of carbon credit came into fashion as part of a Kyoto Protocol meeting, widely accepted. Carbon credits are

authentically issued to nations that mitigate their emission of GHGs, which are responsible for causing the global warming.

8.5 Mitigation of GHGs Emission Through Process Optimization

The GHGs emissions together with the CH_4, NH_3, and N_2O are the main contributor of global warming from the composting. A variety of improvement approach is reviewed to build up the impacts of composting as shown in Table 2. N_2O and CH_4 have a considerably higher global warming than CO_2, while NH_3 was the primary gas for acidification potential. The global warming from the composting process is different approximately 300 kg CO_2 eq. t^{-1} by Couth and Trois (2011).

Based on 1 ton of waste food used as feedstock, Kim and Kim (2010) established that food waste composting had a typical global warming of 123 kg CO_2 equivalent per ton as compared to landfilling (1010 kg CO_2 equivalent per ton). The global warming value varies based on the dry waste basis (200 kg CO_2 eq. t^{-1}) wherever the feedstock waste material was subjected to air-dry and on the wet waste basis (61.2 kg CO_2 equivalent/ton) where the feedstock waste has the wetness content of 70–80%. Therefore, composting is chiefly to lesser the global warming, compared to landfilling. The improvement process to minimize the GHGs generation during composting can comprise the make use of a bulking mediator, aeration arrangement, and matured or chemical composts as covering material. The collection of bulking mediator is based on the available in the vicinity of agricultural residues such as wood chips, sawdust, corn stalk, spent mushroom, and cotton (Santos et al. 2014; Maulini-Duran et al. 2014; Wang et al. 2014a, b; Yang et al. 2013). A bulking mediator aims to develop the airflow inside the compost and to mitigate the CH_4 emission. All the way through the kitchen waste composting, they put together use of sawdust recorded the maximum mitigation of methane and N_2O, which was >97.7 and 72.9%, and then spent mushroom (97 and 28.8%), subsequent to that corn stalks (93 and 46.6%) (Yang et al. 2013). The investigation did

Table 2 Mitigation of GHGs emission through composting at different places of the world

Country	Emission reduction (Ton CO_2 per year)	Ton per day
Bangladesh (Dhaka)	6814	100
China (wuzhou)	7022	93
Ivory Coast (adidjen)	7212	219
Columbia	5570	500
Indonesia (Bali)	27,020	700
Delhi (Okhla)	73,202	1950

Source Kollah et al. (2014)

not demonstrate a noteworthy reduction in ammonia. In a further study to the composting of duck waste, the combined vermin composting (with earthworms additional supplement with the compost) and pre-composting additional with the reed straw drastically lower down the N_2O and methane emission (80.9 and 84.2%) (Wang et al. 2014a, b). Jiang et al. (2015) established the competence of diverse ventilation process and volume of air provides alongside different GHGs emission profiles. For the emission of CH_4, required unbroken aeration with lower aeration showed the highest mitigation where the one with the highest aeration showed the least reduction. On the different, for NH_3, required unbroken aeration with the highest aeration speed performed considerably improved than the one with a minor aeration speed. For N_2O, the intermittent aeration performed better than the forced continuous aeration. The mitigation effectiveness for the gaseous varied all along the heap height. Zhang et al. (2012a, b) showed that the maximum mitigation of CH_4 was achieved at the pile with the less height, but for the case of N_2O, the highest mitigation efficiency was getting to the most heaps.

The phosphogypsum mineral additive, a major by-product of phosphoric acid, is efficiently used for the mitigation of CH_4 in the fresh pig feces composting (Luo et al. 2013) and waste generated from the kitchen (Yang et al. 2015) by enhancing the SO_4 concentration in compost. The mixing of phosphate salts with the magnesium, in the course of the formation of struvite ($MgNH_4PO_4 \times 6H_2O$), mitigates the ammonia emission (Zhang and Lau 2007). N_2O emission was mitigated by the use of dicyandiamide ($C_2H_4N_4$) nitrification inhibitor (Luo et al. 2013). Luo et al. (2014) established the layer where the composting heap with old compost gets a higher degree of GHGs mitigation than by mixing them together. A detailed summary of the mitigation used for every composting operational relating to their effectiveness is included in the additional Table 3. Based on Table 3, for the adding up of the bulking mediator, the kitchen waste composting with three different bulking mediators, namely, sawdust, corn stalk, and spent mushroom substrate, was extensively used to cut down the GHGs generation (Yang et al. 2013). In all three process of treatments and the control (without bulking mediator), the entirety GHGs emission ranged between 33 and 161 kg CO_2 eq. t^{-1} dry mass (DM), with the standard having the uppermost gaseous emission (161 kg CO_2 eq. t^{-1} dry mass) and sawdust treatment having the lowest GHGs emitted (33 kg CO_2 eq. t^{-1} dry mass). Wang et al. (2014a, b) revealed that the bulking agent add into the composting of duck waste, with a 45-day pre-composting followed by a 32-day vermin composting, showed a decrease of GHGs emission approximately 42.1–50.2 kg CO_2 eq. t^{-1} duck manure. For the aeration advance, the spend air reusing system (SAR) functional at different composting heap elevations (2.5, 3.0, and 3.5 m) was most efficiently utilized the compost made from the MSW (Zhang et al. 2012a, b). The sum of GHGs emission recorded ranged between 5800 and 7800 kg CO_2 eq t^{-1} municipal solid wastes. The control (2.5 m pile height, without SAR) formed the uppermost GHGs emission, while the highest heap (3.5 m) with spend air reusing system gave the lowest GHGs emission. Throughout the composting of dairy manure, the heap rotating was well known to enhance the generation of greenhouse gases (Ahn et al. 2011). The greenhouse gases generated from the waste heap being

varied, and the fixed heap was established to be 52.7 kg CO_2 eq. t^{-1} and 33.5 kg CO_2 eq.t^{-1}. Integration and covering the pig manure with the matured compost has considerably mitigated the GHGs emission (Luo et al. 2014; Wang et al. 2016). The free GHGs from not covered, not mixed with matured compost mixed with old compost basically, compost covered with only mature compost mixed, and protected with older compost were 209, 108, 61, and 93 kg carbon dioxide eq. kg^{-1} dry mass. Even though additional mixing can be effective in mitigating the GHGs, the outstanding case was also observed. More information such as the moisture content and the pH waste type of the composting material should be analyzed to produce more conclusive results on the role of mixing or aeration to reduce a particular type of GHGs. The adding of chemical with the superphosphate and phosphogypsum during the course of kitchen waste composting produces the total GHGs emission of 117.9 kg CO_2 eq. kg^{-1} DM to 97.4 kg CO_2 eq. kg^{-1} DM and 109.3 kg CO_2 eq. kg^{-1} DM (Yang et al. 2015).

The chapter reveals that most of the studies were inadequate to small to medium scale of composting and were mostly conducted in a closed system. More center of attention was on the GHGs mitigation for the composting organic biomass. More research is required to analyze diverse scales, operating systems, and waste streams to illustrate more convincing conclusions on the mitigation policies.

9 Technological In-Progress in Cleaner Composting

The composting GHGs savings refer to the avoided emission from the landfill, substitute of motor fuel and peat, and the utilization of compost carbon sequestration. The avoided emission from the landfill includes the CH_4 emission from the anaerobic decomposition of organic waste and the fuel utilization from the transportation of waste. Composting incurs a lesser expenditure of fuel, as the site location is in a more proximate distance from the waste source as compared to the landfill. The replacement of motor fuel and peat reduced the fossil fuel consumption for the production of motor fuel and extraction of peat, and their transportation. Compost utilization can promote carbon sequestration for long-term carbon storage in the soil. The adding together of stable *C* to the soil by the use of compost consumption is one of its kind advantages as compared to other technologies of waste management, like landfill and incineration.

The landfill avoided emission for the organic waste decomposition is frequently calculated from the IPCC (2006). Collection and transportation of waste are frequently excluded by researchers either by excluding them from the system frontier or assumed the travel distance for all waste management scenarios to be the same. Based on the research, usually incorporated the avoided emission from either a motor fuel or peat utilization. Kim and Kim (2010) confirmed that composting may apply a negative effect on the surrounding environment if the record excluded the positive benefits of motor fuel replacement. Boldrin et al. (2010) found that one ton of manure can be used to go back 285 kg of peat, functional at a density of 200–

Table 3 Functional unit study of different waste material compostings (Bong et al. 2016)

Study region	Waste type	Method used	Functional unit	References
Spain	Fruit, vegetable, plant-based waste	Home composting	One ton raw fruit and vegetables composted	Colón et al. (2010)
Denmark	Organic household waste	Home composting	One ton of organic household waste for the assessment time scale of 100 years	Andersen et al. (2011, 2012)
Spain	Fruit, Vegetable, Plant-based waste	Home composting	0.0608 ton/m^2 of horticultural crops of cauliflower	Quirós et al. (2014)
Greece	Food waste	Home composting	One ton of organic household waste composted	Abeliotis et al. (2015)
Taipei	Biodegradable waste	In-vessel composting and home composting	One kg of organic matter	Hermann et al. (2011)
Taiwan	Municipal solid waste	Centralized composting	One ton of solid waste between primary collection and arrival at each treatment method	Chen and Lin (2008)
China	Municipal solid waste	Centralized composting	The 909,160 ton of treatment of MSW by the Tianjin city	Zhao et al. (2009)
Spain	Organic fraction of municipal solid waste	Composting in-tunnel and centralized windrow composting	One ton of Organic fraction of municipal solid waste used	Cadena et al. (2009)
Spain	Organic fraction of municipal solid waste	Composting in-tunnel	Yield of one ton of commercial tomatoes	Martínez-Blanco et al. (2009)
Denmark	Yard waste	Centralized windrow composting	One ton of yard waste being composted	Andersen et al. (2010a)
California	Yard waste	Centralized windrow composting	One ton of source-separated yard waste	Van Harren et al. (2010)
Korea	Food waste	Centralized composting	By-products from the treatment of one ton of food waste	Kim and Kim (2010)

(continued)

Table 3 (continued)

Study region	Waste type	Method used	Functional unit	References
California	Organic fraction of MSW	Centralized windrow composting, aerated static pile	One ton of wet organic matter	Kong et al. (2012)
Pennsylvania State University	Food waste	Centralized windrow composting	Collection, processing, transportation and application of one ton of compost that meets USEPA composting standards over 13 months	Saer et al. (2013)
Sweden	Food waste	Centralized windrow composting	Elimination of one kg of waste food	Eriksson et al. (2015)
Turkey	Food waste and yard waste	Centralized composting (aerated static pile)	1 ton of municipal solid waste generated in Sakarya	Yay (2015)
Iran	Municipal solid waste	Centralized composting	1 ton of municipal solid waste in the region 4 of Tehran municipality	Rajaeifar et al. (2015)
Spain	Municipal solid waste	In-vessel composting → windrow	The household waste collection and treatment in the definite region	Bueno et al. (2015)
African	Biodegradable waste	Centralized windrow composting	1 ton of impurity-free biodegradable waste	Komakech et al. (2015)
China	Municipal sludge and woodchip	Centralized windrow composting	33.33 ton of woodchips and 100 ton of sludge	Zhao et al. (2015)

700 kg m^{-3}. Saer et al. (2013) found that the greenhouses gases release from peat replacement was capable to give back to the environment regarding eutrophication potential (EP), acidification potential (AP), and GWP throughout composting activity. Avoided emission seems to put forth an adverse impact on the overall global warming for composting and should be included in the record analysis.

The assessment and evaluation of the environmental concert on compost and motor fuel substitute are difficult. Compost and motor fuel response in a different way to carbon sequestration, nutrient loss, and heavy metal leaching, which are governed by the local circumstance (Hansen et al. 2006). There is also a high dissimilarity of the nutrient and the eminence of the compost which are dependent on the feedstock and operational environment. Even though composting is seen to reduce peat taking out for horticultural use, there is still limited information on the recognition of farmers or public toward the substitute of peat with compost formed from the organic waste. Used inventory showed that only 21% of the users favored to use home-produced compost in excess of peat, whereas 18% of the users would replace motor fuel with compost (Andersen et al. 2010b). One of the most important concerns is the lack of caution of nutrient quantity and rate of the composted growing medium (Waldron 2005), predominantly in the developing countries where the quality labeling for compost is poor (Fan et al. 2016). There is in addition incomplete information on the ecological performance on the compost post-application, for instance, N_2O release from compost consumption (Boldrin et al. 2010). This results in the elimination of post-application stage in most of the record analysis for composting (Hermann et al. 2011).

Carbon capture via composting has been seen as a key process for the mitigation of GHGs due to the development of humic contents or matter to encourage soil carbon going below the surface (Hermann et al. 2011). Utilization of compost is seen to get improved soil carbon sequestration, a procedure of retaining and enhancing carbon contented in soil with justifying GHGs emission. The procedure contributes to storage of soil carbon and therefore promoting plant enlargement for improved carbon capture in excess of a defined time range (Butler and Hooper 2010). For the GHGs investments through composting consumption, most did not contain the long-term soil carbon storage due to imperfect or partial data. Humic material contributed to the long-term carbon capture on the other hand is tedious to be extracted for quantification. The minimum emission also depends on the management of land activities, function rates, and the period of depth (Butler and Hooper 2010).

10 Future Prospects, Opportunities, and Challenges

Organic Waste Management Sector (OWMS) is a significant contributor to increasing levels of atmospheric GHGs concentrations, and the literature presented in this chapter suggests that there are significant improvements needed for the potential mitigation of GHGs emissions from this sector. Further work is needed to

improve assessments of GHGs emissions from waste management sector, to improve management practices to make certain environmental integrity, to design well-organized policies to implement GHGs mitigation, and to strengthen the potential of waste management to contribute to producing the cleaner approaches. Identifying synergies and co-benefits that may exist in relative to climate change, sustainable development policies, and improvements in ecological quality would make mitigation practices more striking and suitable for the society. Better country-specific data on the mitigation potential of different practices for composting will help countries to design the most appropriate portfolios of mitigation; a practice involves the following:

- Develop simple and user-friendly processes and techniques for the estimation of GHGs emissions and crucial air pollutant emission source;
- Run an indigenous GHGs emissions inventory;
- Develop a process and techniques for the projection of GHGs emissions;
- Develop a methodology for assessing the emissions reduction potential of measures;
- Prepare calculation tool for undertaking cost–benefit analyses of GHGs emissions mitigation measures including environmental externalities;
- Develop a local action plan (LAP) for drastic and long-term GHGs emissions reductions;
- A set of practical guidelines for measures' implementation;
- A monitoring plan and relevant indices for evaluating the progress of the implementation of GHGs emissions reduction measures;
- Development of learning material on climate change directly focuses on the schools (students and teachers);
- Involvement in municipal lighting and municipal buildings, to mitigate their power consumption; and
- Dissemination and fast responsiveness actions.

Financing climate change mitigation and adaptation is a challenging task embedded in a rapidly changing climate change framework. At the identical moment, there is a necessity to take action now in order to meet the challenges connected with climate change. Several national, bi-, and multi-lateral financing mechanisms have been established, which either predominantly finance mitigation or adaptation projects. While climate change is a tremendous challenge, it also may be the source of tremendous opportunities. The steps we take to mitigate GHGs emission impact on our climate are also a potential opportunity to grow the fast economy. There are good jobs from cleaner energy, smarter use of natural resources like organic waste, and adoption of advanced cleaner technologies. By the creation of a market for carbon credit or trading carbon dioxide emissions will be the great opportunity for potentially highly lucrative business in the developing world.

There are numerous issues and challenges which result in a current perceived lack of implementation. Climate change through the GHGs emission from the unscientific waste management practices has become one of the major challenges

for mankind and the degradation of the surrounding atmosphere. Concentrations of greenhouse gases emissions released into the atmosphere are increasing speedily and main accountable for this transform. These climate changes extensively influence the community as well as environmental determinants of health safe drinking water, clean air, secure shelter, and sufficient food. Extreme warmth can account for the casualty. Associated with high concentrations of ozone and other air pollutants can lead to an exacerbation of respiratory diseases. Pollen and other aeroallergen concentration levels are higher under extreme heat. The information on greenhouse gases emissions is held between the great numbers of actors concerned; it can be highly discretized, context-specific, or complicated to access causing fragmentation to be an obstruction. Institutional void is the lack of commonly accepted rules and norms for policy processes to take place, calling into question the legitimacy and efficacy of policy processes. The issues that arise with a system which involves international government cooperation, such as cap-and-trade, could potentially be improved with a polycentric move toward where the regulations are compulsory by numerous small sections of authority as conflicting to one on the whole enforcement agency.

11 Conclusions

Increase of GHGs generation due to continues dumping of organic solid waste has been an ever confront important thing to reduce global warming potential. Municipal corporations from corner to corner the world have imported novel and cutting-edge blueprint to mitigate directly soaring GHGs production. A number of these policies are carbon taxes, energy competence procedure, direction and regulation approaches, and market-based pollution dealings appliance. In this chapter, compared to the GHGs generation from diverse municipal waste management approaches in developing and developed nations, developing nation's citizens do not have a compulsory compulsion to brief GHGs and due to this region has not available of exact statistical figures for solid waste management as well as another way for the quantity of GHGs generation. Due to lack of such statically figure of solid waste generation, a number of presumption have to be hypothesized which influence the certainty of estimation and compose authorization of outcome, a very competitive process. Meanwhile, the GHGs generations from waste disposal in developing nations are assumed to rise gradually. Hence, lower GHGs emission technologies are obligatory to operate the rising production of compost from the organic wastes. Besides waste dump on landfill sites, composting process can help out to recycle household organic waste, while it will mitigate the collection, conveying and handling expenses and energies. Without more regulation among international, national, provincial and district institutions, incorporation into distinctive central priorities and program, and commitment among the private and civic sectors and public, it seems probable that countries in the world will lock in more completely too high-cost, high-carbon expansion route. Because of the global

implication, this would considerably influence our combined competence to escape unsafe altitude of climate change. Strategies for the mitigating of GHGs generation by carbon sequestration and cleaner and sustainable composting processes are the only feasible way to reduce the harmful impact of climate change and give a framework for the improvement and operation of new cleaner technologies for a possible sink of GHGs mitigation.

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Chapter 17
Recent Advances in Composting of Organic and Hazardous Waste: A Road Map to Safer Environment

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Abstract With the rapid development of economy and agriculture, continues to be the fast increase of organic and hazardous wastes such as animal manure, sewage sludge, green waste, antibiotics residue, municipal solid waste, and agricultural waste. These wastes contain lots of organic matter and nutrients, and also contain various kinds of toxic materials or elements (e.g., heavy metals, pathogens, antibiotics and antibiotics gens). The improper disposal of these wastes would result in environmental pollution and potential risk of human health. Composting technology is a kind of biological waste treatments, which has been widely accepted as an alternative method to recycle the organic matter and produce a stable and sanitary soil fertilizer or amendment. Furthermore, many researchers have elucidated the major factors such as temperature, pH, C/N ratio, moisture content, and particle size that are relevant in the monitoring of the composting process. However, the traditional composting still has some drawbacks such as nitrogen loss, leachate generation, odor problem, greenhouse gases (CH_4 and N_2O) emission, heavy metals (HMs) mobility, antibiotic residue, and antibiotic gens diffusion. During the composting process, 9.6–46% initial total nitrogen (N) is lost due to the volatilization of NH_3, which not only decreases the compost quality, but also worsens the air pollution. Meanwhile, the greenhouse gases (CH_4 and N_2O) emission leads to environmental pollution. In addition, the high bioavailability of HMs in compost and the residual of antibiotic and antibiotic gens would also limit the development of composting technology and the land use of compost as well as cause hazard for ecosystem. In order to promote the composting progress and reduce the adverse effect during composting, many viable practical approaches have been applied adjusting the physicochemical parameters (e.g., moisture, C/N ratio, aeration rate and pH), using the different kinds of bulking agents, and adding the chemical agents, mineral additives, and microbial agent. This chapter discusses the benefit

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and challenge of composting of organic and hazardous waste. It will also discuss the current method to promote the compost quality and reduce the environmental risk.

Keywords Composting technology · Antibiotic gens · Greenhouse gases Hazardous waste

1 Introduction

1.1 Background

During the last 10 years, the production of the solid waste was increased to 1.3 billion metric tons (Hoornweg and Bhadatata 2012). These organic wastes normally contain a lot of organic matter and nutrients (nitrogen, phosphorus and potassium, etc.), which could be regarded as the soil amendment or fertilizer. However, the organic wastes also contain various kinds of toxic materials or elements (e.g., heavy metals, pathogens, antibiotics, and antibiotics resistance gens) (Fang et al. 2015; Hargreaves et al. 2008). Heavy metals and antibiotics are widely used in livestock and poultry farms for preventing the animal diseases and promoting the animal growth. The heavy metals and antibiotics cannot be metabolized by animals and get into the farmland soil, surface water, groundwater, and other ecological environment, which will not only cause pollution in ecological environment but also cause serious hazard to human health (Fang et al. 2015; Martínez et al. 2015; Zhang et al. 2015). Hence, the disposal and recycle of these organic wastes have become an urgent issue all over the world.

Aerobic composting has been considered as the effective and sanitary approach to dispose the organic solid wastes and consequently producing the high valued product (Selvam et al. 2012; Wang et al. 2016b, 2017a, b, c; Xie et al. 2016). During the composting process, the moisture and volume of the organic wastes could be significantly reduced. And because of the high temperature in the thermophilic phase during composting process, the pathogens, weed seeds, and antibiotics in the raw materials can be effectively killed or reduced (Qian et al. 2016a). Furthermore, composting can effectively change the factions of N and P in organic wastes, as well as mitigate the risk of nutrient elements migrating into the water body with the rainwater when the organic fertilizer is applied to soil (Lu et al. 2013). Although the composting could effectively recycle the organic waste, it still has some drawbacks such as greenhouse gases emission, nitrogen loss, high mobility of HMs, antibiotic residue, antibiotic-resistant gens enrichment, and the low quality of compost.

In order to improve the composting process and reduce the adverse effect as mentioned above, many practical methods have been proposed such as using different kinds of bulk agents (Dias et al. 2010; Chowdhury et al. 2014a), increasing

the aeration rate (Chowdhury et al. 2014a, b), and adding the microorganisms, chemical agents and mineral additives (Gabhane et al. 2012; Jiang et al. 2015a, b; Awasthi et al. 2016b), etc. With the technology development, using the method of composting to manage the organic and hazardous waste has been widely accepted by people. Hence, this chapter is presented to illustrate the mechanism of composting and the recent advances in composting of organic and hazardous waste.

1.2 Traditional Composting: Advantages and Disadvantages

As an approach of reusing the organic waste and hazardous wastes, aerobic composting has many advantages like low production costs, less pollution, soil improvement, and so on. However, there are still some disadvantages such as the nitrogen loss and greenhouse gas or odor emission during the traditional composting process and the lower maturity, antibiotic residues, salts, and the high bioavailability of toxic metals of its end product (Zhang et al. 2014; Awasthi et al. 2016c). For the aim of improving composting efficiency and their products quality, effective and targeted treatment options are required to be put forward. Several studies reported that co-composting with different additives, such as zeolite (Villaseñor et al. 2011; Awasthi et al. 2016b), rock phosphate (Lu et al. 2014; Zhang and Sun 2017), biochar (Chen et al. 2010; Li et al. 2017; Ngo et al. 2013), lime (Wong and Selvam 2009), bentonite (Li et al. 2012; Wang et al. 2016a), and bacterial inoculation (Wei et al. 2016; Xie et al. 2017) proved to be excellent choices, which is devoted to reduce the environmental risk and improve composting efficiency in organic and hazardous wastes management.

1.3 Markets Demand for Composting

Compared with other treatments, such as sanitary landfill and incineration, the cost of composting is low. For example, in Italy, the use of compost can be divided into three groups: 34% of the well-prepared compost is sold as humus in garden centers, around 62% is treated as an alternative of fertilizers, and the rest 4% is used for soil remediation (Rigamonti et al. 2010). In Berkeley City, the treatment of landfill of green waste costs $40 per ton, while the amount spent for composting is only $26 (Hogg et al. 2002). In South Dakota, United States, the price of 1 cm size of the crushed material composting is 30 dollars per ton and the 0.5 cm size of the product is 25 dollars, the shipments of compost are around 3000–3500 tons per year with the annual income of about 100,000 dollars (Emerson 2005). Composting of the organic waste could not only solve the environmental problems but also bring immeasurable economic benefits.

2 Source and Impacts of Organic and Hazardous Waste in Environment

2.1 Agricultural Waste

According to the United Nations Environment Program, about 2 billion tons of crop straws will be generated in the world every year. Crop straw contains organic matter and nutrients such as phosphorus, potassium, calcium, magnesium, sulfur, and other elements (Ma et al. 2009), which could be potentially renewable biological resources that can be used as fertilizer, feed, and fuel (Diep et al. 2015). However, the unsuitable management of crop straw such as incineration would result in the high consumption, pollution, and low output situation. Burning straw will release a lot of carbon dioxide and increase the content of sulfur dioxide, nitrogen dioxide, and inhalable particles in the atmosphere (Yao et al. 2017). The sulfur dioxide and nitrogen dioxide emissions would aggravate the acid rain and greenhouse effect. In addition, when the concentration of inhalable particle reaches a certain level it could harm human eyes, nose, throat, etc. On the other hand, the smoke from burning the straw can decrease the visible range (Yu et al. 2010), which has a direct impact on civil aviation, railways, highways normal operation, easily leading to traffic accidents and affecting personal safety. The airport is affected by the combustion of straw every day during the harvest season. Sometimes the airport visibility is less than 400 m, which seriously affects the normal takeoff and landing of the airport flight. Meanwhile, burning straw can dramatically increase the temperature of soil, which results in the death of beneficial microorganisms (Yu et al. 2010; Herskin et al. 2016).

2.2 Livestock Manure

On account of the economic development and improvement of people's living standards, the demand for livestock and poultry products has significantly increased. With the expanded scale of production, the livestock and poultry farming model of China has been changed from each household farming to large-scale, intensive farming (Wang et al. 2016a). In 2015, the number of pig, cattle, and sheep in China was about 4.5×10^8, 1.1×10^8 and 1.1×10^8, respectively. The fast development and intensive cultivation also bring serious environmental problems. These livestock and poultry farms are close to water sources, and the problems of environmental pollution caused by livestock and poultry manure are more and more serious, which affect the sustainable development of human and animal health. The untreated livestock and poultry manure are indiscriminate discharge, resulting in the loss of nitrogen, phosphorus, potassium, and other nutrients, consequently, causing the air, water and soil pollutions.

2.2.1 The Effect of Heavy Metals in the Livestock Manure

In order to improve the growth and health of livestock and poultry, many kinds of heavy metal trace elements such as copper, zinc, chromium, lead, and arsenic are added to the feed and veterinary drug. However most of the heavy metals cannot be completely adsorbed by animals, and consequently high concentrations of heavy metals are detected in the manure (Meng et al. 2017; Wu et al. 2016). The application of these kinds of manure to soil would not only lead to the soil deterioration but also cause the water pollution. These heavy metals can also affect the health of the human body through the food chain (Leclerc and Laurent 2017).

2.2.2 Fecal Contamination of Pathogens

Livestock and poultry manures are sources of harmful microorganisms, pathogens, parasites, raw eggs, and other harmful substances. The livestock and poultry manures contain an average of 330,000 and 690,000 coliforms per milliliter and more than 190 roundworm eggs and 100 nematode eggs per 1000 ml of sedimentation tank effluent. Indiscriminate discharge of the livestock and poultry manure not only affects the ecological environment but also harms the human health (Gu et al. 2017).

2.2.3 Odor Pollution of the Livestock Manure

The odor pollution is another problem for disposing the livestock and poultry manure. The main content of the odor is the volatile low molecular weight compound (Zang et al. 2016). These compounds contain organic acids, ammonia, hydrogen sulfide, mercaptans, phenols, and indoles. Among all the compounds, the ammonia emission could not only stimulate the respiratory tract of human and animal but also cause the decline in immunity of livestock and poultry (Holly et al. 2017).

2.2.4 Antibiotic Residues in Livestock Manure

In China, the feed additives contain 17 kinds of antibiotics, antioxidants, and hormone drugs and 11 antibacterial agents. Widely using these feed additives would result in a lot of antibiotics and hormones remain in the manure (Li et al. 2017). Consequently, these pollutants will get into the environment through the excrement, causing environmental and human health risk.

2.3 Humans Activity

With the fast development of the population and economy, lots of municipal solid waste are produced every year all over the world (Wei et al. 2017). In 2015, the municipal solid waste delivering quantity in China has reached 178.6 million tons. According to statistics, the annual discharge of garbage, feces, and sanitation system varies from 40 to 50%, and more than 98% of the garbage and feces are put into the environment without harmless treatment (Waste Manage Res). Furthermore, the accumulation of municipal solid waste in the land would increase the alkalinity of soil, heavy metals contents and decrease the quality of soil (Han et al. 2016). On the other hand, a large number of urban garbage are dump into rivers or lakes, resulting in a variety of harmful substances pouring into the water (Peng et al. 2016). Meanwhile, pouring a large number of garbage into the river can block the river and affect flood discharge.

2.4 Landscaping Waste

Landscaping waste refers to the waste produced by the plant branches, leaves, grass, and other green trim (Amritha and Anil Kumar 2016). With the expansion of urban greening area, landscaping waste also rapidly increased. The landscaping waste is rich in organic matter and also contains some nutrients. Most of the landscaping waste was landfilled or burned, which not only polluted the environment but also increased the cost of transportation and handling. The recycling of the landscaping waste has gradually increased people's attention.

3 Treatment Methods of Organic and Hazardous Waste

3.1 Landfill

Disposal and management of organic and hazardous waste are common concerns all over the world (Artíñano et al. 2017). Because of the economic advantages, landfill is the dominant approach to treat these wastes, especially in developing countries (Narayana 2009). In China and United State, approximately 90 and 54% of waste were disposed by landfill. However, with the rapid increase of the waste, there is no land available enough for landfilling the organic and hazardous waste. Meanwhile, during the process of landfill, it will also lead to the second environmental pollution such as leachate, odor, GHGs emission, and HMs pollution and subsequently caused human health problem (Wang et al. 2017b). Among the all potential pollutions, the leachate and GHGs emission are the most concerned by humans (Renou et al. 2008). Leachate generated from the landfill is a complex mixture of

contaminants, which has the high chemical oxygen demand and ammonium nitrogen concentration as well as the toxic elements (Kai et al. 2016). It will easily leak and then get into the soil system, underground, and surface water. Methane (CH_4) is the main gas formed in landfill site which has been collected for producing the electric power. While most CH_4 will discharge into the atmosphere and lead the greenhouse effect; on the other hand, the accumulation of CH_4 in landfill site also present the risk of explosion (Nair et al. 2017).

3.2 Incineration

Incinerating the organic and hazardous waste is a clean technology, which could obviously reduce the waste and then transfer them to electricity (called waste to energy, WTE) (Mckay 2002; Wang et al. 2017a). The incineration of the waste is a popular and fast treatment, energy recover, and the harmlessness method, and it also become the second large scale to treat the waste all over the world. The investment and maintaining of the incineration plant is costly. And the high moisture content of organic and hazardous waste like poultry manures, sewage sludge, food waste, and antibiotics residue will also increase the expense of incineration plant (Singh et al. 2011). On the other hand, on account of the improper management and the unsatisfactory development status, some problems occurred such as potential toxic emissions (dioxins and HMs) and GHGs discharged (Liu et al. 2017). In addition, the residue of incineration plant should be regulated, because the incinerated residue also contained a lot of hazardous elements, its improper management would have a negative impact on the environment and human health (Sabbas et al. 2003). Consequently, the high investment, lack of reliable technology, and excessive toxic substances in the exhaust gases and residue would restrict the incineration development.

3.3 Anaerobic Digestion

Anaerobic digestion is an approach to transfer the organic carbon to biogas with the help of anaerobic microorganisms under the oxygen free condition (Josse 2017). Biogas (mainly consisting of 60–70% CH_4 and 30–40% CO_2), biogas slurry, and biogas residue are the final product of anaerobic digestion (Igoni et al. 2008). Using this technology to dispose and recycle the organic and hazardous waste is gradually accepted by humans. Compared to the landfill and incineration method, the anaerobic digestion will occupy less land and has much less limitation on the moisture content of the initial substrates. Meanwhile, the biogas slurry and biogas residue could be used for the organic farm, and the CH_4 could be utilized to produce the heat and electricity. However, in the current technology of anaerobic digestion, the productivity of CH_4 is low, and the process of anaerobic digestion is easily

affected by the operating and reactor parameters (Tasnim et al. 2017). And the different source of the waste will also influence the efficiency and productivity biogas formation. Moreover, anaerobic digestion is a mesophilic process, which cannot destroy the pathogen and weeds seeds in the organic waste and consequently restrict the biogas slurry and biogas residue utilization (Chen et al. 2016). For economic aspect, the anaerobic digestions still have some barriers such as inadequate payback and lack of available capital.

3.4 Composting

Composting is a traditional way to manage and recycle the organic and hazardous waste (Taeporamaysamai and Ratanatamskul 2016). The organic matters are broken down to stable and sanitary organic fertilizer (compost) by microbes such as bacteria, fungi, and other microorganisms under the aerobic condition (Wei et al. 2007) in the composting process. And the application of compost could significantly improve the soil nutrition and the quality of the crops (Wong and Selvam 2009). With the increase in demand for the organic farm and organic crops, composting of organic waste gradually increased all over the world. Compared to the other approaches to treat the waste (landfill, incineration, and anaerobic digestion), the operational process is more simple and the initial investment and maintaining of the composting plant is also lower. During the composting, the microorganisms could quickly decompose the organic matter and then produce a lot of heat, this procedure could obviously reduce the volume, decrease the moisture content, and destruct the pathogenic microbes and weed seeds of the wastes (Lohri et al. 2017). On the other hand, the composting also includes the process of organic matter humification, which could transfer the unstable organic matter to a high value-added product (humic substrates) (Zhang et al. 2014). Besides, composting of the hazardous waste and antibiotic residue could be an effective approach to reduce the toxicity of waste.

4 Composting of Organic and Hazardous Waste

4.1 Principle of Composting

Composting is a controlled biological decomposition of organic solid waste in the presence of oxygen, which could stabilize and sanitize the organic waste as well as produce the organic fertilizer (Mustafa et al. 2016). During the composting process, the microorganisms such as fungi, bacteria, actinomycetes, and yeasts could degrade the organic matter and then produce the CO_2, H_2O, heat, and the plant-available minerals (nitrogen, phosphorus and potassium, etc.) (Das et al. 2011). The heat generated from the organic matter degradation could increase the

temperature of the compost and consequently destruct the pathogen and weed seeds (Pandey et al. 2016). On the other hand, these microbes could transfer the part of the organic matter to the chemically humus-like product (Qian et al. 2014). The level of stabilization and humification of the compost depends on the operational process of the composting, and the physicochemical and biological properties of the initial feedstock (Li et al. 2013).

4.2 Physicochemical Characteristics of Composting

The physicochemical parameters of composting are very important factors for the composting optimization. The improper physicochemical parameters would influence the efficiency of composting.

Porosity: The porosity of compost has a great effect on the distribution of air, the suitable porosity (35–50%) of composting process must be maintained during the composting process. The porosity of composting higher than 50% would easily result in the heat loss with the air flow, which leads to the low temperature of the pile. The too little porosity of the compost would form the part of the anaerobic zone in compost pile, and then cause the odor and greenhouse gases emission.

Particle size: The porosity of the compost pile and the surface area for the microbe growth would be affected by the particle size of the substrates. And the particle size has a negative correlation with the surface area of compost (Zhang and Sun 2014). The large particle size would restrict the microorganisms to get into the organic matter and then result in the inadequate decomposition of the substrate. Meanwhile, the too small particle size can reduce the porosity and compact the mixtures, which is harmful for composting (Bernal et al. 2009).

Aeration rate: Composting is an aerobic process and the aeration is critical for the activity of the microorganisms in compost (Chen et al. 2015a, b). And ensuring the suitable aeration is important for the biological process. During the composting, the proper aeration could control the temperature, moisture content, and the oxygen content of the compost (Li et al. 2015a, b).

Moisture content: Moisture is necessary for the microbes to decompose the organic substrates, and the proper moisture content varies from 50 to 60% (Bernal et al. 2009). The higher moisture content (>70%) could inhabit the movement of O_2 and then lead to the anaerobic condition (Turan 2008). During the composting process, the optimum moisture content of compost should be maintained to keep the microorganism activity (Guo et al. 2012).

pH: Generally, the pH at the range of 5.5–8.0 could be beneficial for microbial activity during the composting process (Chen et al. 2015a, b). In most case, the pH of the compost materials is within this range and normally making sure a successful composting. The high pH (>7.5) value will cause the nitrogen loss by the ammonia emission, and the low pH can inhabit the activity of microorganisms (Sundberg et al. 2004).

Carbon to nitrogen ratio (C/N): Carbon and nitrogen are the essential elements for the metabolism and cell differentiation of the microorganisms (Pace 1995). The C/N ratio is a key factor for composting. The suitable initial C/N ratio should be at the range of 25–35 (Huang et al. 2004), which could facilitate the composting process. However, the higher C/N ratio will increase the nitrogen loss (Igoni et al. 2008), and the lower C/N ration will prolong the composting process (Awasthi et al. 2014).

4.3 Biological Characteristics of Composting

Composting is a biological process, where the organic matter degradation and humification are carried out by many different kinds of indigenous microorganisms in compost mixtures (Kaiser 1996). At the beginning of composting, the bacteria (*Streptomyces* spp., *Clostridium thermocellum* and *Bacillus* spp.) are the dominated microbes for decomposing the easily degradable organic matter. Fungi which mainly decompose the hemicelluloses, lignin, complex proteins, and pectin are present during the whole composting process. Compared to the bacteria, Fungi have a high tolerance of the low moisture and pH, and most of fungi need the oxygen to grow (Bondarenko et al. 2017). When the temperature of the pile rises to the 60 °C, the fungi cannot survive. At the later (stabilization and curing) phase of composting, actinomycetes become the dominated microorganisms and could degrade the resistant polymers with the cooperation of fungi.

5 Disadvantages of Traditional Composting Technology

5.1 Greenhouse Gases Emission

The inevitable emissions of methane (CH_4) and nitrous oxide (N_2O) during composting have already attracted public attentions. In the process of composting, 0.2–9.9% of initial total nitrogen and 0.08–10% of initial carbon are lost because of the volatilization of NO_2 and CH_4, respectively. As International Panel on Climate Change (IPCC) found that CH_4 and N_2O are the major greenhouse gases whose global warming potential are 25 and 298 times higher than that of CO_2. Hence, the greenhouse gases (CH_4 and N_2O) emission will not only cause the nutrients loss but also aggravate the global warming (Jiang et al. 2015a, b). At the beginning of composting, the high moisture and low oxygen concentration will generate the anaerobic zone (Blazy et al. 2014); and the high content of nutrients and easily degradable organic materials will accelerate the microbes quickly depleting the biological oxygen. These two conditions will favor the growth and activity methanogenic bacteria and consequently result in the CH_4 formation. N_2O is a

by-product of the aerobic nitrification and the denitrification processes and always occurs at the initial phase or mature phase during the composting process (Awasthi et al. 2016a). For aerobic nitrification, the N_2O is generated at the phase of ammonia-oxidizing bacterial oxidizing the ammonia to nitrite (Shen et al. 2011). While for denitrification, the N_2O is an intermediate of NO_3^- and NO_2^- transferring to N_2 at the oxygen-limited condition. In addition, the characteristics of initial feedstock and the operation condition are also important factors for CH_4 and N_2O release in the composting process (Jiang et al. 2016).

5.2 Nitrogen Loss

According to the published literature, 9.6–46% of initial total nitrogen is lost in the form of ammonia (NH_3) during the composting process. And the NH_3 emission during the composting process can obviously result in the lower agronomic value of compost and cause the eutrophication and acidification of ecosystems (Jeong and Kim 2001). During the composting process, the ammonia volatilization mainly occur in the thermophilic phase which due to the nitrogenous materials quickly decomposed by microorganisms (Wang et al. 2016a). Meanwhile, the NH_3 emission during the composting period is strongly related to some parameters such as C/N ratio, moisture, aeration rate, temperature, pH, and the properties of initial substrates.

5.3 Heavy Metals Mobility

Many kinds of organic waste and hazardous (animal manure, sewage sludge and antibiotic residue) have the problem of high concentration of HMs (Cu, Zn, Pb, Cd, Cr, As, and Hg). The high concentrations of HMs in the organic waste and hazardous will limit the application of the composts from these wastes (Maleki et al. 2009). During the composting process, the HMs in waste cannot be eliminated, while the bioavailability of HMs will be changed. For traditional composting, the changes of bioavailability of HMs are various, as Wang et al. (2016a, b) reported that the mobility of HMs decreased after composting, while Awasthi et al. (2016c) discovered the opposite result. Although, the variations of bioavailability during the composting depend on the initial feedstock, the immobility of HMs in compost is still very high. The utilization of this kind of compost will be harmful for the environment and human health (Wang et al. 2017a, b, c, d).

5.4 Low Organic Matter Degradation and Humification

Composting process mainly include organic matter degradation and humification, these two procedures have an important effect on the compost rate and the final quality (Traversa et al. 2010; Kulikowska et al. 2015). And the organic matter degradation and humification are significantly affected by the composition of initial substrates. During the composting process, the degradation of organic matter could release the CO_2, heat, moisture, and available nutrients for plants (Hermann et al. 2011). The heat produced from the organic matter degradation could be helpful for sanitizing the composting. The unsuitable decomposition of organic matter could not only prolong the composting time but also decrease the quality of final compost. Humic substances, mainly including humic acids and fulvic acids, are the most active components in compost and could indicate the maturity and stabilization of compost (Storino et al. 2016). The utilization of the immature or low humification compost would cause some negative effects for the environment (Mohammad et al. 2012). Hence, the low organic matter degradation and humification will limit the development of industrial composting and the successful utilization of the final product.

5.5 Antibiotic Residue and Antibiotic-Resistant Gens Diffusion

The antibiotic and the antibiotic-resistant gens in the organic and hazardous wastes such as livestock manure, sewage sludge, and antibiotic residue have become an urgent concern around the world (Li et al. 2017; Duan et al. 2017). The diffusion of antibiotic and antibiotic-resistant gens will result in the resistant bacteria generation and consequently harm the ecology and human beings (Qian et al. 2016b). Composting of these wastes can reduce the concentration of antibiotic such as tetracycline, oxytetracycline, chlortetracycline, and penicillin, while the efficiency of antibiotic reduction depends on the duration and temperature of thermophilic phase of composting. The abundance of antibiotic-resistant gens in waste would increase or decrease after composting. As Zhang et al. (2016a, b) reported that the antibiotic-resistant gens in sludge will be enrichment after composting. And the abundance of antibiotic resistant has a strong correlation with the bacterial phylogenetic compositions during the composting. The application of the compost which contains the high antibiotic-resistant gens can lead to the antibiotic-resistant gens spreading in soil. For current composting technology, the antibiotic and antibiotic-resistant gens cannot be effectively eliminated.

6 Recent Advances in Composting

6.1 Adjusting the Physicochemical Parameters

During the composting process, the physicochemical parameters are very important factors which could influence the efficiency of composting. For the sake of improving the composting process and reducing the potential environmental risk of composting, various kinds of approaches were carried out to adjust the physicochemical parameters.

6.1.1 Temperature

The temperature is a visual parameter which could indirectly reflect the composting process. During the composting process, the high temperature (>50 °C) of compost could destruct the pathogen and weed seeds as well as improve the organic matter degradation (Wang et al. 2016a). However, the temperature of traditional composting cannot arrive to the standard, which leads to the low efficiency of organic matter degradation and the long period of composting process. In addition, lots of pathogenic bacteria could not be killed due to the low temperature. In order to ensure the maintenance of high temperature of composting, many approaches have been put forward such as adding mineral additives (Ca-bentonite, medical stone and biochar) (Wang et al. 2016a, b; Awasthi et al. 2017), chemical amendments (jiggery, polyethylene glycol and sugar) (Gabhane et al. 2012), bulking amendment (such as cornstalk, sawdust and wood peat) (Yuan et al. 2017), and microbial agents (*Bacillus altitudinis*, *Streptomyces albus* and *Phanerochaete chrysosporium*) (Xi et al. 2012). The addition of these amendments could not only improve the increase of temperature but also accelerate the efficiency of organic matter degradation.

6.1.2 Moisture Content

In the composting process, moisture content is an important factor which could significantly influence the composting. Generally, the moisture content of the organic wastes such as sewage sludge, animal manure, olive waste, and antibiotic residues is high (80–90%) which is not suitable for composting. Normally, the optimal moisture content of composting ranges from 50 to 60% (Bernal et al. 2009). While, the low moisture content of compost will restrict the microorganism growth (Li et al. 2012); however, the too high moisture content will impose restriction on the diffusion of oxygen, and consequently lead to anaerobic fermentation (Wang et al. 2016b). Hence, adjusting the initial moisture content of compost material is necessary for successful composting. In order to keep suitable moisture content of composting, different kinds of bulk agent (wheat straw, sawdust and green waste, etc.) were added before the composting. Meanwhile, adjusting the aeration rate and

the frequency of turning piles could also regarded as the alternative methods to control the moisture content (Petric et al. 2012). In addition, the mineral amendments such as bentonite (Li et al. 2014), biochar (Li et al. 2015a, b) and zeolite (Awasthi et al. 2016a) could maintain the suitable moisture content during the composting process.

6.1.3 C/N Ratio

The optimal C/N ratio of composting is generally at the range of 25–30 (Huang et al. 2004). If the C/N is too low, especially when the pH and temperature are high, most nitrogen in the materials will be lost in the form of NH_3 and then decrease the quality of the compost and cause the environmental pollution (Awasthi et al. 2014); Meanwhile, when C/N is higher than 35, the microorganisms must undergo multiple cycles of life for oxidizing excess carbon until the suitable C/N is reached (Petric et al. 2015). Moreover, the high C/N ratio will also prolong the composting process and lower the maximum temperature, making it difficult to maintain a long period of high temperature during composting process. Different kinds of bulking agents such as sawdust (Li et al. 2012), straw (Wu et al. 2016), biochar (Awasthi et al. 2016c), and green waste (Yang et al. 2015) were widely used to adjust the C/N ratio to an ideal state. In addition, the suitable C/N ratio can lead to reduce the release of GHGs and ammonia (Jiang et al. 2011) and the availability of HMs (Wu et al. 2016).

6.2 Adding the Mineral Additives

Composting technology provides a simple and promising alternative method to stabilize the organic matter and makes it possible to recycle organic nutrients in agriculture (Liu et al. 2017; Hou et al. 2017). However, the nitrogen loss through ammonia volatilization during composting process and the high bioavailability of HMs in the final product are the major disadvantages of traditional composting (Wong and Selvam 2006; Liu et al. 2017). Meanwhile, the insufficient decomposition of organic matter leads to the production of immature compost (Huang et al. 2006). In addition, the GHGs or odor emission during the composting process has aroused wide attention in recent years (Jiang et al. 2015a, b). These drawbacks as mentioned above restrict the development of organic waste composting.

According to the previous paper, adding the mineral additives is an effective way to overcome the disadvantages as mentioned above. The additives such as zeolite, bentonite, medical stone, lime, and biochar could significantly decrease the nitrogen and GHGs emission as well as reduce the mobility of HMs (Wang et al. 2016a; Awasthi et al. 2016b; Li et al. 2012) (Table 1). Wang et al. (2016b) reported that co-composting of poultry manure with different dosage of medical stone could improve the efficient of composting, and reduce the loss of nitrogen (27.9–48.8%)

Table 1 The mineral additive applied in composting

Mineral addictive	Function	Targets	References
Zeolite	Decrease the salinity and reduce the nitrogen loss	Poultry litter Sewage sludge Food waste	Turan (2008), Villaseñor et al. (2011), Chan et al. (2016)
Lime	Reduce the availability of heavy metals	Sewage sludge	Wong and Selvam (2006)
Biochar	Optimize organic matter degradation reduce the ammonia and greenhouse gas	Pig manure and sewage sludge	Li et al. (2015a, b), Awasthi et al. (2017)
			Awasthi et al. (2016a)
Bentonite	Facilitate the OM degradation, increase TKN content and reduce the availability of heavy metals	Pig manure	Li et al. (2012)
			Wang et al. (2016a)
Medical stone	Reduce the nitrogen	Pig manure	Wang et al. (2016b)

and the mobility of Cu and Zn. Awasthi et al. (2017) pointed out that mixing biochar with sewage sludge could reduce the emission of CH_4 (by 92.85–95.34%) and N_2O (by 95.14–97.3%), while increase the amount of humic substance (by 35–42%). In addition, adding the different amounts of biochar in compost has an obvious impact on decreasing the bioavailability of HMs in multimetal-contaminated wetland soil (Liang et al. 2017). Moreover, the addition of mineral additives could reduce the antibiotic resistance genes and decrease N loss during solid organic waste composting (Zhang et al. 2016a, b; Lim et al. 2017; Chan et al. 2016). Overall, compared to other methods, adding mineral additives is a cheaper and more practical way to improve the composting process.

6.3 Using the Chemical Agents

Compared with other amendments, the addition of chemical agents could not only enhance the composting process but also increase the compost nutrients. Struvite crystallization process which was normally used in the treatment of wastewater to recycle the Mg and P, while this process was also used in composting process in recent years (Jeong and Kim 2001; Lee et al. 2009). During the composting process, the struvite crystallization can decrease NH_3 emission by generate struvite ($MgNH_4PO_4{\cdot}6H_2O$), a high-quality and slow released fertilizer. According to the previous paper, various kinds of magnesium salts such as Mg $(OH)_2$, $MgCl_2$ and $MgSO_4$ and phosphorus (P) salts like H_3PO_4, KH_2PO_4, NaH_2PO_4, and Na_2HPO_4 were widely applied to form the struvite during the composting process and consequently optimized the composting process. Meanwhile, it was recommended that phosphogypsum and dicyandiamide as additives could be used in composting for

the purpose of reducing NH_3, CH_4, and N_2O emissions (Luo et al. 2013). Besides, Zang et al. (2017) pointed out that the addition of nitrogen amendments can control the emission of Me_2S and Me_2SS during the pig manure composting. While Jang et al. (2002) pointed out that adding glucose could make the amount of bacteria and actinomycetes increase, which promote the degradation of the plastic in the food waste composting.

6.4 Introducing the Microbial Inoculam

Composting is a biological decomposition process of organic materials, and microorganisms play very important part during composting process. However, the indigenous microorganisms of traditional composting are usually inadequate and easily restricted by certain environmental factors (Tran et al. 2015). Hence, such composting exhibits a low efficiency and poor end product quality. In that case, adding microbial inoculum can rapidly breakdown these stable organic component and accelerate the composting process (Sarkar et al. 2010). There are lots of research works on inoculating microbes in composting process. Wei et al. (2007) reported that complex microorganisms for instance *Bacillus casei*, *Lactobacillus buchneri* and *Candida rugopelliculosa*) and ligno-cellulolytic such as *Trichoderma* and *White-rot fungi* microorganisms were proved to be efficient to promote the formation of humic substance and maturation process. Similarly, microbial inoculants are beneficial to improve the maturity of compost by adding *Thermoactinomyces vulgaris* A31 (Ke et al. 2010), and accelerate the degradation of organic matter during composting by using *Pediococcus acidilactici* TM14 (Tran et al. 2015).

6.5 Vermicomposting

Vermicomposting has been widely applied in various kinds of organic waste. During vermin-composting, organic materials are decomposed not only by microorganisms but also the addition of earthworms, which is effective on accelerating the stabilization of organic and hazardous waste (Lim et al. 2015). Soobhany et al. (2015) showed that inoculating *Eudrilus eugeniae* during vermin-composting is effective to reduce the total content of HMs. Song et al. (2014) showed that earthworm can promote the degradation of organic matter and reduce the bioavailability of HMs. Meanwhile, Nigussie et al. (2017) also reported that earthworms could reduce the GHGs (N_2O) emissions and accelerate the stabilization of organic materials.

7 Concluding Remarks and Future Aspects

Composting technology is a cost-effective and eco-friendly approach to dispose and recycle the organic waste. And with the development of the advanced techniques such as adjusting the composting parameters, adding the biological, chemical, and mineral additives and improving composting operation, the potential environmental risk of composting is reduced and the quality of the end product is significantly improved. According to the initial characteristics of the raw materials, the different approaches could be selected to improve the composting process and reduce the negative effect of composting. The advanced composting technology could obviously reduce the GHGs emission, odor generation, ammonia volatilization, and the bioavailability of HMs during the composting process, the total content of HMs in compost is still high and cannot be eliminated. The long time application of these composts will result in the soil pollution and then affect the plant growth. Hence, the application amount and object of these composts need to further consider. High concentration of HMs in organic waste (sewage sludge, animal manure and some antibiotic residues) will limit the utilization of the final product of composting. Composting technology can significantly reduce the waste volume and toxicity of the waste which can be regarded as a pretreatment method for landfilling of these wastes. Overall, composting technology can be carried out to as an ultimate treatment or pretreatment of the organic waste, while how to manage the final product should be further evaluated.

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Chapter 18
Integration of Biomarker Approach in Pollution Monitoring Programme of Aquatic Ecosystem

Kanchan Kumari and Ankur Khare

Abstract Water bodies are subjected to a considerable pressure from sewage and industrial wastes. Monitoring methods adopted so far have helped in the assessment level of contaminants in water but not the interaction of these pollutants with living organisms. Water quality testing programmes use two traditional methods for water quality assessment that includes physico-chemical parameters and bio-monitoring. Looking at the limitations of these two traditional methods, a new method known as 'biomarkers of pollution' should be adopted. Evaluating various biomarkers in sentinel species can be of great help in environmental monitoring programme as they forecast various risks and hazards associated with the habitats of aquatic animals. Several countries have adopted Biomarkers in their environmental monitoring programmes; however, to make it a routine and well-recognized tool in the water quality monitoring programme, efforts are still required from scientific communities. The major advantage of Biomarkers is that bioavailability or potential exposure to toxicants can be demonstrated which is not possible in chemical analysis. Persistent organic pollutants (POPs) are chemical substances that do not degrade easily and persist in the environment and detecting some classes of POPs, for example organochlorine compounds, are very difficult as the limits of detection are very low. With the advancement of analytical methods, these chemicals can be now detected in every environmental matrices but changes caused physiologically in living organisms remains unknown. This limitation can be overcome with the help of biomarkers which can detect whether organisms are exposed meaningfully and the physiology is altered in comparison to normal. Whenever any pollutants enter the biological system, it brings molecular changes and the response time of molecular changes are faster than it appears at community level. This leads the scientific communities to start some research work in this area in order to develop some early warning signal or biomarkers. Measurement of molecular changes at the level of body fluids, cells or

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tissues reflecting an alteration in normal functioning/magnitude due to the presence of toxicants forms the basis of selection of particular parameter as a biomarker. This chapter presents the importance of various core biomarkers used as diagnostic and prognostic tools to monitor the water quality assessing the risks associated with the health of aquatic biota. This is high time to focus on the biological responses which are more relevant to predict the health status of any aquatic ecosystem before occurrence of any catastrophic events which are unmanageable.

Keywords Biomarkers · Bioindicator · Xenobiotic · Pollution
Environmental monitoring

1 Introduction

Form the last few decades environmental pollution has increased substantially due to industrial, agricultural, domestic, commercial effluents and other hazardous substances posing a great threat to the environment. Aquatic bodies serve as the sink for a wide variety of pollutants, recalcitrant in nature causing damage not only to the water bodies but to the aquatic life living in the vicinity. In an attempt to measure and define the impact of pollutants on aquatic ecosystems, biomarkers have attracted to be an area of interest. A biomarker may be defined as any physiological, biochemical, behavioural response that can be measured in the organisms due to the impact of any synthetic compound or a group of synthetic compounds. The National Academy of Sciences defines a biomarker or biological marker as changes in cellular or biochemical components or processes, structures or functions induced by any foreign chemical compound that is measurable in a biological system or sample (ENTOX 1996). Biomarkers have evolved as an excellent monitoring tool for evaluating the consequences of environmental pollution in the past decades. They have been known to show an observable endpoint indicating sequential events due to the impact of any toxicants on the organisms.

2 Biomarkers in Environmental Monitoring

Indian waters are subjected to a considerable pressure from sewage and industrial wastes. Monitoring methods adopted so far have helped in the assessment level of contaminants in water but not the interaction of these pollutants with living organisms. Worldwide, apart from physico-chemical evaluation, biomarker approach has been adopted in field monitoring of aquatic ecosystem and their use has been increasingly reported from past few years. This relies mostly on the fact that the cause-effect relationship of any pollutant in the natural habitat helps the regulatory bodies to take appropriate decision in order to protect the ecosystem and choose the appropriate remedial measures or monitoring programmes for the impacted site.

Evaluating various biomarkers in sentinel species can be of great help in environmental monitoring programme as they forecast various risks and hazards associated with the habitats of aquatic animals. Several countries have adopted Biomarkers in their environmental monitoring programmes; however, to make it a routine and well-recognised tool in the water quality monitoring programme, efforts are still required from scientific communities (Schettino et al. 2012). The concept of Biomarkers has its origin from the field of human medicines and it was in the early 1990s, when biomarkers were first introduced in the field of ecotoxicology. David Peakall was the scientist who gave the most simple and often used definition of biomarkers as '*a biological response to chemicals that give a measure of exposure and sometimes, also of toxic effect*'. Biomarker word is most commonly used to refer to quantifiable changes occurring at various levels of any living organisms which can be molecular, physiological or behavioural responses at the level of whole organisms residing in the natural habitats.

In response to toxicants/xenobiotics, biochemical changes in aquatic animals are the most common changes used to give early warning signal to the possible more dangerous scenario of future (Fig. 1) and are very sensitive biomarkers of sublethal stress caused by the toxicants prevailing in the aquatic ecosystem (Huggett et al. 1992).

It is also recommended by many scientist and researchers to include biological markers as an additional tool for ecotoxicological assessment. The recommendation relies on the facts that toxicant-induced alterations at different levels—molecular, cellular and genetic, is due to the stress induced by anthropogenic toxicants, giving early warning signal related to the catastrophic events of aquatic ecosystem (Table 1).

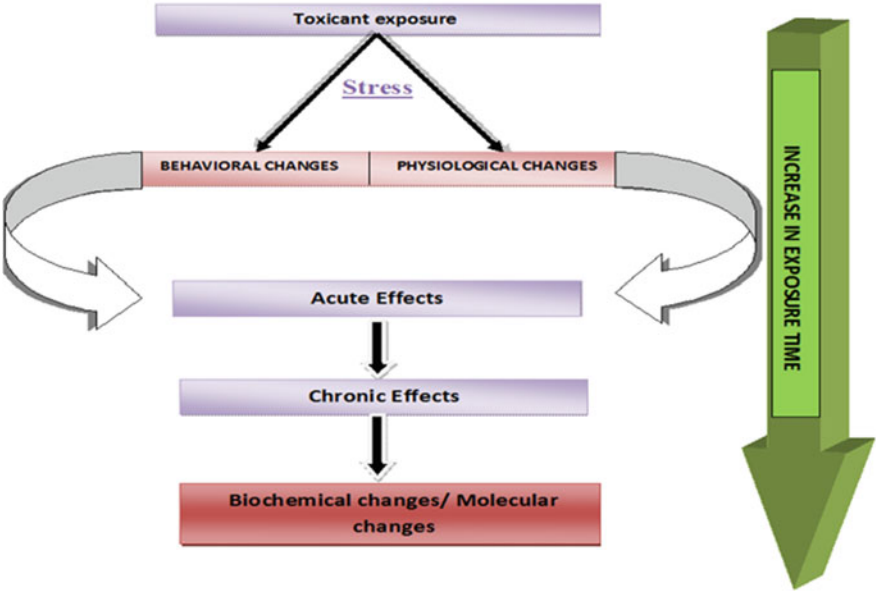

Fig. 1 Various metabolic changes induced by xenobiotics

Table 1 Views of various researcher on biomarkers

Authors	Remarks
Everaarts et al. (1999)	Biomarker: a xenobiotically induced variation in cellular or biochemical components or processes, structures or functions that is measurable in a biological system or sample
McCarthy and Shugart (2005), Kaiser (2001)	Biomarkers help to establish a cause-and-effect relationship between environmental exposure and effects
Hyne and Maher (2003)	Biomarker is more commonly used in a more restrictive sense, namely biochemical sublethal changes resulting from individual exposure to xenobiotics
Hamza-Chaffai et al. (2000), Lam and Gray (2001), Gagne et al. (2006)	Measurements of biomarkers at lower levels of organization have been proposed as sensitive '*early warning*' tools for biological effect measurements in environmental quality assessment
Porte et al. (2000)	Need to couple chemical determinations of environmental contaminants with the use of biomarkers of exposure and/or their effects to provide evidence of a cause-effect relationship and to provide better environmental risk management actions
Hyne and Maher (2003)	Molecular/cellular/organismal biomarkers can be used to anticipate changes at higher levels or biological organization, allowing for planning of biological remedial strategies before the onset of irreparable nature of damage to ecosystem
Van der Oost et al. (2003)	Biomarkers of exposure indicate that an organism has experienced exposure to a toxicant and can be used to identify the specific classes of contaminants
Amiard et al. (2006), Moolman et al. (2007)	Biomarkers can provide information on the relative toxicities of specific chemicals; and biomarkers are applicable in both the laboratory and the field
Kumari et al. (2006)	An advantage of biological sampling versus chemical sampling is that it looks at indicators of conditions which are present in the river over the period of time rather than just at the moment when water sample is collected so as to give water quality value (WQV)
WHO (1993)	Biomarkers-any measurement reflecting an interaction between a biological system and a potential hazard, which may be chemical, physical or biological
Livingstone (2001)	Measurements of body fluids, cells or tissues that indicate in biochemical or cellular terms the presence of contaminants or the magnitude of the host response

Biomarkers can be a used as an early detection tool in the area of toxicology and ecotoxicology by the following ways:

- Estimation of impact of a xenobiotic on specific or target tissue
- Serve as useful markers tool in early stage detection for selection of remedial measures
- Have ecotoxicological endpoints for detection in changes and estimation of the degree of destruction.

Many scientist and researchers have suggested adopting biological markers for the assessment of water pollution. Biological markers always found an added advantage over another kind of monitoring as they shed light on the wellbeing and biological conditions of organisms (Kumari et al. 2014). Even the National Research Council of the USA in 1987 has advocated the use of biomarkers for water quality monitoring. In 1999, the U.S. EPA held a workshop on "Biomarkers: Taking Stock, An EPA/NIEHS In-House Workshop on Applying Biomarker Research" for water quality. Different researchers have suggested to use various biomarkers like AChE, ALP, AST and SDH for monitoring the impact of xenobiotics on biological organisms (Kumari et al. 2010, 2014; Amiard et al. 2006; Lionetto et al. 2003). In the present age, more and more advancement on biomarkers have been accounted as numerous biological markers like biomarkers of gene expressions, DNA damage, lipid peroxidations, biomarkers of oxidative stress, metallothioneins (MT), Heat Shock Proteins (HSP) have been currently used in a water quality monitoring programme in European and American countries. So it is a high time when more and more focus and research needs to be done for promoting and implementation of biological markers for aquatic pollution studies in Indian and Asian subcontinent.

The major advantage of Biomarkers is that bioavailability or potential exposure to toxicants can be demonstrated which is not possible in chemical analysis. POPs are chemical substances that do not degrade easily and persist in the environment and detecting some classes of POPs for example organochlorine compounds, are very difficult as the limits of detection is very low. With the advancement of analytical methods, these chemicals can be now detected in every environmental matrices but changes caused physiologically in living organisms remains unknown. This limitation can be overcome with the help of biomarkers which can detect whether the organisms are exposed meaningfully and the physiology is altered in comparison to normal. Biomarker of exposure clearly establishes the altered physiology and indicates that the organisms are meaningfully exposed. Another important aspect of biomarkers is that it also indicates the non-significant alteration in physiology and in that case, the species is considered not to be significantly exposed to any chemical, even if the toxicant(s) are detected. The potential to differentiate between meaningful and non-meaningful exposure helps the regulatory bodies to decide appropriate remedial actions and associated follow up.

In case of pollutant-induced stress, the responses initiate in a sequential manner within the biological system (Fig. 2). Whenever any pollutants enter the biological

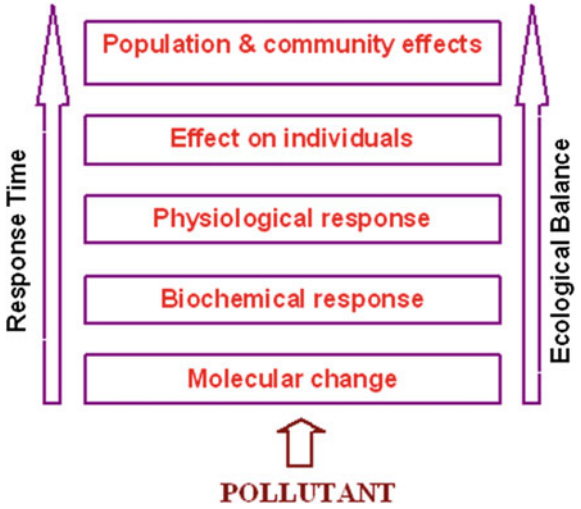

Fig. 2 Biological system showing various interrelated responses to pollutant stress at different level of organization

system, it brings changes which get reflected at the different level of organization. The molecular changes appear fast followed by biochemical changes, physiological changes which affect the individual and finally the population and community. This is linked with the response time i.e. response time of molecular changes are fastest than it appears at the community level. This leads the scientist group to start some research work in this area in order to develop some early warning signal or biomarkers which would reflect the effects of toxicants in aquatic ecosystems. Measurement of molecular changes at the level of body fluids, cells or tissues reflecting an alteration in normal functioning/magnitude due to the presence of toxicants forms the basis of selection of particular parameter as a biomarker.

3 Biomonitoring of Xenobiotics Using Fish as a Bioindicator

With rapid urbanization, environmental pollution has increased drastically in recent decades. The major problem in recent years is the disposal of waste waters created due to population explosion and the rapid pace of industrialization. Waste water includes wastes coming out with domestic discharges; partially treated/untreated effluents of industrial origin supplemented with pollutants like pesticides, various recalcitrant nature of chemicals, heavy metals and many compounds of organic origin which have greatly contributed to massive fish death (Murthy et al. 2013). For example in India, use of pesticides began in the year 1948 using DDT in controlling malaria. India was producing over 5000 metric tonnes of pesticides in 1958, and the production increased to 85,000 metric tonnes (Gupta 2004) however,

it is very surprising to note that percentage of an applied pesticide reaching the target is just 0.1% leaving 99.9% as an unintended pollutant in the environment.

Fishes have been considered as excellent indicators for pollution monitoring as they may accumulate indicative pollutants in their tissue, directly from water through respiration and also through their diet. They are at the top of food chain and may directly affect human health. Exposure of fish to lethal and sublethal concentration of environmental pollutants like pesticides (organochlorines, organophosphates and carbamates), heavy metals, effluents from distilleries, pulp and paper mills lead to the histopathological, cytopathological and ultra-structural changes in cells, tissues and organs and also some behavioural changes that can afford good biomarkers of pollutant stress. Fishes respond with great sensitivity towards the changes in the aquatic environment due to xenobiotics (Kumari et al. 2014) and act as a good biosensor tool for assessment of health of the aquatic ecosystem.

Pesticides/Insecticides are widely used throughout the world in agriculture to protect crops but it can be extremely toxic to aquatic animals. They are classified as extremely toxic to slightly toxic (WHO 2001) and the relative toxicity of various commonly used insecticides to freshwater fishes has been summarized in Table 2.

4 Mode of Action of Different Xenobiotics

There are different types of xenobiotic compounds based on their chemical characteristic and mode of action on the aquatic organisms (Table 3). Each one of them has its own distinct property and degree of impact on the organisms. Pesticides can be classified on the basis of their mechanisms of action. For example, major insecticides like organophosphates (OP), organochlorines and carbamates act mainly by disrupting the function of nervous system. These insecticides are chemically unreactive stable compounds and are characterized by their long-lasting effects (Akerblom 2004). The organophosphorus group of insecticides are also neurotoxic, although this group acts as acetylcholinesterase (AChE) inhibitor in the nervous system. The OP insecticide binds at the active site of AChE protein resulting in the formation of a transient, intermediate complex that partially hydrolyzes, leaving a stable, phosphorylated, and largely unreactive, inhibited enzyme that, under normal circumstances, can be reactivated only at a very slow rate (Ecobichon 1991).

Organophosphates and organochlorines exert acute toxicity by inhibiting AChE activity at the neuromuscular junction, leading to intoxication and respiratory failure. These insecticides are potent acetylcholinesterase inhibitor and exert their impact by disturbing the normal metabolic pathway of the neurotransmitter acetylcholine causing hyperaccumulation of these signalling molecules at the neuromuscular junction. Exposure to these classes of pesticides can produce symptoms like headache, nausea, blurred vision, respiratory difficulty, paralysis, weakness, dizziness, convulsions and coma. The onset of these clinical

Table 2 Relative toxicity of commonly used insecticides to aquatic fishes

Product names	Active ingredient	Relative toxicity
Novodor	Bacillus thuringiensis tenebrionis	Low
Clutch 50 WDG	Clothianidin	
Actara 240 SC	Thiamethoxam	
Assail 70 WP	Acetamiprid	Slight
Coragen	Chlorantraniliprole	
Beleaf 50 SG	Flonicamid	
Admire 240 F, Alias 240 SC	Imidacloprid	
Fulfil 50 WG	Pymetrozine	
Cygon 480EC	Dimethoate	Moderate
Vydate L	Oxamyl	
Delegate	Spinetoram	
Entrust 80 W, Success 480	Spinosad	
Movento 240	Spirotetramat	
Sevin XLR	Carbaryl	High
Lorsban NT, 50W, Pyrifos 15G, Pyrinex 480 EC	Chlorpyrifos	
Ripcord 400, Up Cyde 2.5 EC	Cypermethrin	
Diazinon 50W	Diazinon	
Thionex EC, Endosulfan 50W and 400EC	Endosulfan	
Imidan 50WP	Phosmet	
Matador 120EC	Lambda-cyhalothrin	
Malathion 50EC, 25W, 500E, 85E	Malathion	
Lannate SP	Methomyl	
Dibrom	Naled	
Rimon 10 EC	Novaluron	
Decis 5EC	Deltamethrin	Extremely high
Concept	Deltamethrin + imidacloprid	
Perm Up, Pounce, Ambush 500 EC	Permethrin	
Thimet 15G	Phorate	

Source Mellish (2013)

manifestations usually occurs within 12 hours of exposure due to the formation of methyl paraoxon-cholinesterase bond. If this bond is not cleaved by pharmacological intervention, it leads to irreversible inactivation of acetylcholinesterase in a large amount, causing acute cholinergic effects. The cholinergic activity is typically treated with atropine, an antagonist of muscarinic cholinergic receptors and pralidoxime, a reactivator of AChE (Koshlukova 2010). AChE is found in RBCs and in nicotinic and muscarine receptors in nerve, muscle and grey matter of the brain. It is reported that decrease in plasma cholinesterase results in a decrease of

Table 3 Xenobiotics, mechanism of action and impacts

Type of xenobiotic	Mechanism of action	Impacts
Organophosphates Carbamates	• Inhibition of AChE activity at neuromuscular junction • Alteration in lipid metabolism • Altered transaminase levels in blood	• Sudden jerk/ fast movement • Fin flickering • Poor liver condition
Organochlorine	• Disruption of Na–K pump • Altered Ca metabolism • Alteration in phosphokinase activity	• Immunosuppression • Chromosomal abnormalities
Heavy metals	• Inhibition of AChE activity in gills and brain • Increase in liver size, hyperplasia	• Mucous secretion • Lethargic movement • Decreased SDH activity
Effluents (paper/ distillery)	• AChE inhibition in gills • Increased LDH activity in blood and muscles • Altered creatinine phosphokinase activity	• Lethargic movements • deteriorating health • Elevated levels of ROS within body

Sources Kumari et al. (2006, 2014), Akerblom (2004), Koshlukova (2010), Tripathi and Verma (2004), Scott and Sloman (2004)

cholinesterase activity in the central, parasympathetic and sympathetic nervous system (Kumari and Sinha 2006; Kumari et al. 2010). OP compound interferes with many of the vital physiological functions (Rao and Rao 1981) and consequently alter the levels of various body constituents (Arasta et al. 1996) in fishes. Metabolic changes in carbohydrate and nitrogen induced by pesticide-induced hypoxia have been reported by various researchers. Rao and Rao (1981) reported the depletion of various metabolites, such as glycogen, protein and pyruvate stores from fish tissues such as liver and muscle and, increase in level of lactic acid, protease and free amino acids due to pesticides.

Effect of lethal/sublethal concentration of methyl parathion can be evaluated by

(a) Estimation of brain AChE activity.
(b) Quantification of hepatic detoxification enzyme systems.
(c) Abnormal changes in the hepatic enzyme level.
(d) Modification in the lipid metabolism in various organs (skeletal muscle, cardiac muscle).
(e) Histopathological alteration in various organs (gills, liver, skin, etc.) due to xenobiotic exposure.

Chlorinated hydrocarbon pesticides act by modifying the electrophysiological and associated enzymatic properties of nerve cell membranes, causing a change in the kinetics of sodium–potassium pump, disturbances in calcium transport as well as phosphokinase activities (Watkins and Klaassen 1996). It is a xenoestrogent that enhances the effect of estrogens. Tripathi and Verma (2004) reported decreased activity of citrate synthase (CS) and glucose 6-phosphate dehydrogenase and lactate dehydrogenase (G6-PDH) in the brain, liver and skeletal muscle in fish. Major carps showed decline in glucose level and increase in ALT, ALP on exposure to sublethal

concentration of endosulfan (Saravanan et al. 2010). Paul and Balasubramaniam (1997) reported that the main target organs for endosulfan as liver, kidney, testes and immune system. The pesticide endosulfan is found to be a potent disrupter of semen quality and also promotes cancer in testicles, prostate and also found to induce the male sex organs and increases the incidence of breast cancer (Singh and Pandey 1993).

Carbamate insecticides are neurotoxic primarily and carbaryl falls under this group. It is commercially called 'sevin' and chemically 1-Napthylmethyl Carbamate. It is an odourless, non-corrosive, white to grey crystalline solid (NLM 1997). It is a broad spectrum insecticide used to control a wide variety of pests, including beetles, moths, rats, cockroaches, ticks and mosquitoes (BCPC 2000). They act by interfering the nerve transmission and are the reversible inhibitor of acetylcholinesterase (Fig. 3).

It acts by inhibiting cholinesterase activity, the nerve impulse is transmitted to a specific cholinergic receptor by the release of acetylcholine from a nerve cell terminal across the synaptic cleft. Like other insecticides, carbamates also block the active site of AChE enzyme by adding a carbaryl moiety, thus preventing the interaction of acetylcholine with the enzyme (Watkins and Klaassen 1996). To end the stimulation and restore the sensitivity of the receptor, AChE at the receptor must be eliminated continually which hydrolyzes AChE to choline and acetic acid. Carbamates act by inhibiting these acetylchlolinesterase thus causing their accumulation at cholinergic junctions (Hayes and Laws 1991).

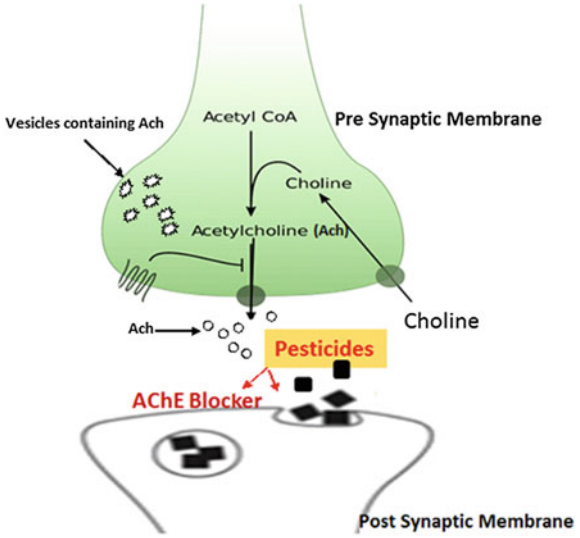

Fig. 3 Showing the action site of pesticides

5 Different Biomarkers in Aquatic Pollution Monitoring

In an aquatic environment where a broad spectrum of chemicals such as domestic, agricultural, commercial or industrial chemicals are entering and being bio-accumulated in aquatic organisms, use of biomarker approach for monitoring their impacts is very important and most appropriate. The biomarkers have attracted a great deal of interest of researchers and scientists in defining and measuring the presence and effects of toxicants in water bodies especially in case of pesticides and heavy metals. The main principle behind the biomarker approach is that it assists in analysis of an organism's pollutant content and compared the concentration with the background pollutant levels. The cause-and-effect relationship between exposure of pollutant and its direct impact on the environment can be very well established with the help of biomarkers.

Biochemical and physiological indicators such as enzymes could be used to identify possible environmental contaminations (Osman et al. 2010). Biomonitoring technique takes an added advantage of the knowledge that toxicants that have entered the organisms leave some indicators/markers that reflect their exposure to the pollutant. The marker may be the chemical, cellular, physiological or an enzymatic biomarker (Table 4). It can also be a breakdown product of the chemical or some biological alterations in the organisms like alteration in normal enzymatic levels or degradation of some enzymes that are a result of the action of the toxicants on the organisms.

To monitor the aquatic pollution, the sentinel species in the aquatic habitats are sampled for the analysis of various biochemical parameters. Enzymes are biochemical macromolecules that control the metabolic processes of the organism, thus a slight change in the enzymatic activity would affect the organism. The measurements of these parameters provide the information about the amounts of a

Table 4 Enzymatic biomarkers, their sources and applications

Enzyme	Principal sources	Principal clinical applications
Lactic dehydrogenase (LDH)	Heart, liver, skeletal muscle	Heart disease, skeletal muscle, haemolysis
Succinic dehydrogenase (SDH)	Liver, skeletal muscle, heart	High aerobic metabolism
Alanine transaminase (ALT)	Liver, skeletal muscle	Hepatic disorder
Aspartate transaminase (AST)	Heart, kidney	Heart disease and muscle disease
Alkaline phosphatase (ALP)	Liver, bone, kidney	Bone diseases, hepato-biliary disease
Creatine kinase (CK/CPK)	Skeletal muscle, heart	Heart disease
Cholinesterase (AchE)	Liver	Organophosphates and carbamate processing

Kumari et al. (2014), Kumari (2006), Kumari and Sinha (2006), Rauf et al. (2009)

pollutant that have entered and remained in the organisms and the corresponding effects induced. These are the specific biomarkers that are indicators of hepatotoxocity, nephrotoxicity, neurotoxicity and muscular toxicity. According to Mayer et al. (1992) and Meyers et al. (1998), the term biomarker has been adopted to refer to the changes occurring at physiological, biochemical and histological levels as indicators of exposure and/or effects of xenobiotics at the sub-organizational level. It is a measurement of body fluids, cells or tissues that indicate in biochemical or cellular terms the presence of contaminants or the magnitude of the host response (Livingstone 1993). These enzymes are called marker enzymes or clinical indicators for those specific tissues, and any increase or decrease in levels of these enzymes is an indicative of some kind of toxicity or abnormality in the organism. Clinical indicators are very specific indicators to stress response, for example organophosphate compounds cause inhibition of acetylcholinesterase in the brain (Coppage 1975). Clinical indicators are useful in the health assessment of an organism due to xenobiotics. The symptom of stress can be assessed using body fluid, serum, etc. as they leach out directly into the bloodstream from the affected organs (Kumari 2006).

Very many laboratory and pilot studies have been conducted by different researchers till date in India on the use and applications of biomarkers but this approach is only limited to research and developmental studies and not being used for water pollution control programme in India. The biomarkers for pollution control has not been implemented till date in the National water monitoring programme but it needs serious attention of the regulatory authorities and biomarkers should be adopted and used in water quality monitoring in India.

The level of environmental pollution and pollutants has changed considerably in the recent decade and water quality assessment by physico-chemical processes are somewhere lacking as they shed no light on the biological status of the aquatic ecosystem. So the use of biological markers has found their way to address a wide spectrum of pollutants (industrial, agricultural, commercial, etc.). The biomarkers nowadays are being used as a prognostic tool for assessing health of the ecosystem as a whole which is giving a clear picture regarding the biological status of the water body. Different types of biomarkers like biomarkers of stress, behavioural biomarkers, cytopathological biomarkers, hormonal biomarkers are being studied in various European and American countries which are giving a clear picture of the type of pollutants to which the organism is being exposed. The biological biomarkers are specific to particular type of pollutants and shows their activity corresponding upon the exposure of particular pollutant like Heat Shock Protein activity is detected in organisms where there is sudden change in the water temperature like water discharge from thermal power plant, alteration in level of vitellogenin is accounted on the presence of progesterone and estrogen-related compounds in the water body. So it is quite evident that rather than focusing on the particular biomarker, a suit of biomarkers has been given due importance in ecotoxicological studies.

5.1 Haematological Biomarkers

Biochemical and haematological parameters can offer valuable indices on different stress responses. Stress response of the fish to toxicants involves all levels of organization from the cell to the individual organism (Barton 2002) to the structure of the population. In response to stressors, a fish will undergo a biochemical and physiological changes in order to compensate the changes that can be measured. Estimation of Haematocrit pigment, estimation of Immature RBCs, Haemoglobin, etc. are potential haematological biomarkers.

5.2 Lysosomal Stability as Biomarkers

Lysosomes found in the epithelial cells in the tissues are the site for storage of heavy metals and foreign synthetic compounds and plays a significant role in detoxifying and eliminating the compounds. The increased level of toxic substances in the water bodies and their uptake by the aquatic organisms has led to changes in the stability of lysosomal membranes resulting in leaching of hydrolytic lysosomal enzymes into the cytosol and into the bloodstream. Recently, different toxicologists have recommended the use of lysosomal membrane stability as a biomarker in the blood cells of different marine organs (Da Ros et al. 2002; Moore 1985). For monitoring the fish liver dysfunction and carcinogenesis in response to toxicants the lysosomal membrane stability has also been recommended as prognostic biomarker in biomonitoring programmes (Broeg et al. 2002; Köhler et al. 2001, 2002).

5.3 Genotoxic Biomarkers

Xenobiotic substances have deleterious effects on the structure and function of DNA. Genotoxicity can cause three types of lesions:

(1) Point mutation, i.e. alteration in nucleotide sequence or base substitution.
(2) Chromosomal mutation-Breaking or translocation of the chromosome.
(3) Numerical changes, i.e. aneuploidy or polyploidy.

Initiation of carcinogenesis is marked by various events inducing pathways leading to DNA damage. Those compounds such as Poly Aromatic Compounds (PACs), alkylating agents that react with the DNA in a direct or indirect way are genotoxic in most cases and called as genotoxic carcinogens, while there are some compounds that induce a multitude mechanism that ultimately leads to carcinogenesis such as pesticides are called epigenetic carcinogens (Cajaraville et al. 2003).

Comet assays (Single-Cell Gel Electrophoresis) for estimation of bond breaking in DNA due to xenobiotics, Micronuclei test (MN test) for measuring structural and numerical changes in chromosomes, chromosomal aberration studies, single cell gel electrophoresis, sister chromatid exchange and many other molecular tools are carried out these days which are genetic biomarkers of pollution.

5.4 Cytochrome P4501A

The subfamily of Cytochrome P4501A (CYP1A) plays a very crucial role for the biotransformation of xenobiotics like PAHs and PCBs, etc. This biomarker (CYP1A) is widely used for the biomonitoring of pollution in both vertebrates and invertebrates. The CYP1A is triggered by cytosolic aryl hydrocarbon hydroxylase receptor (AHH) due to exposure of xenobiotics like PAHs and PCBs. The estimation of CYP1A in terms of 7-ethoxyresorufin O-deethylase (EROD) can be used as a potential biomarker for marine pollution assessment in the fish.

5.5 Behavioural Biomarkers

Monitoring of behavioural changes in the fish can be used as an important biomarker for health assessment occurring due to disease, stress due to pollutant and or environmental changes. The fishes exposed to Xenobiotics show remarkable behavioural changes like yawning, jerks, lethargy, losing balance due to hanging upside down and rolling on the surface of the tank. A first level screening of pollutant exposure can be determined by measuring the condition of a specific organ. Moreover, the interaction of fish behaviour related to the ecology of the habitat can be easily observed even if it can evaluated (Scott and Sloman 2004). This includes (HSI) Hepatosomatic index, which is a morphological parameter, i.e. the ratio between the weight of the liver (gm) and the total body weight (gm): 100. It gives an idea about liver disease or dysfunction (Sloof et al. 1983).

Condition factor (CF) gives an idea about the general condition of fish. It is the ratio of the weight of fish (gm) and length (cm^3). The condition factor expresses the condition of a fish, such as the degree of wellbeing, relative robustness, plumpness or fatness in numerical terms (Kumari et al. 2014). The heavier the fish for a given length, the higher its condition factor. The condition factor may be considered as a useful biomarker when making assessment of fish health. A high condition factor reflects good environmental quality, while low condition factor reflects poor environmental quality.

For instance, the presence of a metal ion in the aquatic environment mediation has found to increase the mucus-like secretion from gill, excessive excretion,

anorexia, Gonado Somatic Index (GSI) and also an alteration in the fin movement (Kumari et al. 2014). These set of behavioural changes in the fishes can be used as general biomarkers for health assessment of carps.

5.6 *Biomarkers of Oxidative Stress*

In the aquatic environment, many toxic xenobiotics exert their effects through redox cycling. Oxidative stress, incorporates both the antioxidant defences as well as oxidative damage, caused by the deleterious effect of the toxicants are common effect in organisms exposed to xenobiotics. During oxidative stress free radical production increases and/or antioxidant level decreases which leads to potential damage of the tissues. This condition can be reflected by an alteration in the level of antioxidant enzyme activity, damage to nucleotides bases, products formed due to protein oxidation and lipid peroxidation.

Figure 4 outlines the antioxidant defence system that plays a major role in inhibiting the formation of oxyradicals includes Super Oxide Dismutase (SOD), Catalase (CAT), glutathione-dependent peroxidase (GPOX) and glutathione reductase (GRED). Based on the results of field studies, many researchers have recommended including antioxidant biomarkers for environmental monitoring in the aquatic ecosystem (Vidal-Liñán et al. 2010; Olakolu et al. 2012) as it gives an early warning signal of the deleterious effect of toxicants/xenobiotics on the habitants at the molecular level before being evidenced at a higher level. The changes in the antioxidant-functioning mechanism serve the purpose of oxidative

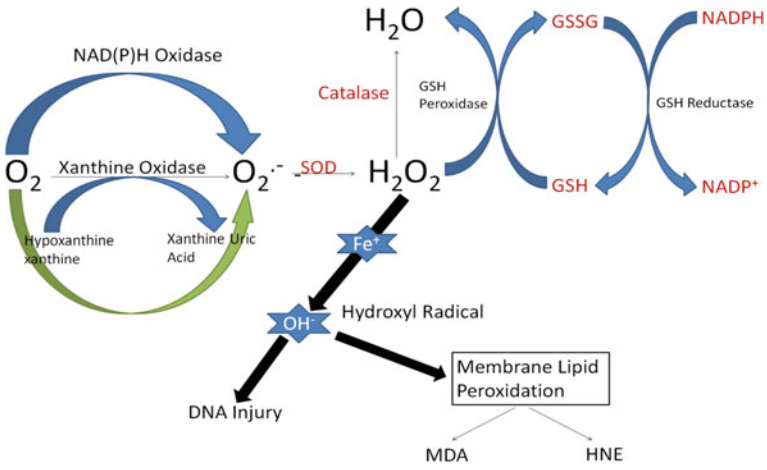

Fig. 4 General diagram of oxidative stress. *SOD* superoxide dismutase, *GSH* reduced glutathione, *GSSG* oxidized glutathione, *MDA* malondialdehyde, *HNE* 4-hydroxy-2-trans-nonenal, *NADPH* nicotinamide adenine dinucleotide phosphate

biomarkers against a variety of xenobiotics. Macromolecules like proteins, nucleic acids, a variety of lipids are affected due to alteration on the oxidative stress within the system (Kumari et al. 2014).

Heavy metal pollution in the aquatic organisms leads to the development of free radical species that are responsible for cellular damage. Metal induce the stress within the body and produce hydroxyl radical ($HO^{\cdot}$) that react with membrane lipids (LH) resulting in the production of highly reactive carbon-centred lipid radical ($L^{\cdot}$) which adds oxygen atom and produce lipid peroxyl radical ($LOO^{\cdot}$). This peroxyl radical abstracts hydrogen atoms from cellular proteins, DNAs, leading to changes in chromosomal activity. The antioxidant α-tocopherol plays a key role to maintain cellular integrity and protect the cellular damage. Many toxicologists have recommended the use of lipid peroxidation test as potential biomarkers for monitoring of aquatic pollution due to metal (Viarengo et al. 1991).

5.7 Metallothioneins as Biomarkers

Metallothioneins (MTs) are cysteine-rich small proteins with low molecular weight that are capable of binding with metals thus by preventing cells from ion-induced damages due to metal conversion or breakdown of metals into its constituents. The thiol groups (–SH) of cysteine residues enable this metallothioneins proteins to bind with the specific heavy metals. It gets induced by both essential and non-essential metals leading to alteration in several biochemical processes and so it can be a potential biomarker for assessing metal pollution in the aquatic ecosystem (Zhang and Wang 2005).

The organisms and tissues which are directly involved in metal uptake from the environment, storage and excretion have a greater efficiency to synthesize MTs. These proteins have also been reported to be found in digestive glands (midgut and hepatopancreas) of aquatic organisms, as well as in the respiratory organs of lower organisms like gills of molluscs and crustaceans (Raspor et al. 2004).

There are various in situ studies conducted validating the role of MTs (Petrovic et al. 2001; Lionetto et al. 2001; Ross et al. 2002; Mourgaud et al. 2002; Rodriguez-Ortega et al. 2002) and the outcomes of these studies are very positive in the condition where metal gradient of pollution are really existing in the environment.

5.8 Histopathological Biomarkers

Histopathology is defined as the microscopic assay of disease processes or alteration in the morphology or structural changes due to the xenobiotic effect that may lead to alterations in the organ system. The alterations in the structure of organ go parallel with the exposure concentration of the xenobiotics compounds within the

Table 5 Summary of histopathological biomarkers and stress indicator

Histopathological biomarkers	Pollutant indicator
Skeletal malformation	Organ malformation due to chlorinated hydrocarbons
Operculum abnormalities	Alteration in organ due to paper/pulp mill effluents
Epidermal hyperplasia	General toxicants (pesticides, heavy metals) and general environmental stresses
Liver histopathology	Changes due to toxic xenobiotics, carcinogens and urban pollution discharges
Gill and kidney histopathology	Stress due to organic pollutants heavy metals, oil discharge, distillery effluents, toxic algae and suspended solids
Embryonic defect	Impact due to organic contaminants, PAHs
Lysosome integrity	Response due to a wider range of pollutants

Kumari et al. (2014), Murthy et al. (2013), Van der Oost et al. (2003)

organisms due to stress caused by the xenobiotics. The changes in histology of an organ are due to the potential of xenobiotic compounds ultimately leading to alteration in enzymatic activity. Histopathological biomarkers of toxicity are useful index of environmental pollution (Palace et al. 2009) as they are easy to determine and they can be linked directly to the health and wellbeing of the individuals that finally relates with the extrapolation of the whole community. Table 5 summarizes the major histopathological biomarkers and stress indicators reported by various authors.

The other histopathological markers include megalocytic heptosis (MH), foci of cellular alteration (FCA), hepatocellular nuclear pleomorphism (HNP) and necrosis, hydropic vacuolation (HV), etc. for assessing chronic toxicity in fishes (Hinton 1994; Murthy et al. 2013).

5.9 Hormonal Biomarkers

Alteration in the hormonal activity or disruption in the endocrinal activity within the organ system has emerged as new area of research these days in terms of both science and public policy. Numerous compounds when discarded in the aquatic environment in an inappropriate manner cause alteration in the endocrine system of the organisms. The chemicals effect, both individual organism and population. The endocrine disrupting chemicals act on every level like synthesis of hormones, secretion, transport, metabolism and site of action.

It is well known from various studies that environmental pollutants have deleterious effects on several hormonal activities. PAHs in the aquatic bodies disrupt the functioning and activities of thyroid hormone (He et al. 2012). The derivatives of PAHs have transthyretin-binding activity, which reduce the level of thyroid

hormone within the body leading to disruption of T3 and T4 level in the blood serum. T3 and T4 hormones are active forms of TH produced mainly in the liver. Stress in the organism can drop the level of T3 and T4 biomarker enzymes within the bloodstream leading to decrease in deiodination activity of the liver system. Several authors have incorporated the essay of T3 and T4 biomarkers for assessment of aquatic pollution due to PAHs (Naderi et al. 2013, 2014).

5.10 Vitellogenin (VTG) in Fish

Vitellogenin estimation in the carps are the most widely used biomarkers all-round the world for estimation of xenobiotics. VTG is a yellow yolk precursor protein found in proteins of oviparous species. The VTG is used for detection of estrogenic depleting chemicals in fishes. The metabolism and functioning of this protein get altered due to xenoestrogens. The amount of VTG is high in female carps while in males it is found in the very low level. As the males and juveniles get affected due to xenoestrogens they start producing VTG similar to females. The ecological relevance of VTG is high for biomonitoring of aquatic pollution and so it can be used as a potential biomarkers for early detection of xenoestrogens in water bodies (Palace et al. 2009).

6 Status of Biomarkers Used in Different Countries

Since a long time, biomarkers have been extensively used for the monitoring of pollution in water bodies round the globe to detect the impact of pollutants on the different vertebrates and invertebrates. Different types of biomarkers have been used according to their suitability while lot many more biomarkers are still under trial to be included in the water monitoring programme. Different types of biomarkers currently in use in various biomonitoring programmes of water bodies in different countries have been shown in Tables 6 and 7. The biomarkers included were based on their effectiveness, suitability, specificity to certain pollutants, their ability to detect different chemicals, ecological relevance etc.

Numerous biomarkers like Gonado Somatic Index (GSI), Vitellogenin in males, EROD activity estimation, Oxidative Biomarkers like GSH, SOD, activity of enzyme Catalase, estimation of blood glucose for metabolism functioning, counting of Macrophages, WBCs counting for Immunal dysfunction, test of lipid content for hepatic toxicity, Ionic balance estimation, etc. are currently being used in biomonitoring programme in different countries like Sweden, Europe, UK, Australia, etc. which are depicting a clear picture with concrete results regarding the status of water quality. Such type of biomonitoring protocols should also be adopted and followed in Asian subcontinent for biomonitoring of water bodies.

Table 6 Different biomarkers in environmental monitoring programme

Country	Classification of different biomarkers									
	Reproduction	Detoxification	Oxidative stress	Genotoxicity	Metabolism/ energy partitioning	Immune defence	Oxygen transport (blood)	Liver function	Growth	Ion balance
Sweden	Gonado somatic index (GSI)	EROD activity	GR activity	DNA-adducts	Blood glucose	White blood cells	Haematocrit (HT)	–	–	Cl^{-}, Ca^{+}, Na^{+}, K^{+}
	Vitellogenin in males	MT2	GST activity	–	Liver somatic index (LSI)	Macrophage centres	Haemoglobin (Hb)	–	–	–
	Primary sex ratio	–	Catalase	–	–	–	Immature (iRBC)	–	–	–
UK	Gonado somatic index (GSI)	EROD activity	–	DNA-adducts	Liver somatic index (LSI)	–	–	Liver pathology	Length, weight	–
	Vitellogenin in males	PAH bile metabolites	–	–	Fish disease	–	–	Liver lipid content	–	–

Source M-88 (2013), www.miljodirektoratet.no/Documents/publikasjoner/M88/M88.pdf

Table 7 Different biological markers used in Norway for monitoring aquatic environment

Types of water bodies	Classification of different biomarkers					
	Detoxification	Reproduction	Endocrine disruption	Metabolism / energy partitioning	Histopathological	Oxidative stress
Lakes	EROD activity (or CYP1A)	Fish vitellogenin	Aromatase activity in fish	Liver somatic index (LSI) in fish	Macroscopic liver neoplasms in fish	–
Coastal water	EROD activity (or CYP1A) and PAH bile metabolites	Fish vitellogenin	Aromatase activity in fish	Liver somatic index (LSI) in fish	–	–
Water bodies located in urban areas	–	–	–	–	Macroscopic liver neoplasms; skeletal deformities and micronuclei test in fish	CAT, GST and GR in fish liver; total oxyradical scavenging capacity (TOSC) in blue mussels

Source M-88 (2013), www.miljodirektoratet.no/Documents/publikasjoner/M88/M88.pdf

7 Conclusion

All the aspects of aquatic pollution like toxicity testing, effects of xenobiotics on organisms and populations, different research findings related to sublethal effects for dose-response relationship can be dealt under a plethora of different biomarkers. The impact of these xenobiotics on organisms creates a cascade of reactions that can be measured using a battery of biomarkers or even a single suitable biomarker for the early detection of pollution before being evidenced at higher level. Estimation and study of biomarker in different sentinel species offers great promises and serves as a valuable tool for environmental monitoring designed for surveillance, hazard assessment, or documenting remediation in confined aquatic environments that serves as a major sink of variety of pollutants and therefore suitable biomarkers should be used as an integral component of environmental monitoring programmes.

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Chapter 19
Bioremediation by Microalgae: Current and Emerging Trends for Effluents Treatments for Value Addition of Waste Streams

Sabeela Beevi Ummalyma, Ashok Pandey, Rajeev K. Sukumaran and Dinabandhu Sahoo

Abstract The development of anthropogenic activities has lead to an excessive disposal of wastes into natural waterbodies, thus affecting the quality of water and polluting the entire environment due to the hazardous chemicals and other nutrients present in the waste, thereby it has a negative impact on the aquatic ecosystems. To avoid these harmful impacts associated with the discharge of wastes into waterbodies, effective remediation processes are required to reduce nutrients like nitrogen, phosphorus, other organic chemicals and heavy metals concentrations in discharged effluents. Current technologies applied for nutrients removal tend to be complex, energy demanding and costly process. Therefore, cultivation of microalgae has appeared as an emerging alternative approach for removing pollutants and heavy metals present in the waterbodies. Biomass production in the alga depends on rapid utilization of the organic content and other nutrients present in the effluent and can be considered as an attractive and eco-friendly means for treating waste streams, other than removing the pollution load, algal cultivation adds value to the process by production of commercially valuable products such as fuels and various chemicals from biomass. This chapter addresses the recent developments

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and perspectives in bioremediation of waste streams by algae for removal of various pollutants for value addition of waste.

Keywords Microalgae · Bioremediation · Wastewater · Biomass Oils

1 Introduction

Problems associated with the pollutions are becoming more severe with the increasing industrialization, urbanization, population and their indirect effects on the entire ecosystem. The consequences include excessive generation of waste streams/wastes, the release this waste streams into the natural waterbodies ultimately affecting the aquatic ecosystems and environment, which are leads to serious challenges for the society, in terms of sustainability of our planet. One of the major sources of pollution of natural waterbodies is the release of wastewater (Nasr and Ismail 2015; Ummalyma and Sukumaran 2014). Each of environment pollution has its own peculiarity which required specific technologies to meet and overcome the challenges associated with pollution. This situation of mixing of wastewater in aquatic bodies is emerging up as one of the major problems that are challenging the stability of developing nations (Renuka et al. 2015; Yang et al. 2008). This is primarily due to the fact that the many of people in developing countries are depends on the freshwater resources for their survival. Water scarcity is a serious issue in many of developing counties including India. Therefore, it is urgent need to preserve our available natural ecosystem with waterbodies and treatment of wastewater is required before it is discharged into the natural water sources. Current wastewater treatment process is a costly and energy-intensive process. Therefore, it is the time to identify cheap, eco-friendly technologies that need minimum infrastructure facilities and easy technology for wastewater treatment. These technologies should be applied at the lab scale to commercial level in the future. Bioremediation of wastewater is a better option compared to conventional methods. Wastewater treatment with microalgae is popularly known as phycoremediation. The phycoremediation process is attractive because it has the potential to solve many problems at a time. Therefore, the cultivation of algae in wastewater bids the combined advantages of greenhouse gas mitigation, wastewaters treatment and simultaneously producing algal biomass. This biomass can be exploited for multiple application as protein supplements, food additives, bioenergy resources (biogas and biofuels), pharmaceuticals, cosmetics and other valuable chemicals (Ummalyma and Sukumaran 2015; Sahu et al. 2013; Pittman et al. 2011; Spolaore et al. 2006).

2 Limitations of Conventional Wastewater Treatment Technologies

Several efforts have been made over the past many years in the designing of different types of wastewater treatment systems based on the specific need and type of wastewater to be processed (Arceivala and Asolekar 2007). Currently, diverse ranges of wastewater treatment plants (WWTPs) are an exit, which is varied based on the design and capacity depending upon the requirement of specific geographical location, the type of wastewater and the amount of water to be processed. The conventional WWTPs mainly include the following treatment steps: preliminary, primary, secondary and tertiary followed by disinfection and disposal of solid sludge generated during different stages of the treatment. The preliminary treatments are basically simple screening of waters for the removal of solid and bigger objects which can clog or damage the further treatment processes, whereas oil and greasy substances along with primary sludge are in the primary treatment using clarifiers and sedimentation tanks. The secondary treatment mainly depends on biological treatment and most of the organic contents are removed due to microbial degradation followed by advance stages of treatment at tertiary level. This step comprises various treatment processes, such as coagulation, flocculation, filtration, reverse osmosis, extended biological nutrient removal and stabilization of oxygen-demanding substances. The treated wastewater is finally disinfected by chlorination, ozonation, ultraviolet (UV) lights, etc., while the process concentrates (biological sludge) generated during the treatment are subjected first for the dewatering and used as fertilizer or subjected to further treatment or disposal which is a big bottleneck of CWWTP technologies.

The installation of WWTPs with the cost of wastewater treatment is considerably higher (McCarty et al. 2011) as the conventional WWTPs need high-energy inputs. Furthermore, the maintenance and operations require technical expertise which also incurs extra costs. Other problems include frequent breakdowns of WWTPs due to mechanical failure or mishandling. The biological wastewater treatment is highly efficient in the nutrient removal; therefore, these systems are extremely effective. However, it adds approximately 3–4 times more costs in overall treatment processes (Abdel Raouf et al. 2012). Moreover, unfavourable environmental situations also lead to the failure of such systems as a major proportion of the treatment is depending on the biological systems. A slight alteration or variation in the physico-chemicals, water or geo-environmental conditions leads to death and decay of microflora involved in the biological nutrient removal processes, whereas excessive growth of filamentous bacteria leads to bulking and foaming (Khin and Annachhatre 2004; De Bashan and Bashan 2004). Therefore, practically this kind of systems has limited application in economically backward nations.

The wastewater treatment could be perfect only if the energy and cost is minimal. In this regard, microalgal wastewater systems are unique and cost-effective. Such systems are being employed for decades for the treatment of various types of wastewater. As these systems are based on the algal source which requires adequate

temperature and sunlight, therefore, these systems are getting due considerations in the temperate regions having suitable light intensity and duration throughout the year and environmentally safe and sustainable process for treating various waste streams.

3 Importance of Microalgae for Wastewater Treatment

Microalgae are photosynthetic microscopic organisms that are found in both marine and freshwater habitat. Their photosynthetic capability is almost similar to terrestrial plants, due to its simple cellular structure and being submerged in an aqueous milieu, where they have efficient access to water, other nutrients and CO_2. They are normally more potent in converting solar energy into biomass. Microalgae are unicellular or multicellular organisms having different size (few microns to hundred of microns), which exists either single cells or as chains or flocs, in both marine and freshwater habitat and identified as one of the oldest life forms (Falkowski and Raven 1997). Microalgae are ancient plants (Thallophytes), i.e. lacking stems, roots, leaves and have no sterile covering of cells around the reproductive cells and their primary photosynthetic pigment is chlorophyll *a* (Lee 1980). Microalgal structures are principally for conversion of energy without any development beyond cellular levels, and their simple development tolerates them to adapt to existing environmental conditions and thrive in the long term (Khan et al. 2009).

The inherent evolutionary advantages of algae are their flexibility to change their nutritional type based on the availability of substrate and light. However, in the context of the substrate, algal growth mode can be autotrophic, heterotrophic or mixotrophic nature. The autotrophic or phototrophic algae require only inorganic carbon in the form of CO_2, salts and light energy source for growth. Phototrophic algae usually gain energy through light by fixing CO_2 from atmospheric. Photosynthesis is a key component of the survival of autotrophic algae and they convert solar radiation and CO_2 absorbed by chloroplasts into adenosine triphosphate (ATP) and O_2 the usable energy form at cellular level, which is then used in respiration process to generate energy to support growth of organism (Falkowski and Raven1997; Zilinskas1976). Heterotrophic microalgae use organic carbon molecule as main energy and carbon source through heterotrophic nutritional mode and assists in high biomass productivities which offer the economical possibility for large-scale production (Perez-Garcia et al. 2010; Behrens. 2005). Some photosynthetic algae can able to grow in mixotrophic condition by performing both autotrophic and heterotrophic mechanisms by absorbing available organic carbon as well as atmospheric CO_2 as carbon and energy source (Lee 1980).

Microalgal cultivation required huge quantities of synthetic fertilizers as well as waters. An alternative to this synthetic fertilizer is to utilize wastewaters which are rich in inorganic nutrient; other organic components can be utilized by the algae for biomass generation for value-added biofuels and chemicals. Cultivation of algae in freshwater is not a viable process due to its increasing demands in the near future.

Therefore, growing of algae in wastewaters or steams are real advantages in terms of remediation of wastes/pollutants from the wastewater and valuable biomass for application in various industries. Algal cultivation needs a huge quantity of water and it has been stated that ~6000 L of water is used for per litre of algal oil production in the conventional system of algal cultivation (Ozkan et al. 2012). Water gives the physical milieu for the growth of the algae and also distributes the nutrients, eliminates waste products and acts as a thermal controller (Murphy and Allen 2011). Therefore integrating algae-based biofuel production along with wastewater remediation has been considered as economic and environmental sustainable process. However, huge amounts of water can be saved and reduced the cost associated with nutrients (P and N) for this kind of waste–water-based microalgal cultivation systems (Hemalatha and Mohan 2016; Singh et al. 2016; Gentili 2014; Ummalyma and Sukumaran 2014).

3.1 Removal of Nutrients from Wastewaters

Wastewaters from various sources, such as agricultural effluent, municipal effluent, industrial effluents and effluent from others are rich in P, N, BOD and COD which will lead to eutrophication of water that affects the aquatic life. Therefore, nutrients must be removed from wastewater before being discharged into waterbodies. Algae have the capacity to eliminate nutrients from the waste streams and give valuable biomass for various applications. Algal biomass production needs the availability of essential nutrients, such as carbon, nitrogen, phosphorus and micronutrients, which are expensive if they required to be added in great amounts (Christenson and Sims 2011). However, the application of wastewater can cut down the expense of algal biomass production and reduce the addition of nutrients. Furthermore, using algae for simultaneously treating wastewater and produce biomass for biofuels or nutraceuticals is also justified by the capability to balance the high costs of wastewater treatment (Christenson and Sims 2011). Microalgae absorb the inorganic nutrients and liberate oxygen through the photosynthetic process; on the other hand, free oxygen is necessary to allow efficient bioremediation of organic molecules by heterotrophic bacteria which in turn, generate carbon dioxide (CO_2) that can be utilized by microalgae for the photosynthetic process (Samorì et al. 2013).

There are many reports are available for the potential of algae to remove N, P and other organic pollutants from wastewaters. Mixotrophic cultivation of microalga *Chlorococcum* sp. *RAP-13* can able to remove organic pollutants, such as COD and BOD of raw dairy wastewater at a ratio of 93 and 82%, respectively (Ummalyma and Sukumaran 2014). Algal cultivation in unsterilized dairy effluent showed removal efficiency of nitrogen, phosphorous and COD at a ratio of 83–99, 89–91 and 84–89% in 2–20% raw dairy wastewater, respectively in the indoor lab-scale experiment for 8 day (Ding et al. 2015). A Recent report on the investigation on the potential of *Chlorella pyrenoidosa* for phycoremediation and dye removal at different concentration of (25–100%) textile effluent showed that

microalgae have potential to grown up to 75% of concentrated effluent and reduces phosphate, nitrate and BOD by 87, 82 and 63%, respectively. The results revealed that algal biomass has the ability to remove methylene blue dye and the absorbing potential was high for wet algal biomass when compared to dry algal biomass (Pathak et al. 2015). *Chlorella vulgaris UMACC 001* cultivated in high rate algal pond (HRAP) enriched with textile dye (Supranol Red 3BW) and effluent from textile industry revealed algae has potential to removed the dye ranged from 42–50% along with nitrogen removal of 44–55%, phosphorous removal of 33% and COD ranged from 38–62%, respectively (Lim et al. 2010). Co-cultivation of microalgae and bacteria is another strategy for fastest removal of pollutants from wastewater. Instantaneous elimination of nutrients and COD was evaluated by co-culture *Chlorella vulgaris* and *Pseudomonas putida*. The co-culture system showed the removal of organic and inorganic molecules than each axenic culture, indicating that nutrients uptake capability of *C. vulgaris* was enhanced in the existence of *P. putida* (Mujtaba et al. 2017). Results revealed the suspended co-culture eliminated 70% of ammonia for 5 days, which is 5 times higher than that by suspended *P. putida* alone. When both microorganisms were immobilized, the removal efficiency of nitrogen was increased to 75%. While, the highest removal of nitrogen was achieved by the cultivation of immobilized C. *vulgaris* and suspended *P. putida*, by which about 85% of ammonia was removed for 5 days. Phosphorous and COD removal efficiency is 66–85%, respectively, when cultivated with co-culture system compared to cultivated alone. A mixed population of microalgae *Scenedesmus species* cultivated in effluents from paper and pulp industry for effluent treatment showed that 60% of effluent was optimum for microalgal cultivation along with maximum removal BOD of 82 and COD of 75%, respectively, when cultivated in the outdoor open pond (Usha et al. 2016). Removal of 65% NO_3–N and 71.29% PO_4–P were observed at the end of cultivation periods. These results underlined that pulp and paper mill effluent could be used successfully for algal cultivation to minimize the freshwater loads and nutrients and removal of pollutants for environmental sustainability. Treatment of Palm oil mill effluent by microalgae *Chlamydomonas incerta* showed algae have potential to consume the organic carbon present in the effluent on the second day of cultivation. The optimal success rate of nutrient removal with *C. incerta* was about 67% of COD for 250 mg/L same effluent in 28 days (Kamyab et al. 2015). From these studies, it is evident that algae can able to remove all the pollutants from the wastewater as a result high-value metabolites and chemicals from the algae biomass can be used for industrial application thereby it will add value to the waste streams.

3.2 Heavy Metal Removal

Growing of industrialization along with urbanization leads to increasing the concentration of heavy metals (HMs) get to alarming toxic levels in the land and water, and hence, suggesting heavy metals richness in many ecosystems to be strongly

related to anthropogenic activities (Herrera-Estrella and Guevara-Garcia 2009; Lasat 2000). A range of reports explained in a different way of traditional uses of heavy metals (Kumar et al. 2015). The generally regular heavy metal contaminants in industrial wastewater include Pb, Cr, Cd, Cu, Hg and Zn which lead to one of the worldwide ecological issues (Hong et al. 2011). Heavy metals in the aquatic environment persists in long term or undergo biotransformations, finally accumulating throughout the food chain, thus it will lead to severe harm to the ecosystem if it is not removed appropriately (Volesky and Holan 1995). However, it will create a serious environmental, public health and economic impact on the ecosystems. Compared to other pollutants elimination of heavy metals from wastewaters are really challenging and cannot be chemically and biologically degraded. Heavy metal disposed into the aquatic environment through wastes has a fatal effect on the aquatic system which destroys the self-purification potential of an aquatic body (Khan et al. 2008).

The currently used physicochemical methods to remove heavy metal ions from wastewaters include precipitation (Charerntanyarak 1999), ion exchange (Dąbrowski et al. 2004), electrokinetic (Yuan and Weng 2006), membrane processing (Qdais and Moussa 2004) and adsorption (Goharshadi and Moghaddam 2015; Lee et al. 2012). A major limitation of this technology is the expense of chemicals associated with industry and metal removal is poor. An alternate approach of this is biosorption of heavy metals by algal species. Biosorption of metal ions from wastewater using algae can offer an ecologically safe, cheaper and efficient method. Algae can be used for biosorption of toxic, radioactive metal ions as well as to recover metal ions like gold and silver (Pohl and Schimmack 2006; Darnall et al. 1986; Mata et al. 2009). However, to attain the preferred level of treatment with algal cultivation systems, it is essential to know the maximum phototrophic production of biomass along with the physiological properties of algae. Different mechanism of heavy metal biosorption by algae is illustrated such as ion exchange, complex formation and electrostatic interaction which occur at micro-scale level (Mata et al. 2008; Demirbas 2008). Among these ion exchange is the most important method of biosorption of heavy metal ions by algae (Michalak and Chojnacka 2010; Mehta et al. 2002). The biosorptions of algae has the following benefit includes fast metal uptake potential, eco-friendly, biomass can be produced in throughout the year, cheap process, ability to bind up to 10% of their biomass with high selectivity, nontoxic waste generation, useful to operate the cultivation system in both batch and continuous operation, applied in waters containing high metal concentrations (Monteiro et al. 2012).

Microalgal affinities towards polyvalent metals help to establish their potential application in cleansing wastewater-containing dissolved metallic ions (de-Bashan and Bashan 2010) particularly; *Chlorella* and *Scenedesmus* potent organisms for metal removal. However, Brinza et al. (2007) elaborated the role of marine algal species as biosorbents for metal uptake. They mentioned use of the following microalgae such as *Chlamydomonas reinhardtii*, *Chlorella salina, Chlorellasorokiniana, C.vulgaris, Chlorellaminiata, Chlorococcum spp., Cyclotellacryptica, Lyngbyataylorii, Phaeodactylum tricornutum, Porphyridiumpur-*

pureum, Scenedes musabundans, Scenedesmus quadricauda, Scenedesmus subspicatus, Spirogyra spp., Spirulinaplatensis, Stichococcus bacillaris and Stigeoclonium tenuea are involved in removal of heavy metals (Brinza et al. 2007). Perales-Vela et al. (2006) also described the metal removal of Cd, U, and Cu by *Chlorella, Scenedesmus* and *P.tricornutum*. Perales-Vela et al. (2006) reported that microalgae and related eukaryotic photosynthetic organisms preferably produce peptides capable of binding heavy metals as results organometallic complexes are formed, which are further positioned inside vacuoles to facilitate appropriate controls of the cytoplasmic concentration of heavy metals inside the cells, this further prevents or neutralizes the toxic effect of the metals (Cobbett and Goldsbrough 2002). Recently, heavy metal absorption by microalgae and its mechanism is reviewed by Kumar et al. 2015. From these observations, it is evident that microalgae act as potent bioremediation tool for effective addressing of elimination of heavy metals from water sources by biotransformation or biosorption (Table 1).

3.3 Bioremediation of Pesticides

Due to the growth of population and the consequential necessity to improve the yield of crops, therefore, utilization of pesticide has been escalated in recent years. This pesticide is runoff from agricultural crops with rainwater and is reported to be a key source of their presence in the aquatic environment. As a result, it has been stated that presence of this compounds in effluents coming from agricultural streams affects the macro-invertebrate communities and ecosystem functions (Schäfer et al. 2007). Some of the pesticides are banned by some government (atrazine, alachlor), other chemical such as diazinon or malathion keep on under continuous monitoring and may be either slowly removed or banned. Several phytoremediation methodologies are applied to overcome these pesticides issues such as microalgae-based ponds and buffer wetlands (Vymazal and Brezinová 2015). Current laboratory-scale experiments on microalgae capacity to remove organic contaminants, such as surfactants, phenolic compounds, polycyclic aromatic hydrocarbons and biocides suggested that microalgae-based wastewater treatment systems can able to remove these compound either by photodegradation, evaporation, biodegradation, or uptake into the biomass (Ho et al. 2014; de-Bashan and Bashan 2010; Haritash and Kaushik 2009). However, limited reports are available mechanism involved for pesticide removal by algae microalgae from effluents. Effluent treatment performance of the algal technology is typically based on the strategies adapted for cultivation system chosen for specific algae. Ho et al. (2014) reported that fed-batch and continuous culture systems have some advantages such as high biomass production and cheap process cost. However, reported experimental studies on pesticide reduction by microalgae have been conducted shake flasks only under controlled conditions (Abargues et al. 2013; Dosnon-Olette et al. 2010). Hence, it is difficult to scale up to pilot-scale, which are generally functioned in continuous mode and nourished with real effluents containing bacteria and other complex

Table 1 Bioremediation potential of microalgae in various effluents

Wastewater source	Microalgae	Types of pollutants	Removal efficiency (%)	References
Food waste effluents	*Chlorella PY-ZU1*	NH3-N TP COD	99 99 68	Cheng et al. (2016)
Piggery wastewater	*C.vulgaris*	TP TN	85.3 89.5	Yang et al. (2016)
Riboflavin manufacturing wastewater	*Chlorella pyrenoidosa*	TP TN COD	94 78 98	Sun et al. (2013)
Dairy wastewater	*Cholorococcum* sp. RAP13	BOD COD	82 93	Ummalyma and Sukumaran (2014)
Municipal wastewaster	*Auxenochlorella protothecoides*	TP TN COD	60 81 90	Zhou et al. (2012)
Domestic wastewater	Microalgal mix	COD Tetracycline	80 93–99	Norvill et al. (2017)
Piggery effluent	Acutodesmus obliquus	TP TN COD	60 70 50	Kim et al. (2016)
Wastewater	*Chlorella* sp., *Scenedesmus* sp.	Lindane, alachlor chlorpyrifos	50–70	Matamoros and Rodríguez (2016)
Pharmaceutical effluents	*Scenedesmus obliquus Chlamydomonas Mexicana*	Carbamazepine	28–38	Xiong et al. (2016)
Secondary effluents	*Chlorella* sp., *Spirulina* sp. *Scenedesmus* sp.	Cu Zn	81.7 94.1	Chan et al. (2013)
Contaminated marine sediments	*Phaeodactylum tricornutum, Nitzschia* sp., *Skeletonema* sp., *Chlorella vulgaris*	Cu Zn	– 	Kwon et al. (2017)
Aqueous solution	*Phormidium* sp., *Pseudochlorococcum* sp., *Scenedesmus* sp.	Hg^{2+} Cd^{2+} Pb^{2+}	97 86 70	Shanab et al. (2012)
Growth medium	*Nostoc muscorum*	Malathion	91	Ibrahim et al. (2014)

components. Additional important aspect in designing an operation strategy in algal ponds is hydraulic retention time (HRT). It is commonly recognized that higher the HRT, possibilities of biodegradation is increased. Photodegradation and biosorption methods occur in biologically based treatment technologies (Garcia-Rodríguez et al. 2014). Meanwhile, there is a gap in information about the potential of microalgae to

remove pesticides from various effluents and how various operation strategies such as batch versus continuous operation along with HRT affecting the removal potential of algae. Matamoros and Rodríguez (2016) conducted studies on the potential of an algal consortium with *Chlorella* sp. and *Scenedesmus* sp. to remove various pesticides under batch and continuous mode of operation spiked with 10 μg L^{-1} of each pesticide. Experiments performed under batch mode showed that the presence of microalgae increased the 50% removal efficiency of pesticides like lindane, alachlor and chlorpyrifos, whereas continuous operation had maximum removal efficiencies for pentachlorobenzene, chlorpyrifos and lindane. Furthermore, efficiency of pesticides removal is increased with higher HRT and minimum HRT for 2 days was capable of removing endosulfan up to 70% along with other pesticides. Hultberg et al. (2016) reported the potential of *Chlorella vulgaris* for removal of wide range pesticide from the water. They have studied the treatments of water with short-term exposure to living and dead microalgal biomass along with long-term exposure to actively growing microalgae. The initial pesticide concentration was 63 μg L^{-1}. A significant reduction of the total amount of pesticides was obtained after the long-term exposure to growing microalgae (29 μg L^{-1}) compared with the long-term control (37 μg L^{-1}). They have evaluated around 38 pesticides, out of those 10 pesticides concentration was significantly removed with long-term algal treatment. These results suggest that effluent treatment with microalgae could be further explored not only for removal of inorganic nutrients but also for removal of organic pollutants present in the water. The use of blue-green algae commonly known as cyanobacteria is used to evaluate their growth and utilization of organophosphorus pesticide such as malathion. The growth of algae was affected by increasing the concentration of malathion. *Nostoc muscorum* is tolerated to various concentrations of malathion and was recorded as an efficient organism for biodegradation (91%) of this compound. Moreover, protein, as well as carbohydrate content of their cells, increased compared to other strains. The algal strains were further subjected to grow under phosphorous limitation along with presence and absence of malathion. Although algal growth under phosphorous limited condition was poor, meanwhile a massive increased in growth and phosphorous content of cells were observed when the medium lacking phosphorous was adjusted with malathion (Ibrahim et al. 2014). This result highlighted that *N. muscorum* with its potential to utilize malathion as a source of phosphorous is considered as a low cost and efficient biotechnological process for remediation of organophosphorus pesticide from contaminated wastewater.

3.4 Removal of Oils

Other environmental issues nowadays are contamination of petroleum hydrocarbon especially in the area of petrochemical industries. Oil pollution can lead to harmful environmental issues in marine ecosystems. Microbial-based breakdown and detoxification of these pollutants are found to be alternative for this issue.

Microalgae and its consortium with bacteria play a vital role in the removal of crude oils pollutants from the environment. Studies conducted with four oil-degrading bacteria along with microalgae tolerant to oil. *Scenedesmus obliquus GH2*, was used to make an artificial microalgal-bacterial consortium for degradation of crude oil (Tang et al. 2010). The result showed that this consortium demonstrated an increased efficiency in degrading both aliphatic and aromatic hydrocarbons of crude oil. It has been reported that crude oil is a mixture of up to 10,000 different toxic hydrocarbons, which causes serious threats to life (Dawes 1998). Moreover, some experimental studies on the effect of crude oil associated with the petroleum products on microalgae have shown contradictory results. Some are reported crude oils product affecting the photosynthetic machinery of microalgae and others reported its helps in pollution removal and enhancing the growth. Microalgae have their inherent capacity to adapt to any harsh conditions. To support this attractive example of the adaptation of microalgae to extreme contamination of crude oil was described in the Arroyo Minero River (AMR) of Argentina (Carrera Martinez et al. 2011; Lopez-Rodas et al. 2009). Underground crude oil has constantly leaked out since 1915 in AMR as an outcome of test drilling for petroleum exploitation. However, four potent microalgae species, such as *Symploca dubia, Chlamydomonas dinobryonis, Scenedesmus obtusus and Scenedesmus sp*. proliferated in contact with crude oil. Other studies on the mechanisms involved in the adaptation of microalgae to crude oil contamination showed that *Scenedesmus sp*. can adapt to low concentrations of petroleum by means of physiological acclimatization. In addition, only specific organisms resistant to crude oil are able to grow under high levels of crude oil. The crude oil-resistant mutants have arisen during rare spontaneous mutation that has happened prior to the exposure of crude oil (Carrera Martinez et al. 2011). Cultivation of *Scenedesmus obliquus* and *Chlorella vulgaris* was conducted under the heterotrophic condition for degradation of crude oil. Experiments were performed by incubating algal cultures with different percentages of crude oil for 15 days. It was found that *Scenedesmus obliquus* and *Chlorella vulgaris* performed the highest biodegradation rate of crude oil when 0.5 and 1% oil was supplemented. The highest growth of *S. obliquus* was obtained with the addition of 0.5% crude oil. While in the case of *C. Vulgaris* it was around 2% under heterotrophic cultivation. Both algae have potential to grow and degrade oil effectively when incubated with low concentrations of oil (El-Sheekh et al. 2013). From this study, it is clear that algae can be applied in crude oil spillage area for removal of pollutants from oils. In order to support this another bioremediation approach using the microalgae/cyanobacterium consortium containing *Anabaena oryzae* and *Chlorella kessleri*. The consortium along with individual of *A. oryzae* and *C. kessleri* was cultivated with various concentrations of crude oil (0.5–1.5%) under mixotrophic conditions. However, for maximum biomass production, 1% of oil is optimum. The pigment content of the algae is varied with the concentration of crude oil in the medium and incubation time (Hamouda et al. 2016). Results showed that these algae can able to degrade some aliphatic compounds, such as heptadecane, octadecane,3-methyl-decane, docosane, nonadecane

and tetracosane and aromatic compounds like cyclohexanebutyl, naphthalene decahydro-2-methyl, benzene-decyl present in the medium.

3.5 Removal of Pharmaceutical Chemicals

Pharmaceutically active chemicals are present in waterbodies is an alarming treat for both humans and animals (Kolpin et al. 2002). Since increasing the number of pharmaceutical industries leads to the dubbing of wastes to the natural waterbodies which cause accumulation of many of Pharmaceutical chemicals in water and have been detected in wastewater over the past decades (Ternes 1998). Several pharmaceutical components such as the antibiotic ciprofloxacin have been found in aquatic environments at a concentration of up to 6.5 mg L^{-1} (Petrie et al. 2015). This higher concentrations of pharmaceutical compounds in higher trophic level organisms leads to long-term effects via biomagnification through food web (Kelly et al. 2007). Among these, the most commonly seen drugs are non-steroidal anti-inflammatory drugs, such as ibuprofen, diclofenac and antibiotics like erythromycin, roxithromycin, ketoconazole, quinolones, fluoroquinolones, antidepressants and antiepileptics (carbamazepine) (Petrie et al. 2015). Microalgae are known to be the primary producers in aquatic food chains and they are the vital indicators of monitoring quality of water and eco-toxicity of pollutants (Stevenson and Graham 2014). Mixotrophic cultivation systems are used for evaluating the bioremediation of wastewater rich in pharmaceutical compounds by algae. Studies conducted for assessing the toxicity, cellular stress and biodegradation potential of carbamazepine (CBZ) by microalgae *Scenedesmus obliquus* and *Chlamydomonas Mexicana* (Xiong et al. 2016). The growth of *S. obliquus* (97%) was significantly inhibited at 200 mg/l of carbamazepine, whereas under the same condition, only 30% inhibition was observed for *C. mexicana*. Moreover, *C. mexicana* and *S. obliquus* could achieve a maximum of 35 and 28% biodegradation of CBZ. They have also found that a mixture of pharmaceutical compounds containing CBZ could strongly affect the activity of ATP synthase in *Pseudokirchneriella subcapitata* (Xiong et al. 2016), suggesting that pharmaceutical chemicals can hamper with energy transduction pathways in chloroplast and mitochondria of algae (Vannini et al. 2011). Recently, studies on the algal consortium containing *Chlorella* sp. and *Scenedesmus* sp. can able to remove 20% of CBZ from synthetic and urban wastewasters (Matamoros and Rodríguez 2016). The removal of pharmaceutical compounds from wastewater by algae-based consortium could be due to biodegradation or bioaccumulation. A study regarding CBZ removal by microalgae showed that some key enzymes such as superoxide dismutase and catalase of phototrophic microorganisms would protect against the reactive oxygen species (ROS) toxicity through regulation of anti-oxidative defence mechanisms (Xiong et al. 2016), which are commonly recognized as the biomarkers (Zhang et al. 2012). Superoxide dismutase activity in *C. mexicana* was increased by 1.3-fold at low concentrations of CBZ whereas at higher concentration enzyme activity is

decreased (Xiong et al. 2016). It is clear that, these pharmaceutical compounds affecting the metabolic pathways and physiology of algae, but this organism is highly evolved to tolerate from all those toxic effects. Considering the fact that, thousands of pharmaceutical chemicals and tons of the compounds containing wastewaters are generated each year from various pharmaceutical industries, it is of vital demand to find effective means to deal with this type of wastewater.

4 Bioremediation of Microalgae in Reactors for Wastewater Treatment

Many of microalgal-based bioremediation studies are conducted in flask level, but it can be more valued its reality with large-scale cultivation in reactors. Microalgal cultivation reactors can be open ponds raceways and photobioreactors. The open pond culture system with and without paddle wheel is usually observed as the most economical route among algae cultivation systems due to its low operating costs, easy operation and maintenance. Open pond system depends on many of the natural environmental condition, such as the location of pond site, evaporation and contamination with other unwanted organisms. Different types of open systems are presently used for algal cultivation with wastewaters are slope system, raceway ponds, spherical ponds and HRAP, etc. Hom-Diaz et al. (2017a) reported 1000 L pilot-scale HRAP for wastewater treatment with degradation of antibiotic ciprofloxacin (CPX). de Godos et al. (2016) studied the performance of HRAP system using anaerobically digested wastewater along with the addition of CO_2. This system efficiently removed nitrogen as NH_4^+ up to 85%. Another report on piggery wastewater treatment with HRAPS which helps in a stable and efficient nitrogen and carbon oxidation performance, with average COD and TKN removal efficiencies of 76, 88%, respectively (de Godos et al. 2009).

Photobioreactors for algae cultivation require high capital and operating costs, however, they are normally more efficient in biomass production due to better control of the culture parameters and contamination. In recent years, photobioreactors are preferred for better performance of wastewater treatment with microalgae. Wastewater-borne cyanobacteria along with a mixed consortium of microalgae are co-cultivated in a closed photobioreactor. This reactor a mixture of urban secondary effluent and digestate are used as a medium for cultivation and run in the semi-continuous mode of operations. The study revealed that total inorganic nitrogen, inorganic phosphorus concentration, phosphorus volumetric load and presents or absence of carbon leaded to different species present in the consortium helps in potential removal of nutrients and biomass production. This study showed that this consortium can be used for wastewater as a nutrient source for their cultivation in photobioreactor (Arias et al. 2017).

Recently, membrane photobioreactors are used for wastewater treatment with microalgae. One of the proposals is annular PBR with ion exchange membranes

(IEMs) for algal cultivation with wastewater to eliminate the harmful effects of pollutants in wastewater, such as heavy metals P, N and suspended solids on algal growth. In this system, the nutrients especially nitrogen and phosphorous present in the wastewater continuously flows into cultures through the membrane for growth, while the pollutants barely leak into microalgae cultures (Chang et al. 2016). Another report on the cultivation of algae in aquaculture wastewater using membrane photobioreactor (MPBR) for pollutants removal along with biomass was evaluated with *Chlorella vulgaris* and *Scenedesmus obliquus*. *C. Vulgaris.* The study showed that better performance was observed with the specific growth rate of 0.17 d^{-1} and continuously cultivated in MPBR. Nutrients present in this effluent were effectively removed in membrane-based reactor, with an average reduction of nitrogen and phosphorous was 82–86%. Unionized ammonia present in aquaculture water which is normally lethal to aquatic animals was also removed with this kind of reactor, with an effluent concentration below 0.002 mg L^{-1} (Gao et al. 2016). Evaluation of algae for treating toilet wastewater containing pharmaceutically active compounds (PhACs) in outdoor pilot-scale photobioreactor of 1200 L capacity showed potential to remove toxic chemical and nutrient present in wastewater along with biomass production (Hom-Diaz et al. 2017b). A tubular PBR is run continuously with the fish farm effluent using microalgae *Tetraselmis suecica,* produced biomass of 0.5 g L^{-1}, nutrients removal (N, P) efficiencies were 49 and 99.0%, respectively (Michels et al. 2014). Another report showed that offshore photobioreators are used for treating incoming raw municipal wastewater of up to 50,000 gal/day by microalgae (Novoveská et al. 2016). They have studied the combination effect of nutrient uptake by algae, aeration from oxygen produced through photosynthesis and harvesting by suspended air flotation. Results showed that algae has removed 75% of total nitrogen, 93% of total phosphorus and 92% BOD from influent wastewater and observed the shifting of the dominance of *Scenedesmus dimorphus* to diverse organisms dominated by genus *Chlorella, Cryptomonas* and *Scenedesmus* sp. which enable efficient nutrient removal. Biomass production rates ranged from 3.5 to 22.7 g/m^2/day through the continuous mode of operation and productivity predominantly determined by temperature and rate harvesting biomass. Study of a microfiltration-based membrane photobioreactor and forward osmosis-based osmotic membrane photobioreactor (OMPBR) is used for cultivation of *Chlorella vulgaris* continuously with tertiary effluent. Both the bioreactors exhibited good biomass accumulation (over 2 g/L), although the OMPBR achieved better nutrients removal due to high rejection properties of the membranes with nutrients removal efficiencies of 86–99% (N) and 100% (P), respectively, whereas the corresponding values in the MPBR were 48–97 and 46%, respectively, but the operating cost for OMPBR is 30–45% higher than that of MPBR (Praveen et al. 2016).

5 Future Perspective

Microalgal cultivation system should be interlinked with wastewater generation industry for addressing the issues associated with its scalability challenges. Coupling of effluents remediation along with high-value biomass from algae for nutraceuticals and biofuel production it will add value addition of waste streams. For improving economic feasibilities and full-scale operation of algae-based wastewater treatment system, application of bio-flocculation, auto-flocculation of cells and other alternative harvesting process could be exploited. Figure 1 illustrates the future integrated algal biorefinery for sustainable production of value-added products from algal biomass utilizing wastewater. Furthermore, identification of potent algal strains resistant to infection by another microorganism, capable of auto-flocculate and aggregate formation that helps to escape from algal feeders with maximize the pollutants removal capabilities should be favoured for the waste valorization. Other challenges in wastewater cultivation in open ponds are the rotifers infection. Therefore, early detection and prevention of this infection by rotifers and others protozoans are needed to control in the algal cultivation systems. Proper management strategies should be focused on reducing carbon emission in the environment. However, many developed technologies are focusing on one particular product without full exploitation of biomass. Therefore, other important metabolites and valuable component present in the biomass are wasted. Hence, an integrated technology for full exploitation of biomass is yet to be developed for improvement of sustainability and exploitation of algal biomass. Integration of algal biorefinery with wastewater remediation along with utilization of waste carbon dioxide will provide an economically viable and sustainable strategy for utilizing biomass as feedstock for biofuels/chemicals and pollution free environment. Therefore, the use of wastewater for the cultivation of algae reduces utilization of

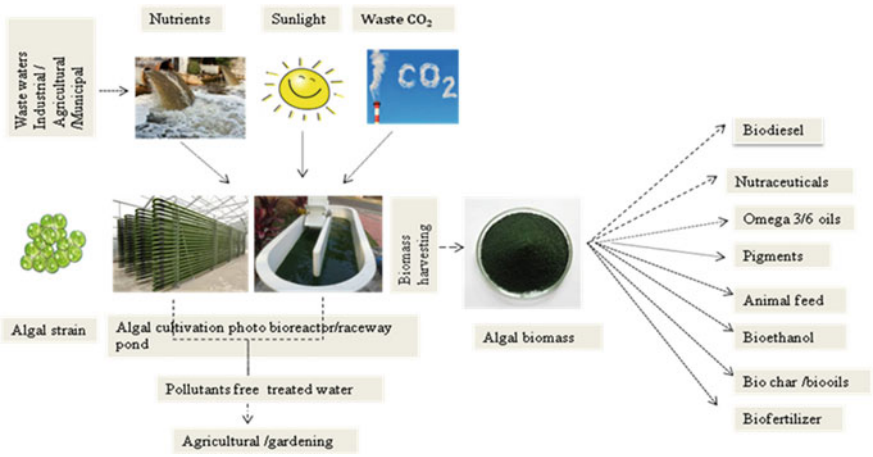

Fig. 1 Future algal biorefineries associated with wastewater treatment

freshwater source and nutrient requirement which significantly reduces the water and carbon footprint along with substantially reduces the cost. However, in view of environmental consciousness, phycoremediation appears as a technology representing wastewater as an important resource, zero-waste concept with minimum water and carbon footprints.

6 Conclusion

Coupling of microalgal cultivation along with wastewater treatment is an economical and environmental possible technology for phycoremediation with cheap biomass production for biofuels and chemicals. For cost-effective large-scale cultivation of algae depends on complete utilization of wastewater for biomass production along with the removal of pollutants present in the water for effective treatment. Wastewater-borne algae can able to perform better than non-indigenous microalgae. Algal–bacterial consortium can also do effective wastewater treatment. Nutrients removal features of algae project that this organism can be a potent source of oil which can be used as feedstock for biofuels (biodiesel) and also as a food or feed supplement. Moreover, algal cultivation in the wastewater could reduce the effluent's pollution load significantly indicating the potential for its use as an effective effluent treatment program with value addition of the waste streams.

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Chapter 20
Environmental Assessment of Biorefineries

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Abstract The development of the biorefinery concept is based on the techno-economic feasibility of the proposed process considering raw materials, process options, mass and energy integration, market constraints, etc. Nevertheless, along with all these considerations, the environmental assessment must be taken into account at the same time, and concepts like sustainability, land uses, environmental impacts, and other related issues must be included in the biorefinery design since the first moment. This work summarizes the main methodologies and aspects concerning the environmental assessment of biorefineries. Different case studies covering a wide range of raw materials and products are also presented.

Keywords Impacts · LCA · Environment analysis · Sustainability

1 Introduction

Fossil fuels have been considered as one of the most important energy sources employed to supply the worldwide energy demand in industry and transport sectors in the last years (International Energy Agency (IEA) 2016; Singh et al. 2016). Nevertheless, international organizations, such as the United Nations and the World Bank, have identified some environmental issues related to the use of this nonrenewable energy sources (Cherubini 2010). The main issues that have been recognized are global warming, ozone depletion, and environmental detriment of natural resources (Yilmaz and Selim 2013; Triana et al. 2011; Zhang et al. 2010). As a consequence, the application of renewable resources to supply a share of the

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worldwide energy demand and its use to produce some goods currently obtained from oil-derivatives has been a key object of research.

The most studied renewable resource to be applied as an energy source, as well as raw material to produce a lot of compounds, is biomass. Nowadays, biomass plays an important role around the world, since it is the most abundant, available renewable resource. Developing countries located at tropical regions have a great potential to produce a high quantity of biomass because of their biodiversity and variety of weathers. Therefore, these countries will become in the most important biomass suppliers in the future. However, these ones do not have the technology for biomass processing well established yet. As a result, developed countries such as the United States or Germany also will play a very important role in the implementation of biomass at industrial level (Mekhilef et al. 2011).

Biomass can be compared with crude oil based on the range of products that can be obtained through its processing in a facility denominated biorefinery. The biorefinery can be defined as a complex system where biomass is integrally processed or fractionated to obtain more than one product including bioenergy, biofuels, chemicals, and high value-added compounds that only can be extracted from bio-based sources. Biorefineries have been the most attractive option in the last years to be implemented due to their benefits from the techno-economic and environmental point of view (Demirbas 2009; Qureshi et al. 2014).

In last years, a diversity of biorefineries with different feedstocks, technologies, and products has been technically and economically evaluated. On the other hand, several approaches focused in the environmental evaluation of biorefineries have been proposed. The most important approaches used to evaluate and define if a biorefinery is feasible from the environmental point of view are: The potential environmental impact (PEI), greenhouse gases emissions (GHG) balance and life cycle assessment calculations. However, there is an enormous problem associated with the conceptual understanding of the above-mentioned approaches causing frequent confusions and inadequate usages or applications.

In accordance with the above, the aim of this chapter is to introduce the main concepts related to green chemistry, environmental assessment, and sustainability (Pfaltzgraff and Clark 2014; Dicks et al. 2015; Moncada et al. 2016). The basics of the biorefinery concept is also reviewed and the different environmental assessment methodologies are discussed, including a selection of recently reported case studies.

2 Biorefinery Concept and Classification

The use of renewable resources to obtain products comparable to petroleum derivatives, with the aim of generating processes and products that are environmentally friendly and economically and socially sustainable, has been a matter of research for decades (Ghatak 2011). In the same way, the de-escalation of the oil-based economy and its possible replacement by a bio-based economy under the sustainable development approach became a priority for government organizations,

academic institutions, and industry in general (Ghatak 2011). The World Commission on Environment and Development (WCDE) defines sustainable development as "*development that meets the needs of the present without compromising the ability of future generations to meet their own needs*" (Ghatak 2011).

To promote sustainable development, biotechnology emerged as the best option, since products were obtained from a renewable material, such as biomass, generating good economic benefits and a lower environmental impact compared to petroleum products (Daza Serna et al. 2016). In addition, with the generation of new jobs and the use of materials from agroindustry, the purchasing power of rural communities was increased. Although at the beginning, and even today, biotechnological processes have great advantages in comparison with petrochemical processes, it was evidenced that the implementation of biotechnological processes for the production of specific products required a large amount of energy and generated highly polluting residues (Moncada et al. 2016).

Taking into account this problem, some researchers stressed the importance of developing an approach to integral use of the biomass (Cherubini 2010; Moncada et al. 2016; Ghatak 2011). This approach implied that residues were fed as feedstocks in other processes, and that both mass and energy integration was a priority of process design (Cardona et al. 2016a, b). Also, the simultaneous production of multiple products through a variety of technologies should be taken into account (Cardona et al. 2016a, b). All this grouping of ideas laid the foundations for establishing the biorefinery concept. Briefly, biorefineries are a network of facilities and equipments arranged for the transformation of the different components present in biomass to added-value products, such as food, biofuels, energy, biomaterials, and marketable chemicals. Some important definitions to give clarity and precision to what is a biorefinery have been given by different organizations. Among the most important ones, that proposed by the International Energy and Bioenergy Agency (IEA) Task 42, defines biorefinery as "sustainable processing of biomass into a spectrum of marketable products (food, feed, materials, and chemicals) and energy (fuels, power, and heat)" (Ghatak 2011). However, some authors understand that this definition is not precise enough, so they have made their own modifications. One of the most cited biorefinery definitions is given by Cherubini (2010), who defines a biorefinery as "able to separate biomass resources (wood, grasses, corn…) into their building blocks (carbohydrates, proteins, triglycerides…) which can be converted to added-value products, biofuels and chemicals. A biorefinery is a facility (or network of facilities) that integrates biomass conversion processes and equipment to produce transportation biofuels, power, and chemicals from biomass."

The concept of the biorefinery is analogous to the oil refinery (Cherubini 2010; Moncada et al. 2016). However, oil refineries and biorefineries have large differences. The first one lies in the raw material, since oil is product of the degradation of millions of years, while biorefineries use living or recently living material; also, the biomass is found in a diversity of places and the quantity and composition of this material depend on the geographic location and climatic factors (Moncada et al. 2016). Biorefineries can be designed at different scales, while refineries in most cases are of high scale (Cherubini 2010). On the other hand, biorefineries integrate a

great diversity of technologies, ranging from emerging technologies to mature technologies, which increases the complexity of the design and the operation of these plants (Moncada et al. 2016). Finally, under the biorefinery concept, products that are not possible from petroleum, such as food, can be obtained (Cardona et al. 2016a, b). Thus, it is possible to propose multiple configurations for one biorefinery, starting from the same raw material.

Biorefineries can be classified according to feedstock fed, the scale and type of products to be obtained (Moncada et al. 2016). For a biorefinery to be sustainable over time, the raw material supply must be reliable and of high quality (Ghatak 2011). Biorefineries according to the raw materials are subdivided based on the source nature, in first, second, third, and fourth generation. First-generation biorefineries are these that use edible crops as raw materials (Moncada et al. 2013). The second-generation feedstocks are those that are obtained as a residue from agro-industrial processes and that can potentially be transformed into added-value products (Mathioudakis et al. 2017). Third generation feedstocks are related to algae (Mata et al. 2010). These organisms have been associated with the production of biofuels such as biodiesel, and the isolation of high added-value products as antioxidants (Mata et al. 2010; Trivedi et al. 2015). Finally, the fourth-generation feedstocks are related with inedible crops and carbon dioxide also (Mata et al. 2010). Since these crops do not compete with food security, and crops such as jatropha and karanja can be grown on land with low amounts of nutrients and water, it allows offering alternatives for the use of arid lands (Moncada et al. 2016). Figure 1 shows the classification of biorefineries according to the feedstocks and scales.

Another classification of biorefineries is based on the scale. The scale of a biorefinery depends on the quantity of products to be obtained (Cardona et al. 2016a, b). In other words, a biorefinery dedicated to produce fine chemicals (e.g., PHB, succinic acid, levulinic acid) ought to be of low scale due to these type of compounds are produced under specific reaction conditions using specialized technology. Furthermore, the market price of these components is higher than the

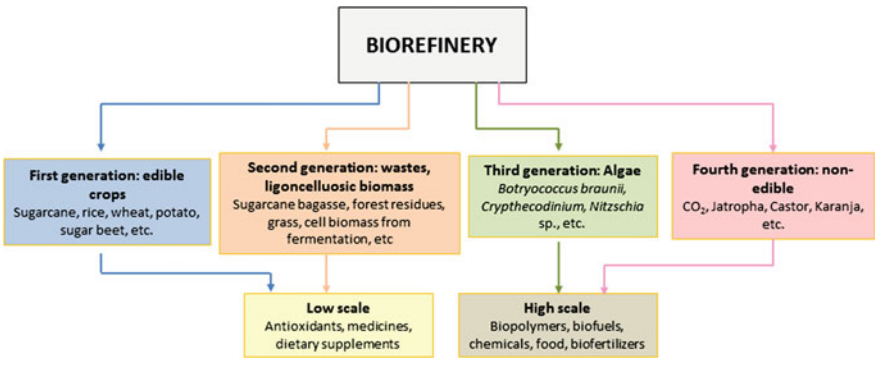

Fig. 1 Biorefinery classification according to the feedstocks and scale

market price of bulk chemicals, such as acetic acid, methanol, and so forth. On the other hand, a biorefinery to produce energy vectors, such as bioethanol, biodiesel, and biomethane must be of high scale due to the market price of these type of products.

3 Methodologies for Environmental Assessment of Biorefineries

3.1 Environmental Assessment

The environmental impact assessment is a procedure to assess the environmental implications given by the use of a certain feedstock to produce some final products. The environmental assessment aims to provide a criterion for deciding, whether it is necessary to change any parameter of the process, with the objective of improving the environmental performance and satisfying the environmental actual policies (Ruiz-Mercado et al. 2012a, b).

The environmental assessment of any process must define some aspects related to the technical analysis such as raw materials flow rate, products flow rate, fuels use, land use, temperature, and so forth. To perform the environmental assessment, a complete understanding of the information obtained from the mass and energy balances of the process is necessary. Once all possible data are collected, the goals of the environmental assessment must be defined in order to define a basis line. Afterward, using the process information, the environmental impacts are predicted through correlations related to objective assessment. Thus, by comparison between the obtained results and the specified environmental goals, it is possible to identify bottlenecks and aspects to be improved in the proposed process (Wathern 1988).

According to the above, the environmental assessment is one of the three main pillars of the processes assessment, together with the technical and economic evaluations. Then, this is an important step that must be performed in order to determine if a chemical process or biorefinery is sustainable. In the next sections, some methodological approaches to perform the environmental assessments are discussed (Dávila et al. 2014; Duque et al. 2015).

3.2 Potential Environmental Impact (PEI)

The environmental assessment of a biorefinery can be performed using the PEI methodology (Young et al. 2000). This approach is related to the impacts of each one of the chemical, biochemical, and thermochemical processes involved in terms of their mass and energy consumptions (Cabezas et al. 1997). In other words, the PEI method is related to the goods consumption.

The PEI can be defined as the effect that a mass or energy release in the environment can have on it. Therefore, this is a quantity that cannot be directly measured (Young et al. 2000). Nevertheless, it is a quantity that can be calculated as the combination of direct or known variables, such as temperature, pressure, feedstock mass flow, and so forth. The PEI calculation can be performed in the same form as the global mass and energy balances of a biorefinery. Then, a potential environmental impact balance can be done taken into account the variable of interest that needs to be evaluated in the environmental analysis. Therefore, the PEI balance, taken into account only material inputs and outputs, is presented as follow (Cabezas et al. 1997; Fermeglia et al. 2007; Chen and Feng 2003):

$$\frac{\mathrm{d}I_{\mathrm{sys}}}{\mathrm{d}t} = I_{\mathrm{in}} - I_{\mathrm{out}} + I_{\mathrm{gen}},$$

where I_{sys}, is the environmental impact content inside the process, I_{in} is the impact rate input, I_{out} is the impact rate output, and I_{gen} is the impact generation rate. However, the accumulative term is not taken into account in the balance for a steady-state analysis. In general terms, this equation can be expanded to include the potential environmental impact related to the energy generation and goods consumption. This expanded equation is presented by Young, Scharp, and Cabezas (Young et al. 2000).

From the above equation, six indexes can be calculated using the mass and energy inputs and outputs streams of a biorefinery. On the other hand, the stream results from the biorefinery must be combined with the overall environmental impact index of each chemical used as a raw material as well as the releases from the process. These indexes are:

(a) Potential environmental impact generated.
(b) Specific potential environmental impact generated.
(c) Process mass inefficiency.
(d) Potential environmental impact released.
(e) Specific potential Environmental impact released.
(f) Pollution Index.

These indexes are calculated taken into account the PEI of the component k in the j stream is calculated using the following equation:

$$I_i = \sum_j I_j^{(\mathrm{i})} = \sum_j M_j^{(\mathrm{i})} \sum_j x_{kj} \varnothing_i + \cdots$$

where M_j is the mass flow rate of the stream j, x_{kj} is the chemical k concentration in the stream j and $\varnothing_i$ is a global coefficient that relates the overall potential environmental impact of the chemical. It is calculated as the weighted sum of the eight environmental impact categories (i.e., Human Toxicity Potential by Ingestion, Human Toxicity Potential by Exposure, Terrestrial Toxicity Potential, Aquatic Toxicity Potential, Global Warming Potential, Ozone Depletion Potential,

Photochemical Oxidation Potential, and Acidification Potential). This weighted sum is performed giving a weight factor to each category according to the user concerns. Finally, the PEI approach to the environmental assessment of chemical processes or other more complex processes such as biorefinery can be estimated using the waste reduction algorithm (WAR) program developed by the US Environmental Protection Agency (EPA) (Young et al. 2000).

3.3 Life Cycle Assessment (LCA)

The approach of to assess environmental impacts throughout the entire life cycle is called a LCA (Reap et al. 2008). This assessment is a methodological tool used to estimate and measure the environmental impact of a product, process or system throughout its life cycle, from the moment the resource is obtained until its recycling or treatment as waste (Reap et al. 2008; Finnveden et al. 2009). According to ISO, "product" is defined as material goods and services. At present, this methodology has been applied to a large number of processes, since it provides a reliable spectrum of the calculation of whole environmental load generated by industrial processes of all type (Finnveden et al. 2009). The assessments with this methodology range from construction of buildings, food products, crops, pharmaceuticals, polymers, fertilizers, chemicals, timber products, meat production from different animals, to the assessment of clothes, shoes, and all kinds of marketable products (Kozderka et al. 2016; Petrillo et al. 2016; Peano et al. 2015; McAuliffe et al. 2016; Hasler et al. 2015; Yay and Suna 2015). The assessment of an industrial process by means of a LCA should be elaborated following a few basic steps, which are: Definition of the goal and scope, the life cycle inventory analysis (LCI), the life cycle impact assessment (LCIA), and the interpretation (Reap et al. 2008). These steps are illustrated below in this document.

3.3.1 Definition of the Goal and Scope

To develop a LCA, the first step is to select the goal and scope of the study (Rebitzer et al. 2004). With the definition of the goal and scope is intended to clarify the limits of the system, as well as the functional unit and the assumptions made to model the system. Also as an important issue, it pretends to establish the target audience for the study and how the results will be communicated (Rebitzer et al. 2004).

In order to recognize the limits of the system, it is necessary to perform a flow diagram with all inputs and outputs of the systems. In addition, it is possible to use the block diagram of each unit operation involved in the raw materials

transformation. However, the above explanation is applicable only in the gate to gate analysis. If the environmental analysis must be performed considering other stages of the life cycle a complete knowledge of all variables is indispensable to perform a more detailed environmental analysis. Figure 2 shows all the possible control volumes that can be proposed to evaluate from the environmental point of a view a process using the LCA methodology.

As for the limits of the system, it can be seen in Fig. 2 that there are four ways of delimiting the system when an environmental assessment is made through a LCA. If it is considered the process of the product inside the plant, regardless of an environmental load of its raw material and its product after it leaves the plant (SimaPro 2013), the LCA is called from "gate to gate". But if, on the contrary, the evaluator decides that the environmental impact that is generated in the production of the raw materials will also be added to the total environmental impact of the product, this type of LCA is called from "cradle to gate". On the other hand, if it is intended to evaluate the entire spectrum of the useful life of the product (i.e., to carry out an environmental assessment from the extraction of resources, through processing and ending with a treatment as waste), this is called a LCA from "cradle to grave". And finally when, between the limits of the system, the product is recycled, entering the life cycle again, this is called an LCA from "cradle to cradle" (SimaPro 2013).

Another important aspect to be considered in defining the goal and scope of an LCA is the functional unit. The functional unit is the measure for the quantification of the environmental impact of a process, product or service (Rebitzer et al. 2004). It allows a level of reference to all the inputs and outputs of the system; in addition, it makes possible that comparisons between LCA studies can be made. The functional unit can be referred to an amount of mass, energy, or in other cases can also be taken with respect to the alternatives of packaging or mode of product delivery (Rebitzer et al. 2004). It is important that the functional unit is properly defined since different functional units can generate different results for the same product

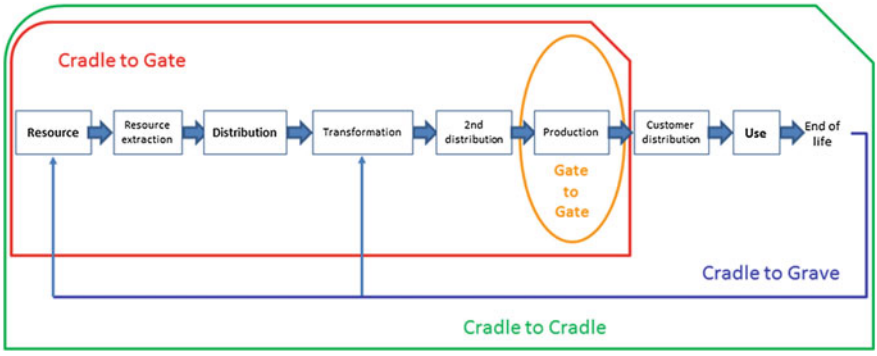

Fig. 2 Possible system delimitations used to perform an environmental analysis using the LCA methodology

system (Reap et al. 2008). After delimiting and defining the functional unit, the next step is to collect the system data. This step is called the LCI analysis.

3.3.2 The Life Cycle Inventory (LCI)

The LCI analysis is the step in which the quantity of raw materials, services required by the system and the products obtained are estimated. In addition, this step also quantifies the amount of waste and emissions generated in the process studied (Iannone et al. 2016). In other words, a quantification of all the inputs and outputs of the system is made. This step is the most laborious and involves greater economic expenses in the study, since the collection of data that are reliable, in many cases can cost a high amount of money (Iannone et al. 2016). In the LCA, two types of data are defined, the foreground and background data (SimaPro 2013).

- Foreground data, refer to specific data that are necessary to acquire to model the system. Typically, they are data describing a particular product system or specialized production system
- Background data, are data used to model the production of generic materials, energy, transport, and waste management. These data can be found in databases and the literature. The distinction between these types of data is unclear and depends on the goal and scope of LCA.

The quality of the data collected depends to a large extent on the depth of the study and the kind of decisions that will be taken with the results of it.

3.3.3 The Life Cycle Impact Assessment (LCIA)

According to ISO 14040, LCA is the step in which understanding and assessing the potential environmental impacts of a system or product is performed, based on the different categories of environmental impact, using the results of the LCI (Finnveden et al. 2009). The LCIA must comply with classification and characterization as mandatory elements, while normalization, weighting, and grouping are optional (Finnveden et al. 2009; Norma Técnica Colombiana 2007a, b).

The classification of environmental impacts is a methodology in which the data obtained in the LCI are assigned to categories of the impact that are appropriate with respect to the goal and scope proposed in the study (Ihobe 2009). It is important to emphasize that, if a substance contributes to different categories of impact, it must be taken into account in each of the categories (SimaPro 2013). There is specialized LCA software and a large number of categories exists (EPA WAR 2017). The categories selected by the researcher are adapted to the conditions of the geographic location, where the impact of the process is to be evaluated. In addition, these ones must be selected according to the information needs (SimaPro 2013; Pennington et al. 2004).

Subsequently, after each substance involved in the LCI is assigned to a category, it must be expressed as equivalent units in which the category is measured. Characterization is the process by which each substance is multiplied by a factor so that the contribution of the substance to the impact category can be aggregated (Ihobe 2009).

3.3.4 Life Cycle Interpretation

Finally, the LCI is the step in which the results obtained in the LCI and LCIA are collected and analyzed to obtain decision elements (Castellani et al. 2017). As a result of the LCI, conclusions should be drawn about the study and the steps in the processing of the product with the greatest environmental impact must be detected. With this interpretation, it is intended that the researcher presents alternatives for environmental improvement of the product or process for which the LCA study was performed (Castellani et al. 2017).

3.3.5 LCA Applied to Biorefineries

Regardless of the type of process to be evaluated, all steps mentioned above must be carried out, unless doing a LCI analysis study is the objective of the study. However, when assessing complex processes or an association of technologies that couples multiple processes, such as biorefineries, we must be careful (Finnveden et al. 2009). Some authors have studied the best way to understand a process, in which multiple inputs of raw material, or multiple outputs of products exist (Finnveden et al. 2009).

The first alternative when having a system with multiple products is to expand the system to each product, so that the environmental load is calculated in relation to each product. However, this alternative is very laborious and can cause the study to become too complex. In this sense, many authors advise that the best option is to divide the environmental impact of products by assigning factors of weight (allocations). This alternative consists in gives weight to the environmental impact of each of the products with respect to its economic cost, or in other cases to physical relations. It is important to mention that when an allocation is designated with respect to a physical or other relationship, it must be supported with clear arguments and must be reflected in the final report of the study (Finnveden et al. 2009).

3.4 Greenhouse Gases (GHG)

Another way to perform the environmental assessment of a biorefinery is through the greenhouse gas analysis. This approach aims at the calculation of the greenhouse gas emissions of the biorefinery. The GHG calculation can be performed

taking into account the emissions generated by the raw materials production and acquisition, the emissions produced from the raw materials transformation as well as the emissions during the transport and distribution of the end-products. Therefore, the greenhouse gas analysis needs to be delimitated because this analysis can be performed in all steps involved in the end-products obtainment (ISCC 2015).

According to the US EPA, the GHG trap heat in the atmosphere generating the so-called global warming. The GHG are: carbon dioxide (CO_2), methane (CH_4), Nitrous oxide (N_2O), and fluorinated gases (e.g., hydrofluorocarbons, perfluorocarbons, sulfur hexafluoride, and so forth). However, another type of chemical releases can be generated during the raw material transformation process and contribute to the global warming. For this reason, the Institution of Chemical Engineers (IChemE) reports the potency factors of different gaseous compounds with the goal to consider them in the greenhouse gas inventory (IChemE 2002).

The greenhouse gas inventory consists on the registration of the gaseous releases to the atmosphere that have a warming burden. This inventory takes into account the greenhouse gas emissions from raw materials production, land use change, production process (including the fuel use to produce utilities), transport and distribution. The emissions produced from the use of any fuel (e.g., natural gas, gasoline, diesel) are evaluated using the emission factors (EF). A special care is necessary when the greenhouse gas inventory is performed for the transformation process because it is necessary to calculate the net greenhouse gas emissions subtracting to the gaseous releases the process savings (i.e. cogeneration systems). Finally, the overall greenhouse gas emissions of a bioenergy supply chain can be calculated as the summation of all GHG sources and savings (ISCC 2015).

An environmental indicator can be calculated from the data used for the greenhouse gas inventory for the transformation process. This one is the global warming potential that is defined by Ruiz-Mercado et al. (2012a, b) as follows:

$$\mathrm{GWP} = \frac{\sum_{i=1}^{I} \dot{m}_i^{\mathrm{out}} \mathrm{PF}_{\mathrm{CO}_2,i}}{\dot{m}_{\mathrm{product}}},$$

where: GWP is the global warming potential indicator, $\dot{m}_i^{\mathrm{out}}$ is the mass flow rate of the gaseous component i (kg/h), $\mathrm{PF}_{\mathrm{CO}_2}$ is the potency factor of the component i for the global warming burden (kg CO_2/kg i) and $\dot{m}_{\mathrm{product}}$ is the mass flow rate of the product.

This indicator is used to compare the environmental impact of different processes using the carbon monoxide as a reference compound and the potency factors (Ruiz-Mercado et al. 2012a, b). Finally, the greenhouse gas emissions inventory and the global warming potential can be used to compare the environmental impact of different processes in terms of the gaseous releases and goods consumption to obtain the end-products.

3.5 *Other Methodologies. Environmental Assessment Through Footprints*

Another way to evaluate the environmental impact of processes that has been widely accepted by the academic community and industry is through a method that groups all the environmental impacts of a process in a single measure. Example of this is the calculation of the carbon footprint (Jianyi et al. 2015). The carbon footprint is a measure of the amount of direct and indirect CO_2 that is related to a specific activity (Jianyi et al. 2015). The carbon footprint comprises both the CO_2 emitted upstream and downstream of the process as well as the CO_2 external to the process. The calculation of the carbon footprint is done by totalizing all greenhouse gas emissions and converting them to equivalent units of CO_2 (Lin et al. 2013).

This concept of carbon footprint is derived from the ecological footprint concept that emerged in 1990. The ecological footprint refers to the total amount of GHG that are released from a process (Fang et al. 2014). Likewise, other methodologies based on the concept of footprint, were developed as a modification to the ecological footprint. Among the most outstanding are: energy footprint, water footprint, emergy footprint, exergy footprint, biodiversity footprint, chemical footprint, the phosphorus footprint, nitrogen footprint, among others (Fang et al. 2014).

However, the method of environmental assessment by means of footprints of different types, have aroused all kinds of suspicions and disagreements between researchers at world level. Many people argue that this mechanism of evaluation and communication of the results of an environmental impact is not adequate, since there are many risks linked to a process being evaluated with a single category of environmental impact (Rugani et al. 2013).

Regarding the environmental assessment of a biorefinery by means of carbon footprint or water footprint, this is an appropriate methodology to give early decision arguments about the possible environmental impact generated by these plants. However, since biorefineries are so complex, it is advisable to use more robust environmental impact quantification methodologies such as LCA.

4 Case Studies

The biorefinery design is supported through the use of simulation software, which contains all the information related to economic parameters to estimate the feasibility of a plant considering indicators, such as the total capital investment, the total capital cost, CAPEX, OPEX, production cost, and so forth. In addition, with the goal to find the best economic scenario for the production of a group of interest compounds, a sensivity analysis, and several comparisons are performed. However, the environmental analysis has been postulated as a fundamental requirement to define the type of process to be implemented. The above, considering the possible effects that the biorefineries could have in our society from a human health point of

view as well as the impact that it could have in natural resources. In this way, more and more works dealing with techno-economic feasibility include the environmental considerations. As an example, Table 1 summarizes recently published articles, where both aspects are considered.

4.1 Comparison of Potential Environmental Impacts Determined in Case Studies

The different possibilities for the estimation of the potential environmental impacts of a new facility make difficult the direct comparison. Nevertheless, there are a number of representative impacts that are usually estimated. The methodology and the database used in each particular case have also an influence on the way environmental impacts derived from biorefineries are presented.

The global warming potential(GWP) is one of the most relevant impacts and it is evaluated in many studies, as shown in Table 2. GWP may be expressed as kg of CO_2 per mass unit of product, per unit of time, or surface of cultivation.

A number of available studies use the WAR algorithm, which considers eight environment impacts or categories: human toxicity by ingestion (HTPI), human toxicity by dermal exposition or inhalation (HTPE), terrestrial toxicity potential (TTP), aquatic toxicity potential (ATP), global warming potential (GWP), ozone depletion potential (ODP), photochemical oxidation potential (PCOP), and acidification potential (AP).

The values of the different potential environmental impacts depend on the nature and composition of raw materials and these of the streams are removed the biorefinery as well as the use of chemicals for the particular process configuration. For example, effluents as stillage with high organic load, solvent purge, and gas emissions were responsible for photochemical oxidation potential (PCOP) to be the most relevant impact in a biorefinery based on the coffee cut stems and sugarcane bagasse. Meanwhile, chemical reagents involved are the main responsible for Human Toxicity Potential by Ingestion (HTPI) and Terrestrial Toxicity Potential (TTP) (Aristizábal et al. 2015). PCOP turned out to be also the most influential impact in the biorefinery concept based on the spent pulp of Colombian Andes Berry (Dávila et al. 2017) and olive stones (Hernández et al. 2014), whose environmental impact is attributed to the organic waste content of the liquid streams leaving the biorefinery. In the case of glucose syrup production from different agroindustrial Colombian residues, HTPI, HTPE (Human Toxicity Potential by Dermal and Inhalation Exposure), and TTP were the most important impacts; in this case, the presence of lignin-derived compounds and others like furfural and HMF trapped in the gypsum formed, are the main responsible for the impacts. On the other hand, the use of the sulfuric acid contributes to the acidification potential (AP) (Dávila et al. 2014). The lowest total Potential Environmental Impacts correspond to raw materials from which the yields of xylose are reduced, such as

Table 1 Relevant examples of environmental assessment of biorefineries

Raw material	Assessment approach	Scenarios and products considered	References
Corn stover	Comparison of different pretreatment methods and conditioning of hydrolysates from corn stover	Use of ammonia, two-stage treatment, and membrane separations reduce environmental impacts when compared to overliming	Pan et al. (2016)
Coffee cut stems and sugarcane bagasse	Five scenarios were compared based on the raw material, economic, and environmental characteristics	Production of ethanol, octane, nonane, furfural, and hydroxymethylfurfural were compared at different levels, conversion pathways, feedstock distribution, and technologies	Aristizábal et al. (2015)
Beech wood	Four biorefinery concepts based on 400,000 tons/year of beech wood are compared. Economic effectiveness and reduction of potential environmental impacts are used as selection criteria	Polymer-grade ethylene; anhydrous ethanol; organosolv lignin; CO_2; biomethane; hydrolysis lignin; and electricity	Budzinski and Nitzsche (2016)
Colombian agricultural residues	Glucose syrup production was evaluated using sugarcane bagasse, plantain husk, cassava husk, mango peel, rice husk, and corncobs	Production cost and yields were compared with and without energy integration. Solid wastes as furfural and HMF influenced the process environmental development	Dávila et al. (2014)
Switchgrass	Results are compared with a fossil reference system giving the same result	Bioethanol, bioenergy, and biochemical from both switchgrass and fossil resources	Cherubini and Jungmeier (2010)
Olive stones	Economic and environmental aspects are compared	Production of xylitol, furfural, ethanol, and poly-3-hydroxybutyrate (PHB) with and without energy cogeneration	Hernández et al. (2014)
Forest biomass (bark)	Assessment of the production of biofoams to be used as building insulation materials using the LCA methodology (cradle to grave approach)	Formaldehyde-free tannin based biofoams to be used as building insulation materials. Further gasification and combustion to be converted into bioenergy (electricity and thermal energy) at end of their life cycle	González-García et al. (2015)

(continued)

Table 1 (continued)

Raw material	Assessment approach	Scenarios and products considered	References
Vetiver leaves	Comparison of using vetiver biomass or coal as energy source in a biorefinery based on the vetiver leaves	Production of bioethanol with and without joint production of furfural are compared to that of conventional petrol and furfural systems	Raman and Gnansounou (2015)
Palm oil biomass	Environmental consequences of including second-generation bioethanol in existing biodiesel production plant	Energy and greenhouse gas emissions are evaluated for biodiesel and bioethanol coproduction. Higher ethanol yields are required for an effective improvement in environmental terms	Lim and Lee (2011)

sugarcane bagasse, rice straw or corncobs, and consequently, furfural and HMF are produced in low amounts (Dávila et al. 2014).

Other studies have been addressed to the direct comparison of biorefineries and petro-refineries using the environmental impact that the last one can produce as a comparison point. For example, Raman and Gnansounou (Raman and Gnansounou 2015) chose one kilometer as a functional unit and compared the production of ethanol and furfural using fossil energy or vetiver, a native to India perennial grass. The Results show that vetiver is a promising feedstock for production of ethanol to be used as a biofuel, or for the production of furfural. From an environmental point of view, the carbon emission reduction of ethanol produced from vetiver leaves is up to 95% when compared to the emissions if crude oil is used (0.01 vs. 0.22 kg CO_2 eq/km, respectively). Concerning fossil depletion, the use of vetiver is 32.5 and 16% compared to the values obtained from crude oil coal, respectively. In another study, the conventional production of diesel, protein, and succinic acid was compared with the use of microalgae, *Chlorella vulgaris*; in this case, the reduction of GHG emissions was 21 and 27% (without or with succinic acid production) compared to the conventional production system. Moreover, the land requirements were reduced by 95% in the algae system (Gnansounou and Kenthorai Raman 2016). The reduction percentage of GHG emissions for other biorefineries is summarized in Table 2.

The way biorefineries can affect the environment can also be assessed by calculating the nonrenewable energy savings for the same productions considered under the biorefinery scheme. In a study on switchgrass as feedstock for an annual production of 110 thousands tons of ethanol, 1.57 thousands tons of phenol, 179 TJ biomethane, 93 TJ electricity, and 16 TJ heat, it was estimated that 3.6 PJ of nonrenewable energy per year were saved, which means 80% savings (Cherubini and Jungmeier 2010). These authors evaluated also the EROI (energy return on investment) index which turned out to be 3.6, that is, the energy output of this biorefinery is 3.6 times higher than the nonrenewable energy invested.

Table 2 Comparison of global warming potential reported in selected works

Subject of study	GWP values	Unit	Relevant results	References
Scenario 1. Xylitol, ethanol, and capsules production in a biorefinery with cogeneration system	4.28	PEI/kg of leaving product $\times 10^{-2}$	For leaving PEI, the most affected environmental categories were Human Toxicity Potential by Ingestion (HTPI), Terrestrial Toxicity Potential (TTP), and Photochemical Oxidation Potential (PCOP)	Dávila et al. (2017)
Scenario 2. Sc. 1 + energy integration	1.59			
Scenario 3. Xylitol, ethanol, and capsules production in a biorefinery with mass and energy integrations	2.72			
Scenario 4. Sc. 3 + cogeneration system	5.30			
Maize	3119 (315)	kg CO_2 eq/ha (kg CO_2 eq/t dm)	These GWP values (including soil C change) would be 40–66% lower if indirect land use change impacts (ILUC) were not taken into account	Parajuli et al. (2017)
Grass-clover	2728 (354)			
Ryegrass	3588 (410)			
Winter wheat-straw	492 (152)			
Switchgrass-based biorefinery producing bioethanol, phenols, biomethane, electricity, and heat	60.5	kt CO_2 eq/year	Fossil reference system value, 281 kt CO_2/year for 1.45 kt dry biomass per day	Cherubini and Jungmeier (2010)
Activated carbon (AC) production from coconut shells. Four different scenarios:		Person equivalent	Reference value, GWP (100 years): 2.1×10^{-11}. Most relevant impact: Human Toxicity Impact	Arena et al. (2016)
1. Coconut shells for electricity in Indonesia	1.5×10^{-10}			
2. Distillate products released into the atmosphere	2.4×10^{-11}			
3. Coconut shells sent to New Zealand for AC	8.2×10^{-12}			
4. AC production in Indonesia and electric energy	4.3×10^{-11}			

(continued)

Table 2 (continued)

Subject of study	GWP values	Unit	Relevant results	References
Ethanol production from spruce wood chips	59	%	The higher the ethanol yield, the lower the Percentage reduction of GWP for onsite enzyme production environmental impact. Production and use of enzyme are the main contributors to GWP regardless the process configuration	Janssen et al. (2016)
Comparison for onsite enzyme production at 12, 20, and 30% solid concentration	62			
30 different process configurations using different detoxification strategies	65			
Corn stover hydrolysates production. Different pretreatment and conditioning methods:		kg CO_2 eq/t dry biomass	Only membrane separations offer negative net environmental impacts according to the endpoint assessment [eco-system quality (EQ) and human health (HH)]	Pan et al. (2016)
Scenario 1. Dilute acid + overliming	22			
Scenario 2. Dilute acid + ammonia addition	18.9			
Scenario 3. Two-stage pretreatment + ammonia	7.6			
Scenario 4. Dilute acid + membrane separations	6.2			
Vetiver leaves. Two schemes:		%	Percentage reduction of GHG emissions compared to conventional system. Increase of the fertility of soil	Raman and Gnansounou (2015)
Scenario 1. Bioethanol production (C6 and C5 sugars to ethanol and lignin as a solid fuel)	95			
Scenario 2. Biorefinery process of ethanol and furfural production (C5 sugars to furfural, C6 sugars to ethanol, and lignin as a solid fuel)	99			
Chlorella vulgaris		%	Percentage reduction of GHG emissions respect to the conventional system. Land required in conventional system was 95% higher than the algae systems	Gnansounou and Kenthorai Raman (2016)
Scenario 1. Biodiesel and protein production	21			
Scenario 2. Biodiesel, protein, and succinic acid production	27			

(continued)

Table 2 (continued)

Subject of study	GWP values	Unit	Relevant results	References
Willow chips production and transport. Maximal yield, transport 25, 50, and 100 km	70.33 78.77 95.67	kg CO_2 eq/t dm	These GWP values would be 53–73% lower if organic carbon sequestration in soil under the willow plantation was included in the calculations. The greatest impact is Freshwater toxicity	Krzyzaniak et al. (2016)
Citrus waste		kg CO_2 eq/t dry biomass	GHG emissions reduced by 53% (market allocation) and 59% (energy allocation) for ethanol production and use as E85 fuel (large biorefinery)	Pourbafrani et al. (2013)
Scenario 1: large (>200,000 t/y; ethanol, biomethane, limonene, and digestate)	79.9			
Scenario 2: small (50,000 t/y; biomethane, limonene, and digestate)	83.3			
Palm oil biomass		%	Percentage reduction of GHG emissions respect to fossil system. Including bioethanol processing into palm oil LCA decrease the overall GHG savings	Lim and Lee (2011)
Scenario 1: the current palm oil biodiesel process	86			
Scenario 2: Sc. 1 + to convert palm oil fronds and empty fruit bunch (EFB) into bioethanol	52			
Scenario 3: Sc. 2 + bioethanol conversion using shell and fiber	64			
Chlorella vulgaris. 5 Scenarios:		t CO_2 eq/t biodiesel	In GHG emissions of the whole biodiesel production system, the most important stage is the cultivation Integration with another plant for use their co-products (CO2, stillage, and electricity) would improve the environmental feasibility of microalgal biodiesel on large scale	Maranduba et al. (2015)
1: biodiesel (1 t), glycerine (0.05 t) and cake (1.35 t)	5.12			
2: biodiesel (1 t), glycerin (0.05 t), and pyrolysis oil (0.8 t)	5.10			
3: biodiesel (1 t), glycerin (0.05 t), and biogas with 70% CH4 and 30% CO_2 (0.01 t)	4.88			
4: Sc. 2 + integration with an ethanol distillery	2.38			
5: Sc. 3 + integration with an ethanol distillery	1.85			

(continued)

Table 2 (continued)

Subject of study	GWP values	Unit	Relevant results	References
Base case: Conventional sugarcane farming + sugar milling + ethanol production + electricity generation	38	kg CO_2 eq/t dry biomass	Mechanized farming reduce the net GHG emissions respect the burnt in the field	Silalertruksa et al. (2015)
Scenario 1: Mechanized sugarcane farming + sugar milling + ethanol production + electricity generation	32			
Scenario 2: Sc. 1 + 50% cane trash recovered from the field for electricity production	23			
Scenario 3: Sc. 2 + vinasse utilization as fertilizer	23			
Rapeseed and carinata	49	%	Percentage reduction of GHG emissions respect to fossil system. Without energy allocation of co-products, GHG emission saving is lower than 26% for rapeseed or carinata biodiesel production (diesel 83.8 g CO_2 eq/MJ)	D'Avino et al. (2015)
Biodiesel production	50			

4.2 Combined Environmental and Economic Sustainability

To simultaneously take account of both economic and environmental aspects in the design and operation of biorefineries, the eco-efficiency indicator was introduced by the World Business Council for Sustainable Development (WBCSD). This indicator, which is highly dependent on the economic and environmental performance

Table 3 Comparison of combined environmental and economic sustainability indicators reported in selected works

Subject of study	Relevant results and comments	References
Spruce wood chips. 30 different ethanol production process configurations using different detoxification strategies	Impacts show a high sensitivity to ethanol prices, e.g., changing from 600 to 415 €/t ethanol results in 2–30% decrease of impacts, and low sensitivity to changes in methane price (e.g., increasing methane price from 579 to 750/t produced a reduction of impacts of 1.7%)	Janssen et al. (2016)
Citrus waste. Two different biorefinery configurations producing ethanol (large, 200,000 t/y or small, 50,000 t/y), biomethane, limonene, and digestate	Percentage reduction of GHG emissions respect to fossil system: Large: 53 (ethanol as E85); 92 (biomethane for electricity), small: 90 (biomethane for electricity) Allocation of GHG emissions based on the market values compared to system expansion result in higher GHG emissions assigned to the biofuel	Pourbafrani et al. (2013)
Beech wood Scenario 1. Ethylene, organosolv lignin, biomethane, hydrolysis lignin Scenario 2. Same as Sc. 1 + carbon dioxide Scenario 3. Ethanol, organosolv lignin, biomethane, hydrolysis lignin Scenario 4. Same as Sc. 3 + carbon dioxide	Net present value (NPV), M€: −9.12, −5.35, 86.97 and 100.59 for scenarios 1–4 PEI, ReCiPe points/yr: −2.33E + 03, −2.43E + 03, −2.25E + 03 and −2.35E + 03 Biorefinery 4 fulfills both criteria simultaneously, economic effectiveness and reduction of potential environmental impacts	Budzinski and Nitzsche (2016)
Base case: Conventional sugarcane farming + sugar milling + ethanol production + electricity generation Scenario 1: Mechanized sugarcane farming + sugar milling + ethanol production + electricity Scenario 2: Sc. 1 + 50% cane trash recovered from the field for electricity production Scenario 3: Sc. 2 + vinasse utilization as fertilizer	Eco-efficiency, US$/kg CO_2 eq: 1.7, 2.0, 2.8, and 2.8, respectively Compared to base case, the use of cane trash and vinasses show the highest eco-efficiency	Silalertruksa et al. (2015)

in each particular case considered, can be calculated as the ratio of the value of a particular product and its environmental impact (Silalertruksa et al. 2015). For example, for sugarcane biorefineries, the eco-efficiency indicator has been defined as the gross value added (US$) divided by the total GHG emissions (kg CO_2/t). This indicator can be used for comparison of different biorefineries based on the same raw material and in the same geographical location, i.e., the same country. The scenarios considering the use of sugarcane crop residues for power generation and the production of fertilizers from vinasse increased the value of the eco-efficiency indicator by 65%, Table 3 (Silalertruksa et al. 2015).

The economic performance of the biorefinery can be assessed by determining main economic parameters, such as Net Present Value (NPV), the Internal Rate of Return, and the Amortization Period, while the environmental aspect can be considered through the comparison of LCA and the substitution method, i.e., using a reference system for producing the same products with conventional, fossil-based technology (ReCiPe methodology). Following this approach, Budzinski and Nitzsche (Budzinski and Nitzsche 2016) compared four biorefineries based on beech wood, and were able to order the biorefineries according to NPV and the variation of potential environmental impacts compared to reference systems.

Environmental impacts can also be evaluated considering the influence of product price (allocation of impacts), as reported in the case of 30 different ethanol production process configurations using different detoxification strategies, where the impact categories were highly dependent on the price of ethanol (Janssen et al. 2016). The same idea was applied to a biorefinery based on the beech wood, where the allocation of GHG emissions was based on the price market for the main products, i.e., ethanol, biomethane, limonene, and digestate (Pourbafrani et al. 2013).

5 Conclusions

Comparing technological options for biorefinery development is usually done based on the economic criteria, but environmental aspects have gained a key role in addition to economic factors for the selection of the best production scheme. Although different approaches, methodologies, and databases are available, the combined consideration of economic and environmental factors can be used as a right tool for decision-making.

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