Suraja Kumar Nayak ·
Bighneswar Baliyarsingh ·
Ilaria Mannazzu · Ashutosh Singh ·
Bibhuti Bhusan Mishra *Editors*

Advances in Agricultural and Industrial Microbiology

Volume 1: Microbial Diversity and Application in Agroindustry

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Editors
Suraja Kumar Nayak
Department of Biotechnology
Odisha University of Technology and Research
Bhubaneswar, Odisha, India

Bighneswar Baliyarsingh
Department of Biotechnology
Odisha University of Technology and Research
Bhubaneswar, Odisha, India

Ilaria Mannazzu
Department of Agricultural Sciences
University of Sassari
Sassari, Italy

Ashutosh Singh
School of Engineering
University of Guelph
Guelph, ON, Canada

Bibhuti Bhusan Mishra
Department of Microbiology
Odisha University of Agriculture and Technology
Bhubaneswar, Odisha, India

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About the Editors

Suraja Kumar Nayak obtained Ph.D. from Odisha University of Agriculture and Technology in 2013 and is presently working as Assistant Professor, Department of Biotechnology, Odisha University of Technology and Research (formerly College of Engineering and Technology), Bhubaneswar, Odisha, India. Dr. Nayak has 9 years of teaching and research experience in the field of microbiology and biotechnology. His areas of teaching and research include general and environmental microbiology, soil and agricultural microbiology, industrial and food biotechnology, and microbial biotechnology. Dr. Nayak has 20 publications including book chapters in various journals and publishers of national and international repute (h-index: 7 and Citation: 300 in Google Scholar). He has also submitted six accession numbers to NCBI, USA, and has presented papers in various national and international conferences. He has edited three books in microbiology and biotechnology published by national and international publishers. Five M.Tech. and more than 15 B.Tech. students have successfully completed their dissertation under his guidance, and presently two more are actively working.

Bighneswar Baliyarsingh, Ph.D., is presently working as Assistant Professor, Department of Biotechnology, Odisha University of Technology and Research (formerly College of Engineering and Technology), Bhubaneswar, Odisha, India. He obtained one of his postgraduate degrees, M.Sc. in Biotechnology, from Ravenshaw University, Cuttack, India, followed by M.Tech. in Biotechnology from West Bengal University of Technology, Kolkata, India, sponsored by the Department of Biotechnology (DBT), Govt. of India, in the year 2007. He also obtained his Ph.D. degree in Biotechnology from Ravenshaw University, Cuttack, India. He has gained research and industrial work experience by serving in different institutes like ICAR-NRRI (Cuttack), Bose Institute (Kolkata), and AIIMS (New Delhi) with brief industrial exposure through BCIL-BITP, sponsored by DBT, Govt. of India. He qualified CSIR-UGC National Eligible Test (NET) in the year 2007. He has successfully supervised a good number of M.Tech. and B.Tech. dissertation works, continuously. He has published more than 15 articles including research papers, reviews, and book chapters in various journals and publishers of national and international repute (h-index: 4 in Google Scholar).

Ilaria Mannazzu, Ph.D. in Microbial Biotechnology, was promoted to Associate Professor in Microbiology at the University of Sassari, Sassari, Italy, in 2001 and Professor of Microbiology in 2014. She obtained the National Academic Qualification as Full Professor of Microbiology. Her teaching activity mainly involves general microbiology, microbial biotechnology, and food microbiology and includes master and doctoral seminar courses. Her research interests cover the biodiversity, physiology, and genetics of microorganisms of biotechnological relevance, mainly *Saccharomyces cerevisiae* and nonconventional yeasts. Also, her research activity focuses on (i) microbial interactions for the development of natural antimicrobials; (ii) *Saccharomyces cerevisiae* and non-Saccharomyces yeasts in wine and beer; (iii) fermentative stress response in wine yeasts; (iv) biotechnological production of primary and secondary metabolites; and (v) heterologous expression of microbial proteins. She has coauthored 75 publications in international peer-reviewed journals with 4244 citations (h-index 27, www.scopus.com, as of today), 10 book chapters, and more than 100 communications, proceedings, and posters presented at national and international conferences and papers in technical journals. She has been invited as a speaker at many national and international conferences. She is also a reviewer of many international journals, member of the editorial board of Fermentation (MDPI), Microorganisms (MDPI), and World Journal of Microbiology and Biotechnology (Springer), etc. Being a member of the teaching board of the School of Doctorate in Agricultural Sciences of the University of Sassari, she participates in the preparation of the doctoral degree study program in biophysical chemistry of Brno University of Technology, Czechia. She represents the University of Sassari in the General Assembly of MIRRI IT-JRU, and she is a member of Società di Microbiologia Agraria, Alimentare e Ambientale (SIM3A), and the Italian Group of Wine Microbiology.

Ashutosh Singh is presently working as Associate Professor in the School of Engineering at the University of Guelph, Canada. After completing B.Tech. in Biotechnology from Vellore Institute of Technology, India, in 2007, he obtained M.Sc. and Ph.D. degrees in Bioresource Engineering from McGill University, Canada, in 2010 and 2014, respectively. Before joining the University of Guelph, he worked as a Postdoctoral Fellow at Dalhousie University, Canada, from 2014 to 2015 and later joined McGill University as a Research Associate. Dr. Singh's research work involves the development of novel food processing methods and the use of physical, chemical, engineering, bioinformatics, and biotechnological tools to improve the understanding of the nutritional component of food at the molecular level. In recent years, his research group has expanded the research areas to include the development of nondestructive food quality and safety testing techniques using ATR-FTIR and NIR. His research group also works in the area of design, fabrication, and application of microfluidic electrochemical, quartz-crystal microbalance (QCM), and colorimetric biosensors to identify food allergens and toxins. Till date, he and his research group members have published over 50 research articles (Citation: 1004, h-index: 18, and i10-index: 23 (Google Scholar)).

Bibhuti Bhusan Mishra is presently working as President, Odisha Bigyan Academy under Science and Technology Department, Government of Odisha, India. He obtained M.Phil. and Ph.D. degrees in 1983 and 1987, respectively, from Berhampur University, Odisha. He served as ICAR-Emeritus Professor at PG Department of Microbiology, College of Basic Science and Humanities, Odisha University of Agriculture and Technology, Bhubaneswar, Odisha, India, after superannuation in 2018 for 3 years and has been Emeritus Professor at OUAT from 2021. He has more than 40 years of teaching and research experience. Thirteen students have been awarded doctoral degree under his supervision in the field of environmental, soil, and agricultural microbiology from various universities across India, and currently two are actively working. In addition, he has supervised more than 30 PG students. He has more than 75 publications including book chapters and research manuscript in various journals of national and international repute (h-index: 22 and Citation: 1635 in Google Scholar). He is credited with 25 accession numbers submitted to NCBI, USA. He has contributed research articles and book chapters in many more edited books pertaining to environmental, soil, agricultural, and industrial microbiology. He has edited eight books in microbiology and biotechnology published by national and international publishers and one textbook on botany practical. He successfully completed one major project funded by the University Grants Commission (UGC), Govt. of India, and was the Chief Nodal Officer of a project funded by the Rashtriya Krishi Vikas Yojana (RKVY) on "Establishment of Biofertilizer Production Unit," Govt. of Odisha, amounting to Rs. 150 lakhs. He is a recipient of "Best Teacher Award" from the University in 2012, from the College of Basic Science and Humanities in 2015, and from Odisha Botanical Society in 2018. For significant contributions in microbiology, he was conferred with Prof. Harihar Pattnaik Memorial Award by the Orissa Botanical Society in 2016.

Soil Fertility and Sustainable Agriculture

1

Kalaivani K. Nadarajah

Abstract

The productivity and well-being of crops and plants are largely dependent on soil fertility. Soil fertility is determined through the interaction and intercalation of three matters: physical, chemical, and biological. Biological fertility is determined by the organisms that live in the soil and their interaction with other alike components. Their relationship with each other and with plants eventually controls the overall complexity and dynamics of the given ecosystem. However due to the complexity of the microbe–microbe, microbe–organisms, microbe–plant, and the multifactorial relationship between all, biological fertility becomes one of the least understood components. In addition, to soil fertility, soil microorganisms play a very essential role in the nutrient cycles that are fundamental to life on the planet. Fertile soil teems with microbes, where the most dominant microbes in soil are the bacteria, actinobacteria fungi, soil algae, and soil protozoa. A better understanding of soil microbiology is essential if agricultural production is to meet the needs of a growing world population. Therefore, it is important for us to understand how microorganisms may be useful in maintaining soil fertility and to determine its application in sustainable agriculture.

Keywords

Soil fertility · Biology · Growth · Yield · Beneficial microbes

K. K. Nadarajah (✉)
Department of Biological Sciences and Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan, Bangi, Malaysia
e-mail: vani@ukm.edu.my

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1.1 Introduction

Soil fertility is an important component that enables sustainable plant growth and optimized crop yield. In agricultural practices, soil fertility has been maintained through the application of organic and inorganic fertilizers to the soil. Considering the importance of soil fertility in advancing food security, an integrated soil fertility management system is required to maximize crop production with minimal depletion of soil nutrient reserves, and destruction of the soil's physical and chemical properties that may lead to land degradation and soil erosion and loss of soil fertility (Nadarajah 2016a, b).

An efficient and integrated soil fertility management system would aim at optimizing use of nutrients while improving crop yield and productivity. There are many agricultural practices of old and new that may be incorporated to achieve soil fertility and sustainable agriculture. Soil fertility may be restored to soil through biological, chemical, and physical interventions. Practicing sustainable agriculture and maintenance of soil fertility includes many components. In times past, nature was the natural remedy to soil health, where soil fertility and health was maintained through natural events (Bargaz et al. 2018; Nadarajah 2017).

The current day conventional farming methods have contributed to changes in the biology, chemistry, and physics of soil environment. The chemical inputs have resulted in the deterioration and degradation of soil health and fertility. These changes clearly cause a change to the soil biological composition and subsequently result in the detriment of soil well-being. In farming, certain biological processes positively and negatively affect plant growth. While it is possible to provide the same beneficial effects as soil biological processes through chemical inputs, this approach has a detrimental effect to the environment and does not provide an avenue for soil biological activities to feed into soil nutrition (Havlin and Heiniger 2020).

In studying these soil biological processes, interconnectivity was observed in some while others remain distinct and separated. Since it has been reported that each teaspoon of soil has millions of microorganisms, therefore it is insufficient to analyze the status of soil health and fertility without discussing the contribution of the biological components and more specifically the contribution of microorganisms in soil fertility (Kibblewhite et al. 2008; Baliyarsingh et al. 2017). It is anticipated that when microbial diversity is altered, it will result in processes being altered positively, negatively, or not at all. Proper utilization of the knowledge derived will help in decision-making and achieving sustainable agriculture. The biological characteristics of the soil are affected by soil type, nutrient management systems, agricultural practices, and the surrounding vegetation (Zachary et al. 2020).

Sustainable agriculture will depend on the balance between natural replenishing of soil nutrient content by the biological components of the soil through nutrient cycling and the input provided either chemically or organically. It is however observed and recommended that these soil nutrient enhancers such as fertilizers should be organic and less of chemically derived fertilizers as these negatively impact the biological components in the soil. The microbial populations have a wide and varied contribution from improving growth, to induction of defense, and

protection against diseases. Therefore, optimization of agricultural practices needs to be studied further to derive mechanisms by which soil fertility may be maintained with limited erosion of soil health (Tahat et al. 2020).

Some sustainable agricultural practitioners have also recommended that for soil fertility we need to copy nature in returning fertility where practices such as composting, remineralization, and organic fertilizers may be among the ways by which soil may be enriched and at the same time the fertility of the soil is improved. The following sections in this chapter will look into the utilization of agricultural practices and the ways by which soil microbial community may enrich soil and contribute significantly to soil fertility and sustainable agriculture (Nadarajah 2017; Zachary et al. 2020).

1.1.1 Some Agricultural Practices that Contribute to Soil Fertility

Nature has a way of creating biologically diverse and rich soils naturally. Natural practices that contribute to the soil positively, including composting, humus, grazing, cultivation practices, and organic nutrient input, have been studied by several research groups. It would be beneficial to note on natural factors and means that contribute to richly fertile soils and ecosystems with little by way of inputs and human intervention (Soumare et al. 2020; Tahat et al. 2020).

Nature has a way of returning nutrients to the soil through cycles. Carbon is returned to the land from feeding and waste production and also through the reduction of grasslands to carbon from forest fires and other human activities. These carbon compounds are then utilized by the soil microorganisms in their biological functions in the soil. In addition, composting is a process that aids with the decaying of organic substances that returns organic material into simple carbohydrates and amino acids that may be utilized by trees, grasses, and crops (Gougoulias et al. 2014; Huera-Lucero et al. 2020).

There is also the contribution of trace elements that have been shown to impact the soil microbiota. Microorganisms increase in number when boron is fed into the soil food web. Humic acid and other humic sources have been utilized as food source or concentrated by actinobacteria and mycorrhizal fungi which are the group of organisms playing a major role in nutrient retention and delivery in soil. Trace elements when combined with other soil compounds will assist in soil bulking and form the soil nutrient network necessary to retain the soil food web (Soumare et al. 2020).

1.2 The Plant Microbe Interaction

Soil is a source of nutrient to plants, and a region of active biological activity through the many macro- and microorganisms that inhabits it (Müller et al. 2016). The soil microorganisms engage in a variety of processes that are parasitic, mutualistic, symbiotic, inhibitive, or neutral (Mendes et al. 2013). Further, nonbiological

activities are also able to impact the soil microbial interactions through submergence, drought, salinity, heat, cold, and other environmental stresses (Jacoby et al. 2017; Meena et al. 2017).

The vastly diverse microbes in the soil congregate into small communities or pockets which constitute a very small percentage of soil mass (Young et al. 2008). These colonies then establish microhabitats that result in colonies and or biofilm which gives rise to soil bulking (Kuzyakov 2009). Within the soil there are microbial hotspots that may be further characterized into four groups which are defined as (i) rhizosphere, a region identified as a location surrounding the root system and is in interaction with the root and is also the location within which most of the root exudates are formed; (ii) detritusphere, this region is actively involved in the process of litter decomposition which results in a rich turnover of organic material in the soil and is a factor that contributes toward soil richness and fertility; (iii) biopores, is a zone in the soil which has deep growing roots, and microfauna, and finally (iv) the soil aggregate surfaces (Kuzyakov and Blagodatskaya 2015; Mohanram and Kumar 2019). These regions collectively contribute to the richness in soil complex such as the availability of moisture, oxygen, and nutrients. Together they affect the microbial population of the soil and their activities (Kuzyakov and Blagodatskaya 2015).

The rich content of labile carbon in the abovementioned regions is a contributing factor toward these areas becoming central to activities such as nutrient cycle, respiration, gas exchanges, and others (Richter et al. 2011). These rhizodeposition found in the region surrounding the root systems is made up of low or high molecular weight compounds including organic acids, sugars, secondary metabolites, vitamins, and polysaccharides (Badri and Vivanco 2009). These rich deposits make up a large portion of synthetically fixed C and N (Jones et al. 2009; Kuzyakov and Blagodatskaya 2015). Researchers have reported that these exudates are responsible for the shaping of the rhizosphere by recruiting the microbial population according to the compounds secreted. The altered soil chemistry as a consequence of these depositions plays a vital role in determining the population. Plant types, soil type, and farming practices have a quantitative and qualitative role to play in the deposition. Plant growth stages and development influence rhizodeposition. Further, the type of soil affects the plants and thence as a consequence affects the plant exudates. The agricultural practices alter the chemical, physical, and also the biological components of the soil. This will impact the soil microbial population, though maybe not directly as a consequence of rhizodeposition (Hartmann et al. 2009; Malusà et al. 2016; Yang and Crowley 2000).

Overall, the above factors place a strong selective pressure on the rhizosphere, which determines the kind of population that is present in any given rhizosphere. However, research has shown that out of the very rich and diverse microbial population in the soil, only lesser than 5% is directly related to plant growth. In natural selection, plants select beneficial organisms that will assist them in stressed conditions (Antoun and Kloepper 2001; Lareen et al. 2016). In addition to the colonization of soil, microbes also colonize roots and plant tissues (phyllosphere and endosphere) (Thapa and Prasanna 2018). These relationships can be mutualistic, symbiotic, or parasitic. Therefore, it is common to be able to isolate microorganisms

on or in the plant tissues. The entire microbial genome of the community referred to as the microbiome has a role to play in the natural processes of plants and soil communities, from nutrient uptake, respiration, abiotic tolerance, disease suppression, and metabolic capabilities (Sessitsch and Mitter 2015; Jacoby et al. 2017). Therefore, studying the microbiome is extremely important in understanding the regulation of stress in plants, nutrient uptake, and the growth and development of plants for sustainable agriculture.

1.2.1 Soil Associate Microbes and Plant Growth

Plants in the field are not seen as an individual entity as they live in interaction with soil microbiota. These microbiota have a role to play in the growth and development of the plant either positively or negatively. As reported by other researchers, the rhizosphere is a region that is extremely rich in soil microbiomes, which have been identified and studied through traditional and modern-day metagenome analysis (Mendes et al. 2013). Generally, through the dissection of the soil microbial diversity, important microbial groups that are zeroed in on are the organisms involved in plant nutrient cycling (N, P, S, K), mycorrhizal fungi association, plant growth-promoting rhizobacteria (PGPR), and microorganisms with biocontrol abilities. Through the advent of next-generation sequencing (NGS) platforms, the diversity, density, and profiles of the organisms in a particular soil location may be identified. Hawkes et al. (2007) identified many hundreds of species of microorganisms that have colonized the soil and root systems of different plant species through NGS studies and meta-analysis of libraries. The largest groups of microorganisms found in the soil are from the Proteobacteria group. These microbes assisted with the stress management of plants against both biotic and abiotic (Gopal and Gupta 2016).

In the past decades there has been more focus on studying the ecological impact of soil microorganisms on plant growth promotion. Organisms such as Rhizobium, mycorrhizae, and various genera of bacteria have been implicated in the nodulation process of legumes and have contributed to improved growth and yields. These findings have resulted in some farmers including them (*Pseudomonas*, *Azospirillium*, *Azotobacter*) in pre-treatment of seeds, while others use them in soil amendments to improve the quality of growth and yield. These organisms were then labeled as plant growth-promoting bacteria (PGPB) and have since been targets for growth promotion and yield (Di Benedetto et al. 2017; Nadarajah 2016a; Nadarajah 2017; Mishra and Pahari 2021).

As the science in this field progressed, researchers began to realize that it was no longer sufficient to be focusing on individual microbial strains in addressing their growth and yield improvement quest. Eventually root microbiomes were studied through metagenomics, which has resulted in the identification of microbial taxa with potential for crop growth and yield improvement. Further, in the recent years, focus was concentrated on the assemblage of synthetic communities made up of important taxa that can contribute toward better soil health, yield, growth, and alleviation of stress (Busby et al. 2017). From these studies it is hoped that a better

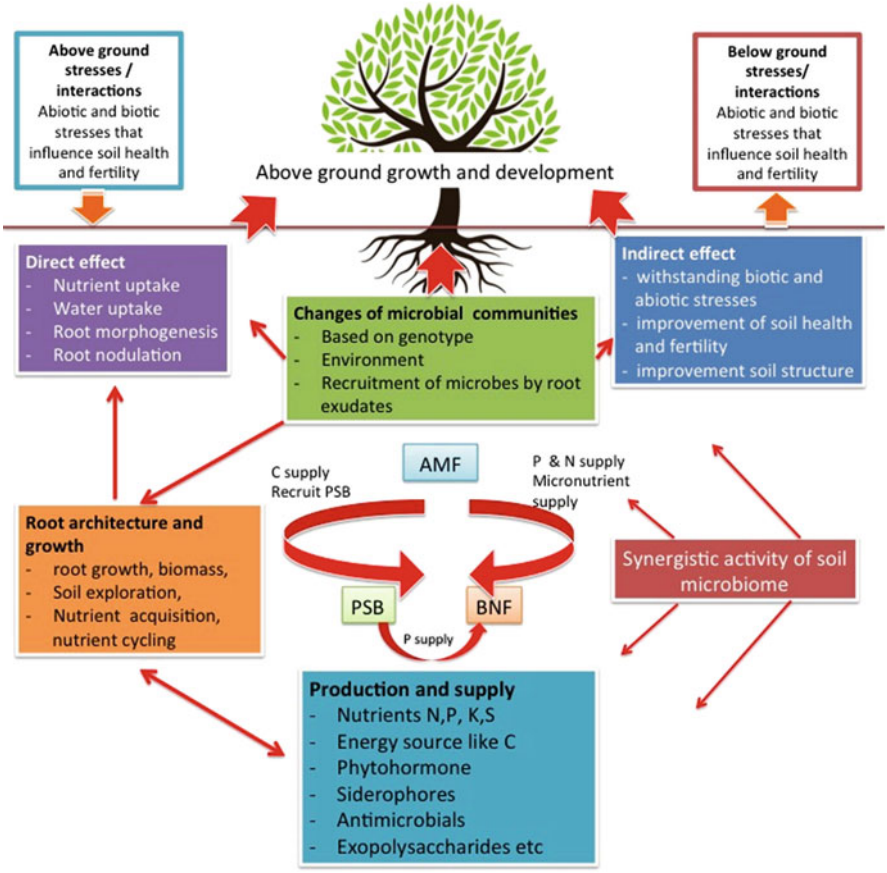

Fig. 1.1 Soil microorganisms involved in enhancing plant growth and development

understanding of the microbe–microbe, microbe–plant, and microbe–environment interaction may be gathered so that better microbial communities may be designed to carry out specific functions in agriculture (Nadarajah 2019a, b). Figure 1.1 shows how microbes play a role in plant growth and development.

1.2.2 Soil Associated Microbes and Nutrient Availability

Many research groups have worked on the mechanisms adopted by microorganism in making nutrients available to plants. From many available literatures and reviews it is confirmed that microbes are central in nutrient cycling in soil. Nutrients and growth-promoting factors are essential components that encourage growth and development of crops. Verbon and Liberman (2016) listed three major mechanisms by which microbes may boost plant growth, which include (1) manipulation in

hormone regulations, (2) removal of competing microbes, and (3) increase in the bioavailability of soil-borne nutrients. These mechanisms are especially useful for plants to access nutrients that require metabolism into easily absorbed forms (Schimel and Bennett, 2004). In natural ecosystems, nutrients such as N, P, and S, which are not easily available to the plant, will require metabolism within the microbial cells. Plants are unable to utilize gaseous N_2 and therefore require a complex four-step cycle that involves N_2 fixation, nitrification, denitrification, and N_2 mineralization. Besides fixing nitrogen, microorganisms also significantly improve the utilization of nitrogenous fertilizers (Gupta et al. 2012). Further, another growth limiting nutrient phosphorus is either available in organic or inorganic form. However, this nutrient binds with other metals and thus is not readily available to the plant. Phosphorus is also of low solubility and is not taken up efficiently by the plant (Nadarajah 2019a). Certain PGPRs such as *Pseudomonas* and *Acinetobacter* were able to also enhance the uptake of Fe, Zn, Mg, Ca, and K in plants (Jacoby et al. 2017). Once metabolized, the ammonium, nitrate, phosphate, sulfate, and various other minerals obtained through metabolism are then ready for plant utilization in supporting growth and development (Bonkowski 2004; Jacoby et al. 2017). These microbial nutrient transformations are important to promote plant growth and become a rate-limiting factor in determining ecosystem productivity. The microbial diversity involved in nutrient acquisition is complex and is made up of many genera.

In understanding the process of plant–microbe interaction and nutrient absorption, several plant microbiome studies have been initiated where observations were made on how organisms such as rhizobiums and mycorrhizae are able to strike up a symbiotic relationship with leguminous plants and other crops. Literature is rich on how rhizobacteria are common and most useful organisms that are involved in the fixation of atmospheric nitrogen in the root systems of leguminous plants. The N_2 gas is fixed into form that can be utilized by the plant (Hunter 2016, Nadarajah 2019b). The mycorrhizae, otherwise known as arbuscular mycorrhizal fungi (AMF), on the other hand increases surface area and releases hydrolytic enzymes from the plant root system that leads to nutrient absorption from the soil. One further benefit of the mycorrhizae is that it also improves soil structure in addition to nutrient translocation by creating stable soil aggregates. While both rhizobacteria and mycorrhizae show similar signaling and cross talk in their processes, these symbionts have distinct mechanism by which they benefit the plant (Begum et al. 2015; Azcón-Aguilar and Barea 2015). The difference between these two systems is important in achieving the desired goal of developing mechanisms that are able to fix nitrogen effectively and provide sustainable nutrition for crops (Geurts et al. 2012). PGPR have also been implicated to play an important role in germination, root growth, yield, nutrient uptake, stress tolerance, and disease resistance (Akhtar and Siddiqui 2010). These organisms are also involved in enhancing P availability and N_2 fixation, iron sequestering, hormone production (gibberellins, cytokinins, and auxins), and in the synthesis of ACC deaminase (Akhtar and Panwar 2011; Gupta et al. 2012; Yadav et al. 2011).

There are other free-living and endophytic organisms that are actively involved in nitrogen fixation. Genus such as *Azotobacter*, *Azospirillum*, *Bradyrhizobium*,

Pseudomonas, *Bacillus*, *Achromobacter,* and *Burkholderia* are some of the important groups that have been associated with N_2 fixation and impact on the crop through increase in growth and development (Di Benedetto et al. 2017; Igiehon and Babalola 2018; Soumare et al. 2020). In addition to making N available to the plants, phosphate-solubilizing bacteria (*Alcaligenes*, *Aerobacter*, *Bacillus*, *Pseudomonas*) and fungi (*Aspergillus*, *Penicillium*, *Fusarium*) are involved in mineralizing P for use by the plant (Kalayu 2019; Sharma et al. 2013). Other than N, and P as mentioned above, rhizosphere microorganisms facilitate the absorption of trace elements. These organisms have siderophores that are able to chelate Fe^{3+} and turn it into soluble Fe^{2+} (Mendes et al. 2013). Some siderophore components are pyoverdine, enterobactin, ferrioxamines, and ferrichromes that are produced by both bacteria and fungi (Elias et al. 2016; Kalayu 2019; Sharma et al. 2013). Pseudomonads have siderophores and function well in nutrient absorption for Graminaceous and dicotyledonous plant species (Shirley et al. 2011). Whiting et al. (2001) reported that zinc mobilization via gluconic acid production was observed in *Pseudomonas*, *Stenotrophomonas*, and *Streptomyces* (Costerousse et al. 2018). Other groups of organisms such *Trichoderma harzianum*, and *Phanerochaete chrysosporium* are linked to processes such as organic matter breakdown and improvement of soil fertility.

From the initial studies using single inoculums, research soon progressed into dual or co-inoculation studies to compare and contrast the contribution to yield and growth. Studies by Wu et al. (2005) on maize showed better results on growth and nutrient uptake when *Glomus* sp. were co-inoculated with a free-living nitrogen fixer, *Azotobacter chroococcum*. Similarly, inoculation with *Pseudomonas fluorescens* ACC50 and *P. fluorescens* ACC73 provided better nutrient uptake for wheat plants (Shaharoona et al. 2008). When PGPRs were used in combination on oil palm and pomegranate, the growth, nutrient uptake, and biomass production were increased (Aseri et al. 2008). It is important to optimize the right inoculum or combination of inoculum for application to specific plants and in specific soil types.

Application of PGPR and AMF was reported to improve phosphate solubilization and mineralization (Tawaraya et al. 2012). Studies have also shown that there are phosphate transporters at the AM hyphae that initiate the process of phosphate transfer from the fungi to plant. In a study by Ruiz-Lozano and Bonfante (2001) involving *Burkholderia* sp. and AMF, P was metabolized by a shunting mechanism that resulted in phosphate being transferred from AMF to the plant. Further, PGPRs such as *Bacillus* sp. and *Pseudomonas* sp. worked in concert with AMF to better utilize nutrients in the soil. These microbes were able to improve N, P, Fe, Ca, and Mn uptake (Amir et al. 2005). These series of experiments provided a basis to hypothesize that there is an elaborate interaction between the bacteria and AMF to enable the plants to better acquire and utilize nutrients (Akhtar and Panwar 2011). In addition to better nutrient utilization PGPRs and AMF also improved the plant's ability to cope in mitigation of biotic and abiotic stresses (Yang et al. 2016).

1.2.3 Soil Associate Microbe and Disease Control

The rhizosphere is also reported to be a zone, where microorganisms can have inhibitory effects on other microbes. These organisms have been a useful source of cell wall degrading enzymes (CWDE) or antibiotics that may result in the inhibition or reduction of other microbes. They also compete with the microbial populations for nutrients and space (Raaijmakers and Mazzola 2012; Caravaca et al. 2015). Moieties like pyrrolnitrin, pyoluteorin, and phenazine-1-carboxylic acid have been reported as antimicrobials (Wackett 2013). Several strains of *Pseudomonas fluorescens* produce 2,4-diacetylphloroglucinol, which suppress soil-borne microbes like *Fusarium* sp. through antimicrobial activity (Meyer et al. 2016). CWDE like chitinase and β-1,3 glucanase are able to degrade fungi and other soil microbes. From several studies on organisms that have shown antimicrobial activities, their arsenal is made up of more than one compound and each component may have a specific function, or identical functions at different levels of inhibition. Broad and narrow spectrum antibiotics and iron chelators play an important role in the inhibition of growth of pathogenic microbes. Chelators through sequestering iron limit growth of pathogens (Raaijmakers et al. 2010). The wilt disease caused by *Fusarium* has been managed efficiently through *Bacillus subtilis* siderophores (Yu et al. 2011). Many fungal strains have been reported with the ability to produce siderophores like *Aspergillus niger*, *Penicillium citrinum*, and *Trichoderma harzianum.* These organisms have been developed into biocontrol agents for diseases in various types of crops (Yadav et al. 2011). Furthermore, in a study conducted to compare the abilities of siderophore production between bacteria, Ferreira et al. (2019) reported that *A. vinelandii*, *B. megaterium,* and *B. subtilis* had the most efficient iron complex formation ability and produced both catechol and hydroxamate as siderophores.

In addition to the production of enzymes, antimicrobials, and siderophores, rhizobacteria produce elicitors that are able to induce systemic resistance in plants against pathogens. A major organism in the soil, *Pseudomonas aeruginosa*, has the ability to induce systemic resistance in plants. This organism also produced pyoverdine and pyochelin as siderophores. Together with these siderophores and salicylic acid these organisms are able to negate the disease-causing effect of *Botrytis cinerea* on bean and tomato, and that of *Colletotrichum lindemuthianum* on bean (Meziane et al. 2005; Yu et al. 2011). Another well-studied organism *Serratia marcescens* 90–166 also produced catechol as a siderophore and is able to reduce disease incidences caused by cucumber mosaic virus, *Colletotrichum orbiculare*, *Erwinia tracheiphila*, *Fusarium oxysporum*, and *Pseudomonas syringae* (Van Loon et al. 1998). The rhizobacteria have been reported to trigger responses in plants through signaling pathways that involve jasmonic, ethylene, or salicylic acid. The activation of these pathways leads to the activation of the defense mechanisms in plants that thereafter triggers the physical or chemical defenses in plants. These changes may involve the fortification of cell walls, callose deposition, lignification and the production of defense enzymes such as phytoalexins, phenolics, phenylalanine ammonia lyase, lipoxygenases, peroxidases, chitinases, PR proteins and various

other stress induced gene products as a means of elicited chemical defenses (Whipps 2001; Heil and Bostock 2002; Yi et al. 2013). Therefore, besides moderating the interactions below ground, these groups of organisms also play an important role in activating the plant's immune response. The complex interaction in the soil is further complicated by the web of pathways and genes that are activated in the solicitation of defense within the plant. Due to this trait, rhizobacteria are a much sought after candidate for development of biocontrols.

1.2.4 Soil Associated Microbes in Mitigating Abiotic Stresses

As mentioned above, the rhizobacteria are an important group in mitigating disease spread and inhibiting pathogens while activating the plant's defense mechanisms. In this portion we look at how rhizobacteria are able to reduce the effect of abiotic stresses in plants. It is believed that as in biotic stress mitigation, the rhizospheric microorganisms have specialized metabolic and genetic capabilities that will assist with the negation of stress (Gopalakrishnan et al. 2015). PGPR such as *Azotobacter*, *Burkholderia*, *Bacillus*, *Pseudomonas*, *Enterobacter*, and *Trichoderma* have been implicated in various managements of abiotic-related stresses (Atieno et al. 2012; Meena et al. 2017; Sorty et al. 2016). One such example is *Burkholderia phytofirmans* PsJN which has been reported to induce tolerance to drought by increasing photosynthesis, and grain yield under water deficit conditions in wheat while providing heat tolerance to tomato plants (Naveed et al. 2014). *Pseudomonas putida* NBR10987 was able to reduce drought stress in chickpea through the production of exopolysaccharides, which resulted in better water holding capabilities (Srivastava et al. 2008).

In addition to heat tolerance afforded by rhizospheric organisms, some strains have also shown tolerance to low temperature via mechanisms that result in quicker response of producing stress-related proteins and metabolites (Theocharis et al. 2012). Some unique rizhospheric organisms with the ability in stress tolerance are such as *Brachybacterium saurashtrense*, *Zhihengliuella* sp., and *Brevibacterium casei* (Jha et al. 2012). *Trichordema* sp. have shown some promise in improving nutrient uptake in plants like rice, wheat, and corn. As reported in multiple studies, Pseudomonas, Microbacterium, Actinobacteria, and fungi such as mycorrhizae are implicated in remediation of soil by affecting the mobility and bioavailability of metals and by so doing improve their uptake into plants (Kawasaki et al. 2012; Yang et al. 2016; Cruz-Hernández et al. 2012). *Trichoderma* were also linked to antioxidant and osmolyte accumulation which enables salinity stress tolerance (Ahmad et al. 2015). The ability of *Pseudomonas* sp. to produce siderophores, exopolysaccharides and antimicrobials show that the exopolysaccharides excreted are components necessary for salt tolerance (Sen and Chandrasekhar 2014). *Bacillus subtilis* GB03 was reported to induce salt tolerance in Arabidopsis through reducing the amount of salt uptake in the tissue (Zhang et al. 2008).

1.3 Future Prospects and Conclusions

The growing world population places great stress on the demand for agricultural produce. A large chunk of agricultural land is operated under conventional agricultural practices, which results in environmental pollution (water, soil, and GHGs), degradation of soil, poor soil health and fertility, and increased production cost. Therefore, it is beneficial to find alternatives, which will alleviate the above detrimental effects in agriculture. To produce high yields while not resulting in an overall negative effect to soil fertility, efficacious microbial strategies need to be applied concurrently with fertilization (preferably organic). It is recommended that to improve crop productivity, the utilization of mineral fertilizers will provide higher eco-efficiency that will meet the needs of the world. Furthermore, to tie in sustainability to agricultural practices, optimized function of rhizospheric organisms in the various biological processes is important to provide enrichment to the soil. From all the available data and the new technologies, it is important to focus on understanding the mechanism of nutrient production and its uptake from soil to plant system. Through the already available knowledge on the roles played by microorganisms in nutrient solubilization, mineralization, and mobilization, new avenues must be addressed to determine more efficacious microbiological resources that will provide the agricultural industry with a profitable integrated plant nutrient agro-system. Isolation, identification, efficiency, co-inoculums, carrier, dosage, and many other factors need constant illumination.

The above continues to be tested and formulated in various laboratories, fueled by the demand of the agricultural industry to find profitable solutions to increasing yield in line with the ever-increasing global food demands. In this chapter we looked briefly at the role of microbiomes in growth, nutrient uptake, disease suppression, and abiotic stress modulation. The diverse microbial groups have a specific niche that they fit into and provide a particular function in soil biology and activity. Besides focusing on the effect of these microbes on yield and growth, it would be most beneficial to identify microbial taxa that may be directly or indirectly involved in abiotic and biotic stress modulation. The mechanism by which these microorganisms achieve their respective roles individually and in synergy with other organisms in the soil is still inadequately understood. Through the utilization of advance technologies and platforms we hope to understand these interactions in depth right down to the cellular and molecular level. With this wealth of information, it would be easier to create designer microbial solutions specific to host and environment.

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Bacterial Community Structure and Function in Acid Soil Ecosystem

2

Madhumita Barooah, Dibya Jyoti Hazarika, and Archana Deka

Abstract

The soil, composed of living and nonliving materials, forms the crust of the earth's surface and is the base of all terrestrial ecosystems. The pH level of the soil determines the fate of several parameters including nutrient availability and the complex trophic interactions influencing microbial diversity, community structure including metabolic activity and functions. In this chapter we discuss the factors leading to the formation of acidic soils and its impact on the mineral availability, vegetation including crop growth and microbial ecology. The use of high-throughput technologies along with traditional methods to study the microbial diversity specifically bacteria along with their community composition and abundance is discussed. We consider experimental evidences to reveal narrow diversity of bacteria and predominant bacterial group in soils affected by low pH. Soil bacteria being drivers of several ecological events including the nutrient and carbon cycling, decomposition of organic matter, and overall soil health, we examine how these microbial functions particularly those related to agricultural aspects are influenced by acidic soil condition. In spite of the importance of soil acidity in regulating microbial community composition and function, our current knowledge needs further elucidations on the mechanisms underpinning several aspects pertaining to microbial ecology in acidic soils. We draw conclusion by discussing the recent advances and future prospects of increasing our understanding on ecosystem processes that may be possible through use of modern tools and development of experimental methods.

M. Barooah (✉) · D. J. Hazarika · A. Deka
Department of Agricultural Biotechnology, Assam Agricultural University, Jorhat, India
e-mail: madhumita.barooah@aau.ac.in

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Acid soil · Bacillus · Metagenomics · Microbial diversity · Next-generation sequencing

2.1 Introduction

The soil is a mixture of nonliving materials including minerals, organic matters, inorganic matters, gases, water, etc. along with living organisms like algae, bacteria, and fungi. These living and nonliving materials together form the crust of the earth's surface and called as the pedosphere. The building block of pedosphere, i.e., soil, is the base of all terrestrial ecosystems in earth which supports all the living communities including plants and animals. Soil characteristics differ based on their chemical, physical, and biological compositions, geographical locations, and altitudes. These characteristics of soil define the vegetation of a particular geographical region. The soil is the primary constituent for fulfillment of all the livelihood practices of human including agricultural practices as such; its quality has major influences on crop productivity. Soil property is determined by the various components that make the soil. Components like mineral particles (clay, silt, and sand), organic matter (dead or living), water, and air contribute to the development of different soil properties. Among these, soil pH which is defined as the negative logarithmic scale (base 10) of the concentration of H^+ ion in soil plays a pivotal role. Soil pH or soil reaction is used to specify the soil acidity (or alkalinity). It is measured from acidic to alkaline scale of pH 1–14, with 7 being considered as neutral. As the soil pH is measured in a logarithmic scale, soil having the pH of 4.0 is 10 times more acidic than that of pH of 5.0, and 100 times more acidic than the soil having pH of 6. Likewise, the acidity of soil with pH 4.0 is 1000 times more than a soil with a pH 7.0. Soil pH is an important trait that determines whether a soil is neutral, acidic, or alkaline as it impacts the solubility and availability of several important compounds, the relative ionic exchange, and the microbial activities in soil (McLean 1983). The pH of soil has often been called as the master variable, since it can show a prevailing effect on numerous physical-, chemical-, and biological-properties and processes (Brady et al. 2010). The soil pH in a particular area may change over time depending on several parameters, such as parent material, climatic changes, and applied agricultural practices.

2.2 Acid Soils

2.2.1 Prevalence and Factors Leading to Soil Acidity

Soil acidity is greatly influenced by soil composition, chemical exchange of the cations, and hydrolysis reactions linked with the various inorganic and organic soil components, as well as by the concentration of CO_2 in soil (Thomas and Hargrove

1984). Based on the intensity of acidity in soil, acid soils have been classified as: slight acidic (pH range 6.1–6.6), medium acidic (pH 5.6–6.0), strong acidic (pH 5.1–5.5), very strong acidic (pH 4.6–5.0), and extremely strong acidic (pH 4.5 or lower). Increase in the amount of hydrogen ion in the soil decreases the soil pH and thus makes the soil more acidic. Soil pH may vary considerably from one spot in a field to another. To determine the average pH of soil from a field, collection of soil should be carried out from several locations and individual samples should be combined to one composite sample.

There are a number of estimates regarding the worldwide distribution of acidic soils. As described by Wambeke (1976), acid soils covers 11% (≈1455 million ha) of the world's land, while according to Haug and Foy (1984) around 30–40% of the world's arable soils are acidic, which covers up to 70% of potentially arable soil. vonUexküll and Mutert (1995) estimated the global vastness of the acid soils with pH <5.5 in their surface layers to be approximately 30% (comprising 3950 million ha) of total ice-free land in the world. A similar estimate was calculated by Eswaran et al. (1997) who reported that around 26% of total ice-free land in the world is constrained for crop production due to soil acidity. Acid-affected soils are found both in the northern belt and the southern hemisphere of the globe. In the northern belt, acid-affected soils occur in cold humid temperate zone covering South Asia, North America, and Russia. While, in the southern belt, regions that have humid tropical climate coupled with high rainfall, such as several areas of South Africa, South America, Australia, and New Zealand, tend to have acidic soil. Soil acidity affects approximately 38% of agricultural land in Southeast Asia, 20% in East Asia, 31% in Latin America, 56% of Sub-Saharan land in Africa, and parts of North America. About 1616 million ha of American land (mostly in South America) is affected by soil acidity. Likewise in Australia and New Zealand, about 239 million ha of land under cultivation is acidic, and China and India holds approximately 212 million ha (12%) of agricultural land which is acidic in nature.

2.3 Factors Leading to Acidification of Soil

Acidification of soil is a natural phenomenon. The nature of the parent material leading to soil formation has a profound impact on the type of soil. Soft rocks such as sand, stone, shale, sultone, and conglomerate are easily weathered and corrodible. High rainfall with hilly terrain creates favorable conditions for very deep weathering of rocks that leads to the heterogeneity in the soil characteristics and promotes acidic soil formation. The presence of sulfate soil also contributes to the soil acidification. The considerable amount of loss of bases from the soil under the influence of high rainfall also aids in turning the soil acidic. In the regions of high rainfall, the soluble basic salts, e.g., those of Ca, K, Mg, and Na, are leached out by drainage water and insoluble acidic residues composed primarily of oxides, as well as silicates of iron, silicon, and aluminum are left leading to the acidification of soil. Additionally, slightly acidic pH of rainwater due to the formation of carbonic acid from CO_2 in the atmosphere also contributes to soil acidity. Large-scale industrial growth and

dependence on the use of coal and crude oil distillates have been increasing concentration of gases like SO_2 in the atmosphere, which leads to acid rain and upon falling into the soil heavily impacts the soil acidity. An important contributor to the acidification of soil is the unbalanced and inefficient use of chemical fertilizers, especially ammonium-based fertilizers. Adoption of intensive agricultural production system with widespread application of fertilizers and soil amendments has resulted in turning the soil more acidic (Kennedy 1992). Ammonium-based fertilizers like ammonium sulfate and ammonium chloride have the greatest effect on soil acidification by generating two H^+ ions for each ammonium molecule nitrified to nitrate. Repeated production and harvest of high-yielding crops play the most substantial role in soil acidification. During growth, these crops rapidly absorb basic elements such as calcium, potassium, and magnesium to meet their nutritional requirements. As crop yields increase, plants require larger amount of the basic nutrients and are thus removed from the field leaving the soil acidic. Moreover, most plant material is slightly alkaline and removal by grazing or harvest deposits the residual hydrogen ions in the soil. Over time, as this process is repeated, the soil becomes acidic. The decay of organic matters in soil produces H^+ ions, which are also responsible for soil acidity. The temperate regions or hilly areas covered with conifers have the tendency to turn acidic due to the release of organic acids as a result of decomposition of leaf litter. Increase in temperature, changes in the rate of precipitation, and rise of the sea level due to climate change also have a role in acceleration of this process.

2.4 Effects of Soil Acidity

2.4.1 Availability of Nutrients

The availability of nutrients to plants is greatly affected by soil pH. In acidic soils, the availability of the major plant nutrients, viz. nitrogen, phosphorous, potassium, sulfur, magnesium, and the trace element molybdenum, is restricted and may be insufficient for plant nutrition. In addition to being chemically less available to plants, the positional availability of nutrients may also be less because of deprived root growth in acidic soils. Due to restricted root growth, plants are unable to spread over sufficient soil volume to compensate for the reduced chemical availability. In such case, higher nutrients than the regular necessary level would be needed for optimal plant growth; however reduced root growth also limits the access to the water present deeper in the subsoil. The availability of copper, iron, zinc, manganese, and aluminum is increased in acidic soils. In many parts of the world, toxic level of aluminum is a problem. Manganese toxicity can also create problem for plants in acidic soil. However, this depends on the concentration which is rarely high enough.

2.4.2 Plant Growth

Soil pH affects plants growth in several ways that ultimately leads to reduction in crop yield. Most plants thrive in neutral to slightly acidic soil, but some crops like rice, cassava, cashew, citrus, mango, pineapple, blueberries, cowpeas, and some of the grasses thrive well in acidic soil condition. Very limited numbers of crop plants can grow well in strong acid soils. Soil pH below 5.5 is usually injurious to plants. Plant roots are severely affected if the pH value exceeds limits of tolerance for particular crops. Acid soils have a major effect on plant productivity due to low availability of nutrients when the soil pH falls below 5.0. In plant communities it has been recorded that richness of plant species is greatly controlled by soil acidity, and it is the highest at relatively high pH levels. Increased soil acidity results in reduced crop yield due to increased concentration of aluminum and iron, deficiency of bases (calcium, potassium and magnesium), reduced availability of phosphorus caused by the high fixation capacity of soil, decrease of biological activities in soil, impairment of nitrogen fixation by legumes, aluminum, iron, and manganese toxicities and deficiency of molybdenum in submerged soils, etc. Metal toxicity negatively impacts growth of plant roots in acidic soils and is the key reason for controlling soil pH. In acidic pH, certain metals including Al, Fe, and Mn are released into the soil solution. These metals, particularly Al, cause damages to the plant roots by interfering with cell wall formation and cell division, and also hamper the uptake, transport, and utilization of Ca, P, and few other nutrients. Aluminum is not actively taken up by plants as it is not a plant nutrient, but when present in the soil solution, it can passively enter plant root system through osmosis. The aluminum stress response is primarily observed in the roots (Taylor 1988; Jayasundara et al. 1997). The roots exposed to Al-stress are stubby and brittle. Root tips and lateral roots become thicker and turn brown. The main symptom of Al toxicity is rapid inhibition of root growth, and such roots are inefficient in uptake of nutrients and water.

2.4.3 Soil Microbial Diversity and Functions

Soil is a heterogeneous and structured system with complex trophic interactions that houses a great diversity of microbial populations (Nannipieri et al. 2003). The diversity and abundance of soil microorganisms is a critical environmental aspect and has been a subject of intense study. Soil microbes have drawn increased attention because apart from the chemical composition, the soil fertility also depends on the qualitative and quantitative nature of the microbial communities inhabiting the soil. The pH of the soil greatly determines the diversity of microorganisms in soil, especially the diversity of bacterial communities (Fierer and Jackson 2006; Nicol et al. 2008; Lauber et al. 2009; Chu et al. 2010; Liu et al. 2014). Beneficial soil microbiota and plants generally prefer a near-neutral pH range of 6.0–7.0. Therefore, increase in soil acidity shifts the microbial community structure and their activities. The study of the composition of fungi and bacteria in agricultural soils (Bardgett et al. 2001) and forest (Bååth and Anderson 2003; Blagodatskaya and Anderson

1998) revealed strong influence of pH on the microbial community with bacterial population and their diversity being impacted the most. Earlier studies on phospholipid-derived fatty acid (PLFA) pattern in soil microbial population indicated more abundance of Gram-positive and fewer Gram-negative bacteria in acidic soil condition (Frostegård et al. 1993). Bacterial abundance and diversity was maximum between pH 4.0 and 8.0. Bacterial community structure was more variable across a change in the pH range compared to the fungal community composition which was only weakly affected. The pH ranges for optimal growth of bacteria are narrow which is why they may be more influenced by pH, while fungi can generally grow over wider pH ranges. In a C-13 incorporation assay, absence of C-13 incorporated 18: 1 omega-7, 16: 1 omega-7, i15: 0, a15: 0, i17: 0 and a17: 0 (the major fatty acid in many soil bacteria) in low pH, and the presence of C-13 incorporated 16: 1 omega-7, 18: 1 omega-7 i15: 0, a15: 0, i17: 0 and a17: 0 in neutral pH indicated the inhibition of soil bacterial growth at low soil pH as compared to pH 7 and 8 (Arao 1999). Among the bacterial community, the shift in their structure from ammonia-oxidizing bacteria to archaea has been reported in soils with low pH and lower NH_3 content (Xu and Gao 2011). Low acidic pH affects the growth and activity of nitrogen fixing bacteria which results in reduced ammonification, nitrification, denitrification, as well as symbiotic and non-symbiotic nitrogen fixation (Robson and Abbott 1989). Increased soil acidity has been reported to significantly reduce nodulation and its functioning including N-fixing capabilities within the roots of legumes crops (Tang and Thomson 1996; Bordeleau and Prévost 1994; Ferguson et al. 2013). Highly acidic soils (pH $<$ 4.0) often show low availability of phosphorus, calcium, and molybdenum and high levels of soluble aluminum and manganese creating toxicity for both symbiotic nitrogen-fixing partners. In soils with pH below 5.0, nodules per soybean plants were reported 40–60% reduction, compared to a soil with a pH value above 6.0 (Lin et al. 2012). In low pH, legume plants having ability to secrete the required signals into the rhizosphere further attract rhizobia and cause delaying in nodulation resulting in reduced plant vigor and crop yield. Since the taxonomic diversity is significantly and positively correlated with functional gene diversity, decrease in taxonomic diversity leads to reduced microbial functional diversity (Fierer et al. 2013). The microbes mediate the decomposition and mineralization process through a variety of microbial enzymes and metabolites. Microbial enzyme production and activity depends upon several factors including pH, temperature, oxygen content, enzyme cofactors, and enzyme inhibitors (Burns et al. 2013). Since most of the enzyme activities have a pH optima that veers around neutral to alkaline range, low pH decreases the microbial enzyme activity and metabolism (Nayak et al. 2012). Microbial decomposition of organic matter is reduced in acid soil condition (Wakelin et al. 2008). Most microbial processes, including the degradation of organic matter and recycling of nutrients, are restricted in acidic soil due to the reduction in growth and reproduction of the soil microbes, primarily bacteria. Microbial mediated activities such as phosphate solubilization, zinc solubilization, siderophore production, etc. are constrained in acidic soil which affect plant growth and development. Soil acidity disturbs the favorable environment for the growth and activity of earthworms and many other soil

organisms, which also alters the diversity of microbiota in acid soil. Under more appropriate pH levels, the activity of detrimental soil microbes can also be increased and may need to be managed.

2.5 Study of Soil Bacterial Diversity

The soil is habitat to a large number of microbes which differs both in taxonomic and functional diversity. A typical soil may contain 1×10^9–10^{10} microbes/g of soil (Torsvik et al. 1990; Gans et al. 2005). The estimated number of microbes in individual soil samples in a global scale would amount 26×10^{28} microbes in terrestrial habitats (Whitman et al. 1998). Despite their abundant existence in nature, several thousands of microbial species remain to be isolated and described. This is largely because we are yet to understand the cultural conditions favoring their growth and reproduction in the laboratory. The methods employed to study the cultivable bacteria vary from those which are uncultivable. Traditional method of studying bacteria is dependent on isolating bacteria on culture medium. Diversity in bacterial communities in soil are usually determined by phenotypic characterization of isolated pure cultures obtained through repeated sub-culturing. Conventional techniques require the knowledge of suitable growth media, optimum growth conditions, and other parameters of microbes (Trevors 1998; Tabacchioni et al. 2000). Another problem with phenotypic characterization is that phenotypic methods can be employed only for those bacteria which can be isolated and cultured. So far only 1.5–10% of the total bacterial population present in soil has been estimated to be isolated through traditional cultivation method. Carbon source utilization profile, BIOLOG and Community Level Physiological Profile (CLPP) are modern variation of the traditional methods and rely on biochemical parameters such as carbohydrate utilization pattern. These methods provide the initial idea of the physiological profile such as the nutritional profile and the nature of the products produced by the organism. The use of signature lipid biomarkers (SLB) like PLFA and the sequence information of their nucleic acids, respectively, also provide information about the bacterial community structure. However methods based on the biochemical analysis sometime fail to give the actual taxonomic identity of bacteria. Despite of the advancement in microbiological culture techniques, it is still not possible to isolate a majority of bacterial species using the standard laboratory culturing methods. So, most bacteria are excluded when phenotypic diversity is estimated. Most of the traditional physiological and biochemical methods for soil microbial diversity analysis have depended on cultivation of the microbes and/or evaluation of their phenotypic characters such as respiration, enzyme activity, and catabolic potential (Bing-Ru et al. 2006). Due to low expression of genes in test conditions, use of biochemical test kits often shows fairly common negative results (Torsvik et al. 1998). The polyphasic system of identifying bacteria is based on the information obtained at phenotypic, genetic, and phylogenetic level. Several phenotypic data including cellular fatty acid composition, cell wall composition, polyamines, etc., together with other expressed characters based on the nucleic

acids (DNA and RNA) viz. sequences of 16S rDNA, %GC content, DNA–DNA relatedness, etc., are taken into consideration for studying taxonomic diversity of cultivable bacteria (Vandamme et al. 1996). Bacteria can also be classified up to genus or species level through different genetic serological typing, fingerprinting techniques, ribotyping, and phage typing (Vandamme et al. 1996). The approaches to study microbial diversity in soils have been broadly categorized as the culture-independent approach and the culture-dependent approach (Kirk et al. 2004).

2.5.1 Culture-Independent Approach for Analysis of Microbial Diversity in Acid Soil

Biochemical profiling of soil microbiota can be carried out to study the microbial diversity in different soil ecosystems. The use of SLB like PLFA has been able to provide quality information about the diversity of soil microbes. Phospholipids are an important structural constituent of all microbial cell membranes. The PLFAs are the building block of the phospholipid molecule and can serve as suitable biomarkers to determine the living microbial types and their richness in the soil. In PLFA analysis, total phospholipids from soil samples are extracted and quantified using gas chromatography (GC) or gas chromatography-mass spectrometry (GC-MS).

The extensive use of PCR and 16S rDNA sequencing has played a crucial role in characterization and identification of bacterial isolates and the discovery of novel bacterial taxa from various sources. In case of bacteria with unusual phenotypic profiles, slow growing bacteria, rare bacteria, uncultivable bacteria, and culture-negative infections, the 16S rDNA sequencing particularly play important role for characterization. The similarity in the 16S ribosomal RNA (16S rRNA) gene has been extensively used to characterize bacterial community compositions in a variety of ecological niches. This approach even includes the study of host associated communities, such as the endogenous human microbiome (Arumugam et al. 2011) and free-living communities, such as those in soil and ocean environments (Polymenakou et al. 2005). The 16S rDNA sequencing is very suitable for identification of unusual bacteria that are challenging to identify by conventional methods, providing identification at the genus level in >90% of cases, and identification at species level in 65–83% of cases (Drancourt et al. 2000; Mignard and Flandrois 2006). The rRNA sequences are mainly used in ranking phylogenetic nomenclature including that of microorganisms (Hwang et al. 2011). A number of studies have compared the effectiveness of 16S rDNA sequencing with conventional or commercial techniques for the identification of diverse groups of clinically important bacteria. In general, 16S rDNA sequencing offers a greater percentage of species identification than the conventional or commercial techniques. Depending on the bacterial group and the criteria used for species definition, species identification by 16S rDNA sequencing has success rates ranged from 62% to 92% (Hall et al. 2003; Bosshard et al. 2003, 2004). The concept of 16S sequencing can be used to categorize the bacteria into new species (Kawanami et al. 2011). The comparison of 16 s rRNA from different strains and different species indicates that there exists high

species-specific homogeneity (Kawanami et al. 2011). Early bacterial community analyses typically used the entire 16S rRNA gene for sequencing, but their capability to sample the full array of bacterial diversity was restricted by depth of sequencing. With the arrival of massively parallel next-generation sequencing technologies, focus has shifted from full 16S rRNA gene sequencing to shorter sub-region sequencing of the gene at great depth (Caporaso et al. 2011). It is observed that the nucleotide sequences of some portions of the 16S ribosomal deoxyribonucleic acid (rDNA) are highly conserved. However, other regions of this gene are hyper variable. The 16S rRNA identifies organisms by comparing certain locations on a 16S rRNA molecule with a database of previously identified organisms whose 16S rRNA mark is known. These sequences also enable the identification of microorganisms because the 16S rRNA contains variable sequences that change according to different species.

The soil metagenome is the sum of the total microbial DNA isolated from a soil sample; it represents the collective DNA of all the native soil microorganisms (Handelsman et al. 1998; Rondon et al. 1999). Phylogenetic analysis is further carried by PCR amplification of one or more biomarker genes, such as 16S rRNA genes (for prokaryotes) and 18S rRNA gene/internal transcribed spacer (ITS) region (for fungi and other lower eukaryotes), to identify the microorganisms present in that soil DNA sample. These analyses allow profiling and comparison of the microbial diversity in various soil habitats and the comparative study of variations in community structure related to altered environmental factors (Lloyd-Jones and Hunter 2001; Dunbar et al. 2002). Other marker genes used to evaluate the microbial diversity are amoA (ammonia monooxygenase), dnaK (HSP-70-type molecular chaperone), etc. (Yap et al. 1996; Webster et al. 2002). Fierer and Jackson (2006) demonstrated a continental-scale diversity of soil bacterial communities and the environmental factors that influences the biodiversity. They reported that bacterial diversity was highest in soils having neutral pH and lower in acidic pH. Microbial diversity in the acidic soil of Brahmaputra River basin (Assam, India) has been studied through metagenomic approach. Amplification of 16S–23S ribosomal DNA inter-genic spacers of bacteria was used for metagenomic analysis which revealed the presence of α-, β-, and γ- subdivisions of Proteobacteria, along with *Acidobacterium* and member of Comamonadaceae (Bhattacharyya et al. 2014). Abundance of bacteria, belonging to the Acidobacteria, Gammaproteobacteria, and Betaproteobacteria, has been reported in acid soils (Lauber et al. 2009; Shen et al. 2013; Yun et al. 2016). Increasing acid rainfall further decreases the microbial abundance and diversity (Wu et al. 2006; Xu et al. 2015; Zhalnina et al. 2015). Cho et al. (2016) also described that the bacterial compositions in acidic soil were different compared to the neutral soil. Proteobacteria (49%) were dominant phyla in acidic soil compared to other ten phyla, whereas four main phyla, viz. Actinobacteria, Bacteroidetes, Proteobacteria, and Cyanobacteria, cumulatively dominated 94% of the soil microbiota in neutral soil (Cho et al. 2016). These findings indicate the narrow diversity of bacteria and predominant bacterial group in soils affected by low pH. The Illumina sequencing of 16S rRNA genes from different sites of karst landscape in central China revealed bacterial community variability to be

significantly correlated with pH. Surface soils were dominated by Acidobacteria, Verrucomicrobia, and Planctomycetes; however, the diversity significantly declined considerably with acidic pH values (Yun et al. 2016). The relative abundance of the dominant phylum Actinobacteria also decreased in lower pH compared to that in higher pH; in contrast, the abundance of Proteobacteria and Acidobacteria increased with decreasing soil pH (Wang et al. 2019).

2.5.2 Culture-Dependent Approach for Analysis of Microbial Diversity in Acid Soil

The culture-dependent approach of microbial diversity analysis involves selective plating and direct viable counts. This approach enables the isolation of culturable microorganisms in enriched media followed by their characterization based on their morphological, biochemical, and molecular information. This approach is suitable for isolation of dominant bacterial species in a particular soil ecosystem followed by in vitro screening and analysis of biological potential of selected microbial strains for application as bioformulation. However, efficiency of this approach for soil microbial diversity analysis is limited to the culturable microbial species. Conventional microbiological characterization has been subjected to debate, as the ability of the bacterial strains to grow under specific environmental parameters decides their chances for characterization. Classical microbiological techniques are indirect and therefore produce artificial alterations in the microbial community structure and as such, most bacteria are excluded when phenotypic diversity is estimated. Most of the traditional physiological and biochemical methods for soil microbial diversity analysis have been dependent on cultivation of the microbes and/or analysis of their phenotypic characters such as respiration, enzyme activity, and catabolic potential (Bing-Ru et al. 2006). A number of studies have reported the microbial diversity in different acid soil ecosystems through culture-dependent approach (Table 2.1). For instance, the genotypic diversity among rice rhizospheric plant growth-promoting (PGP) bacteria in acidic soils of Kerala (pH varying from 6.3 to 6.8) revealed most isolates as belonging to the *Bacillus* genus, including *Bacillus humi*, *B. megaterium*, *B. drentensis*, *B. pocheonensis*, and few others (Yadav et al. 2011). The diversity and their functional properties of bacteria prevalent in acidic tea garden soils (pH 3.8–5.5) studied both by culture-dependent studies and PLFA analysis suggested a high richness of Gram-positive bacteria. Further, 70 acid-tolerant bacterial isolates were characterized using a polyphasic taxonomy approach grouped them as belonging to the genus *Bacillus*, *Lysinibacillus*, *Staphylococcus*, *Alcaligenes*, *Aeromonas*, *Brevundimonas*, *Enterobacter*, *Escherichia* and *Klebsiella* (Goswami et al. 2017). Wilhelm et al. (2011) studied the microbial diversity in an acidic wetland from the Canadian High Arctic with comparison between the active layer and permafrost. They reported that the active layer contained approximately 100-fold more aerobic viable cell counts than those from the permafrost. However, species diversity was for cultured microbes from permafrost, as determined by 16S rRNA gene sequencing. There were substantial differences between the bacterial

Table 2.1 A list of culturable bacterial diversity reported from various acid soil ecosystems

Sl. no.	Bacterial diversity	Source	Location	References
1	*Azospirillum brasilense*, *A. amazonense*, *Bacillus* sp., *Bacillus circulans*, *B. pantothenticus*, *B. megaterium*, *Pseudomonas aeroginosa*, *P. pieketti*, *P. fluorescens*, *Streptomyces anthocysnicus*	Rice rhizosphere (acidic soil)	Different locations of Assam, India	Thakuria et al. (2004)
2	*Mycobacterium* sp.	Aromatic hydrocarbon-contaminated acidic soil	Former manufactured gas plant, Belgium	Uyttebroek et al. (2007)
3	*Bacillus humi*, *B. arbutinivorans*, *B. aestuarii*, *B. drentensis*, *B. megaterium*, *B. pocheonensis*, *B. niacini*, *Brevibacterium casei*	Rice rhizosphere (acidic soil)	Acidic soils of Kottayam and Alappuzha, Kerala, India	Yadav et al. (2011)
4	*Bacillus* sp.,*Pseudomonas* sp.	Acidic soil from the rhizosphere of grapevine	Storm (Fırtına) Valley, Turkey	Karagöz et al. (2012)
5	*Burkholderia seminalis*, *B. thailandensis*, *Sphingomonas pituitosa*	Acid sulfate soil of rice rhizosphere	Semerak, Kelantan, Malaysia	Panhwar et al. (2014)
6	*Arthrobacter* sp., *Achromobacter* sp., *Pseudomonas* sp.	Rhizosphere of grassland	La Araucanía Region, Chile	Campos et al. (2015)
7	Members of *Bacillus*, *Lysinibacillus*, *Staphylococcus*, *Aeromonas*, *Alcaligenes*, *Brevundimonas*, *Enterobacter*, *Escherichia*, *Klebsiella*	Tea garden soil	Different locations of Jorhat, Assam, India	Goswami et al. (2017)
8	*Bacillus amyloliquefaciens* and other *Bacillus* sp.	Acidic soil from open ground	Diphu, Assam, India	Deka et al. (2019)

communities in each layer, especially within Actinobacteria. The 16S rRNA gene sequence comparison between the samples of Arctic permafrost and cold-temperature wetlands revealed Acidobacteria, Actinobacteria (families Intrasporangiaceae and Rubrobacteraceae), and Chloroflexi as commonly occurring taxa (Wilhelm et al. 2011).

2.6 Mechanism of Acid Stress Resistance

To survive in an acidic environment, microbes especially bacteria employ different signaling and/or enzymatic machineries available in their genome. The bacteria that lack these machineries are unable to tolerate the acid stress. There are several effective mechanisms used by those acid-tolerant bacteria to survive under acidic environment. The most usual mechanisms are the GAD system, proline production, biofilm formation, the F_1–F_0–ATPase proton pump, alkali production and protection or repair of macromolecules.

2.6.1 GAD System

The GAD system for acid tolerance is present in a diverse bacterial species including *Shigella flexneri* (Waterman and Small 2003), *Listeria monocytogenes* (Cotter et al. 2005), *Lactobacillus reuteri* (Su et al. 2011), and *Escherichia coli* (Kanjee and Houry 2013). This system involves one/two Gad enzymes (pyridoxal phosphate-dependent Gad: GadA and GadB) and a Glutamate/γ-aminobutyrate (GABA) antiporter (GadC) containing 12 transmembrane segments (Ma and Lu 2012). The Gad enzymes catalyze the conversion of protonated Glutamate to GABA, followed by exportation to the outside of cell in exchange of a new extracellular Glutamate molecule (Fig. 2.1). Glutamate is the prime uncharged ion species at intracellular pH below 4.25, and deamidation of glutamine consumes one intracellular proton (Feehily and Karatzas 2013; Teixeira et al. 2014). This process requires protons, which subsequently increases the intracellular pH, thereby protecting the cell from acid stress.

2.6.2 Proline Production

Proline is considered as a compatible osmolyte that protects macromolecules under osmotic stress. Several reports have demonstrated the biosynthesis of proline during osmotic stress (Saum and Müller 2007; Hoffmann et al. 2012; Hoffmann et al. 2013). Godard et al. (2020) demonstrated the mechanism of proline production during high salt concentrations. Goswami et al. (2018) described the involvement of proline during acid stress management in *Bacillus megaterium* (Fig. 2.1). Transcriptome analysis suggested the upregulation of proline biosynthetic genes under acidic condition. The extracellular and intracellular proline content of *B. megaterium* culture increased during acid stress suggesting its involvement in acid stress management (Goswami et al. 2018). However, actual mechanism of proline in acid stress response is still unclear.

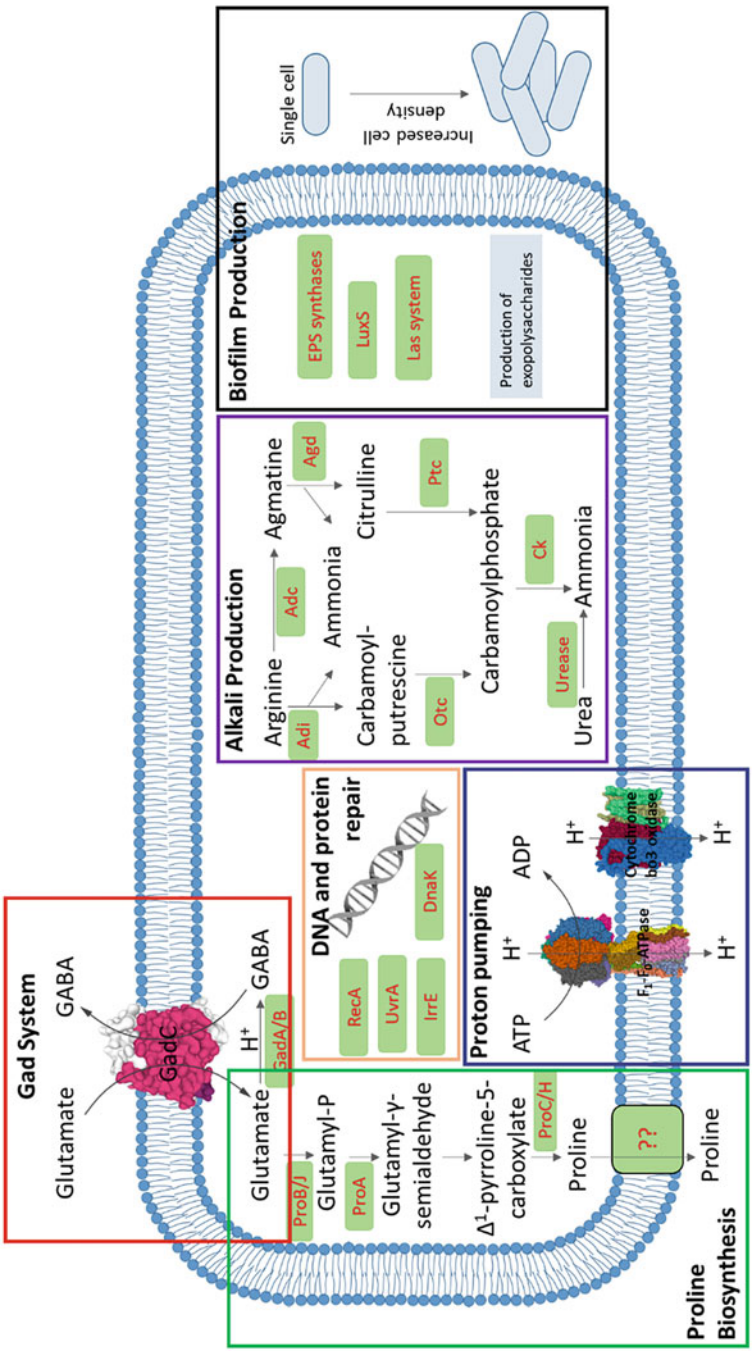

Fig. 2.1 Functional overview of the molecular responses used by acid-tolerant bacteria in response to acid stress

2.6.3 Biofilm Formation

Bacteria that attach to surfaces aggregate in a densely packed hydrated polymerizing matrix of their synthesis to form biofilms. Biofilms are rigid structures formed through cell-to-cell attachments, which offers the cells to tolerate the environmental stresses. Biofilm formation is an important strategy of bacteria to show resistance against antibiotics, as well as to low pH. Formation of biofilm is facilitated by the attachment of bacterial cells to a surface. In a biofilm the cell aggregates to form a compact cellular layer, where the cells in the outer surface of biofilm provide a protective shield to the innermost cells. Thus, the innermost cells remain unaffected from acid stress or antibiotics. Several bacterial species form biofilms under acid stress conditions. Biofilm formation enables *Staphylococcus mutans* cells to resist highly acidic pH (Welin-Neilands and Svensater 2007). Lactic acid bacteria (LAB) such as *Lactobacillus plantarum* are also able to tolerate acetic acid and alcohol through biofilm production (Kubota et al. 2008). Biofilm formation enables soil bacteria to survive under acid soil ecosystems (Fig. 2.1). Bacterial exopolysaccharides, the important components of biofilms, perform several vital functions and thereby provoke acid tolerance response and salt tolerance response. Exopolysaccharides production in *Bacillus amyloliquefaciens* enables the bacterium to tolerate acid stress (Deka et al. 2019). The bacterium alters the exopolysaccharide compositions under acid stress, as arabinose is found as prime sugar monomer of biofilms in acidic pH in place of galactose in neutral pH. The *epsB* gene (encodes tyrosine autokinase involved in exopolysaccharide biosynthesis) inactivation in *B. amyloliquefaciens* resulted in drastic reduction in exopolysaccharide production and thereby became susceptible to acidic pH (Deka et al. 2019). Exopolysaccharides production by soil bacteria have crucial role in soil health and plant growth. Exopolysaccharides of bacterial biofilm help soil aggregation and increase the water-holding capacity of soil under draught stress, salinity stress, and acid stress conditions (Sandhya et al. 2009; Qurashi and Sabri 2012; Deka et al. 2019).

2.6.4 Alkali Production

Production of alkali molecules to neutralize the acids is an important defense strategy employed by some bacterial species. Alkali generating enzymes such as urease and arginine deiminase produce ammonia from urea and arginine, respectively, which further utilize intracellular proton to neutralize intracellular pH (Fig. 2.1) (Burne and Marquis 2000; Griswold et al. 2004). The urease system is widely distributed among soil bacteria that tolerate acidic pH. This system has been reported in *Bacillus* (Mahdavi et al. 2017; Phang et al. 2018), *Staphylococcus* (Moosazadeh et al. 2019), *Enterobacter* (Liu et al. 2012), and many other genera. Some of the neutrophilic autotrophic soil bacteria (e.g., *Nitrosospira* sp.) utilize urease system to tolerate acidic pH (Allison and Prosser 1991). Another system, i.e., arginine deiminase (Adi) or arginine dihydrolase system (Ads), has been reported in

few bacterial species including members of *Lactobacillus* (Arena et al. 1999; Rollan et al. 2003), *Streptococcus,* and *Pseudomonas aeruginosa* (Marquis et al. 1987).

2.6.5 F_1-F_0-ATPase Proton Pump

The F_1-F_0-ATPase is a multi-subunit protein complex containing a hydrophilic enzyme (F_1) and a hydrophobic transmembrane channel (F_0). The F_1 complex is composed of α, β, γ, δ, and ε subunits, and is involved in intracellular ATP hydrolysis or synthesis. The F_0 complex is composed of a, b, and c subunits. It plays significant role in translocation of protons across the membrane. Several species of bacteria use this mechanism to maintain intracellular pH under acid stress condition by translocation of H^+ ions to extracellular matrix (Fig. 2.1). This process is facilitated by hydrolysis of ATP molecule. Expression of the F_1–F_0ATPase is induced by exposure of bacterial cells to acidic pH. Several soil inhabiting bacteria including *Bacillus megaterium*, *B. subtilis*, *B. licheniformis*, *B. flexus*, *Rhizobium leguminosarum*, and many others involve F_1–F_0ATPase system for acid tolerance (Chen et al. 1993; Shobharani and Halami 2014).

2.6.6 Cytochrome Oxidase Activity

Under acid stress conditions, the neutrophilic *Escherichia coli* and other bacteria increase the expression of respiratory chain complexes that facilitates pumping of protons out of the cell. This can be evidenced by upregulation of cytochrome *bd* oxidase and cytochrome *bo3* oxidase in different bacterial species (Chai et al. 2009; Goswami et al. 2018). Together with NAD (P)H-dependent dehydrogenases the cytochrome bd oxidase has been suggested to be involved in an alternative electron transport chain (Chai et al. 2009).

2.6.7 Macromolecule Repair and Protection System

Macromolecular stability is a crucial factor for acid tolerance in bacteria, as low pH tends to disturb the regular structure and functions of macromolecules like membrane transporters and enzymes. Therefore, the stability of membrane protein oxidases can be correlated with acid tolerance of bacteria (Trcek et al. 2006, 2015). DNA repair system containing RecA has been recorded to play significant role in biological processes (Adikesavan and Katsonis 2011). During acid stress RecA is involved in homologous DNA repair and recombination as well as the SOS repair mechanisms (Fig. 2.1). A RecA-mutant of *Helicobacter pylori* was reported to be sensitive to DNA-damaging agents resulting in a decreased survival in acidic environments (Amundsen et al. 2008). Other acid stress-responsive genes include *uvrA*, *iirE*, *dnaK*, *SmnA*, etc. (Liu et al. 2015).

2.7 Next-Generation Sequencing (NGS) Technology for Identification of Acid-Tolerant Genes

Previous approaches to assess the soil bacterial communities mostly relied amplification of 16S rRNA followed by subsequent sequencing and comparing with the existing libraries. This approach allows detection of only certain dominant microbial groups (Chen et al. 2013). Recent advances in NGS methods enable massively parallel analysis of nucleic acid data from PCR amplicons or environmental nucleic acids, and provide a more direct way of detecting microbial taxa, especially those with low-abundance species changes (Oberauner et al. 2013). The whole community structure of Tapovan hot spring soil analyzed through next-generation sequencing revealed the occurrence of different microbial species. A total number of 14 phyla comprised the bacterial community. The dominant phylum belonged to the Firmicutes (63.18%) followed by Proteobacteria (19.99%), Thermobacteria (12.79%), Bacteroidetes (1.83%), and Aquificae (1.51%) (Rawat and Joshi 2019). Transcriptome sequencing provides a descriptive portfolio of gene expression in bacterial cells in response to a particular stress condition (van Vliet 2010). Transcriptome analysis of the acid soil bacterium *Bacillus megaterium* during acid stress indicates that the bacterium uses several mechanisms for surviving in acid stress, including maintenance of cell integrity, GAD-dependent pH homeostasis, and alternative energy generation, as major mechanism of acid stress tolerance. Moreover, upregulation of oxidative stress and osmotic stress related genes at pH 4.5, especially the proline biosynthetic genes, indicated the presence of a connection between acid stress and other stresses (Goswami et al. 2018). Another study of RNA sequencing has differentiated the gene expression patterns of *Salmonella enteritidis* biofilm forming and planktonic cells grown in acidic pH (Jia et al. 2017). Differentially expressed small regulatory RNAs and tRNAs were also reported in *Lactococcus lactis* in response to acid and other environmental stresses (van der Meulen et al. 2017). The NGS data serve as a foundation to characterize stress-responsive genes, which can be further validated through number of techniques, such as quantitative real-time PCR, western blot, and mutagenesis approach.

2.8 Conclusion

Although soil acidification is a natural process, the rate of its acidification has increased over the recent times. Increased anthropogenic activities including industrialization and agricultural activities have accelerated the process which is now a becoming a major global concern for agricultural production system. Low soil pH restricts the bacterial diversity and community structure by limiting their growth reproduction disruption of their functioning. Establishment of potential mechanisms of acid stress tolerance in bacteria has opened up new possibilities towards development of microbial genetic engineering based bio-inoculum. Plant growth promoting neutrophilic bacteria can be redesigned to increase their biological potential under

acidic agro-ecosystems, thus enhancing the agricultural productivity and contributing towards sustainable agriculture.

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Soil Enzymes and Their Role in Soil Health Improvement

3

Rini Rahul, Pallavi Sharma, Ashutosh Singh, Joginder Singh, and Manoj Kumar

Abstract

Soil is the most vital part of terrestrial biota. Since all the related ecological communities thrive on it, the protection, maintenance and improvement of soil is of high importance. The quality of soil to function in a dynamic equilibrium with the thriving biosphere to sustain plant, animal and human life is now being considered as soil health. Assessment of soil quality solely depends on biological, chemical, physical indicators, as all of these contribute in maintaining soil health. The quality of soil can be evaluated directly by reviewing the soil enzymes. Soil enzymes are the fundamental factors of the soil system that are critical for the maintenance of ecosystem functioning and nutrient recycling. They have the capability of efficiently catalysing the decomposition of organic components in the soil system, which helps in maintaining the life processes of soil microorganisms and structural stabilization of soil. Enzymes present in soil are mostly of microbial origin, but can also be of plant or animal origin. These enzymes can be extracellular or intracellular depending on their location, and depending on their origin of derivation, they can be intracellular, free enzymes or

R. Rahul · M. Kumar (✉)
Department of Life Sciences, Central University of Jharkhand, Ranchi, India
e-mail: manoj@cuj.ac.in

P. Sharma
School of Environment Science and Sustainable Development, Central University of Gujarat, Gandhinagar, India

A. Singh
School of Engineering, University of Guelph, Guelph, ON, Canada

J. Singh (✉)
Department of Microbiology, Lovely Professional University Punjab, Phagwara, India
e-mail: joginder.15005@lpu.co.in

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cell-associated. There are vast number of enzymes that have great efficiency in soil improvement and maintenance of soil health. This chapter provides a detailed review of all the major soil enzymes and their activities in maintaining the health of soil and their prospective role in soil improvement.

Keywords

Biological indicators · Nutrient recycling · Soil enzymes · Soil health · Soil quality

3.1 Introduction

American Soil Science Society elaborates soil as a blend of organic substances, minerals, liquids, gases, and a variety of organisms with life supporting attributes (Karaca et al. 2011). Soil health, a soil quality indicator, is considered as the evaluation of a set of factors affecting the functionality of soil in terms of productivity of plants and animals, environmental quality, and the ability of the environment to sustain life (Karaca et al. 2011). The concept of soil health, initially, was restricted to soil characteristics, their analysis and evaluation of soil conditions (Doran and Safley 1997), but in due course of time, it has now become clear that analysing properties of soil alone is insufficient to assess soil quality (Bastida et al. 2008). As a result of the on-going development in science and technology, the management of soil and interactions with different ecological systems, the term "soil quality" has been modified in numerous formats (Rao et al. 2017). Today's standard framework for assessing soil quality depends on a set of facts, such as processes, soil functions, indicators, methodology and attributes. Soil function can be described as the characteristic aspects of a soil usage at any stage in this stepwise evaluation, and soil processes can be described as the answer to the query of "what the soil does". The soil qualities that affect the soil's ability to perform different functions are referred to as soil quality attributes. The characteristics and indications of the soil's physical and chemical processes are frequently thought to be sufficient for evaluating man-made changes in the health of soil; conversely, the constantly evolving soil properties are also few inferences that can be generated (such as nutrient balance, organic matter pool, and soil composition), due to the fact that a number of changes in soil physical and chemical conditions occur over time. A soil quality assessment is used for examining the huge changes in soil physicochemical parameters and can be operated based on these characteristics (Gil-Sotres et al. 2005). Contrary to popular opinion, the biological–biochemical characteristics of soil have been shown to be more vulnerable to slight changes in land use and soil management practices (Sena et al. 2002; Karaca et al. 2011). There have been numerous biological indicators proposed, each with its own set of benefits and drawbacks. The well-reported biological indicators so far are substrate and basal-induced respiration, microbial biomass, enzyme activity, mineralizable nitrogen, abundance of microflora abundance (actinobacteria, fungi, bacteria, algae), soil fauna (microfauna, macrofauna, mesofauna), soil (functional and structural) biodiversity, root disease, food web

structure, plant biodiversity and plant growth (Bruggen and Semenov 2000; Alkorta et al. 2003). Soil microorganisms are one of the indigenous biological components of soil that play a vital part role in biochemical processes, including soil biological activities, energy transfer, sulphur, carbon, phosphorus and nitrogen cycles (Baliyarsingh et al. 2017; Karaca et al. 2011). The enzymes, which play a significant part in defining soil health and the environment, are one of the essential components of the soil biological indicators and can thus aid in improving soil quality. Soil enzymes are mostly microbial, but they can also come from plants or animals (Calderon et al. 2000). These enzymes can be extracellular or intracellular depending on their location, and they can be free or cell-associated enzymes based on their source of origin. A certain balance of biological, physical, and chemical components maintains soil health. As a result, all of these components must be measured in order to estimate soil quality (Das and Varma 2010). They are sensitive to natural or anthropogenic stimuli because of their property to measure all kinds of soil nutrient cycling along with a plethora of crucial microbial reactions and they can easily measure. Enzyme activities of soil have been reported as sensitive indicators of soil ecological quality and as a useful tool of soil biochemical quality. Furthermore, assays for a large range of soil enzymes activity have been well documented; they are the recommended method for assessing soil health (Rao et al. 2017). This chapter gives a thorough sight on the role of soil enzymes in defining soil health, soil biochemical processes, and soil quality improvement, as well as a deeper understanding on soil activity determinants and tools and procedures for measuring enzymatic activity.

3.2 Soil Enzymes

They are a class of enzymes found in the soil that help to maintain chemical and physical properties, soil ecology, fertility, and overall health of soil. The mineralization of organic materials biochemically in the soil system is guided by soil enzymes (Sinsabaugh et al. 1991). All soil types have a class of enzymes that control the biochemical reactions (Adetunji et al. 2017) of the soil, which are influenced by its biochemical, chemical, physical, and microbiological properties. The quantity of enzymes present in soil varies due to the presence of distinct amount of composition, organic matter, and microbial activities, in addition to a variable intensity of biological functions. Soil enzymes can either be constitutive, those that are always released and found in cells, regardless of whether or not there is addition and/or presence of any particular enzyme, or can be inducible, the ones that are not always available in the soil, but promptly created and secreted by cells with any substrate addition (Fig. 3.1) (Bakshi and Varma 2010). Taking into account the actual situation, biochemical reactions are generally catalysed by enzymes and a myriad of substrates that contribute as sources of energy for microorganisms (Fuhrmann 2021).

In response to such a condition, the classification of enzymes is based on the nature and type of reaction catalysed by them. According to this, there are six different types of enzymes:

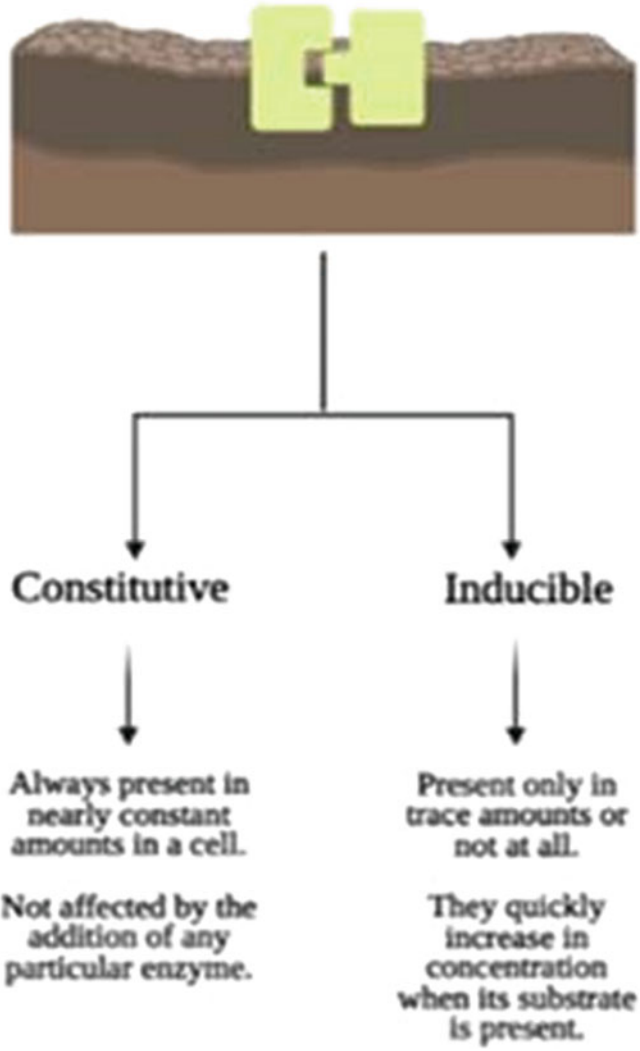

Fig. 3.1 Describing the two different kinds of soil enzymes and their properties

- Oxidoreductases are those that catalyse the process of oxidation and reduction (e.g. Dehydrogenase, Catalase and Peroxidase).
- Transferases are those enzymes that transfer atoms between donor and acceptor molecules (e.g. Aminotransferases, Rhodonese).
- Hydrolases are enzymes that cleave bonds in a hydrolytic manner (e.g. Phosphatase, Cellulase and Urease).
- Lyases, other than hydrolysis and oxidation, can cleave bonds (e.g. Aldolase).
- Isomerases play a role in the isomerization process.

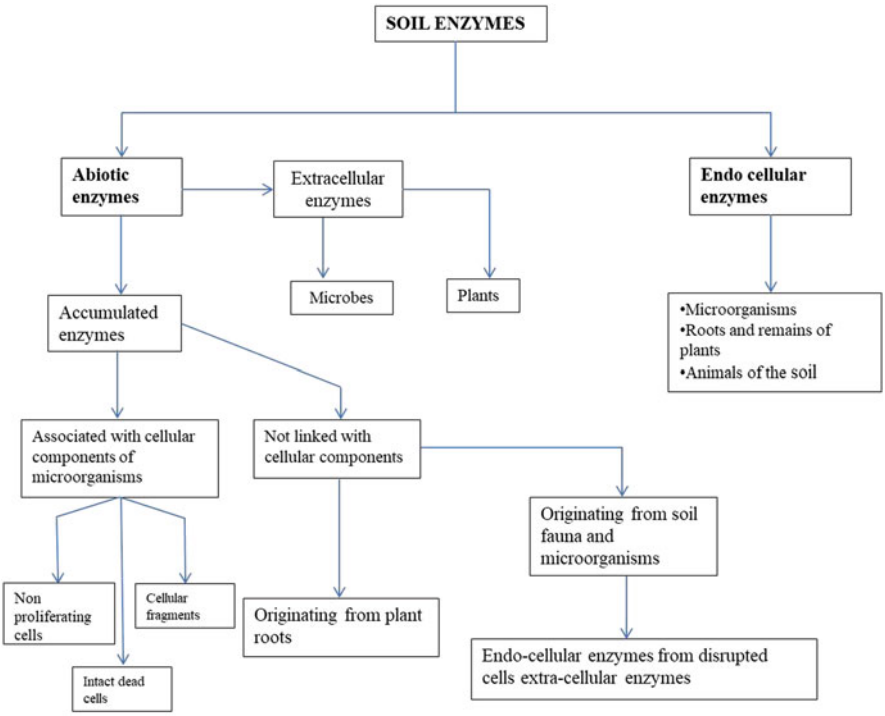

Fig. 3.2 Sources of enzymes in soils (Adapted from Karaca et al. 2011)

- Ligases are enzymes that cleave ATP to create bonds (for example, acetyl-CoA carboxylase).

They usually belong to class hydrolases and the remaining belongs to other classes such as lyases, transferases, and oxidoreductases (Dick and Tabatabai 1987). Microbes (both alive and dead) serve as the most common source for the origin of enzymes, but they can also come from plant roots and wastes as well as soil fauna (Fig. 3.2) (Bandick and Dick 1999; Das and Varma 2010). Different kinds of enzymes are manufactured from soils (Gupta et al. 1993; Ganeshamurthy et al. 1995), organic substances, microbial populations (James et al. 1991; Richmond 1991), plants and animals. These consist of arylsulphatases, amylase, β-glucosidase, chitinase, cellulose, phosphatase, dehydrogenase, urease and protease (Das and Varma 2010). The recycling of organic components in soil system is facilitated biochemically by the soil enzymes (Rao et al. 2017). They are vital for the nutrients release into soil through breakdown of organic matter, for identification of soil, for identification of microbial activities, and as strong monitors of environmental change (Das and Varma 2010). The most common applications of soil enzymes include determining the successional stage of an ecosystem, associating

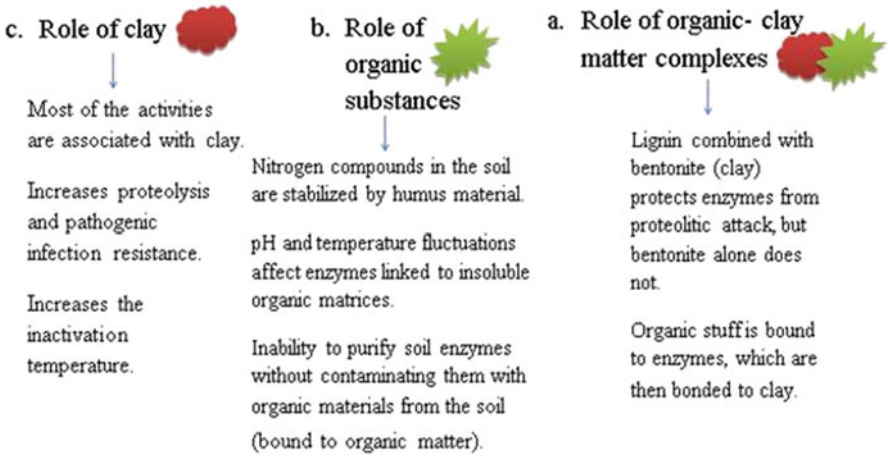

Fig. 3.3 Showing the different factors affecting the state of enzyme in soil

with soil quality, microbial biomass, and biochemical cycling of carbon, nitrogen and sulphur in soil assessing the degree of environmental pollution.

The existence of enzymes in soil is best described by a phenomenon known as state of enzymes (Dick and Tabatabai 1987; Karaca et al. 2011). When an enzyme comes in a contact with soil system, they are present in different states in soil system. These states include: microencapsulation, adsorption, copolymer formation, cross-linking, entrapment, adsorption, covalent attachment and ion-exchange. These mechanisms are considered as the protective effect of soil on extracellular enzyme activity (Fig. 3.3). Clay minerals adsorb enzymes, which acts as a protective barrier against pathogen attack (Fomina and Skorochod 2020). They form complexation with humic colloids and stabilized on organic matter as well as on clay surfaces (Boyd and Mortland 1990). They are highly stabilized by organic components rather than inorganic matters (Dick and Tabatabai 1992). The enzyme activity is inversely proportional to soil organic matter concentration (Yarwood 2018). Furthermore, free enzymes can interact and form complexes with humic substances, which are highly resistant to enzymatic and heat destruction rather the free enzymes only (Serban and Nissenbaum 1986; Karaca et al. 2011).

3.3 Functions of Soil Enzymes

The following is a list of soil enzymes and their involvement in maintaining health of soil in Table 3.1. According to Rao et al. (2017) enzyme activities are used to calculate rates of reaction for soil processes and fertility, activity of microbes, and pollutant inhibition.

Table 3.1 Major soil enzymes and their functions (Adapted from Rao et al. 2017)

Enzyme	Source	Potential indicators	Catalytic activities	Output
α-Amylase	Microbes, animal and plants	C cycling	Hydrolysis of internal α-1,4-glycosidic linkages starch	Glucose, maltose, and maltotriose units.
β-Amylase	Mainly plants	C cycling	Hydrolysis of α-1,4-glycosidic linkages starch	Maltose and/or Glucose
β-Glucosidase	Plants, animals, fungi, bacteria, and yeasts	C cycling	Hydrolysis and biodegradation of β-1,4 linkage of various β-glucosides that are present in plant debris	Glucose
Arylsulfatase	Microorganisms, plants, and animals	S cycling	Sulphate esters hydrolysis	Sulphate (SO_4^{-2})
Cellulase	Microbes	C cycling	Decomposition of cellulose polysaccharide	Glucose
Chitinase	Plants and microorganisms	C and N cycling	Degradation and hydrolysis of chitin polymer chain	Inorganic nitrogen and carbohydrates
Dehydrogenase	Microorganisms	C-cycling, microbial oxidative activity	Organic compounds oxidation	Conversion of H to NAD (nicotinamide adenine dinucleotide) or NADP (nicotinamide adenine dinucleotide phosphate)
Phenol oxidase	Plants and microorganisms	C cycling	Lignin hydrolysis	C compounds (humic substances)
Phosphatase	Bacteria, plants and fungi	P cycling	Hydrolysis of anhydrides of phosphoric acid and esters	Phosphate (PO_4)
Protease	Microorganisms and plants	N cycling	N mineralization	Amino acids and peptides
Urease	Microorganisms, invertebrates and plants	N cycling	Hydrolysis of urea	CO_2 and NH_3

3.4 Factors Affecting Soil Enzyme Activities

The absence and presence of an activator and an inhibitor, temperature, pH and ionic state are the determining factors of the soil enzymatic activities (Xian et al. 2015). Every enzyme has a pH range where it works best and lowers its activity above or below this range. Temperature has a distinct effect on enzyme activity than chemical processes. The rates of chemical reaction are folded with increase in temperature (10 °C), but reaction rates of enzyme are increased till optimal pH is reached, then lowered (Tabatabai 1994). Enzymes perform a specific function and excess temperature of about >50 °C causes the enzyme denaturation (Mathews and van Holde 1995; Biederbeck et al. 2005). Enzyme activity declines when an inhibitor is present, but rises when an activator is present (Table 3.2).

3.5 Soil Health

Soil health refers to a soil's ability to support plant, animal and human productivity and diversity, as well as maintains or improves quality of water and air (Acton and Gregorich 1995). The essential premise behind the phrase "soil health" is that soil is more than just a growth medium; it is a biological, evolving, and ever-changing ecosystem (Pankhurst et al. 1997). Using the human health model, a healthy soil can be characterized as the one which contains composite well-being in terms of physical, biological, and chemical qualities, that is not ill or infirmed (i.e. neither deteriorated nor decomposing), with each of those attributes of working together to ensure that the soil realizes its maximum potential and resists deterioration while performing a diverse set of tasks (particularly water, carbon, and nutrient cycling), and that it maintains this ability in the future (Doran and Safley 1997). Soil health has the potential to affect many factors like animal health, atmospheric balance, microbial health, plant health, human health, soil ecosystem health leaching and surface run-off to groundwater (Fig. 3.4) (Das and Varma 2010).

3.6 Soil Enzymes as Biological Indicators of Soil Health

Soil enzymes, the biological indicators of soil, are of high importance. Enzyme activities are frequently linked to soils organic matter, soil physical and microbial qualities, activities and it transforms considerably quicker than other metrics, suggesting primary alterations in health of soil (Dick et al. 1996). According to Tate (1995) enzyme activities inside the soil can also be used as markers of pollutant inhibition, soil productivity, and microbial activity as shown in Fig. 3.5. Significant number of soil enzyme activities can be studied by simple, easily doable and well-documented tests that are already available (Tabatabai 1994; Dick et al. 1996).

Table 3.2 Soil enzymes and factors affecting their enzymatic activity (Adapted from Rao et al. 2017)

Enzymes	Potential determinants of enzymatic activities	References
α-Amylase and β-Amylase	Vegetation types, management practices, soil types and environment	Makoi and Ndakidemi (2008), Bakshi and Varma (2010)
β-Glucosidase	pH, soil management and environmental contamination	Makoi and Ndakidemi (2008), Bakshi and Varma (2010), Vinhal-Freitas et al. (2017)
Arylsulfatase	pH, organic matter content, pollution, and sulphate esters availability	Deng and Tabatabai (1995), Alkorta et al. (2003), Karaca et al. (2011), Guangming et al. (2017), Vinhal-Freitas et al. (2017), Holik et al. (2019), Adetunji et al. (2020)
Cellulase	pH, temperature, location and quality of organic matter, water, O_2 contents, fungicides and mineral elements	Caldwell (2005), Makoi and Ndakidemi (2008), Rao et al. (2017)
Chitinase	Availability of N, soil depth, atmospheric CO_2 levels, etc.	Karaca et al. (2011), Makoi and Ndakidemi (2008), Rao et al. (2017)
Dehydrogenase	Temperature, soil water content, pesticides, management practices, trace elements, pollution, etc.	Karaca et al. (2011), Makoi and Ndakidemi (2008), Rao et al. (2017), Sherene (2017)
Phenol oxidase	pH, mean annual temperature and, precipitation soil organic matter content, N enrichment, management practices, etc.	Caldwell (2005), Karaca et al. (2011), Rao et al. (2017)
Phosphatase	pH, organic matter content, pollution, management practices, crop species, etc.	Bakshi and Varma (2010), Nannipieri et al. (2011), Chen et al. (2019), Xu et al. (2020)
Protease	C and N bioavailability, humic acid concentration, etc.	Caldwell (2005), Makoi and Ndakidemi (2008), Rao et al. (2017), Dotaniya et al. (2018)
Urease	Organic matter content, management practices, cropping history, soil depth, heavy metals, temperature, pH, etc.	Guo et al. (2012), Adetunji et al. (2017), Sherene (2017), Han et al. (2019), Hossain et al. (2019)

3.7 Role of Soil Enzymes in Soil Health Maintenance

Potential role of major soil enzymes in soil health maintenance have been discussed below.

3.7.1 Amylases

α -Amylase and β -amylases are recognized to constitute the amylase. Starch have α 1, 4 linkages to join glucose monomers and thus create a long chain of polymeric

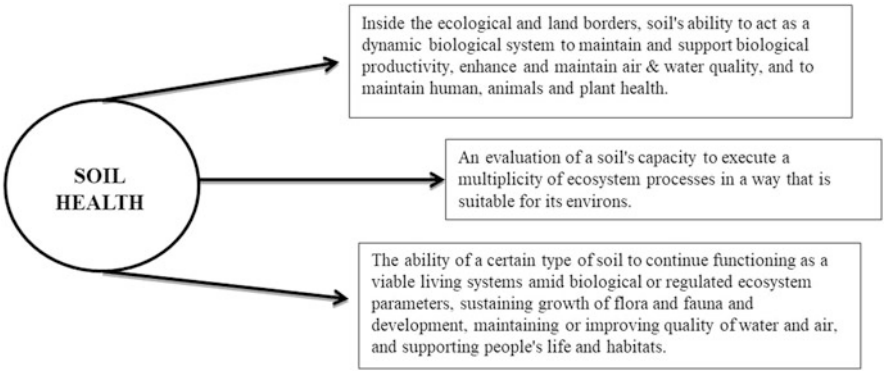

Fig. 3.4 Various ways to describe soil health

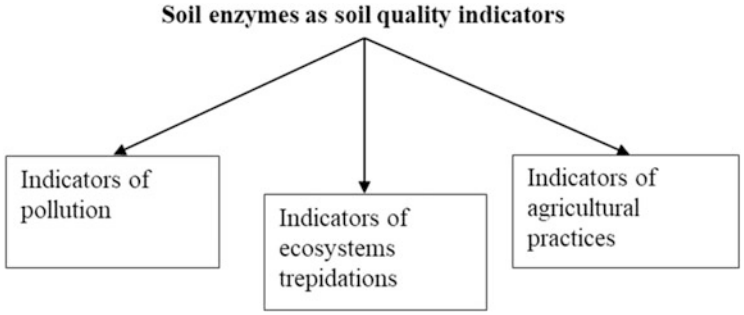

Fig. 3.5 Division of soil enzymes as soil quality indicators in three distinct areas

carbohydrates. Plants, animals, and microbes all produce α-amylases, whereas plants produce the majority of β-amylase (Dotaniya et al. 2018). α-Amylases, transforming starch-like substrates to oligosaccharides and/or glucose and β-amylase, responsible for converting starch to maltose, are commonly distributed in soils and plants (Makoi and Ndakidemi 2008). According to Wirth and Wolf (1992), several factors such as agricultural practices, floral types, environment, and types of soil might influence the activities and functions of α and β-amylase enzymes. Plants, for example, might impact the amylase enzymes' activities in soil by providing directly enzymes through their remains or eliminated chemicals, or by indirectly facilitating the synthesis pathway of microorganisms (Makoi and Ndakidemi 2008; Dotaniya et al. 2018).

3.7.2 Arylsulphatases

In plants, sulphur is absorbed as inorganic sulphate (SO_4), and its bioavailability is regulated through its mobilization or mineralization (Adetunji et al. 2020) from

R-O-SO_3- (esters of aromatic sulphates). This is because it bounds with organic components and are available indirectly to plants. According to Tabatabai (1994) the hydrolysis of the extracellular or intracellular esters and oxidation of organic materials ingested by microbes to give carbon and energy structures for biological synthesis, both of which produce by-product SO_4-S, influence the availability of these enzymes, because both of these reactions have a common condition to occur and that is arylsulphatase (Alkorta et al. 2003; Adetunji et al. 2020). In nature arylsulphatases are commonly found in biosphere particularly in soils (Ganeshamurthy et al. 1995; Alkorta et al. 2003; Vinhal-Freitas et al. 2017). The breaking of sulphate esters using water molecules (Kertesz and Mirleau 2004) is the reaction product of this soil enzymes; this is now followed by the release of hydrolysed esters into the environment with the aid of microorganisms in sulphur insufficiency reaction (Alkorta et al. 2003; Adetunji et al. 2020). The microbial biomass and sulphur immobilization rate are the direct indicators of the enzyme in nature (Klose and Tabatabai 1999; Vong et al. 2003). A chemical equation (Tabatabai 1994; Whalen and Warman 1996) to illustrate the participation of this enzyme in the breaking of sulphate esters by the reaction with water from R-O-SO_3- to phenols (R-OH) and sulphate (SO_4^{-2}) as products, or sometimes sulphate sulphur (SO_4-S) also is mentioned in Eq. (3.1).

$$R - O - SO_3^- \xrightarrow[\text{Arylsulphatases}]{\text{Hydrolysis}} R - OH + SO_4^- \tag{3.1}$$

A number of ecological elements impact the generation and sulphate discharge in the soil from sulphate esters (either soluble or insoluble). This can also be observed during heavy metal contamination which changes the soil pH (Acosta-Martínez and Tabatabai 2000). The amount of sulphate esters in organic form in the soil also inhibits the activity of arylsulphatase enzyme (Kunito et al. 2001; Karaca et al. 2011; Holik et al. 2019).

3.7.3 β-Glucosidase

In soils, β-glucosidase is a dominant and common enzyme (Eivazi and Tabatabai 1988; Tabatabai 1994). It gets its name from the nature of bond it hydrolyses. This enzyme is significant in soils because it catalyses the biodegradation and hydrolysis of different β-glucosides found in plant matter decaying in the soil system (Ajwa and Tabatabai 1994; (Esen 1993). An important carbon energy source for soil bacteria is glucose, which is its end output (Martinez and Tabatabai 1997). β -Glucosidase is also reported to stabilize the organic matter present in the soil (Bandick and Dick 1999; Ndiaye et al. 2000). Thus the test of checking the quality of soil has become easier (Bandick and Dick 1999). In addition, β-glucosidase activities also change the organic carbon long before other conventional methods can reliably quantify it (Dick 1994; Dick et al. 1997; Wick et al. 1998). However, some studies have found that it has phyto-pathological consequences in the ecosystem (Almeida et al. 2015;

Adetunji et al. 2017). Aglycons are the precursors of poisonous compounds responsible for inducing soil sickness are found in soil where monoculture plant growth system is followed (Maurya et al. 2020). The enzyme β-glucosidase is extremely sensitive to variations in pH (Kuperman and Carreiro 1997; Bandick and Dick 1999; Acosta-Martínez and Tabatabai 2000) and the soil conservation measures (Dick et al. 1997; Bergstrom et al. 1998; Leirós et al. 1999; Madejón and Burgos 2001). In conditions where this enzyme is active, this feature can be employed as an effective biochemical biomarker for measurement of ecological changes caused by soil acidification. Contamination of heavy metals, such as copper and others, is known to block the β-glucosidase enzyme (Deng and Tabatabai 1995; Haanstra and Doelman 1991). Plant waste, for example, does not disintegrate β-glucosidase activity when comes in contact with soils polluted with heavy metal, according to research (Geiger et al. 1993).

3.7.4 Cellulases

The most prevalent organic substance in the biosphere is cellulose, accounting for about half of all biomass produced by CO_2 fixation photosynthetically (Eriksson et al. 1990). Microorganisms which are vital in soils rely on carbon supply found in the soil to grow and survive (Deng and Tabatabai 1995). Cellulases enzymes must digest cellulose in plant detritus into cellobiose, glucose, and oligosaccharides before carbon can be released as a source of energy for microbes (Maurya et al. 2020). Cellulases catalyse the breakdown of cellulose, which is a polysaccharide made up of β-1,4 linked glucose units (Deng and Tabatabai 1995). Soils cellulases are primarily formed from plant detritus that has been integrated into the soil, with a small percentage coming from bacteria and fungi in the soil (Richmond 1991).

Several factor such as oxygen content, organic matter structure, water, temperature, soil profile horizon, pH, quality of plant waste and soil mineral components, as well as trace elements from fungicides, have been shown to influence cellulase activity in agricultural soils. From the studies made by Srinivasulu and Rangaswamy (2006) it has been observed that black soil has substantially more cellulase stimulatory effects when compared to red soil. Gong et al. (2015) found that increasing the temperature reduced cellulase activity by roughly 30% in grassland soils. The breakdown of cellulose by cellulases attributes to different mechanisms. In the presence of chitin, cellulose stimulates the production of lytic enzymes and chitinase, which aids in the generation of β- glucosidase into the culture environment (Sherene 2017). Cellulase activities can be utilized to get a rough idea of some of the chemical-physical features of soil, making arable soil management tactics easier. Because cellulases enzyme is responsible for cycling most prevalent polymer, cellulose, it is necessary to have a better understanding of this enzyme so that it can be employed more frequently in various soil fertility programmes as a forecasting tool.

3.7.5 Chitinase

Chitinase, also known as chitinolytic enzymes, is an enzyme that degrades and hydrolyses chitin. Chitins are fundamental structural constituent of fungal cellular wall of several fungi that utilize hyper-parasitism mechanisms to defend themselves against pathogenic microorganisms and parasites (Chet 1987). Antibiosis and competitiveness are examples of some of the other methods in use by these biological agents to diminish disease-producing organisms (Olander and Vitousek 2000). Various organisms, comprising plants and microbes, generate or release this enzyme which is quite essential in terms of agriculture (Deshpande 1986). It can be detected in a variety of ecosystems and this has proved its efficacy in regulating soil-borne diseases in beans and cotton, such as southern blight of crops and plant pathogenic fungus (Shapira et al. 1989). One of the hypothesized methods includes the chitinase, which degrades harmful fungi's cell walls (Nayak et al. 2020; Singh et al. 1999). In addition to its role as biological pest managing enzyme, there are other numerous directions for using this enzyme to maintain soil health and, as a result, boost plant development and ultimate harvests, which is possible due to its eco-friendliness (Das and Varma 2010).

3.7.6 Dehydrogenases

The biological activities of soil measure can be determined with the help of the dehydrogenase enzyme activities. Though this enzyme does not gather extracellularly in the soil, yet it is thought to be a necessary element of intact cells. These enzymes have the tendency to oxidize soil organic matter. This process involves the transferring of positive and negative subatomic particles from substrates to acceptors. Such kinds of responses are highly dependent on air-water and soil type conditions and are found in the soil microorganisms' respiratory routes (Glinski and Stepniewski 1985; Kandeler 1996). For the reason that these processes are part of soil microorganisms' respiration pathways and can reveal the soil's ability to support biochemical processes, research being carried out, to study the various kind of dehydrogenase activities enzyme in soil system, is critical in understanding soil fertility and health. These enzymes can also serve as possible signs of oxidative activities of microorganisms and biomarkers for the redox systems of soil microbes (Wolinska and Stepniewsk 2012; Kumar et al. 2013). Dehydrogenase enzyme can also be utilized as a direct indicator of soil microbial activity (Garcia and Hernández 1997; Das and Varma 2010) and is also used to quantify the disturbances caused by management practices in the natural soil profile, trace elements or pesticides, etc. (Reddy and Faza 1989; Wilke 1991; Frank and Malkomes 1993). With the help of this enzyme, the intensity and severity of soil pollution can be simply measured. The polluted soils caused due to the release of pulp and paper mill effluents have been reported with elevated levels of dehydrogenase enzyme (McCarthy et al. 1994) whereas the level was low in fly ash-polluted soils (Pitchel and Hayes 1990). Likewise, dehydrogenases share an inversely proportional relation with the

pesticides. Higher activity at low doses of chemicals and reduced activity at larger concentrations of pesticides have been observed by Baruah and Mishra (1986). Dehydrogenases have created a niche in the biological oxidation of soil humates because they have the ability to transport hydrogen from biological molecules to chemical acceptor compounds. Dehydrogenases transfer hydrogen to NAD or NADP in a variety of ways (Subhani et al. 2001).

3.7.7 Phosphatases

Phosphoric acid esters and anhydrides are hydrolysed by the catalytic property of a certain group of enzymes known as the phosphatases. Bakshi and Varma (2010) suggested that they play an eminent role in the phosphorus cycle that operates in soil ecosystem. This theory was further established through proofs that showed their involvement in phosphorus stress and hence plant growth. The phosphatases are among the good soil fertility indicators along with their ability to maintain the soil ecosystem (Dick et al. 2000). This can be proved by an example of phosphorus deficiency. During such a condition, a signal is generated indicating such a deficiency. This leads to an increased production of phosphatase enzymes from roots of plant to curb the deficiency and boost the solubilization and remobilization of phosphate molecule (Versaw and Harrison 2002); this mode of action prepares the plant's defence mechanism to survive the phosphate stressed conditions (Mudge et al. 2002). The activity of enzymes changes as the temperature varies. Gong et al. (2015) studied the effect of temperature influences the activity of phosphatase and increased it by 36%. The knowledge regarding the enzyme activity dynamics in soil systems is vital for forecasting their interactions, as their actions may affect uptake of nutrient and plant development, and both of the features are crucial for maintaining soil health.

3.7.8 Proteases

The process of nitrogen mineralization that regulates the quantities of accessible nitrogen to plants (Landi et al. 2011) and plant growth involves an important part of proteases in it. Proteases are always linked to both inorganic and organic colloids and they are commonly found in soil systems (Nannipieri et al. 1996, 2002). Proteases are basically carbohydrate molecules. Because of their activity and nature they are found in soil (Patil and Shastri 1985; Vranova et al. 2013) in soils from a forest or perennial grassland (Sidari et al. 2008; Nannipieri et al. 2011) in compost from solid municipal garbage (Rad et al. 1995); and in productive land (Gianfreda and Ruggiero 2006; Gramss et al. 1999). The activity of extracellular enzyme is indicative not just of soil's biological capability for enzymatic substrate conversion, which is independent of microbial activity, but also of the microbial ecology. Several biotic and abiotic variables influence the activity of proteases. Low amounts of neutralized soil humic acids, for example, block some protease activity while

stimulating others through mechanisms involving 2-nitrobicyclo[2.2.1]hept-5-ene-2,3-dicarboxylic acid carboxyl groups (Shan et al. 2010). Activity of pronase enzyme is inhibited regardless of the charge of the hydrolysed substrate, implying that humic acid combines with the enzyme rather than the substrate, hence an inhibition in the activity rate (Hester et al. 2017). Quantitative examination of the effects of substrate concentrations and humic acid on carbobenzoxy-glycyl leucine pronase hydrolysis reveals that inhibition is not caused by humic acid and substrate anions together (Makoi and Ndakidemi 2008).

3.7.9 Ureases

The hydrolytic enzyme in charge for the conversion of urea fertilizers into ammonia and carbon dioxide is ureases; this relates to a spike in pH of soil (Andrews et al. 1989; Antonious 2003). As a result, fast nitrogen loss to the atmosphere via ammonia volatilization occurs (Simpson and Freney 1988; Guo et al. 2012). The presence of urease activity in soil regulates the nitrogen supply cycle in terms of plants following urea fertilization (Han et al. 2019); hence researches are now being directed towards the study of their activities in soil. Plants and microbes are the principal sources of soil urease (Dilrukshi and Kawasak 2016), which can be found as both intra- and extracellular enzymes (Mobley and Hausinger 1989). Urease produced from microorganisms, or plants, conversely, is swiftly destroyed in soil by the enzymes that are proteolytic in nature. This shows that extracellular urease, which is maintained by restriction on organic and mineral soil colloids, can be held responsible for a large portion of ureolytic activities in the soil. According to Yang et al. (2006) and Hossain et al. (2019), history of cropping, soil's depth, amount of soil organic matter, presence of heavy metals, amendment of soil and other ecological variables like temperature are among few of the numerous factors that affect urease activity in soils. Urease also gets affected by the harmful heavy metal doses amended in soil (Yang et al. 2006; Liu et al. 2020). In general, urease activity rises as the temperature rises. Higher temperatures are expected to increase enzyme activity coefficient. As a result, applying urea at periods of the day when temperatures are low is recommended (Sherene 2017).

3.8 Overview

Soil forms the most basic and important component of land-based ecosystems and the primary framework for agricultural production (Bastida et al. 2008). It is critical to have proper methods to identify and prognosis the possible soil changes in order to identify and comprehend the functioning of soil and to prevent soil damage caused by both human and natural-caused influences, including agricultural management methods. Biological indicators, such as soil enzymes, are frequently employed to assess the state of the soil ecosystem (Balezentiene 2012). The study of soil enzymes is important because these enzymes play important biochemical roles in organic

matter synthesis and degradation, along with biogeochemical cycles in the soil ecosystem, soil structure stabilization, and pollutant degradation, all of which are crucial in agriculture (Makoi and Ndakidemi 2008; Liao et al. 2014). Due to the extreme limitations of soil enzymology approaches, there is a big delineation between the extracellular and intracellular activities of the soil (Brookes 1995; Dick 2011; Burns et al. 2013; Dlugosz and Wilczewski 2014). Soil enzymes typically have different characteristics than other enzymes from sources such as plants and water. When exposed to soil protease assault and in extreme soil conditions including a wide range of soil temperature and its response, they often show a definite range where they behave stably. Soil enzymes often have lower Vmax values and a larger Km constant, because they generally have differing kinetic characteristics as compared to their counterparts in pure form. This feature indicates towards a lessened catalytic efficiency and a poorer substrate affinity (Kujur and Pater 2014). A combination of natural and anthropogenic influences may have a considerable impact on soil enzymatic activity. Enzymes have been discovered to be a valuable indication of soil probable changes since their response to diverse circumstances is more obvious and quicker than other properties of the soil (Utobo and Tewari 2015), as an example, physicochemical cycles. Soil enzymes are primarily influenced by changes in their production and consistency in natural conditions by natural factors such as microbial biomass, seasonal changes in soil moisture and temperature, topographical distribution, location of the domain, content of mineral particles that are clayey in nature, physicochemical properties, and the amount of available forms of nutrients and amount of organic matter. The immobilization of extracellular enzymes is aided by the physicochemical characteristics of the soil and even by the high presence of clay and humus (Demkina et al. 2017). Patra et al. (2006) and Yang et al. (2006) have highlighted the fact that varied vegetation cover might have a significant impact on environmental variations. Changing the temperature could affect the nature and kinetics of specific extracellular enzymes in either a synergistic or antagonistic way. Enzymatic activity increases with increase in temperature, according to Steinweg et al. (2013), and additional increase can lead to enzyme denaturation. It is well understood that the distribution of reaction products, enzyme proteins, and substrates in soil are influenced by soil texture (soil type) and moisture (Gong et al. 2015). Enzymatic activity is low when soil moisture is low, and extended droughts frequently impede enzyme secretion as a result affecting their activities. However, in dry soils, decreased enzymatic activity combined with continuous creation of enzymatic proteins, even in tiny amounts, may result in the maintaining of total enzymatic pool size throughout the dry period (Burns et al. 2013). Because of cell lysis and soil aggregate rupture, increased soil moisture accelerates organic matter transformation and availability. As a result, the microbial biomass may be activated, and its transformation may lead to a transient rise in overall soil enzymatic activity (Spohn et al. 2013). Various natural and anthropogenic influences can impact soil enzymatic activity directly or indirectly, allowing them to be employed as good markers of potential variations in the soil quality (Dlugosz 2019).

3.9 Conclusion and Future prospects

Soil enzymes are significant soil components that are linked to the soil biological and physicochemical properties. Anthropogenic activities, agricultural practices, and pollution, on the other hand, have a substantial influence on their existence and activities in soil. As they are more sensitive to changes than other soil variables, soil enzymes are often employed as dependable markers for fertility, soil health, and production, as influenced by various natural and human causes. Understanding the existence and activity of enzymes in soil can enhance the knowledge of the researchers to understand the transformation of organic substances and cycling of ecological nutrient elements. However, due to a scarcity of well-resourced laboratories and trained personnel to conduct research in this field, responsible for influencing the activity of soil enzymes, there is scarcity of data to understand the factors. There is a crucial need to develop new techniques to quantify enzymatic activities with high precision in this complex area. In this perspective the advances in the study of proteome, comprehensive metabolic studies and RNA related studies hold great potential. To make the measurements more exact and practicable, the restrictions associated with in situ enzyme activity must be overcome. Temporal and spatial investigations of soil enzyme activities are required to understand the elements that regulate enzyme activity and to determine the ideal conditions for soil enzyme metabolism. Soil enzyme profile under various management approaches can aid in the development of connections between soil enzyme activity, productivity, and ecosystem soil health. Abiotic and biotic stressors generated by climate change have an impact on soil enzyme activity. Basic and strategic research is needed to establish coping mechanisms for maintaining soil enzyme activities under climate change conditions, which will aid in soil improvement. Future research should look at the global, widely used soil fertility/productivity index, which is established based on a complicated scientific calculation with a number of enzymatic and physicochemical characteristics being included. Such an approach will result in a more accurate portrayal of the soil environment's complexity which would be based not just in terms of the condition where it was created. Soil enzyme activity is a key signal of most changes in soil health and thus soil quality, yet it has been determined that location-specific evaluation parameters are required for a more precise use of soil enzyme activity as a marker of soil quality.

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Soil *Bacillus* as Biocontrol Agent: Prospects and Applications

4

Swapnarani Nayak, Suraja Kumar Nayak, Bighneswar Baliyarsingh, Avishek Pahari, Debasish Dash, and Bibhuti Bhusan Mishra

Abstract

Soil, amongst the natural resources is of superlative important, nurturing innumerable microbes essential for maintaining soil fertility, crop protection vis-à-vis crop productivity. Productivity of crop mostly decreases due to disease caused by wide range of phytopathogens. The use of bioantagonist as biocontrol agents has been of utmost importance to combat phytopathogens instead of agrochemicals/pesticides. Biocontrol of crop phytopathogens comprehended curtailment in pathogenic inoculum concentration or restricted infectivity. Using few bacteria is an alternative option against rice phytopathogens as well as agrochemicals for an affirmative socio-ecological impact. Species of soil *Bacillus* sp. offers advantages over others in biocontrol of rice pathogens via production of array of broad-spectrum antibiotics and resistant endospores. Advancement in molecular biology revealed the molecular pathways and mechanisms involved in

S. Nayak
Fish Genetics and Biotechnology Division, ICAR-Central Institute of Freshwater Aquaculture, Bhubaneswar, India

S. K. Nayak (✉) · B. Baliyarsingh
Department of Biotechnology, Odisha University of Technology and Research, Bhubaneswar, India

A. Pahari
Department of Preventive Medicine, College of Veterinary Science & Animal Husbandry, Odisha University of Agriculture and Technology, Bhubaneswar, India

D. Dash
Department of Botany, College of Basic Science and Humanities, Odisha University of Agriculture and Technology, Bhubaneswar, India

B. B. Mishra (✉)
Department of Microbiology, Odisha University of Agriculture and Technology, Bhubaneswar, Odisha, India

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antagonism of *Bacillus* sp. aiding disease reduction, remediation, improvement and wider use of biocontrol agents. The pathway of biocontrol prospective involves parasitism, competition for nutrients and space, antibiosis, induced disease resistance and other cellular interactions. In the present chapter an attempt has been made to implement *Bacillus* sp. as a biocontrol agent to combat rice bacterial pathogens including its in depth physiology, mode of action, genetics, limitations and enhanced applications for disease management.

Keywords

Rice (*Oryza sativa* L.) · Bacterial pathogens/disease · Biocontrol · *Bacillus* sp.

4.1 Introduction

Unimpeded application of agrochemicals to crop fields for protection against arrays of bacterial phytopathogens is a current drift globally instead of huge research on its environmental toxicity. Many governments have ban on pesticides and bactericides because of higher cost and resistant developments in pathogens (Rahman 2013). Technological advances in agricultural realm leads to successful utilization of microbial antagonist as a natural resource for biocontrol prospective. Antagonistic characteristic of biocontrol agents stand as an opposite alternative on use of toxic chemicals and can be used as a suitable option for pathogenic control of bacterial plant pathogens (Thomashow 1996). Bacterial close association with the plants might be an excellent and effective biocontrol for sustainable agriculture. Soil is the most important and an essential resource that harbours numerous microbes which are vital to maintain soil fertility as well as crop productivity and also represents as a possible treasure of biocontrol bacteria (Bloemberg and Lugtenberg 2001). Soil substrates have gone through various processes of degradation and other processing for release of secondary metabolites. Due to the dominating role of sedentary organisms and presence of heterotrophy in soil (chemolithoautotrophs as an exception), biotic interactions in soil vary from other resources/systems. For this only soil microbial diversity remains as a lead eye-catching resource for decades. The natural products like as antibiotics and chemosimilars derived from soil microbial strains are having economic value (Daniel 2004). Soil microorganisms are distributed throughout the soil (inside and outside of microaggregates) and are able to establish soil as a most diverse and heterogenous mixture with an array of various microbial niches.

Bacterial disease in rice (*Oryza sativa* L.) is not only a serious concern in Asia but also in other parts of the world (Rabindran and Vidhyasekaran 1996). As staple food, rice serves half of the world population. The warm and humid conditions of the tropics and subtropics are optimum for bacterial growth; hence lots of bacterial plant diseases are frequent particularly in this region. Generally the bacterial pathogenicity outbreaks for plant cell-wall-degrading enzymes, polysaccharides, bacterial secretion systems (types I-IV), toxins, proteinases, melanin, hormones and siderophores which are having utmost importance for virulence & infection and produced during

host plant and pathogenic bacteria interaction(s) (Agrios 2005). The efficacy of antibacterials and host pathogenic susceptibility is evaluated by the cultural and environmental interactions particularly in root and foliar diseases (Dean et al. 2005). In few cultivars the pathogenic bacteria also develop resistance to chemicides due to genetic modifications. A number of soil bacterial isolates like *Bacillus* sp. and *Pseudomonas* sp. gives biocontrol on bacterial diseases (*Xanthomonas* sp., *Burkholderia* sp.) in field trials. Organisms enable to interfere with pathogenic survival, growth, development and infection by various direct or indirect mechanisms are referred as antagonists.

Mostly soil bacteria expresses antimicrobial activity due to production of various lytic enzyme complexes which enable them to intake as substrates (De Boer et al. 1998). Only due to antibiotics production capability they have been used as biocontrol agents against phytopathogenic bacteria (Yilmaz et al. 2006). Reports on antibacterials harangued on half of the antibacterials certified after 1994 are from biological source. Some beneficial soil bacteria (esp. *Bacillus* sp. and closely related *Paenibacillus* sp.) living in and around normal and rhizospheric soil are of particular interest in this regard. *Bacillus* sp. is extensively available in nature, nonpathological, innocuous and harmless to plants (Nayak et al. 2017). Also produces antimicrobial compounds such as (lipo)peptides antibiotics (Stelle and VlamiM de Souza 2002) and antibacterial proteins in vitro (Shrestha et al. 2016). Additionally, *Bacillus* sp. offers advantages over others against bacterial plant pathogens due to endospores formation and synthesis of broad-spectrum antibiotics. The *Bacillus* endospores have ability to resist at abrupt temperature and elevated chemical concentrations and have extensive storage and marketability. Previously, *Bacillus* sp. were well documented as engaged in soil fertility and optimization of plant growth and nutrition apart from that also involved in bacterial phytohormones production, phosphate and other essential mineral solubilization (de Freitas et al. 1997).

4.2 *Bacillus* sp.: An Emerging Soil Bacteria

Amongst all, soil is contemplated as one of the apposite environment connect to microbial growth and prosperity (Cavalcanti et al. 2006). During the late 1970s the occurrence and importance of antagonistic microbial interaction in soil were established. Natural inhibition of microorganisms was carried out in both treated and non-treated soils for various kind of phytopathogens (mostly bacterial and fungal). Moreover, for any particular case in conducive soil there is a continuous synthesis of chemosimilars which involve in suppression of specific group of pathogens. Soil bacteria are beneficial as involved in N_2 fixation, production of various phytohormones, array of antistress enzymes synthesis, siderophore production, solubilization of potassium and zinc and disease management through inhibition and reduction in phytopathogenic load (Kumar et al. 2012). Moreover, the biocontrol bacteria secrete extensive range of exometabolites as a result of diverse

secretion mechanisms with involvement of typical lytic enzymes and effectuating various biochemical reactions (Das et al. 2006; Nayak et al. 2017).

Numerous soil-inhabiting microorganisms have been previously described as possible biocontrol agents such as *Pseudomonas* sp., *Streptomyces* sp., *Trichoderma* sp. including *Bacillus* sp. to control various bacterial diseases (Nemutanzhela et al. 2014). The *Bacillus* sp. solely controls in between the range of 30–50%. Genus *Bacillus* described by F. Cohn in 1872 is taxonomically included in the family Bacillaceae, order Bacillales and class Bacilli. *Bacillus* sp. are very diverse group of organisms (pathogens to beneficial) with spore-forming, Gram-positive, aerobic/(facultatively) anaerobic respiration characteristics. Present day taxonomy reveals more than 266 numbers of species are ubiquitous in nature. Since past century *Bacillus* have been studied because of their of antibiotics production capability (Nayak and Mishra 2020) with agricultural relevance. However, they also have exceptional colonization ability with an effective sporulation, adapt and resist to adverse environmental conditions and diseases suppression for which they signify the candidature of potential biocontrol agents. Additionally, they are simple to multiplication and metabolites production (non-pathogenic), economically viable and effective. Apart from soil, few strains also colonize in the rhizosphere, promote plant growth and destroy pathogens and their spores (Basha and Ulaganathan 2002). Genus *Bacillus* produces more than double dozens of antibiotics with different structure and function (Stein 2005), amongst them mostly are of peptides (Mannanov and Sattarova 2001; Stein 2005).

Presently researchers have been involved in isolating various soil *Bacillus* sp. and identifying their bioactive compounds and their potential to produce multistructure inhibitory substances (Stein 2005). Furthermore, a wide range of antibacterial antibiotics are produced by *B. thuringensis*, *B. cereus*, *B. weihenstephanensis*, *Paenibacillus* sp. (erstwhile *Bacillus polymyxa*), *B. subtilis* and other *Bacillus* sp. Research was more focused on finding and isolating soil microorganisms antagonistic to plant bacterial and fungal pathogens that caused specific crops diseases in the mid-nineteenth century (Shrestha et al. 2016). As mentioned earlier, the peptide antibiotics are the dominant class. The (lipo)peptides (a group of microbial-based peptides) often enable the plant for activation of defence mechanism. Disturbance of lipid bilayers, decline in surface tension and many similar surface alterations are the main qualities of *Bacillus* lipopeptides. Systemic-induced resistance (SIR) and other resistances including the signal transduction pathways are accelerated in plants defence mainly due to *Bacillus* sp. (Fig. 4.1) (Shafi et al. 2017; Nayak et al. 2017).

4.3 Major Rice Bacterial Pathogens

Disease proliferation can be another measure of soil fertility index (Station TAE 1996). Phytopathogenic bacteria are the leading cause of lower yield while few of them even produce anthropogenic toxic compounds. However, these pathogens are also considered as economically important, responsible for both severe economic loss and harvest yields (Aye and Matsumoto 2010). Apart from the quantity, quality

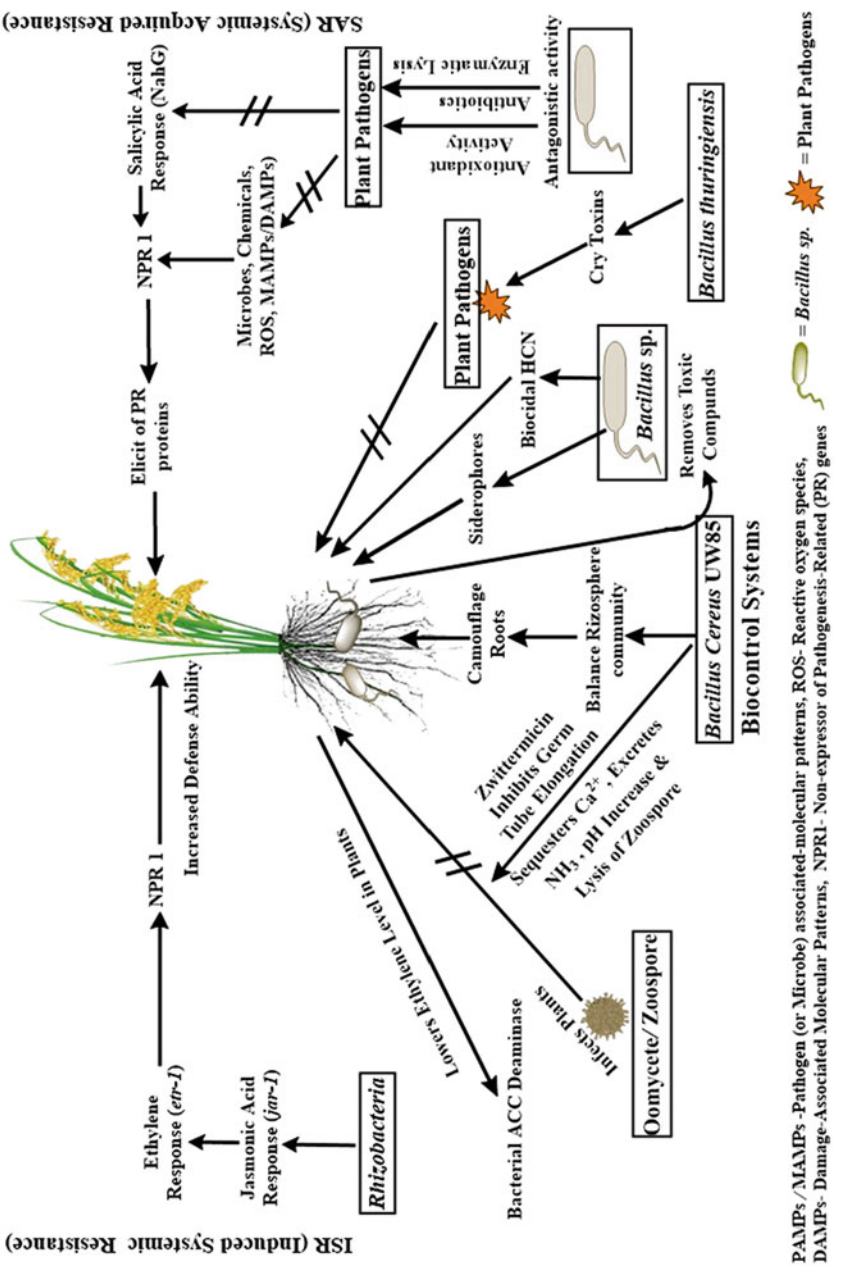

Fig. 4.1 Biocontrol of rice bacterial pathogens by *Bacillus* sp. and other soil bacteria

of the staple food is also sacrificed because of any pathogenic group/individuals. Rice diseases due to various phytopathogens and their pathovars appear to be proliferating at exponential increasing rates (Table 4.1). Further the diseases profile and pathogenicity of rice has changed over time due to ample changes in global climatic conditions, diversified variants and cultivation practices as region specific. Many diseases previously considered as obscure have become sinister. Reports are available for more than 70 diseases to occur on rice, out of which 15 are of bacterial origin and rest are of fungal, viral and other nematodes (Saha et al. 2015).

Of these diseases, Bacterial Leaf Blight (BLB)/Bacterial blight (*Xanthomonas oryzae* pv. *oryzae*; synonym: *X. campestris* pv. *oryzae*), Bacterial leaf streak (*X. oryzae* pv. *oryzicola*), Bacterial brown stripe/Bacterial stripe (*Acidovorax avenae* subsp. *avenae*; erstwhile *Pseudomonas avenae*; *Pseudomonas. syringae* pv. *panici*) and Bacterial panicle blight of rice (BPBR) (*Burkholderia gladioli*; synonym: *Pseudomonas gladioli*; *Burkholderia glumae*; synonym: *Pseudomonas glumae*) are the vital bacterial diseases and cause remarkable losses, quantitatively as well as and qualitatively (Cui et al. 2016). Additionally, Seedling blight (*Burkholderia plantarii*; synonym: *Pseudomonas plantarii*), Sheath brown rot (*Pseudomonas fuscovaginae*; erstwhile: *Pseudomonas marginalis* [*Pseudomonas fluorescens biovar II*]), Grain rot/Seed rot (*Burkholderia glumae*; synonym: *Pseudomonas glumae*; *Burkholderia gladioli*) and Seedling rot (*Burkholderia glumae*; synonym: *Pseudomonas glumae*), Halo blight (*Pseudomonas syringae* pv. *oryzae*), Bacterial palea browning (*Pantoea agglomerans*; synonym: *Erwinia herbicola*; *Pantoea ananatis*) and Bacterial sheath rot (*Pseudomonas syringae* pv. *syringae*; synonym: *Pseudomonas oryzicola*, *Erwinia carotovora*) have worsen the scenario as addendum. Unexpected diseases such as (bacterial) Foot rot (*Dickeya chrysanthemi*; erstwhile: *Erwinia chrysanthemi*), Black rot (*Xanthomonas campestris* pv. *oryzae*, *Pseudomonas itoana*), Cinnamon speck of rice grains (*Xanthamonas cinnamona*) and Black-eye spot (*Xanthomonas atroviridigenum*) have become important as region-specific diseases and are becoming notable problems where they were quondam known as of minor importance (Ou 1985; Nayak et al. 2017).

4.3.1 Bacterial Leaf Blight (BLB)/Bacterial blight

Xanthomonas oryzae pv. *oryzae* is a causative agent for bacterial blight disease which is one of the most socioeconomically important rice diseases round the globe and particularly in the tropical and temperate areas (Ou 1985). It is also called as the monsoon disease of rice. The severity of the disease depends on cloudy, drizzling and stormy weather with rainfall. Additionally, application of high nitrogen fertilizers leads to mineral imbalances in soil and ultimately intensifies pathogenic load. Reports are available on the infection of the disease also by *Pantoea agglomerans* (erstwhile *Enterobacter agglomerans* or *Erwinia herbicola*), a member of Enterobacteriaceae family (Lee et al. 2010).

Table 4.1 Antibacterial activity of soil *Bacillus* sp. against rice bacterial pathogen

Name of disease	Causative agent/organism	Symptoms	Parts infected/ affecting	Antagonistic *Bacillus* sp.	References
Seedling blight	*Burkholderia plantarii* (syn. *Pseudomonas plantarii*)	(a) Basal chlorosis (b) Withering of the second or third leaves (c) Infected Seedlings which become reddish brown	Seeds and Leaves	*Bacillus* sp.	Saha et al. (2015), Amaki et al. (2011)
Bacterial brown stripe/ Bacterial stripe	*Acidovorax avenae* subsp. *avenae* (erstwhile *Pseudomonas avenae*) *Pseudomonas. syringae* pv. *panici*	(a) Inhibition of germination (b) Brown stripes found at the midrib or leaf margins (c) Abnormal elongation of mesocotyl	Seedling and Leaf margins	*B. laterosporus* B4	Saha et al. (2015), Xie et al. (2011), Kakar et al. (2014a)
Bacterial Leaf Blight (BLB)/ Bacterial blight	*Xanthomonas oryzae* pv. *oryzae* (syn. *Xanthomonas campestris* pv. *oryzae*) *Pantoea agglomerans*	(a) Lesions with light brown to faint red spots on upper leaf blades (b) Transparent stripes on leaves and become brown and opaques when dry condition	Leaves, Seedlings, Glumes and Stripes	*B. subtilis* A15, *B. amyloliquefaciens* D29, *B. methylotrophicus* H8 *B. polymyxa*	Ou (1985), Saha et al. (2015), Lee et al. (2010), El-shakh et al. (2015)
Bacterial leaf streak	*Xanthomonas oryzae* pv. *oryzicola* (syn. *Xanthomonas campestris* pv. *oryzicola*)	(a) Entire leaves turn brown, then greyish white and die (b) Brown or black discolouration of florets and seeds	Leaves, Seeds and Flowers	*B. amyloliquefaciens* Lx-11	Saha et al. (2015), Zhang et al. (2012)
Halo blight	*Pseudomonas syringae* pv. *oryzae*	(a) Pale green to yellowish brown lesions on leaf blades (b) Dark brown spot in the centre	Leaf blades	*B. subtilis*	Kuwata (1985), Saha et al. (2015) Bhattacharjee and Dey (2014)
Sheath brown rot	*Pseudomonas fuscovaginae* (erstwhile *Pseudomonas*	(a) Discolouration of the leaf sheath which may spread to the midrib or veins of the leaves	Leaf sheath, Leaf midrib,	*Bacillus amyloliquefaciens* Bk7	Saha et al. (2015), Razak et al. (2009), Kakar et al. (2014b)

(continued)

Table 4.1 (continued)

Name of disease	Causative agent/organism	Symptoms	Parts infected/ affecting	Antagonistic *Bacillus* sp.	References
	marginalis [*Pseudomonas fluorescens biovar II*])	(b) In the mature plants, leaf sheath become dry and panicle withers	Leaf veins and Panicle		
Bacterial sheath rot	*Pseudomonas syringae* pv. *syringae* (syn. *P. oryzicola*) *Erwinia carotovora*	Rotting occurs on leaf sheath	Leaf sheath	*B. thuringiensis*	Rostami et al. (2010), Saha et al. (2015), Goto (1979), Dong et al. (2004)
Grain rot/Seed rot	*Burkholderia glumae* (syn. *Pseudomonas glumae*) *Burkholderia gladioli*	(a) Wilting or soft rot of the leaves (b) Grains are shrunken and pale green on the panicle	Leaf and Panicle	*B. amyloliquefaciens*, *B. methylotrophicus*, *B. subtilis*	Ura et al. (2006), Maeda et al. (2004), Saha et al. (2015), Shrestha et al. (2016)
Bacterial palea browning	*Pantoea agglomerans* (syn. *Erwinia herbicola*) *Pantoea ananatis*	(a) Light brown, water soaked lesions occur on the lemma or palea (b) Discolouration of palea (c) Infected panicles have immature and lighter grains at harvest	Lemma, Palea and Panicle	*B. cereus*	Azegami (2013), Stabb et al. (1994)
(Bacterial) Foot rot	*Dickeya chrysanthemi* (erstwhile *Erwinia chrysanthemi*)	(a) Dark brown decay of leaf sheaths (b) Wilting and yellowing of leaves (c) Nodes, culms and crown become rotted and turn black (d) Roots attached to infected nodes decay and fall off	Leaves sheath, Nodes, Culms, Roots	*B. thuringensis*, *B. cereus*, *B. weihenstephanensis*, *B. subtilis*	Saha et al. (2015), Krzyzanowska et al. (2012)

		(e) Infected plants have an unpleasant odour			
Black rot	*Xanthomonas campestris* pv. *oryzae* *Pseudomonas itoana*	(a) Partial blackening on the grain at the apex (b) Aleurone layer of endosperm turns black	Endosperm	*B. megaterium*, *B. subtilis*	Mew et al. (1993), Kannan and Bastas (2015), Monteiro et al. (2005)
Seedling rot	*Burkholderia glumae* (syn. *Pseudomonas glumae*)	(a) Brownish discolouration of seeds (b) Seedlings may also have darker rot at the base of the plant (c) Seedlings are stunted, turn yellow, and eventually could die resulting in poor stand	Seedlings	*B. thuringiensis*	Maeda et al. (2004), Wamishe et al. (2013) Cho et al. (2007)
Cinnamon speck of rice grains	*Xanthamonas cinnamona*	(a) Grains produce distinct odour and disintegrate (b) Grains become cinnamon coloured	Damage to grains	*Bacillus* sp.	Ou (1985)
Black-eye spot	*Xanthomonas atroviridigenum*	(a) Grains are smaller than normal (b) Grains are discoloured from dark-yellowish brown to dark grey (c) Embryos are damaged	Embryos	*Bacillus* sp.	Ou (1985)
Bacterial panicle blight (BPBR)	*Burkholderia gladioli* (syn. *Pseudomonas gladioli*) *Burkholderia glumae* (syn. *Pseudomonas glumae*)	(a) Blank florets found across the panicles (b) Up right blank florets can easily be identified early or late in the season	Rice flowerings	*B. amyloliquefaciens*, *B. methylotrophicus*, *B. subtilis*	Li et al. (2017), Shrestha et al. (2016)

X. oryzae pv. *oryzae* sustains mainly with in preinfected grain seeds, stubbles, straw, ratoons, self-sown plants and rhizosphere of few winter crops (Sundar and Dodan 1989). It invades through wounds or any natural openings on leaves (hydathodes and stomata) and turns to systemic in the xylem of rice (Devadath and Rao 1975) indicating as a vascular disease. Under advantageous circumstances non-resistant varieties undergo more than 70% loss of yield (Mew et al. 1993; Mew and Vera Cruz 2001).

4.3.2 Seedling Blight

Burkholderia plantarii (syn. *Pseudomonas plantarii*) is the causative agent of rice bacterial seedling blight disease. In 1985, it was first detected in Chiba Prefecture, Japan (Azegami et al. 1985), associated with the seedling in nursery boxes and subsequently spread throughout Japan. Conditions of high temperature and relative humidity are favourable for infection. In addition, tropolone, a chemical produced by the pathogen, is responsible for increasing the disease symptoms like leaf chlorosis and root growth (Azegami et al. 1987). In rigorous infection, root growth is retarded and seedlings easily lodge.

4.3.3 Bacterial Brown Stripe/Bacterial Stripe

Bacterial brown stripe, otherwise known as bacterial stripe, is caused by *Acidovorax avenae* subsp. *avenae* (erstwhile *Pseudomonas avenae*) and *P. syringae* pv. *panici* (Shakya et al. 1985; Kadota 1996). The pathogen was primarily described by Manns (1909) as the causative agent of blade blight of oats in Ohio, United States. Disease spreads in upland and wetland areas (nurseries). However, it is mostly distributed in the rice-growing countries (Shakya et al. 1985) and considered as a minor disease of rice. *A. avenae* ssp. *avenae* is seed-borne and *P. syrinage* pv. *panici* is likely to be seed-borne and can easily be recovered from seeds.

Indiscriminate symptoms create difficulty in detection and diagnosis. After the infected seedlings were transplanted, symptoms were obscure. Natural occurrence of the disease was not seen after the tillering stage, except transplants in submerged condition (Kadota and Ohuchi 1983). Moreover, similar symptoms were carried out by several other rice bacterial pathogens like *P. fuscovaginae*, *B. glumae* and *P. syringa*e pv. *syringae* and making detection tests difficult for interpretation (Song et al. 2004).

4.3.4 Bacterial Leaf Streak

Bacterial leaf streak (BLS) is another severe bacterial disease of rice caused by *X. oryzae* pv. *oryzicola*, in high temperature and high humidity areas. Feng and his co-workers in 1957 named the disease as bacterial leaf streak which was earlier

termed as stripe disease (during 1918) in Philippines. Disease prevalence is in tropical and subtropical regions of Asia, Africa (including Madagascar), Australia and South America in both low land and upland areas and absent in temperate countries (including Japan). It is one of the most important rice diseases in China (PRC) in hybrid rice varieties (mostly) and cannot be coherently managed (Zhang et al. 2005). The yield loss ranges from 8–17% and 1–3% during wet and dry season, respectively. Under preferable conditions BLS may affect entire fields and causes the grain weight loss up to 32% (Ou 1985).

Pathogen has the ability to infect the host plant in various growth stages. The mature plants may easily recover the disease with minimal yield losses while the early growth stage tillering to panicle initiation if infected affects the most. All the wild *Oryza* sp. may be infected by *X. oryzae* pv. *oryzicola* and also serve as reservoirs of pathogenic inoculum. The pathogen hibernates under the glumes in mature seeds and surpasses seasonal variation up to appropriate condition. Bacterial exudates from lesions are dispersed primarily by windblown rain and splashing and also by direct (leaf) contact and water irrigation (Zhang et al. 2012).

4.3.5 Sheath Brown Rot

As a devastating bacterial disease, Sheath brown rot is caused by *Pseudomonas fuscovaginae* (erstwhile *P. marginalis* [*P. fluorescens biovar II*]). It was first detected in Japan and further spread throughout the world mostly in temperate regions of Asia (including China), Africa, Australia and Latin America (Cother et al. 2009) as seed-borne disease (Duveiller et al. 1990).

During infection at early seedling stage the seedlings died and in later stage infection the (whole) leaf sheaths become necrotic followed by discoloured, i.e., light green or yellow to brownish or dark brown, and empty panicles and/or grains (Cottyn et al. 1994). The level of disease incidence varies (5–62%) along with the level of yield loss (up to 72.2%) (Cahyaniati and Mortensen 1997). Use of infected rice seeds in cropping fields is the vital cause of disease spreading (Razak et al. 2009).

4.3.6 Bacterial Sheath Rot

Bacterial sheath rot is considered as one of the major diseases of rice caused by *Pseudomonas syringae* pv. *syringae* (syn. *P. oryzicola*) and outbreak throughout the world. This disease is prevalent in Australia, Hungary and the tropical regions of Asian continent (Zeigler and Alvarez 1990). Brown and elongate spots on the sheaths are generally regarded as the disease symptoms and somehow similar to sheath brown rot caused by *P. fuscovaginae*. The disease causes grain sterility and serious yield loss in rice grown on upland areas (Rostami et al. 2010).

4.3.7 Grain Rot/Seed Rot

Burkholderia glumae (syn. *Pseudomonas glumae*) and *Burkholderia gladioli* (syn. *Pseudomonas gladioli*) are responsible for the bacterial grain rot/seedling rot. The disease mostly affects rice cultivation in South-East Asian and oriental countries like Korea, Japan and Taiwan (Chien et al. 1983). In the epidermis of the plumules of growing seedlings disease appears. It can be found in the upper leaf sheaths, including the flag leaf sheaths prior to heading stage, although symptoms are absent on leaf blades or leaf sheaths. It then invades the flowering spikelets, multiplies rapidly and finally causes the disorder (Hikichi et al. 1994; Maeda et al. 2004).

4.3.8 Bacterial Palea Browning

Bacterial palea browning disease is a region-specific disease and found in oriental countries. *Pantoea agglomerans* (syn. *Erwinia herbicola*) and *Pantoea ananatis* are the main causative agents of the infection. Temperature in 30–35 °C range and prolonged rain may increase the severity. Anthers are the optimal proliferation site of *P. ananatis* during the flowering stage (Azegami 2013). The disease discolours the palea to dark brown, but often the lemmata are also discoloured. The infected spikelets produce brown unpolished rice of lower quality. The disease incidence ranges between 15 and 32% (Saha et al. 2015).

4.3.9 (Bacterial) Foot Rot

Rice (Bacterial) foot rot is mainly caused by *Dickeya chrysanthemi* (erstwhile *Erwinia chrysanthemi* Burkh., McFadden and Dimock). It mostly occurs in tropical countries including India, Bangladesh, Korea (North and South) and the Philippines (Goto 1979). Earlier it was confused with the "kresek" phase of bacterial leaf blight due to similar in pattern of disease symptoms. The pathogen invades the base of culm followed by crown. It was also restricted to the leaf sheaths of few tillers (Goto 1979). The roots attached to infected nodes gradually decay and fall. In some cases, the young leaves of tillers that show no sheath browning may wilt as a result of systemic infection of the crown alone. Particularly in Japan, plants grown around the rice fields serve as inoculum source as the pathogen has multihost infecting capacity. In addition, the bacterium is also found in rhizosphere of healthy plants expected to be there due to irrigated water (Saha et al. 2015).

4.3.10 Black Rot

Xanthomonas campestris pv. *oryzae* and *Pseudomonas itoana*, n. sp. cause black rot disease of rice. The disease occurs least in basal part, often in middle part and mostly in apical portion. The pathological change occurs in the seed coat and the upper parts

of the endosperm, while the blackening/rot certainly not advances into the inner parts of the grain. The affected tissues turn black in colour and died. However white spots appeared as a disease symptom on the leaves of the host plants (Monteiro et al. 2005).

4.3.11 Seedling Rot

The infection of seedling rot is due to *Burkholderia glumae* (syn. *Pseudomonas glumae*). Flowering and seedling stage are the most appropriate growth stage for pathogen invasion. Rotting of seedlings occurs near the base in the breeding seedling box(es). As a symptom leaves and leaf sheaths turn white to light brown and then gradually turn yellowish brown and leaves sometimes become abnormally stretched. The pathogen is also known to cause browning on leaf sheaths. The disease is confined to Asian crop fields (Cho et al. 2007).

4.3.12 Halo Blight

Halo blight is a minor disease of rice caused due to *Pseudomonas syringae* pv. *oryzae* infection. In the year 1985, it was first reported at districts of Aomori Prefecture, Japan. During tillering stage, the disease developed and further dormant in the booting stage. In infection small lesions appeared on leaf blades and further united to large blotches on further development process (Kuwata 1985).

4.3.13 Cinnamon Speck of Rice Grains

As minor disease of rice this is confined to Asian continent only as per reports available. It is caused by *Xanthomonas cinnamon* (Miyake and Tsunoda) Muko and optimum temperature required is 20–45 °C. The pathogen usually targets the embryo. The grains appeared cinnamon in colour, exerts a distinct odour and are brittle (Ou 1985).

4.3.14 Black-Eye Spot

Xanthomonas atroviridigenum (Miyake and Tsunoda) Tagami and Mizukami causes black-eye spot, a minor disease of rice. The infected, unhulled grains are comparatively smaller in size, dark yellowish-brown to black in colour in case of severe infection (Ou 1985). Similarly, hulled rice is also discoloured partly or entirely, yellowish-brown, brown or black. Kernels may also be shrunken, embryos black or damaged.

4.3.15 Bacterial Panicle Blight (BPBR)

Bacterial panicle blight of rice (BPBR), an emerging rice disease, is caused due to *Burkholderia gladioli* (syn. *Pseudomonas gladioli*) and *Burkholderia glumae* (syn. *Pseudomonas glumae*) infection worldwide. Gradually increase in temperature and high rainfall during cropping season increases the disease epidemics. In 1956, the disease was identified in Japan and further spread throughout the world as a result of fluctuations in climatic conditions, changes in cultivation systems, abrupt use of fertilizers and underplanned water management as well as mismanaged swapping of rice varieties/hybrids/combinations and ultimately leads to yield loss substantially in Asia, Africa and American continents (Li et al. 2017).

B. glumae cells colonized the surface of the lodicule and the inner surface of lemma as well as infect almost every part of the plant. They can also incubate as endophytes prior to booting stage of the host. In addition, the pathogen can also colonize abundantly on glume hairs during the early infection of glumes. The bacterial cells multiply on the surface of glumes and penetrated inside to the palea and lemma (Tsushima et al. 1987; Hikichi et al. 1993). It produces phytotoxin (yellow pigmented), toxoflavin and similar as a main virulence factor/s (Sato et al. 1989).

4.4 Pathogenesis: A Quick Molecular Approach

Each year million metric tons of crops get loss due to vast quantity of microbial disease. Based on the rapid strength of plant response to pathogens plant resistance is further separated into two types, partial/quantitative resistance and complete/qualitative resistance (Kou and Wang 2010). Complete resistance is having pathogen specific and can be strengthened through presence of a single *MR* gene also called as major disease resistance gene. The partial resistance is carried out through multiple gene complexes and are pathogen nonspecific. Due to most resistance and stress-free manipulation commonly the complete resistance has been widely targeted for field applicability.

During onset of pathogenic attack, the host plant resisted through a double line defence innate immune mechanism. The primary one is known as pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and the other one is effector-triggered immunity (ETI) (Jones and Dangl 2006). During host–microbe interactions pathogenic microbe -produced PAMPs and/or damage in host plants created by plant peptides or cell wall fragments released during pathogen invasion recognized by plant pattern recognition receptors (PRRs) localized in membrane part to initiate PTI. The self-defence through this kind is generally weak and acts as community-nonspecific and thus also recognized as basal resistance (Thomma et al. 2011). Non-susceptible pathogens can surpass PTI using effectors secreted in cells of host plant. Host plants having resistance (R) proteins can initiate ETI by direct or indirect sensitivity of specific effectors. ETI is pathogen community-specific and exerts a maximum resistance generally, and thus it is also called gene-for-gene or

community-specific resistance. Partial resistance can be regulated by either R genes functioning in ETI or PRR genes functioning in PTI. Genes initiating complete resistance are denoted by MR.

4.5 Biocontrol Soil Bacteria Against Rice Bacterial Phytopathogens

Plant bacterial diseases management need a thorough understanding of the pathosystem for identification in apposite timings of host susceptibility as well as pathogens multiplication. An integrated management approach of cultivating more resistance cultivars or utilization of plant host resistance, application and regular upgradation of biological and or chemical controls, and inoculum reduction by modification in cultural practices represents the best strategies for useful and sustainable disease management. Overall, these multidisciplinary integrated approaches depend on the availability of suitable host plants, efficacious controls (chemical and biological), and feasible cultural practices and lastly economically acceptable so that growers will practically use them (Sundin et al. 2016).

Biocontrol simply denotes suppression or inhibition or control of harmful pathogens by other bioforms/lifeforms/antagonistic organisms. The bacteria as biocontrol agents occupy a certain ecological niche, and their functions are dependent and target specific. Biocontrol bacteria can be isolated from paddy water, diseased and healthy plants (both from uplands and irrigated lands), healthy and contaminated plants and rhizospheric soils. Both culturable and unculturable methods indicated maximum biocontrol bacterial population in rhizosphere soil followed by paddy water.

Bacillus bacteria as a biological control agent exhibited antagonism on a wide range of plant pathogens and also promoting plant growth. Reports are available on *Bacillus* sp. isolated from soil near or from rice fields (irrigated) and rice rhizosphere/endorhizosphere develop $<70\%$ protection against rice bacterial diseases in comparison to isolates from other different sources (Yang et al. 2008). *Bacillus* sp. executed biocontrol activity roughly through two approaches, i.e. direct approaches and indirect approaches and sometimes a cumulative approach of it (Fig. 4.2). Furthermore, the direct approaches are as a result of antagonism/antibiosis, competition, lytic enzymes, rhizosphere colonization and plant growth promotion and stress effects through toxic compounds while indirect approaches inclusive of elicitation of plant-mediated response for resistance (induced and acquired). Physical contact and/or a high degree of selectivity for the pathogen by the mechanism(s) expressed by the biocontrol agents are basics for direct approach whereas indirect approaches stimulates plant host defence pathways by non-pathogenic plant chemical agents while not targeting any particular pathogen.

Fig. 4.2 Soil bacterial interactions with rice pathogens

4.5.1 Antagonism/Antibiotics

Antibiotics are the chemical compounds produced by bacteria for antagonism even if at low concentration. For optimum biocontrol effect it must be produced in ample quantities in disease premises near the pathogen (Weller et al. 2007; Mavrodi et al. 2012). Sometimes the antimicrobials synthesized by antagonistic bacteria are of 50 amino acids (small antimicrobial peptides) or smaller which are involved in interpopulation competition and by plants as part of the innate immunity system in response to microbial challenge. Mostly these peptides are cationic and involved in protein or nucleic acid production (Brogden 2005). Research is still going on for more than two decades involving strategies of prolonged applications of small peptides for the management of rice bacterial pathogens and use of indigenous antagonists that secrete peptides as a biological control agent.

Bacillus sp. excretes antimicrobial compounds like zwitermicin-A, kanosamine, lipopeptides and polyketides active against an array of rice bacterial pathogens (Stein 2005). Surfactin is another polypeptide that decreases surface tension of water and exerts detergent-like actions on biological membranes (Carrillo et al. 2003). The role of bacilysin A (dipeptide antibiotic) is to block glucosamine synthetase, an essential enzyme for biosynthesis of cell wall. *B. amyloliquefaciens* secreted surfactin, bacillomycin and fengycin, three lipopeptides which induce systematic resistance against *X. oryzae* pv. *oryzicola* pathogens (Kakar et al. 2014b.

4.5.2 Competition

Inter- and/or intra-species competition is the fundamental characteristic of microorganisms. Pathogens and biocontrol bacteria compete for nutrients, space, etc., and fundamentally leads to biocontrol. Soil and rhizosphere have nutritional limitations. So for successful colonization a microbe must effectively compete for nutrients bioavailable (Shafi et al. 2017). Biocontrol agents are able to decrease the availability of resources to the pathogens because of their efficient and quick utilizing capacity. Siderophore is an Fe chelating compound produced by biocontrol bacteria under iron-limiting conditions for competitively acquiring Fe^{3+} (Whipps 2001). Phosphate solubilization and Zinc solubilization are few more competitive advantages of biocontrol bacteria over pathogens. Similarly, rhizosphere competence is considered as a prerequisite of effective biocontrol.

4.5.3 Lytic Enzymes

Enzymes play an important role in the biocontrol ability of bacteria and are improved by transformation with lytic enzymes. These are extracellular and hydrolytic in nature and suppress the plant bacterial pathogens. These hydrolase(lytic) enzymes utilize the nutrient in substrate and transform unavailable form to bioavailable form. *Bacillus* sp. is capable of producing enzymes like β-1,3-glucanase, superoxide dismutase, phenylalanine ammonia lyase (PAL) and polyphenol oxidase (PPO) and similar defence-related enzymes. PAL has an important role in the formation of phenolic compounds like lignin and flavonoid. It also plays a key role in phenylpropanoid biosynthetic pathway (Hahlbrock and Scheel 1989), while PPO acts as catalyst in phenolic compounds oxidation and creating an unfavourable environment for pathogen development. As per reports, the synthesis of phenolic compounds in plants due to infection is associated with resistance and increased accumulation of phenolics and increases in PAL activity which involves disease protection (Jayaraj et al. 2004). Catalase, an oxygen-scavenging enzyme, protects cells from the toxic effects of H_2O_2 during development (Choodamani et al. 2009). Finally, the extracellular enzymes in combination with some other compounds overcome the competition and other pathogenic hindrances.

4.5.4 Rhizospheric Colonization and Plant Growth Promotion

Rhizospheric colonization capacity and potential to grow and proliferate over a substantial time period, in the presence of the indigenous microflora, results in close associations which provides a selective adaptation directly (Lugtenberg and Dekkers 1999; Parke 1991). In addition, the biocontrol agents have ability to grow on various sites/substrates, whether shoot region (fruit, seed surface, shoot area or stump) or root region, and provide protection for pathogenic infection (Parke 1991). Plant growth promoting rhizobacteria (PGPR) biocontrol through are competition

for an ecological niche and substrate, production of allelochemicals with inhibitory effect and through ISR to a broad spectrum of rice bacterial pathogens (Compant et al. 2005). *Bacillus* sp. as PGPB is found to suppress *X. oryzae* infection (Chithrashree et al. 2011).

4.5.5 Stress Effects Through Toxic Compounds

The mechanism of disease control is also involved in suppression of pathogens by stress effects produced by biocontrol agents for sustainable disease management. Volatile organic compounds (VOCs) like HCN are able to suppress the pathogens solely or in assembly with other compounds. Additionally, cyanide producing bacterial strains induce resistance in the host and restrict the pathogenic invasion (Devi and Kothamasi 2009). The bacteria are able to hydrolyse starch, produce cyanide, and also have glucanase activity. Glucanase activity is likely to play a role in direct antagonism (Gustavo et al. 2012).

4.5.6 Systemic Resistance

Indirect approach for plant defence mechanism is also exhibited by some biocontrol agents. It is represented in two different categories/ways: systemic acquired resistance (SAR) and induced systemic resistance (ISR). Practically, SAR is induced in the presence of pathogens while ISR is salicylic acid (SA)-independent and is induced through nonpathogens (*van Loon* et al. 1998*)*. During infection salicylic acid (SA) is frequently produced and proteins such as PR-1, PR-2 and some peroxidases are also expressed (Kageyama and Nelson 2003; Park and Kloepper 2000; Ramamoorthy et al. 2001). As a mechanism of protection PR proteins destroy the infected cell, support cell membranes to resist further infections, and/or induce localized cell cessation. Certain cases peroxidase, phenylalanine ammonia lyase, phytoalexins, polyphenol oxidase, and/or chalcone synthase enzyme accumulation increases instead of PR proteins and increases higher accumulation of total phenols (Chen et al. 2000; Jain et al. 2011; Nagendran et al. 2013) in infection.

However, certain nonpathogens or specifically as PGPR strengthen plant cell wall, activate various cellular defence responses and strengthen defence-related gene expression through altering in host plant physiology and metabolism (Nowak and Shulaev 2003; Ramamoorthy et al. 2001). ISR involves the priming of rice plant defence against *P. syringae* and *X. oryzae* pv. *oryzae* through the actions of certain rhizospheric *Bacillus* sp., and is conferred through plant hormone-mediated signalling (Pieterse et al. 2014). Surfactins and fengycins lipopeptides from *Bacillus subtilis* S499 and *B. amyloliquefaciens* act as ISR elicitors (Ongena et al. 2007) and increase resistance for *Xanthomonas* sp. infection (Kakar et al. 2014b). Application of *B. amyloliquefaciens* increased yield of rice by >50%, due to enhanced plant health for ISR, and also inhibited the *P. fuscovaginae* infection in vitro (Vasudevan et al. 2002). Under demanding conditions resistance level was higher

than in non-demanding situations; therefore, ISR is highly advantageous for biological control of rice bacterial pathogenic diseases.

In addition to the above explained mechanisms few more techniques are employed by the biocontrol bacteria. Biofilm formation and suppression of plant pathogens is another technique of biocontrol along with alter in plant hormone concentration and increase in biological N_2 fixation. *Bacillus* sp. are expert in biofilm formation and reports suggested that the endophytic colonization and biofilm formation improves the ability to act as a biocontrol agent against plant pathogens (Nayak et al. 2020; Kakar et al. 2014b).

4.6 Mode of Action

The understanding on biocontrol mechanism is vital for its triumphant development and to progress its efficacy. Spread in use for suppressing different plant diseases basically involves various mode of action and their possible combinations with other control methods (Correa et al. 2009; Sharma et al. 2009). Biological control is considered as a positive response amongst all the explicit and vague biointeractions. Biointeractions like amensalism and/or commensalism, competition, mutualism, neutralism, parasitism, predation and protocooperation naturally occur and *Bacillus* sp. employs these for bacterial biocontrol (Schallmey et al. 2004). Frequently the biocontrol agents control not only through single mechanism of action, but also with synergism, where the importance of each one is relative and is conditioned by few natural factors. Simply the mode of action has been considered significantly in the biocontrol of rice bacterial pathogens with *Bacillus* sp. have been divided into two categories such as (1) deterrent colonization and (2) antagonism through noxious by-products. Each of these mechanisms are interconnected and affects the interactions and accuracy for intime application(s).

The activation of host defences also has a role against antagonistic microorganisms (Droby and Chalutz 1994). Direct interaction of the *Bacillus* sp. with the pathogen hyphae does not tacit higher antagonistic activity, whereas in vitro extracellular enzyme (β-1,3-glucanase and few types of chitinases) activity might account for higher levels of armamentship. In few instances the pathogens are inhibited by metabolites through obstructing protein synthesis in lieu of nucleic acid synthesis. A better understanding of the antagonists' mode(s) of action is an important prerequisite both for improving their performance and to establish screening criteria in the search for emerging isolates.

Competition is a defensive mechanism of action for nutrients and space suitably applied to pathogenic inhibition without affecting self. In connection to that the assimilation of crucial nutrients such as nitrogen compounds, Fe, P, Zn, N, C, or O confers the capacity to limiting the pathogen growth. Similarly, direct interaction like hyperparasitism interferes with the growth and progress of the diseases. Quorum quenching or quorum sensing inhibition approach is another factor obstructing pathogenic spread. N-Acyl-L-homoserine lactones (AHLs) are the most common and best known small signalling molecules amongst pathogens. Some species of

Bacillus are able to produce AHL lactonase, an enzyme for rapid hydrolysis of the AHL ring. The quorum-sensing-dependent virulence of *Erwinia carotovora* has been repressed by soil isolate *B. thuringiensis* (Dong et al. 2004).

4.7 Advantages and Limitations

Chemical control of rice disease is a traditional as well as economically feasible process which is needed for food security and agri-based industries. As per environment is concerned the prolonged use will affect the ecological balances, bioaccumulate and affect the organisms of higher tropic level. Biocontrol agents have been applied with a slender focus on preparations containing microorganisms (living), through to a wider definition which includes microbial compounds/metabolites and chemosimilars. The *Bacillus* sp. based products have longer shelf life and have advantage of endospore production. Since no major direct effect exerted on pathogen and, efficacy lowered in high pathogens innocula in soil or against very competitive soil-borne pathogens. In several *Bacillus* sp. the highest efficacy is observed on growing stages as seeds or plantlets, because of the advantageous combination of antagonism, resistance and growth promotion.

The biocontrol agents have several advantages and other benefits.

1. No or meagre toxins are produced, so safer both for the environment and applying person(s).
2. It is economically possible with less production cost than chemical agents and quite simple in manufacture.
3. Target specificity and used as synergism with biofertilizers.
4. Having a wider range of pathogen affectivity.
5. Safe for the host plant, enhances the host resistances and involved in PGPR activity. Also mobilizes plant nutrients and make it bioavailable for the plants.
6. Microbial biocontrol is self-regulating, does not require any stringent management practices and also retains the ecosystem integrity.

As every merit has some drawbacks so the biocontrol agents have also few adverse effects on environment. An efficient biocontrol agent applied beyond its native area may behave as a predator for some indigenous beneficial organisms. Few more are listed below.

1. As it is solely depending on environmental conditions, minute variations in surrounding affect its efficiency.
2. Biocontrol agents are mostly preventive measure rather than a curative measure against diseases.
3. Shifting of host, i.e. spreading out host range, attacks native organisms.
4. The efficiency of control varies with place and time, pathogenic location in host & symptom and cause of disease involve.

5. Few legal and ethical issues are cognate with the release of genetically modified/engineered organisms (GMOs) in practical field application as an environmental concern.

4.8 Effects on Soil Health and Rice Productivity

For the last century fertilizers are being used for better production. This ultimately leads to influence the soil microbial community. Mostly the N and P fertilizers are applied to the fields either in single or in complex mixture forms. As these chemicals are continuously applied to soil it is necessary to manage their effects on soil and surrounding environment (Nayak et al. 2018).

The indigenous soil microbial community and their activity for the soil ecological processes are straightly affected as well as inhibited by fertilizers, bactericides, fungicides, etc. (Grant and Wu 2008). Soil microbiota influence more than 80% of soil biotic processes in terms of fertility maintenance, nutrient cycling, disease control, etc. Moreover, the bactericidal application for foliar and shoot diseases triggers few antimicrobials and VOCs production in the rhizosphere and the above ground parts, respectively (Nayak et al. 2017). Reports are sparsely available on microbial diversity and soil quality. Scientifically the total soil microbial community including the quiescent microbes, i.e. having functional redundancy, maintains soil health and functionality. Determination of diversity structure and function of microbial communities is a usually referred index of soil status (Baliyarsingh et al. 2017).

Soil microbial community in influenced by soil properties and vegetation. However, it also acts in response to minor variation in the environment naturally or manmade. Due to pathogen-specific interaction, soil *Bacillus* sp. have minor to transient effect on the soil-inhabiting microflora and microfauna as biocontrol agents. These are specific bioaccumulate, biodegradable and with having less/no half-life. So superior strategic implementation is required for pathogenic inoculum reduction and amelioration in soil and plant health which is resulted after inoculation of biocontrol agents. The inoculated agents inhibit bacterial pathogens, improve some abiotic and physiological stress responses and enhance plant growth and yield. Also attenuates the other microbes due to buffer effect on available nutrients helps to recover the originally existed native population. In comparison with chemical fertilizers increase total soil microbial load/biomass, active synergism effect and also enzyme activities and biomass carbon (Bajsa et al. 2013). The field trial of biocontrol formulations should be expeditious to rectify the effect on excipients. Presently, *Bacillus* sp. as biocontrols used against rice bacterial phytopathogens extensively is a clear indication of the safety and usefulness of it.

4.9 Conclusion

The indigenous soil microbial communities along with the exotic ones and native as well as exotic soil microbial communities affect soil health, nutrient balancing, plant growth and yield and ecosystem functioning in depth. Thus, it is vital to know the value of interrelationship in between agricultural practices and soil microbial communities. Predominantly plants are contaminated by soil pathogenic bacteria present in the rhizospheric region. The matter of extreme concern is to protect rice, the necessary food for more than half human population from overwhelming bacterial phytopathogens with altered levels of pathogenicity. Scenario is worsened by agrochemicals added which not only contaminate the environment but also increases pathogenic resistance. Soil dwelling *Bacillus* sp. paves an accurate, alternate, economically and eco-friendly path to alleviate the complication. By updated mechanisms, mode of action and modified physiology it is significant to reveal that members of *Bacillus* sp. always combat against different rice bacterial pathogens comprehensibly as throughout described.

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An Overview of Soil Bacteria for CO_2 Sequestration

5

Muhammad Kashif Shahid, Ayesha Kashif, Prangya Ranjan Rout, and Younggyun Choi

Abstract

The emission levels of carbon dioxide (CO_2) and other greenhouse gases into the atmosphere are increasing rapidly due to different anthropogenic activities. This increased concentration of CO_2 is the foremost reason of climate change and global warming. Carbon sequestration is known as long-lasting storage of carbon in soils, oceans, vegetation, and geologic configurations. CO_2 capture and sequestration include several techniques for reducing the CO_2 emissions from the atmosphere and to manage the carbon cycle. The soil carbon cycle significantly depends on the activity of CO_2 fixing bacteria. This chapter discusses the soil microbial communities that contribute in CO_2 sequestration. The structure characteristics of bacteria and the mechanisms/pathways involved in CO_2 are discussed. A special attention is given to the effect of ecological factors (e.g., soil characteristics, pH, soluble organic carbon, soil organic carbon, total nitrogen and cation exchange capacity) on the CO_2 sequestration. The influence of long-term exposure to CO_2 on the characteristics of soil microbial community is also

Muhammad Kashif Shahid and Ayesha Kashif contributed equally with all other contributors.

M. K. Shahid (✉)
Research Institute of Environment and Biosystem, Chungnam National University, Daejeon, Republic of Korea

A. Kashif
Department of Senior Healthcare, Eulji University, Daejeon, Republic of Korea

P. R. Rout
Department of Biotechnology, Thapar Institute of Engineering and Technology, Patiala, India

Y. Choi
Department of Environmental and IT Engineering, Chungnam National University, Daejeon, Republic of Korea

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deliberated. Overall, this chapter explains the nature, characteristics, and activity of soil microbial community involved in CO_2 sequestration with special focus on geological factors and sequestration mechanism.

Keywords

Bacterial communities · Carbon sequestration · CO_2 emissions · Enzymatic actions · Geological changes · Soil organic matter

5.1 Introduction

Climate change is the major challenge of the time (Fekete et al. 2021). The increasing level of greenhouse gases (GHG) emissions in the atmosphere and their adverse effects are the primary contributor in climate change (Gernaat et al. 2021). Beside other GHG, carbon dioxide (CO_2) covers the foremost area in the technosphere and significantly disturbs the ecosystem. The only solution to global warming is to minimize the CO_2 level in atmosphere and subsequently establishing an ecofriendly and sustainable system (Takht Ravanchi and Sahebdelfar 2021). To meet this objective, scientists and researchers are working on the multiple approaches for lowering the level of atmospheric CO_2, such as CO_2 capture and storage (Ko et al. 2021; Li et al. 2021a).

Carbon sequestration is the long-term storage of carbon in soils, plants, ocean, and the geological formations (Wang et al. 2021a). Both natural and anthropogenic activities can be used to accomplish the carbon sequestration. Generally, this process deals with the storage of carbon, which has the instantaneous tendency to turn in CO_2. Considering the challenges of climate change due to the rising level of atmospheric CO_2, substantial attention has been given to the carbon capture and storage perspectives (Oelkers and Cole 2008). Several physical, chemical, and biological processes have been introduced and applied to capture the atmospheric CO_2 (Cannone et al. 2021; Kundu and Sarkar 2021).

CO_2 discharge from soil can be controlled by the implementation of such practices that can increase carbon input in soils and likewise can decrease the potential of organic matter decomposition (Zimmerman et al. 2011). Generally, the exhaustive agricultural practices cause significant loss in soil carbon and soil degradation, especially because of unfortunate crop and soil management applications (Lehmann et al. 2006). In terms of agricultural soils, carbon sequestration signifies the rise in soil carbon storage. Major agronomic and associated practices, which can contribute in carbon sequestration, are highlighted in Table 5.1.

Agriculture areas can contribute significantly in reducing GHG due to the higher potential of agricultural soils to serve as sink for CO_2 sequestration (Kelland et al. 2020). It is noteworthy that the soil with greater organic content can display greater potential of CO_2 sequestration (Haque et al. 2020). Soil microbial activities

Table 5.1 Major agronomic and associated practices and the advantageous of soil carbon sequestration

Main agronomic practices	Advantageous
Assimilation of shelter crops; no or minimum tillage adoption; ecofriendly and soil health friendly cultivation setup; mulch consumption (synthetic material or crop residue); consumption of organic amendments; reduced water and soil losses via erosion and surface runoff; integrated nutrient management to improve soil fertility; upgraded farm forestry	Reduces GHG and CO_2 emissions; decreases the atmospheric temperature; minimizes the nutrient loss; maintains appropriate biotic habitat; improves water conservation; enhances soil health; minimizes soil erosion; facilitate and maintain root growth

facilitates the biological carbon sequestration due to their role in enhancing the soil biological, chemical, and physical properties of the soil (Shi et al. 2021).

The soil biota, a living portion of soil, contains a higher number and a variety of microbes (Remke et al. 2021). These organisms interact with plants and with each other, straightforwardly offering nutrition and other assistances (Smith et al. 2021). They are also liable for decomposition of organic matter and for the conversions of organically bound N and minerals, which are accessible to plants. Owing to the biological control pathways, these organisms control their own growth rate and of arriving microorganisms. Micro- and macro-organisms, such as bacteria, are of fundamental importance in regulating ecosystem function, and their growth and populations are considerably influenced by the diverse crop management practices (Wang et al. 2021b). Bacteria are influenced by the soil management in the forest and agricultural ecosystems (Guron et al. 2021). Different soils assist differently in persistence and development of distinct bacterial species. A study reported the greater value (49.9 g C kg^{-1}) of carbon sequestration for the soil rich in bacteria (Bailey et al. 2002). Hence, the utilization of range of microorganisms that are advantageous for soil and environment can improve soil carbon sequestration and the yields of crops.

5.2 CO_2 Storage and Sequestration

Carbon sequestration discusses natural and managed procedures that either eliminate CO_2 from the atmosphere or distract from origin of emission, and preserve in the geological formations, oceans, and terrestrial environments such as soils, sediments, and vegetation (Wu et al. 2021; Davies et al. 2021). The decomposition of dead animals and plants is the natural practice responsible for releasing CO_2 in the atmosphere. Anthropogenic activities, including burning of fossil fuels, are responsible for increasing CO_2 level into the atmosphere through exploitation of its enduring geologic storage as natural gas, petroleum, and coal (Shahid et al. 2020; Ahmad et al. 2021).

The history of an increased atmospheric CO_2 concentration goes back to the early footsteps of industrialization associated with an excessive consumption of fossil

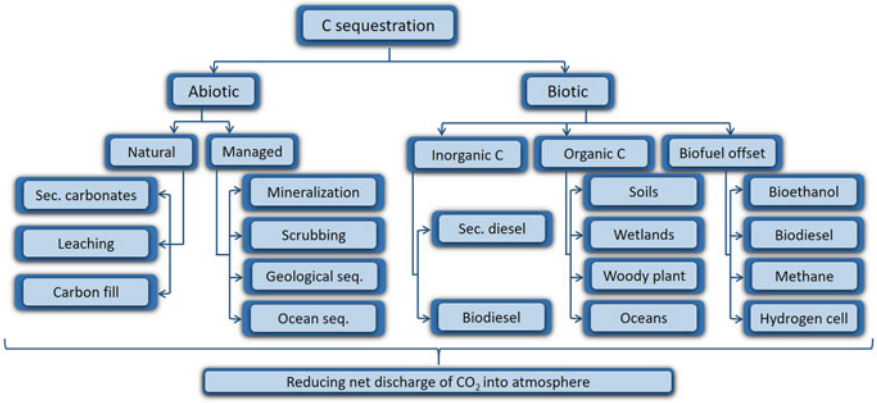

Fig. 5.1 Biotic and abiotic technologies applied for carbon sequestration

fuels. CO_2 is a major GHG and it absorbs the infrared (IR) energy released from the surface of earth. As level of CO_2 increases in the atmosphere, CO_2 possess the higher potential of absorbing greater IR energy (Li et al. 2021b). This causes an increase in the average temperature of lower atmosphere of earth, referred as global warming.

Prior to industrialization that resulted in excessive GHG and CO_2 emissions, the global carbon cycle was well managed by nature, i.e., there was a balance between intake and discharge of CO_2 to the atmosphere. Whereas, now the higher discharge of CO_2 into environment deliberated the need for the systems, which integrate the lowering discharge and increasing storage of CO_2. Many processes are known for the CO_2 sequestration. The selection of an individual or integrated process is imperative for voicing the energy strategies for future economic development at domestic and international scales. These choices can be categorized into biotic and abiotic carbon sequestration (Fig. 5.1). Abiotic sequestration contains several physical and chemical processes coupled with or without engineering practices, in the absence of any involvement of living entities such as microbes and plants. Biotic sequestration involves the microorganisms and plants to confiscate the atmospheric CO_2.

To reduce the level of CO_2 in the atmosphere, a variety of reservoirs are used to retain and store the carbon in natural or other way. Such reservoirs, known as carbon sink, significantly contribute in reducing the CO_2 level in the atmosphere (Loisel et al. 2021). Considering the global perspective, ocean and vegetation are two major carbon sinks (Phyoe and Wang 2019). For instance, forests work as carbon sink; therefore, forestation is the type of carbon sequestration. However, deforestation results in the higher carbon discharge into the atmosphere.

Naturally, carbon moves from atmosphere to the terrestrial carbon sinks via photosynthesis (Du et al. 2021). It is noteworthy that carbon sequestered in above ground vegetation and soils can also be entered back to the atmosphere due to the climate change or land use. For example, decomposition (results from microbial actions) or combustion (caused by fires) can result in the discharge of stored carbon

to the atmosphere. In both cases, CO_2 forms due to the interaction of atmospheric oxygen and carbon stored in plant tissues.

Over 66% of the organic carbon preserved in terrestrial ecologies is a part of soil organic matter and the flux of carbon through soil to the atmosphere is about 60 Pg (Peta gram) per annum (Schlesinger 2005). As compared with virgin and uncultivated soil, the higher carbon loss is estimated from the cultivated soils due to an excessive cultivation (Cole et al. 1997). The tendency of an agricultural soil to recover the lost carbon can assist in enhancing the soil fertility, lowering erosion, and inhibition of CO_2 discharge. Several studies reported an interaction between decomposition of soil organic matter and soil microbes (Błońska et al. 2021). It is highly important to determine the effect of different microbial communities on development and stabilization of diverse soil organic matter constituents found in an agricultural soil.

Generally, the carbon level of soil is estimated using balance among organic matter additions (roots, root exudates, plant residue) and the loss of organic matter due to leaching, erosion, and decomposition. Usually, over 90% of soil microbes is composed of bacteria and fungi which contribute in the decomposition of soil organic matter (Zhang et al. 2021). As the soil microbes are main controllers of dynamics of soil organic matter and nutrient accessibility, changes in composition and function of the microbial community (in result of various agronomic practices) can contribute significantly in estimating the carbon loss from the soil.

5.3 Bacteria-Based CO_2 Sequestration

The fundamental biological process involves the decomposition or breakdown of animal and plant residues in the soil. This process involves the transformation or conversion of different matters into other forms, such as ammonium is generated from nitrogen and the recycling of carbon takes place as CO_2 (Munira et al. 2016; Cheng et al. 2017). Being a sink and source of mineral nutrition, microbes contribute significantly in nourishing the soil productivity (Srivastava et al. 2020; Muñoz-Arenas et al. 2020). The natural carbon cycle is greatly reliant on microorganisms. The microbial communities perform key roles in stimulating plant growth, fixing atmospheric carbon, and conversion of organic matter present in the environment (Ishii et al. 2015). Currently, the greater quantity of organic carbon is wrapped in tropical forests, grassland soils, high-latitude permafrost, and other ecologies (Guillaume et al. 2021; Mishra et al. 2021). The microbial communities of soil perform basic role in estimating the durability and constancy of such carbon in these environments.

The application of biology in CO_2 sequestration not merely resolves the issue associated with higher consumption of energy, but also provides a variety of bio products such as biofuels, alcohols, and carboxylates (Adeniyi et al. 2018). Photosynthetic microorganisms have achieved notable attention to capture CO_2 and light energy to produce a range of products (Larkum 2010). These organisms may go through anoxygenic or oxygenic photosynthesis as the main pathway for CO_2

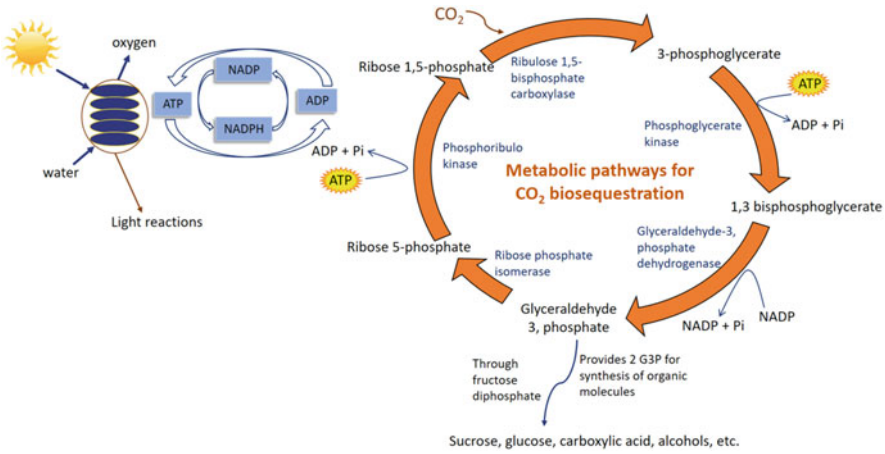

Fig. 5.2 The photosynthetic metabolic routes for CO_2 biological sequestration

sequestration. Photosynthetic bacteria are the major contributor in the CO_2 sequestration. These bacteria take reducing equivalents in addition to energy (ATP) that expedites the conversion of CO_2 to bio products as highlighted in Fig. 5.2 (Angermayr et al. 2015). Bacteria sequester CO_2 via carbon concentrating pathway by means of ribulose-1,5-bisphosphate carboxylase and carbonic anhydrase (Bharti et al. 2014). The photosystem of photosynthetic bacteria is quite simple as compared with algae and plants; however, their metabolic routes are distinctive and complex (Blankenship 2014).

Heliobacteria and green-sulfur bacteria that have type I photosynthetic reaction centers (use Fe-S clusters as electron acceptors) assist the reducing equivalents in the reverse oxidative tricarboxylic acid cycle (TCA cycle) for CO_2 fixing. Whereas, the cyanobacteria use the Calvin cycle for autotrophic CO_2 fixation (Tang et al. 2011). In both chemoheterotrophic and photoautotrophic organisms, glyceraldehyde-3-phophate (G3P) plays a fundamental role. In chemoheterotrophic bacteria, it functions as a prime intermediate for several anaerobic catabolic routes such as solventogenic, homolactic, and ethanolic fermentation (Angermayr et al. 2009).

Under specific environment, anaerobic bacteria sequester the CO_2 for metabolism (Agler et al. 2011). Contrary to the photosynthetic bacteria, these chemoautotrophic bacteria obtain energy and reducing equivalents via channeled enzymatic actions. CO_2 sequestration is assisted via catalytic action of gamma (γ) and zeta (ζ) carbonic anhydrase enzymes, which typically exist in anaerobic CO_2 sequestrating bacteria. The metabolic activity can result in the production of several products including alcohols, biogas, carboxylic acids, etc. based on catalytic activity of specific enzymes. Bioelectrochemical or microbial electrosynthesis systems also achieved a huge attention for CO_2 sequestration, where the chemolithoautotrophic bacteria used CO_2 as a source of an inorganic carbon (Salek et al. 2013; Ishii et al. 2015).

A study reported the anaerobic sulfate-reducing bacteria for improving the conversion rate of CO_2 into solid minerals for enhancing the long-standing carbon

storage (Paul et al. 2017). The anaerobic sulfate-reducing bacteria were found to have threshold level for the carbonate mineralization associated with CO_2 utilization. For instance, such study highlighted the potential for carbonate mineralization up to 14.7 psi pCO_2, whereas no carbonate mineralization is achieved at 20 psi pCO_2. This happened due to the mitigation of bacterial metabolism triggered by excessive CO_2, which induced the acidic environment. CO_2 contribute remarkably to lower the pH and turn environment in acidic conditions (Shahid et al. 2017). Another study reported the effectiveness of *Bacillus altitudinis* bacteria for CO_2 sequestration in soil (Nathan and Ammini 2019). The bacterial carbonic anhydrase enzyme is found to contribute in lowering the CO_2 level.

In conclusion, soil bacteria meaningfully assist in the transformation of organic matters and dead plant tissues into CO_2 or soil organic matter that can be located in terrestrial ecosystems for numerous years (Wang et al. 2021a). Soil microbial communities also enhance the soil aggregation that physically shields soil organic matter and thus circuitously affects the carbon cycle (Wei et al. 2021). Subsequently, interfaces among the structure of bacterial community, quantity of microbial biomass, microbial byproducts, and soil characteristics including clay mineralogy, aggregate dynamics, texture, and pore-size distribution effect the sequestration of carbon in terrestrial ecosystems (Juhos et al. 2021). Hence, to achieve the higher rate of CO_2 sequestration in soils, it is highly important to consider the reactions of bacterial community and ecological conditions governing the conversion process of organic carbon in soil.

5.4 Factors Effecting the CO_2 Sequestration

As multiple conditions are associated with the CO_2 sequestration such as pH, temperature, organic matter, and bacterial species, all these characteristics influence the CO_2 sequestration process either individually and/or conjointly. In the earlier studies, soil pH is appeared as a leading constraint in assessing the bacterial abundance, priming effect, and CO_2 discharge (Sheng and Zhu 2018). In addition to soil pH, other factors including soil organic carbon and carbon-to-nitrogen ratio also play an important role in shaping the composition of bacterial community (Xu et al. 2014; Zheng et al. 2016). For example, when sulfate-reducing bacteria were used, the acidic conditions caused by excessive CO_2 resulted in low carbonate mineralization. Generally, the potential of bacteria to protect themselves from an acidic environment or CO_2 induced toxicity, via EPS production and biofilm, can make sure an improved activity and superior survival.

An earlier study reported the biofilm generation, when bacteria is exposed to the high level of CO_2 (Mitchell et al. 2009). It is established fact that at supercritical CO_2 concentrations, it is not possible for bacteria to survive. Temperature and pressure are also two major constraints influencing the metabolic activity of bacteria. Pressure originated from the overlying soils has an adverse impact on the bacterial activity in the pore cavities. For the shallow sequestration localities, the swift pressure variation (because of CO_2 intake) can be challenging for the survival of bacteria. The bacterial

activity can be restricted in deep zones and under high temperature conditions (Dupraz et al. 2009). For instance, in case of calcite precipitation, bacteria can exhibit higher metabolic activity at shallower depths, where temperature remains low.

A study determined the potential influence of salinity on the soil microbial communities, microbial activity, and the potential of soil carbon sequestration (Hu et al. 2016). The high salinity controlled the decay capability of soils by altering the bacterial community structure and hindering bacterial actions, which might improve its carbon sequestration. Furthermore, the effect of salinity also depends on the type and nature of microorganisms. For instance, the low saline conditions were found favorable for survival of some specific microorganisms (e.g., β-Proteobacteria), whereas high salinity is appeared fine for other microorganisms such as halobacteria.

Biochar significantly influences the bacterial community of soil through changing the physicochemical characteristics of soil. Subsequently, it alters the CO_2 discharge and priming effect of soil organic carbon (Maestrini et al. 2015). The biochar addition rate into the soil affects the acceleration and suppression of CO_2 emission based on organo-mineral interactions (Sheng and Zhu 2018). It is noteworthy that soil pH increases due to the alkaline nature of biochar that can result in lower abundance of gram-positive bacteria and higher proportion of gram-negative bacteria (Aciego Pietri and Brookes 2009). Furthermore, the solubility of nutrients (phosphorus and nitrogen) and some small compounds (formic acid, succinic acid, lactic acid, etc.) in biochar can be influenced by soil pH, which may causes serious effect on bacterial growth (Lin et al. 2012; Smith et al. 2013).

5.5 Future Perspectives

At one side, the global fossil fuel resources are decreasing rapidly and, on the other hand, climate change due to GHG emissions is a challenging issue with respect to both economic and health perspectives. The microbial CO_2 sequestration is an auspicious process for concluding the carbon. The application of existing biotechnology techniques can assist in improving the ability of CO_2 fixation in heterotrophic and autotrophic microbial communities and the issue associated with microbial CO_2 discharge can be moderately resolved.

Conventional technologies to sequester CO_2 including CO_2 separation (e.g., membrane separation and adsorption) (Chou 2013), CO_2 capture (e.g., oxyfuel combustion and post-combustion) (Hicks et al. 2017), and CO_2 storage (e.g., offshore geological formations and saline aquifers) (Leung et al. 2014) are of greater importance for inhibiting CO_2 discharge into the atmosphere. But such processes have some prominent insufficiencies as compared with microbial sequestration of CO_2 due to the higher operational costs, energy intensive applications, and/or byproduct generation.

The rapid growth rate is the distinctive feature of microbial CO_2 sequestration, which makes it more important as compared with plant-based CO_2 sequestration.

Moreover, the photosynthetic microbial communities such as cyanobacteria can be cultured without competing with agronomic practices for terrestrial resources (Goli et al. 2016).

To enhance the efficiency of microbial CO_2 sequestration, the possible areas of research and advancements include (a) discovery of new autotrophic CO_2 fixing framework; (b) developing more competent genetic techniques for autotrophic hosts; (c) scheming innovative synthesis paths to reduce the ATP requirement; (d) merging carbon-efficient routes with CO_2-discharging routes to attain maximum carbon yield during chemical biosynthesis in heterotrophic microbial communities; (e) revealing the mechanism involved in transport of electron from the extracellular to intracellular section; and (f) optimization of bioprocess conditions during CO_2 fixation.

5.6 Conclusion

The industrial revolution and ceaseless anthropogenic activities have agitated the CO_2 balance in the atmosphere leading to rising sea levels, increasing global temperatures, transferences in ecosystems, and amplified incidence of wildfires. These environmental variations created a space for research and development of methods to control the emission level of GHG and CO_2 in the atmosphere. An instantaneously accessible choice of lowering CO_2 levels in atmosphere is the carbon sequestration. This chapter details the role of soil bacterial communities to sequester atmospheric CO_2. The carbon sequestration ability of bacteria depends on multiple factors including nature and characteristics of bacteria species, environmental conditions, and the soil properties. Studies reported the adverse effect on the bacterial activity due to the long-term exposure to the CO_2 or in the presence of excessive quantity of CO_2. Overall, this chapter describes the nature, characteristics and activity of soil bacteria in CO_2 sequestration vis-à-vis emphasizing the geological factors.

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Soil Verrucomicrobia and Their Role in Sustainable Agriculture

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Bighneswar Baliyarsingh, Byomkesh Dash, Swapnarani Nayak, and Suraja Kumar Nayak

Abstract

Soil as a natural resource embodies innumerable microbes regardless of their species. Amongst all phylum Verrucomicrobia harbours in various soils and the effect of both biotic and abiotic factors are still indistinct. The members comprise few cultural and mostly unclutural members and play pivotal role in biodegradation of complex chemicals and promote plants/crops yield by availing nutrients and preparing a conducive environment. Soil pressure as well as root pressure, temperature, nutrients, and moisture content are some of the few abiotic stresses which decide the community growth, activity and composition. Apart from these their unique structure helps them to survive in other stress conditions. Utilization of various organic matter is a privilege for the members to survive in stresses and makes them suitable agents to move forward for agricultural sustainability.

Keywords

Soil microorganisms · Verrucomicrobia · Soil pressure · Sustainable agriculture · Spartobacteria · PVC superphylum

B. Baliyarsingh · S. K. Nayak (✉)
Department of Biotechnology, Odisha University of Technology and Research, Bhubaneswar, India

B. Dash
Crop Improvement Division, National Rice Research Institute, Cuttack, India

S. Nayak
Fish Genetics and Biotechnology Division, ICAR-Central Institute of Freshwater Aquaculture, Bhubaneswar, India

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6.1 Introduction

Soil personifies incalculable number of microorganisms enormously involved in soil improvement leading to fertility vis-à-vis crop yield. As a natural resource it encompasses diverse metabolism of indigenous microflora which leads to the cycling of micro- and macro-nutrients besides from many other soil services and affects tasks of soil ecosystems. This resource fluctuates substantially, due to the climatic conditions, dwelling organisms, land forms and parent materials. Till now the major challenges in soil microbial ecology are related to understanding the changes in soil bacterial community structure over time (Ge et al. 2008). Certainly, climatic fluctuations often divulge microbial consortia and its abundance, and thus lead to have a very strong correlative effect on crop productivity and soil health (Allison and Martiny 2008). Several works have been performed throughout different soil layers; microbes exist in all and are bountiful in surface regions, root area and similar (subsurface soils) and macropores (Baliyarsingh et al. 2017). Soil microorganisms and their consortia constitutively work as heterogenous tropic level agents and the consortia composition substantially influences microbial processes (Baliyarsingh et al. 2017; Mikola and Setala 1998). It is now widely accepted that bacterial consortia are composed by an assembly of resident taxa, those being slightly affected by soil biotic and abiotic factors and by occasional/fluctuating taxa, those varying amongst samples (Logares et al. 2013; Bacci et al. 2015). Material decomposition and soil weathering processes are also included in the services of these members. Thus, study of microorganisms in soil necessitates an overall understanding on all soil layers, which further leads to development of a fully functioning ecosystem (Pham and Kim 2012). At present the physiology, mechanism and characteristics of poorly studied but largely available soil bacteria are of the main concern for researchers belonging to soil microbiology realm (Rappe and Giovannoni 2003). Additionally widening the fundamentals on diverse bacterial community is likely to expand fundamental insight on soil bacterial communities and embellishes new microbial processes, mechanism of actions, adaptations, product and utilizations which present heretofore new solutions for agricultural utilization and sustainability (Aislabie and Deslippe 2013).

Reports revealed approx. one million of total species diversity found per 10 g of soils. Both culture-independent and -dependent studies revealed the major bacterial phyla are Proteobacteria (alpha, beta and gamma), Acidobacteria, Actinobacteria (Gram positive, high G + C content bacteria), Bacteroidetes, Chloroflexi, Cyanobacteria, Firmicutes, Gemmatimonadetes, Planctomycetes and Verrucomicrobia (Trivedi et al. 2016). Amongst the various phyla, members of Verrucomicrobia phylum are progressively figured as agriculturally and environmentally significant soil bacterial community (Sangwan et al. 2005). The phylum Verrucomicrobia forms a cluster, i.e. superphylum, known as PVC superphylum (Planctomycetes-Verrucomicrobia-Chlamydiae) with similar other phyla as Planctomycetes, Chlamydiae, Lentisphaerae and the Candidate phylum Omnitrophica (Rinke et al. 2013), and some other uncultured bacteria (Wagner and Horn 2006). These superphylum shared at least a single common

superphylum-specific signature protein and is which is publicized by phylogenetic and indel analysis technique (Gupta et al. 2012; Lagkouvardos et al. 2014). According to Weisburg et al. (1986) Planctomycetes and Chlamydiae relationship was recognized by 16S rRNA oligonucleotide gene sequencing. Verrucomicrobia are of immense significance to understand the bionetwork of soil microbial communities and their function as they are quantitatively significant component of soils round the globe. The very first isolated members are aerobic heterotrophs. Further scientific investigation exposed occurrence of enormous diversity in Verrucomicrobia such as combination of both the acidophilic and thermophilic methanotrophic physiology obtained in *Methyloacidiphilum* species. This species significantly helps in understanding the bacterial methane oxidation and others involved in global methane cycling (Fuerst 2019). Each of these aspects of the verrucomicrobia are dealt briefly later in this chapter.

Anoxic flooded rice fields to bare lands, and in extreme environments including the Antarctic soils, hot springs (60 °C) and in pH 2.0–2.5 conditions (Hou et al. 2008) they are able to prosper. Also the spatial variability in the abundance could be foreseen from environmental conditions and were most abundant in intermediate temperature and precipitation soils. In last decade only few members have been found as cultural representatives and further as more accurate only four subdivisions. They also utilize an array of carbon sources as referred as saccharolytic fermentative to polysaccharolytic (Schlesner et al. 2006). The representatives of Verrucomicrobia are involved in vast roles in soil ecosystems. Furthermore they have remarkable role for methanogenesis in soil, nutrient cycling, production of metabolites, mitigation of pollutants and reduction of pathogens are few to mention (Shen et al. 2017). Moreover, Verrucomicrobial plays important role in plant health improvement and soil quorum sensing in rhizospheric region. Technological progress in omics technology facilitates acquaintance on how it differs from other rhizospheric colonizers and promotes plant growth. Phylum Verrucomicrobia are still an interesting subject for in detail study in future to soil microbiologists because of their complex interaction, meagre availability and limited idea on the culturability. Knowledge on this phylum in coming decades will help in development of microbial inocula for sustainable agriculture and developing economy like India.

6.2 Abundances and Occurrences of Soil Verrucomicrobia

Naturally as a resource, soil shields the earth surface and nests a variety of microorganism both in single cells and in consortia which further results in both taxonomical and functional diversity. Besides, it stands in as the confederate state of soil bio-geological materials, soil water and soil air (air in soil pores). Several works have been performed inspecting bacterial community variation both in cross-sectional (different sites at the same time) and longitudinal studies (the same site studied over time) (Bartram et al. 2014; Chen et al. 2016; Logares et al. 2013; Bacci et al. 2015). Due to its extensive complexity and genetic heterogeneity, strong interest on soil microbial communities still exists. On an average 4 thousand to

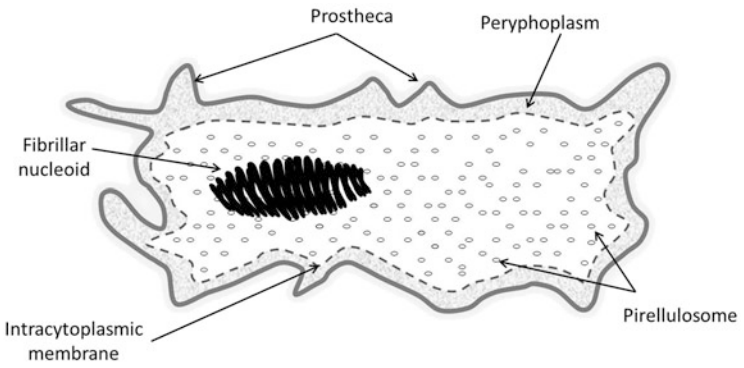

Fig. 6.1 Verrucomicrobial cell structure

40 million species are found per gram and more than a half century of phyla have been identified in last decade. Traditional in vitro culture based method reported non-accessibility to more than 95% microbial community due to occurrence of meagrely cultivated or uncultivated members (Nichols 2007). As a solution (Meta)-genomics, (Meta)transcriptomics and/or (Meta)proteomics like omics approaches (cultivation independent) paves the path and clears the route by analysing the functional genes to study soil bacterial community along with their physiologies and metabolisms (Tringe et al. 2005). Many studies on the composition and community structure of soil microbial communities have been performed by using DGGE, TGGE, PLFA and SSCP profiles techniques and low-resolution molecular methods (Correa-Galeote et al. 2016). In addition, the bacterial community structure was analysed in detail by tracking the ribosomal RNA (rRNA), relatively abundant.

Soil Verrucomicrobia appears to be relatively abundant (23.5%) in comparison to other dominating phyla as Actinobacteria (13%), Acidobacteria (20%) and Proteobacteria (39%) since last three decades its discovery (Janssen 2006). *Verrucomicrobium spinosum,* the very first cultural representative, leads to name the phylum Verrucomicrobia (Schlesner 1987). The cell surface projections are alike to the warts in human skin (Verrucomicrobium is derived from the Latin word verruca meaning a wart). These surface projections are now known to be true "prosthecae" or projections of the cell wall having some cytoplasm in them instead of external appendages (Fig. 6.1). Verrucomicrobia are relatively abundant in top layer and up to A horizon of soil region. This phylum comprises active members and is positioned second and represents 1.0 to 9.8% of the soil bacterial 16S rRNA (Buckley and Schmidt 2001, 2003; Felske et al. 2000). Sometimes it is observed that this phylum sustains in consortia with others.

According to the reports it is noteworthy to mention that 10^6 to 10^8 nos. of verrucomicrobial population/cells persists in per gram of dry soil (Felske and Akkermans 1998), which is further strengthened by availability of 9000 no. 16S rRNA gene sequences in the Ribosomal Database Project (http://rdp.cme.msu.edu). As per 16S rRNA sequences phylogeny Verrucomicrobia is distributed into five

subdivisions/subphyla/class (Jones et al. 2009; Schlesner et al. 2006) and commonly comprises uncultured species with a few cultivated members (Janssen 2006). Firstly it was recognized as a separate phylum about 35 years ago, comprising 28 genera in soil. Currently, a total 65 confirmed isolates are classified under 28 genus, out of which 18 nos. are soil isolates. Through 16S rRNA it is clear that the Verrucomicrobial isolate of subdivision 2 (unidentified) is highly phylogenetically related to uncultivated bacteria obtained from forest as well as pasture soils of the USA (Lee et al. 1996), pasture soil representative and *Brassica napus* L. rhizospheric member of the United Kingdom (Macrae et al. 2000), cropping land soil in Sweden (Sessitsch et al. 2001), a grass land soil in The Netherlands (Felske et al. 1998), and a forest soil in Australia (Liesack and Stackebrandt 1992).

Commonly in soil communities, Class Verrucomicrobiae is found in less frequent (Kielak et al. 2008; Bergmann et al. 2011). The members are profusely associated with rhizospheric soil of *Solanum tuberosum* L. and leek (*Allium porrum* L.), etc. (da Rocha et al. 2010). Maximum representatives are uncultured members with few deviations. Rhizosphere competence was dominated by a new genus Candidatus genus *Rhizospheria* from Rubritealeaceae family and *Luteolibacter* sp. (da Rocha et al. 2010). *Verrucomicrobium* sp. like *V. spinosum* was the first isolate cultured and identified from soil resource. Further *Prosthecobacter* sp., another isolate, is one of the cultural representatives from the Australian pasture soil (Janssen 2006). Later this subdivision is being distributed in three distinct clades. The cultural isolates of the Verrucomicrobiae are also well characterized in Bergey's manual of Systematic Bacteriology (Garrity et al. 2003).

Class Spartobacteria is most abundant and dominating in all types of soil ranging from pasture (Janssen 2006) to tall grass prairie soils, USA (Joseph et al. 2003), and ranging to 10–50 cm depth as subsurface soil horizons (Bergmann et al. 2011). Spartobacteria contains free-living taxa, as well as a number of associated endosymbionts of *Xiphinema* sp. (nematode) (Vandekerckhove et al. 2002; Wagner and Horn 2006). Order Chthoniobacterales is the only order from this class that represents cultural representative/s, *Chthoniobacter flavus,* a free-living aerobic heterotrophic soil isolate (Sangwan et al. 2005). Chthoniobacteraceae family represents ten isolates; amongst them *C. flavus* Ellin428 isolate from rye grass and clover (*Lolium perenne* L.) pasture, Australia, utilizes phytopolysaccharides (Sangwan et al. 2005; Kant et al. 2011a). There are several types of stains available in soil also. An anaerobe *Terrimicrobium sacchariphilum* isolated from paddy field soil has indistinctive order or family. *T. sacchariphilum* having 89.6% 16S rRNA gene sequence similarity with *C. flavus* proves clade similarity (Qiu et al. 2014). Apart from this, some uncultured environmental clones also have been noticed, i.e. Candidatus Xiphinematobacter in this class. Class Spartobacteria dominates verrucomicrobial communities in nearly all biomes including both O and A horizons and some more in-depth regions. Members of Spartobacteria were present 2×10^8 cells per soil gram (Lee et al. 1996) which is a 4–9% of the total soil verrucomicrobial gene population (Sangwan et al. 2005).

Another class Opitutae, second most abundant from this phylum, collectively contains both cultural and uncultural representatives from soil (mesophilic to

psychrophilic). The isolate *Opitutus terrae,* taxonomically representative of Opitutales order and Opitutaceae family, was isolated and identified from paddy rhizosphere (Chin et al. 2001). It is a strict anaerobe that grows by nitrate reduction or fermentation using all form of plant saccharides (Hernández et al. 2015). *O. terrae* PB90–1, an isolate from rice cultivated soil, produces propionate via utilizing plant polysaccharides (Chin and Janssen 2002). However, as a common dependant on hydrogen partial pressures interacts with methanogens locally (Janssen 1998; van Passel et al. 2011). The Bergey's manual of Systematic Bacteriology also well mention cultural isolates the Opitutaceae (Garrity et al. 2003).

Only verrucomicrobial phylum contains aerobic methanotrophs apart from Proteobacteria is in class Methylacidiphilae comprises of few cultural representatives which optimum growth obtained in pH 2.0–2.5 and isolated from acidic soil as well as alkaline environment proving their immense presence (Morris et al. 2002; Lin et al. 2004). Basically it is involved in methane oxidation and uses methane as a sole source of energy and carbon. Moreover, Methylacidiphilae is more diverse than others and is also involved in C1 utilization metabolic pathways (Dunfield et al. 2007). Hou et al. 2008 studied an extremely acidophilic methanotrophic *Methylokorus infernorum* V4 from soil of methane-emitting geothermal field, New Zealand. Two other thermoacidophilic Verrucomicrobia with >98% of 16S rRNA sequence similarity were also simultaneously isolated from Solfatara volcano mudpot, Italy, as *Acidimethylosilex fumarolicum* SolV and from an acidic hot spring in Kamchatka, Russia, as *Methyloacida kamchatkensis* Kam1 (Islam et al. 2008). Moreover *M. infernorum* V4 has similarity in genome with autotrophic bacterium and contains simple signal transduction pathways with potential of limited gene expression regulation.

Few more members are also present in the unclassified /miscellaneous verrucomicrobia and sparse in soil environment and represent <1% of the soil bacterial community. They do not cover the full phylogenetic breadth of class which blurs the complete picture of members from same phylogeny (Sangwan et al. 2005). Some mesophilic acidophilic verrucomicrobial methanotrophs were isolated from volcanic soil, Italy, and showed 97% - 98% 16S rRNA sequence similarity with each other and related (89–90% 16S rRNA) to the thermophilic genus *Methylacidiphilum* and proposed as *Methylacidimicrobium*, a new genus. Few noteworthy members from the novel species are *Methylacidimicrobium fagopyrum*, *M. tartarophylax* and *M. cyclopophantes,* and these are well adapted to specific niche within their geothermal environment (van Teeseling et al. 2014).

All Verrucomicrobia are mesophilic with few omissions, strict aerobic, facultatively or obligately anaerobic, saccharolytic and having oligotrophic nutrition (Janssen 1998; Chin et al. 2001; da Rocha et al. 2010). Characteristically soil Verrucomicrobia may have extremely small overall cell dimensions leading to access soil pores and develops predators escapism mechanism (Wright et al. 1995). All the isolates of this phylum are morphologically rods or coccus and divide through binary fission or irregular cell division and possess wart-like cellular protrusions and are negative to Gram staining (Schlesner et al. 2006). Through TEM analysis it is clear that the cells consist of membrane-coat-like proteins and

condensed DNA like as of nucleoid. Fimbriated prosthecae, a cellular extension in all directions from cell surface, are also found in *Prosthecobacter* sp. and few others (Hedlund et al. 1997).

It is fascinating that microbial communities varied with depth even after tillage (soil homogenization). Various soil parameters such as total organic carbon, soil pH, total nitrogen, temperature and soil moisture have been decreased with increase in depth in agricultural fields with no significant change due to increases in tillage intensity (van Gestel et al. 1992). Perhaps microbial community composition is otherwise influenced by long-term management practices and cropping community composition (Buckley and Schmidt 2003).

6.3 Environmental Factors and Verrucomicrobia Distribution

The role of environmental factors involved in the regulation of diversity and abundances of phylum Verrucomicrobia is unclear though it occupies diverse soil ecological niches both in category and in depth. However a vast number of data are generated by researchers and amongst them few are accessible and discussed here which clears the concept regarding their distribution. The different subdivision/class/subphylum are dependent on few abiotic factors including pH, temperature, pressure, environmental factors as soil type (Singh et al. 2007), and biotic factors as phytotypes (Chow et al. 2002; Sanguin et al. 2006) and others are described briefly in Table 6.1.

6.4 Role of Verrucomicrobia in Soil

Ecosystem functioning vis-a-vis crop yield have been notably controlled by soil native microflora (Baliyarsingh et al. 2017). Even if the detail ecophysiology is sparsely understood it is reported that Verrucomicrobia appears to be one of the prevailing soil bacterial communities round the globe (i.e. from West to far west including far south), with significant role and interactions in soil environment (Fig. 6.2).

An array of things occur in subsurface soil where enormous chemicals were released by the roots and triggers rhizospheric soil plant roots releases a series of chemicals leading to rhizodeposition and ultimately triggers beneficial symbioses, impedes rhizocompetition, improves a carbon- and energy-rich environment and build ups quorum sensing for intact colonization (Walker et al. 2003). In this active zone they are also involved in releasing of siderophores and chelators like molecules for plant growth promotion in addition to protection against soil-borne diseases (Idris et al. 2004; Pahari et al. 2017). 16S rRNA gene clones data reveals rhizosphere abundantly comprises particularly Subdivisions 2, 3 and 4 (Chow et al. 2002).

Verrucomicrobia are also involved metabolism of soil fertility factors particularly total nitrogen, phosphorus, potassium and some of bases limiting to calcium and magnesium (Wertz et al. 2012). Few Verrucomicrobia isolates dynamically process

Table 6.1 Factors influencing the distribution of Verrucomicrobia

Factors	Role	Verrucomicrobial distribution	References
Abiotic			
Soil pH	It signifies as one of the strongest factors and influences microbial community diversity and composition of both culturable and unculturable. Verrucomicrobia are higher in no. in mild acidic pH (i.e. 5.0–6.0) and outnumbers with high-pH (6.0–7.5) conditions	The mentioned pH range suits not specifically any Verrucomicrobia but all order except the methanogenic members	Bartram et al. (2014)
Soil temperature	Verrucomicrobial community growth and activity significantly alters by fluctuations in soil temperature. Starting from paddy field to forest soil everywhere the mean temperature in the range of 25–35 °C favours the growth of this particular phylum	The moderate temperature is ambient for growth of verrucomicrobia, some members of methanogens (class Methylacidiphilae) have isolated from soils with high temperatures	Dunfield et al. (2007), Islam et al. (2008)
(Soil)plant root pressure	Soil pressure which is more or less in synergy with root pressure, exerted by plant roots that govern not only the verrucomicrobial communities but also its abundance and composition. They are also interdependent with water/moisture content and soil nutrients. These pressures are often the results of interactions between consortia and root respiration processes	The rhizosphere of Lodgepole pine (*Pinus contorta*) in loamy soil and Maize (*Zea mays* cv. PR38a24) in sand loam soil selectively increase the verrucomicrobial community	Chow et al. (2002), Sanguin et al. (2006)
Soil moisture	The positive correlation is dependent on soil depth, soil sampling time and soil management history. Increase in soil moisture have been associated with enhanced nutrients diffusion and microbial	Anaerobic Verrucomicrobia intensifies the anaerobic environment linked to soil moisture content and favours the growth of community. Class Spartobacteria exhibited	Sierra and Renault (1998), Treves et al. (2003), Maestre et al. (2015)

(continued)

Table 6.1 (continued)

Factors	Role	Verrucomicrobial distribution	References
	predation and reduction in oxygen tension. Surprisingly it is clear that the resource availability and soil connectivity influence the moisture content. High moisture soils favour the community along with heat waves and drought	variable response to soil moisture, resulting that they have multidimensional role in soil	
Seasonal variability	It is coupled with soil organic matter, i.e. C and N, with soil moisture and temperature. Especially, Verrucomicrobia, Archaea and Acidobacteria alter mechanism to adopt the changes in the environmental conditions	Due to sparse information available on verrucomicrobia at present it is difficult to discuss the specific causes of the temporal variability	Nayak and Mishra (2020)
Elevation gradient	The soil bacterial community strongly influenced by elevation. In particular with increasing elevation from 1050 to 2550 m and increased at 2750 m the Verrucomicrobial diversity decreases monotonously. This is mainly due to decrease in organic carbon and other nutrients	Verrucomicrobial richness linearly decreased with increased elevation and diversity exhibited a unimodal pattern with elevation	Shen et al. (2017), Zhang et al. (2015)
Soil depth	The diversity and abundance are related to the availability of organic nutrients which resulting in maximum no. of microbial cells in topsoil/ surface soil (up to 10 cm in depth). This no. gradually decreases while moving down in soil	Verrucomicrobia may be relatively abundant in subsurfaces due to their oligotrophic nutrition. Class Spartobacteria did not change significantly with increase in depth while changes in number were found in Class Verrucomicrobiae	da Rocha et al. (2010), Sangwan et al. (2005)
Soil Air/Oxygen concentration	The exact role of soil air on Verrucomicrobial community is sporadically present. The	They are strict anaerobes, facultative anaerobes and strict aerobes	Buckley and Schmidt (2001)

(continued)

Table 6.1 (continued)

Factors	Role	Verrucomicrobial distribution	References
	soil air is also linked with soil moisture which is explained as due to anaerobic respiration of Verrucomicrobia they increase the anoxic environments linked to increase in soil moisture content, which further favours their growth and metabolism in soil		
Salinity/ acidity/ Ion concentration	It plays a lead role in biogeochemical cycles and that is due to the variation in size and the activity of soil microbial community and biomass involvement	Their abundance decreased with the lift in salinity and wealthy community were observed in low-salt than high-salt soil. Due to high salinity bacteria intimately associated with the soil organic matter, and perhaps with a significant advantage in the soil carbon cycle	Rietz and Haynes (2003), Yang et al. (2016)
Soil available nutrients/ Soil fertility	Fertilization results in variation of physiology and metabolism thus they expresses differently to available nutrients and are basically due to increasing in N and P inputs. The induced shifts in either copiotrophic or oligotrophic nutrition have significant implications for soil C cycling. The community abundance increases in connection to C and shifts in C dynamics can be correlated with expression of carbohydrate metabolism genes. They are higher in the forest than in adjacent pasture soils. At some instances the forest-pasture conversion changes the soil chemistry which further	*V. spinosum* (a facultative anaerobe) and *Prosthecobacter* sp. are able to grow on sugary compounds instead of on amino or organic acids compounds	Hedlund et al. (1997), Janssen (1998), Wieder et al. (2013), Fierer et al. (2013)

(continued)

Table 6.1 (continued)

Factors	Role	Verrucomicrobial distribution	References
	expedites an environment for Verrucomicrobial new members		
Biotic			
Plant-derived nutrients	They are utilizing various plant-derived carbon compounds in soils as their primary nutrients and energy sources. Apart from this the plant and other soil consortia composition and diversity also affects the possibility of novel soil Verrucomicrobia population	Mostly the oligotrophs grow outnumber in the rhizospheric soil	Ranjan et al. (2015), Stevenson et al. (2004)

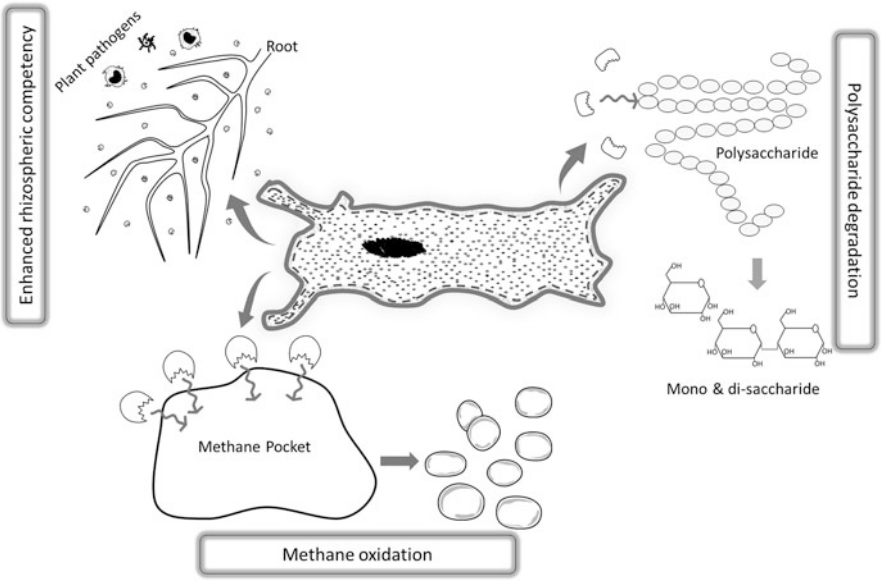

Fig. 6.2 Verrucomicrobia and their role in soil environment

the methane oxidation (Dunfield et al. 2007) and (biological) nitrogen fixation (Khadem et al. 2011) and signify their immense role in soil environment. In soil *Methylacidiphilae* are thermoacidophilic and acidophilic methanotrophs (use methane as energy source) capable of both methane oxidation and N_2 fixation (Dunfield et al. 2007; Shen et al. 2017). This N_2 fixing activity helps them to sustain in pasture

soil and uprises the soil N_2 content (Ranjan et al. 2015). Some members are also present as endophytes and increase plant disease resistance (Mostajeran et al. 2007). Spartobacteria, an abundant soil dweller, use glucose, pyruvate and chitobiose as substrates for growth and metabolism. Synergism is also noticed in this class for survival because of sparse utilization to amino acids and vitamins. The proteasome organelle denotes participation in other metabolism/activities (Brewer et al. 2016).

Moreover they degrade/utilize cellulose and/or cellulolytic substrates into simpler compounds both in the presence and absence of soil oxygen. In flooded rice paddy soil (anoxic condition) cellulose is the main substrate to use by the methanogenic verrucomicrobes (Chin et al. 2001). *V. spinosum* have cellulose enzyme/protein meant for cellulose degradation. The cellulolytic capabilities are immensely required for the fullest implementations in the process of organic farming, i.e. utilizations of organic amendments like compost, manure, and slurry for crop growth and yield as well as disease control. Spartobacteria may play important roles in cellulose, hemicellulose and lignin biodecomposition and leads to act significantly in cellulose turnover worldwide. Biodegradation of noncellulosic polysaccharides is also confirmed through studies and data avail in public domains. Cellulases, chitinases, sulfatases, peptidases, carbohydrate lyases, and esterases are some of the biocatalysts employed for various polysaccharide hydrolysis (Dash et al. 2020; Martinez-Garcia et al. 2012). Few members are also specifically involved in production of extracellular laccase for phenolic and non-phenolic lignin-related compounds oxidation (Kunamneni et al. 2008). Similarly *C. flavus* Ellin428 is capable to degrade polysaccharide as per the genome sequencing data (Kant et al. 2011b). van Passel et al. (2011) revealed *O. terrae* degenerate the plant polysaccharides for the production of propionate in fermentation process.

However, in addition to the foresaid activities the verrucomicrobial genome revealed the presence of some of the conserved signature indels (CSIs) in the proteins depicting their role in active electron transport (Cyt c oxidase), efficient repair mechanism in varied soil conditions (UvrD helicase), utilization of polycarbon and ammonical compound for substance (urease) and increase in nucleic acids/cell numbers in unfavourable conditions (Gupta et al. 2012).

6.5 Intercommunity Interaction for Sustainable Agriculture

Various interactions occur within plant, plant roots and Verrucomicrobia due to appearing as massive rhizosphere colonizers (Kielak et al. 2008). Rhizosphere is a valuable interface for microorganism-soil-plant interaction, signalling, protection and production following quorum sensing (alteration of signals), adsorption/absorption of nutrients and effects of metabolism through various biocatalysts. In rhizosphere various interactions occur as (a) commensalism where neither one is hampered and new species are added, i.e. through production of exopolysaccharides and plant hormones, (b) microbial stasis, i.e. through production of secondary metabolites and antagonism (Nayak et al. 2017) and (c) mutualism, i.e. through degradation and detoxification of recalcitrant herbicides and polycyclic

hydrocarbons and it is utilized as main carbon sources for both plant and associated microorganisms (Nayak and Mishra 2020).

The verrucomicrobial communities varied during developmental stages of the plant species and by other biotic and abiotic factors as discussed in Table 6.2 and in some instances alleviate the same. Concurrently plants also regulate its rhizosphere communities via amending root exudates (Chen et al. 2016). Some soil (rhizosphere) culture independent analysis revealed that Verrucomicrobial were detected approx. at par to 50% of total bacterial rRNA gene sequence (Filion et al. 2004). The root environments represent greater proportions of aerobes(strict) while both facultative and strict aerobes are the rhizospheric organisms (Hernández et al. 2015). Literature available that higher no. of community in continuous cropping land than in bulk soil was observed as the soil environment determines community structure and composition in the rhizosphere (Mishra et al. 2021). Marginal population density has been noted at young roots and root tips. On the contrary, mature roots, root hairs and root tips drive enormous community population and that is may be because of different rhizosphere selective forces (DeAngelis et al. 2009). Carbonaceous compounds as root exudates along with the native soil influence the community density and played a role in metabolism. Nitrogen as an essential nutrient for plant growth and soil available nitrogen and added N fertilizer have a major role in verrucomicrobial diversity. However it diverges with plants as the Verrucomicrobia are abundant in *Artemisia frigida* Willd. (a dominant temperate grass sp.) rhizosphere and very sparse in *Stipa krylovii* Roshev. rhizosphere. It is also profusely found in the paddy soil with less information available on their function (Do Thi et al. 2012). In addition to this verrucomicrobia also support important metabolic processes for plant growth, development and yield.

According to Hernández et al. (2015) Spartobacteria along with *Opitutus* sp. colonizing on the rice rhizosphere soil and roots. Few members from *Pedosphaerales* family are also abundant in cotton rhizosphere, utilizing the root exudates and important role in plant metabolism (Qiao et al. 2017). Rhizospheric bioremediation is also carried out by few members (Kawasaki et al. 2012; Nayak et al. 2018) and leads to conversion of fertile nontoxic land for agriculture. Also phytodisease suppressiveness was marked during shifting from bulk soil to cropping land because of microbial consortia (Mendes et al. 2013). The distribution and role of this specific phylum with biotic and abiotic factors/stress in its environment provide important indication for understanding the organisms' basic physiology, consortia interaction and its inherent role with the environment. Though culture-independent studies help to go inside the community function in biome but precise studies and prolong involvement and invention of new techniques will help to explore it in future and incorporate for agricultural sustainability.

The plant genotype plays an important role in accessing the phyto-associated microbial communities and determining its biological outcome (Smith and Goodman 1999; Ding et al. 2013). In maize-cultivating farms it is found that genera Gp4, Flavobacterium, Subdivision3 genera incertae sedis of the Verrucomicrobia phylum, *Dechloromonas*, *Parcubacteria* incertae sedis, *Rhodoferax* and *Spartobacteria* were abundant. Whereas, the maize-bur clover consortium increased relative abundance

Table 6.2 Soil Verrucomicrobia and their role in agriculture

Sl. no.	Isolates	Class/subdivision	Role	Type	Sampling site	References
1	*Chthoniobacter flavus* *Terrimicrobium sacchariphilum*	Spartobacteria	Degradation of plant saccharides	Aerobic and anaerobic	Pasture to grass field (10–50 cm depth); Endosymbiotic to *Xiphinema* sp. (nematode)	Wagner and Horn (2006), Qiu et al. (2014)
2	*Opitutus terrae*	Opitutatae	Nitrate reduction; polysaccharide, monosaccharide degradation	Anaerobic	Rhizospheric soil	Chin et al. (2001), Hernándaz et al. (2015)
3	*Verrucomicrobium spinosum* *Prothecobacter vanneervenii*	Verrucomicrobiae	Metabolizing methane	Aerobic	Rhizospheric soil of crops; pasture soil	Jenkins et al. (2002), da Rocha et al. (2010)
4	*Methylokorus infernorum* *Acidimethylosilex fumarolicum*	Methylacidiphilae	Methane oxidation	Acidophilic Thermoacidophilic	Acidic-alkaline soil; Geothermal field; Acidic hot spring	Hou et al. (2008), Lin et al. (2004), Islam et al. (2008)
5	*Methylacidimicrobium fagopyrum*	Unclassified	Methane oxidation	Mesophilic Acidophilic	Volcanic Soil	van Teeseling et al. (2014)

of Flavobacterium, Subdivision3 genera incertae sedis of the Verrucomicrobia phylum, *Dechloromonas* and *Parcubacteria* incertae sedis because of utilization of various phytoextracts by the members (Correa-Galeote et al. 2016).

6.6 Concluding Remarks

More than two decades substantial studies have been going on utilization of soil microbial communities for to benefit the human race by maximize the agroyield. Few microorganisms are already engaged in the service and a substantial number of microbes are in the members, few more are in the process owing to their indigenous harsh habitat, slow and low frequency growth and lesser information on their physiology and metabolism. Culture-independent methodology makes it possible to study, explore and utilize the maximum microflora. Soil Verrucomicrobia comprises both culture-dependent (few, till now) and -independent members with important role in agronomy due to inter(microbe)/intra(plant) interactions, role in soil, degradation of agrochemicals, utilization of complex polymers for nutrient resources, etc. Advances in molecular techniques will increase much knowledge on diversity and applications of members from the phylum. This further facilitates utilization of it for agricultural sustainability through reliable development, higher productivity and safe and controlled management.

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7 Agricultural Wastes as an Alternative Source for the Production of Antibiotics: Recent Developments and Future Perspectives

Ayesha Kashif and Muhammad Kashif Shahid

Abstract

Antibiotics are bioactive compounds that selectively kill or mitigate the growth of microorganisms. The increasing human population, development, and industrialization resulted in an increased demand of antibiotics. The annual consumption of antibiotics round the globe has reached over 200,000 tons. Hence, alternative and cost-effective sources for the production of antibiotics are inevitable. Agricultural wastes, i.e., corn cobs, sawdust, rice hulls, and groundnut shell, are rich source of bioactive compounds. Therefore, the agro-waste can be utilized for industrial production of various value-added products including antibiotics. The composition, quantity, and quality of antibiotics produced from agro-waste depend on both starting material/substrate (raw waste) and the processing steps. By applying appropriate fermentation techniques, agro-waste can be used in cost-effective production of antibiotics. Recent studies reported the production of neomycin, oxytetracycline, and rifamycin using agro-wastes as substrate by solid state fermentation (SoSF). Several microorganisms were used for the production of these valuable products. In addition, the external energy sources were supplied to enhance the production of antibiotic.

Keywords

Agro-waste · Antibiotics · Solid state fermentation (SoSF) · Mechanism of action · Process optimization

A. Kashif
Department of Senior Healthcare, Eulji University, Daejeon, Republic of Korea

M. K. Shahid (✉)
Research Institute of Environment and Biosystem, Chungnam National University, Daejeon, Republic of Korea

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7.1 Introduction

Antibiotics are known antimicrobial substances that treat and prevent humans and animals against bacterial infections by either killing or mitigating bacterial growth (Rocha et al. 2021). In nature, antibiotics are generated by fungi, bacteria, and actinobacteria (Chandra and Kumar 2017). About 70% of effective antibiotics are produced by actinobacteria and *Streptomyces* attribute to >80% of the global antibiotics (Al Farraj et al. 2020). Beside natural pathways, several studies reported the alternative routes for the production of antibiotics. The potential of cellulose agro-waste for antibiotics production has been identified in many studies (Asagbra et al. 2005). The groundnut shell, sawdust, corn cobs, rice hulls, and different other agricultural wastes have been used for the production of antibiotics (Sadh et al. 2018).

Solid state fermentation (SoSF) is known as a promising method for the production of wide-ranging antibiotics. The media composition and the fermentation conditions (e.g., pH, temperature, fermentation time) significantly affect the production rate of antibiotics; therefore, the optimization of nutritional factors and the fermentation conditions is necessary to attain high productivity (Al Farraj et al. 2020).

This chapter gives an overview of antibiotics, agro-waste, and selection of appropriate fermentation process for the production of antibiotics using agro-wastes as substrate. The analysis of SoSF process and subsequent experimental design including substrate treatment, process optimization, and isolation conditions can ensure highest quantity and quality of the target product. The appropriate technology can provide a systematic and valuable information for antibiotic producing industries on one side and helps to reduce pollution risks (posed by agriculture waste) on the other side.

7.2 Antibiotics

Antibiotics are low molecular weight bioactive compounds having selective antimicrobial (antibacterial, antifungal), antiviral, and even antitumor properties. Antibiotics have been derived from certain organisms, i.e., bacteria, fungi, algae, lichens, and green plants, by fermentation process, and inhibit the growth of certain other microorganisms. Antibiotics, for example, penicillin, are produced by molds and have high selective toxicity against many human pathogens. These bioactive compounds are used in a range of industries including pharmaceuticals and food industry.

7.2.1 Chemical Structure and Classification

On the basis of toxicity or range of effectiveness, antibiotics can be classified into broad spectrum (effective against diverse group of microorganisms) and narrow

spectrum (effective against a limited group of microorganisms). Other classifications are based on microbial spectrum, nature of biological activity, source, chemical structure, and characteristics (Walsh 2003). Based on molecular or chemical structures, the most common classes of antibiotics include aminoglycosides, quinolones, macrolides, β-lactams, sulfonamides, tetracyclines, oxazolidinones, and glycopeptides. β-Lactams are the major group of highly selective commercial antibiotics having the β-lactam ring. Penicillin G ($C_{16}H_{18}N_2O_4S$), cephalosporins, ampicillin ($C_{16}H_{18}N_3NaO_4S$), and monobactams are the main representative of this group (Elander 2003). They selectively kill the target microorganism, i.e., *Streptococcus*, *Meningococcus*, and *Diphtheria*, by impairing their normal cell wall synthesis process, mainly by interfering with peptidoglycan structure. Some bacterial strains develop resistance to β-lactam antibiotics by producing enzyme β-lactamase (Drawz and Bonomo 2010). In some cases, to overcome resistance, β-lactamase inhibitors, e.g., clavulanic acid ($C_8H_9NO_5$), sulbactam ($C_8H_{11}NO_5S$), and tazobactam ($C_{10}H_{12}N_4O_5S$), are often effective.

Aminoglycosides are broad-spectrum antibiotics composed of an aminocyclitol ring linked with two or more amino sugars via glycosidic bond. They exhibit high antimicrobial activity against facultative and aerobic Gram-negative bacteria like *Mycobacteria*, *Staphylococci*, *P. aeruginosa*, *Acinetobacter*, and *Enterobacter*. This group comprises tobramycin ($C_{18}H_{37}N_5O_9$), gentamicin ($C_{21}H_{43}N_5O_7$), kanamycin ($C_{18}H_{38}N_4O_{15}S$), neomycin ($C_{23}H_{46}N_6O_{13}$), and streptomycin ($C_{21}H_{39}N_7O_{12}$). However, these antibiotics have unwanted side effects including nephrotoxicity and ototoxicity. Some semisynthetic aminoglycosides with distinct toxicology for resistant strains include amikacin, dibekacin, and netilmicin (Mingeot-Leclercq et al. 1999).

Tetracycline has class-specific and intrinsic antibiotic-resistance mechanisms against Gram-positive and Gram-negative bacteria, spirochetes, obligate intracellular bacteria, as well as protozoan parasites. This group includes tetracycline ($C_{22}H_{24}N_2O_8$), minocycline ($C_{23}H_{27}N_3O_7$), tigecycline ($C_{29}H_{39}N_5O_8$), omadacycline ($C_{29}H_{40}N_4O_7$), and eravacycline ($C_{27}H_{31}FN_4O_8$). Tetracycline arrest synthesis of protein through binding with 30S ribosomal subunit, thus interfering with the docking of aminoacyl-transfer RNA to mRNA-ribosome complex. These antibiotics also exhibited anti-inflammatory activity, immunosuppression, mitigation of collagenase and lipase activity, wound curing, and treatment of range of sexually transmitted diseases.

Sulfonamides are the modern bacteriostatic antibiotics that act synergistically against range of bacteria comprising both Gram-positive and Gram-negative bacteria, some types of protozoa and fungi. They block microbial folate synthesis process by competitively mitigating the transformation of p-aminobenzoic acid (PABA) to dihydropteroate. Macrolides are bacteriostatic antibiotics composed of macrocyclic lactone ring of different sizes linked with one or more deoxy-sugars. This class of antibiotics includes erythromycin ($C_{37}H_{67}NO_{13}$), clarithromycin ($C_{38}H_{69}NO_{13}$), and azithromycin ($C_{38}H_{72}N_2O_{12}$). These are effective against Gram-positive bacteria whereas slightly active against penicillin-resistant staphylococci, enterococci, and

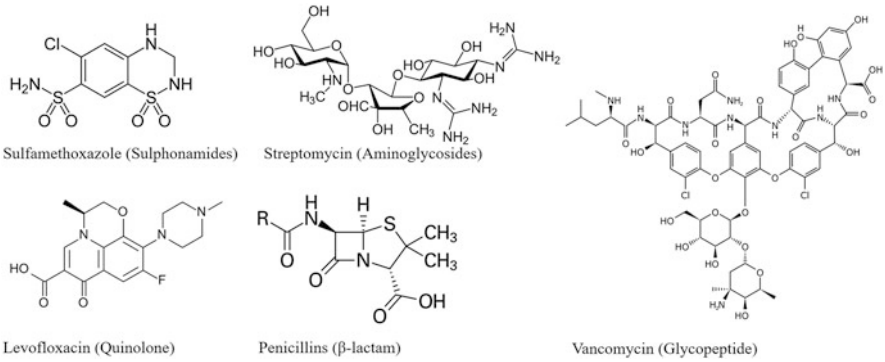

Fig. 7.1 Chemical structures of some major antibiotics with class name in parenthesis

Table 7.1 Some common antibiotics and their mode of actions

Representative antibiotic	Mode of action
Polymyxins, polyenes	Disrupting the plasma membrane
Penicillin, cephalosporin, bacitracin	Inhibition of cell wall synthesis
Sulfonamides	Inhibition of enzymatic activity
Streptomycin, tetracycline	Interference with protein synthesis
Rifamycin, ciprofloxacin	Interference with nucleic acid synthesis

most Gram-negative bacteria. They arrest the protein synthesis by binding reversibly to 50S ribosomal subunits of microorganisms (Dinos 2017).

Glycopeptides are the glycosylated cyclic (or polycyclic) non-ribosomal peptides, which are categorized as first-generation antibiotics (e.g., vancomycin ($C_{66}H_{75}Cl_2N_9O_{24}$), ramoplanin ($C_{119}H_{154}ClN_{21}O_{40}$), and teicoplanin), and second-generation semisynthetic antibiotics (e.g., dalbavancin ($C_{88}H_{100}Cl_2N_{10}O_{28}$), oritavancin ($C_{86}H_{97}Cl_3N_{10}O_{26}$), and telavancin ($C_{80}H_{106}Cl_2N_{11}O_{27}P$)). They disrupt the cell wall synthesis of microorganisms through preventing peptidoglycan incorporation (Blaskovich et al. 2018). Glycopeptides antibiotics are key weapon in the fight against drug resistant bacteria including multi-resistant *Staphylococci* (MRSA) although they have limited activity against different Gram-positive microorganisms. Figure 7.1 shows the chemical structure and classes of some common antibiotics.

7.2.2 Mechanism of Action

Antibiotics interfere with the normal cellular function of the target microorganisms, in microstatic mode (simply prevent growth) or microcidal mode (directly kill microorganisms), while leaving the host cell unaffected. The major modes of action by most commonly used antibiotics are described in Table 7.1. Antibiotics generally target the cell wall, cell membrane, nucleic acid synthesis, protein synthesis, and

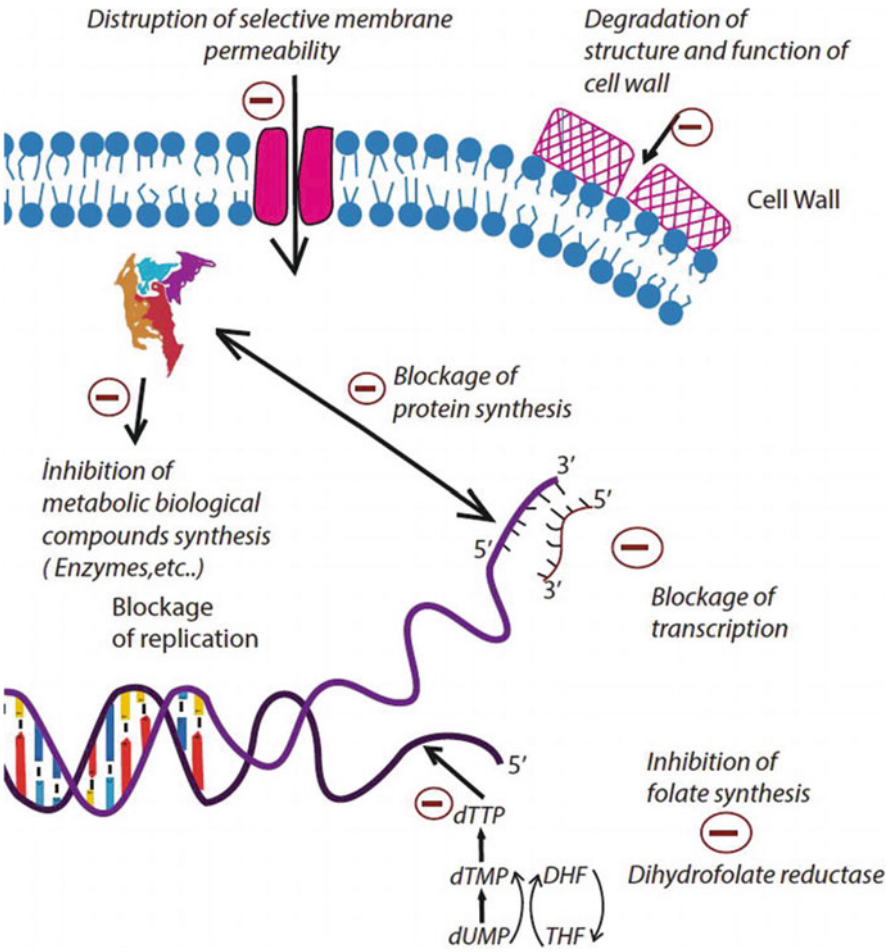

Fig. 7.2 Targets of antibiotics (Kırmusaoğlu et al. 2019). Image reused under the Creative Commons Attribution License

synthesis of biological metabolic compounds. A depiction of antibiotic actions is presented in Fig. 7.2. The excessive consumption of antibiotics, gene mutations, carrying resistance genes in plasmids and chromosomes, taking resistance genes from transposons, insertion sequences, and conjugation lead bacteria to progress resistance against antimicrobials.

The major group of antibiotics that work by inhibiting the assembling of cell wall are β-lactams (penicillin, cephalosporins) and glycopeptides (vancomycin and Bacitracin). β-Lactams prevent the final cross-linking of the newly synthesizing peptidoglycan layer by irreversibly binding to the active site of transpeptidases. Bacitracin interferes with the dephosphorylation of membrane carrier molecules such as bactoprenol pyrophosphate. Eventually faulty peptidoglycan assembly

results in weaker bacterial cell wall, which is prone to swelling and lysis in the hypotonic environment. Polymyxins are the important class of antibiotics that act by disturbing the structural integrity of the plasma membrane and resulting in leakage of important cell contents. Polymixin B binds to the phospholipids and thus disrupts the permeability of cell membrane.

Rifamycins are the class of antibiotics that interfere with mRNA production by inhibiting bacterial RNA polymerase. Other antibiotics including ciprofloxacin and trimethoprim inhibit DNA synthesis by binding with topoisomerase, preventing the super coiling of DNA. Owing to the ability to penetrate tissues and selective toxicity these are widely used in chemotherapy. Antibiotics such as streptomycin, erythromycin, gentamycin, tetracycline, chloramphenicol, and spectinomycin inhibit the protein synthesis via binding with bacterial ribosome (50S or 30S subunit) or mRNA. Some antibiotics block important metabolic pathways occurring inside the microbial cells. For example, folic acid is crucial for purine and pyrimidine synthesis, sulfonamides inhibit the folate (folic acid) biosynthetic process in some microbes including bacteria, thus acting as bacteriostatic antibiotics.

7.2.3 Production and Purification

Antibiotics are secondary metabolites mostly produced by fermentation process in which high yielding microbial strains are cultured under optimum conditions. This commercial production is brought about by manipulating classical and molecular genetics as well as nutritional regulations to enhance the production of target metabolites and to bypass and/or remove negative regulatory mechanisms. The first step in antibiotic production is identification and isolation of appropriate source or organism followed by managing its optimum growth conditions in a sterile environment and finally extraction and purification to a crystalline product. Table 7.2 summarizes the mostly applied methods for the production of antibiotics.

7.3 Agro-Waste Products

An increasing global population also increased the need of food and agricultural products. This caused rapid growth of agricultural industry and subsequently excessive waste production, a serious threat to environment. The term agro-wastes is generally referred to the by-products or waste produced during agricultural practices. As per an estimate 1/3 of the food production (~1.3 billion ton) is wasted per annum (Ravindran et al. 2018b). About 40–50% of the global food waste contains vegetables, fruits, tuber, and roots reaching up to 0.65 billion ton/year. In the European union, the food wastage is 89 million ton/annum including 39% share of waste generated during manufacturing. Whereas, total production of agricultural waste reaches 367 million ton/year that includes crop residues.

There are several ways for classification of agro-waste; however, primarily they are divided in two major categories including industrial residues and agricultural

Table 7.2 Commonly used techniques for the production of antibiotics

Production category	Applied method and examples
Natural production of antibiotics	Fermentation is the widely used technique for the natural production of antibiotics. Penicillin is the common example that is produced by *Penicillium chrysogenum*
Semisynthetic production of antibiotics	It is the combination of natural fermentation and lab-scale efforts to enhance the efficiency of antibiotic. Methicillin and ampicillin are the common examples of semisynthetic production of antibiotics. The addition of an extra NH_2 group to the distinguished R group of the penicillin results in the formation of ampicillin. The presence of an additional NH_2 group offers ampicillin a wider scale of application as compared with penicillin. Methicillin is also the derivative of penicillin and its structure differs from penicillin due to the presence of two methoxy ($O–CH_3$) groups. The methoxy groups enable methicillin to be applied against penicillinase (a specific form of β-lactamase) forming bacteria, which would otherwise be impervious to the penicillin
Synthetic production of antibiotics	Although majority of antibiotics are obtained from natural or semisynthetic ways, there are also some antibiotics that are entirely synthesized in the laboratories. Quinolones are the common examples of antibiotics produced via this method. The common quinolones are fluoroquinolones including lomefloxacin ($C_{17}H_{19}F_2N_3O_3$), ciprofloxacin($C_{17}H_{18}FN_3O_3$), ofloxacin ($C_{18}H_{20}FN_3O_4$), norfloxacin ($C_{16}H_{18}FN_3O_3$), moxifloxacin ($C_{21}H_{24}FN_3O_4$), and levofloxacin ($C_{18}H_{20}FN_3O_4$)

residues. The industrial residue includes the agricultural waste generated at commercial scale such as fruit peels, vegetable peels, soybean oil cake, groundnut oil cake, etc. Agricultural wastes or by-products are further divided in two categories known as process residues (molasses, bagasse, husk) and fields residues (stalk, stems, seeds, etc.) (Lehmann et al. 2006; Sadhukhan et al. 2019). Generally, the agro-wastes have high nutrition value and they may serve as breeding bases for pathogenic microbes if remained unprocessed and poorly treated. Amusingly, these wastes can be applied as a potential source of renewable energy or as substrate for the production of several valuable products. Cellulose nanocrystals are appropriate for range of progressive medical applications including drug delivery, tissue engineering, emulsion stabilization, enzyme or protein immobilization, etc. (Wijaya et al. 2017).

Table 7.3 shows the chemical composition of widely used agro-wastes. Cellulose is the basic constituent of plant matrices and the plant cell wall is composed of stiff cellulosic microfibrils entrenched into a matrix of soft hemicelluloses and lignin. Cellulose units are organized to form close, intermolecular and/or intramolecular H-bonds, which are stabilized to produce compression. These microfibrils contain basic fibers responsible for the stem strength in plants. Due to high composition of carbon, the agro-waste is widely used for many applications such as biosynthesis of nanoparticles, raw material for biotechnology (Terrone et al. 2020), biofuel production (Falarz et al. 2018), biochar synthesis (Jung et al. 2016), water treatment (Shahid et al. 2020), and manure production.

Table 7.3 The chemical constituents and their percentage share in common agro-waste

Agro-waste	Lignin	Protein	Fat	Crude fiber	Carbohydrate	Ash	References
Cassava peel	1.92	1.7	3.1	11.2	75.5	2.4	Adeniran et al. (2010)
Yam peel	–	1.8	–	4.1	74.7	4.3	Adeniran et al. (2010)
Rice bran	25.63	38.2	30.4	26.9	14.1	3.4	Ravindran et al. (2018b)
Coffee waste	23.90	17.44	2.29	60.46	55.53	1.30	Ravindran et al. (2018a)
Banana peel	6.4	0.6	3.0	9.3	79.0	2.7	Adeniran et al. (2010)
Wheat bran	5.6	13.2	3.5	33.4	56.8	3.9	Onilude et al. (2012)
Sugarcane bagasse	17.79	2.3	–	–	66.48	8.80	Veana et al. (2014)
Citrus waste	1.0	7.9	–	–	30	1.7	Biz et al. (2016)
Brewing grains	30.48	2.4	–	3.3	79.9	7.9	Francis et al. (2003)

7.4 Production of Antibiotics Using Agro-Wastes

Agro-waste can be efficiently used for the production of antibiotics. Appropriate fermentation techniques such as SoSF can be employed to produce cost-effective antibiotics at large scale from agro-waste. Many antibiotics such as neomycin, oxytetracycline, and rifamycin can be produced using agro-waste as substrate in SoSF process. Beside the appropriate fermentation technique, other factors including raw material (starting agro-waste as substrate) and the optimized processing steps also contribute to the composition, quality, and quantity of the antibiotics.

In general, the SoSF is known as the process in which the growth of organisms takes place on solid substrates or non-soluble materials in the absence of free water. The widely applied substrates in SoSF includes wheat bran, cereal grains, legume seeds, lignocellulose materials, and a variety of animal and plant materials. The absence or near absence of water in SoSF provide numerous benefits such as cost-effective production, easy product recovery, small fermenter-size, abridged downstream processing, and low energy demand for sterilization and stirring. The proficiency of SoSF process is associated with range of process variables including substrate, microorganisms, aeration, temperature, and fermenter type.

The microbes applied in SoSF can be of different types such as single pure culture, mixed recognizable culture, or a consortium of diverse indigenous microbes. SoSF accomplishes in several steps including substrate selection, pretreatment of substrate, hydrolysis of basic polymeric matter (proteins and polysaccharides), fermentation, and purification of final product. Several studies reported the

application of agro-waste including blacked eye pea, rice, peanut press cake, ground nut oil cake, apple pomace, etc. in SoSF processes (Sadh et al. 2018). The composition of substrates varies from one to another, and hence their usage is product oriented.

A study evaluated the capability of *Streptomyces rimosus* NRRL B2659, *Streptomyces alboflavus* NRRL B1273, *Streptomyces* sp. OXCI, *Streptomyces rimosus* NRRL B2234, *Streptomyces vendagensis* ATCC 25507, and *Streptomyces aureofaciens* NRRL B2183 for the production of tetracycline utilizing domestic agro-waste (corncob, cassava peels, peanut shell, and corn pomace) as growth media for SoSF (Asagbra et al. 2005). Peanut shells were the highly efficacious substrate (4.36 mg/g) as compared with other agro-waste components including cassava peels (2.16 mg/g), corn pomace (1.99 mg/g), and corncob (2.64 mg/g). For the production of tetracycline, the optimal solid state medium composition was 100 g peanut shells, 0.5 g KH_2PO_4, 0.5 g $MgSO_4$ $7H_2O$, 1 g $(NH_4)_2SO_4$, 0.5 g NaCl, 0.5 g $CaCO_3$, 10 g soluble starch with 65–68% moisture content. The initial pH was in range of 5.3–6.3. 1 g dry wt of substrate is inoculated with 1×10^8 conidia/mL and incubated at 28–31 °C for 6 ± 1 days, generating 13.18 mg/g of tetracycline. The production of tetracycline is initiated on third day and the maximum level is achieved on day 5.

Mahalaxmi et al. (2010) examined the production of rifamycin B through isolated *Amycolatopsis* sp. RSP 3 using agro-wastes (corn husk, corn cobs, and wheat bran) as substrate material in SoSF (Mahalaxmi et al. 2010). The application of corn husk resulted in fourfold higher production of Rifamycin B than corn cobs and wheat bran. Another study reported the production of neomycin using range of agro-waste (cotton seed meal, apple pomace, wheat bran, and soybean powder) in SoSF (Vastrad and Neelagund 2011). The use of apple pomace substrate resulted in the maximum production of neomycin, i.e., 2.7 mg/g substrate. This study also evaluated the effect of critical parameters (e.g., particle size of substrate, inoculum size, initial pH, incubation temperature, moisture contents, incubation time, surplus nitrogen and carbon sources) on the production of neomycin using *Streptomyces fradiae* NCIM 2418. The maximum production is achieved with optimal values of 2×10^6 CFU/g inoculum size, 1.2 mm size of substrate particle, 30 °C incubation temperature, pH 8.0, 70% moisture, 1% w/v fructose, 1%w/v L-glutamine, 1% w/v $(NH_4)_2HPO_4$, and 10 days of incubation period. An optimization in process/parameters results in 2.6-fold enhancement in production of neomycin.

Another study assessed the proficiency of *Streptomyces* sp. SD1 for the production of antibiotics using range of agro-wastes (Kalaiyarasi et al. 2020). This study used wheat bran, pineapple peel, apple pomace, tapioca powder, rice bran, green gram husk, and orange peel as substrate for production of antibiotics. *Streptomyces* sp. SD1 exhibited the maximum production of antibiotics when green gram husk is used as substrate. The effect of several carbon sources on the antibiotic production is also evaluated. It is found that production is increased when maltose and starch were supplied, whereas glucose and sucrose caused an adverse effect on the production of antibiotics. The supplementation of calcium chloride, manganese chloride, and magnesium sulfate improved the production of antibiotics while addition of mercuric chloride and cobalt chloride left negative impact on production of antibiotics.

Factually, reported studies highlighted the cost-effectiveness of SoSF for the production of antibiotics.

7.5 Conclusion

This chapter discusses the potential of agricultural waste to be used for the production of antibiotics. Agro-wastes including sawdust, green gram husk, corn cobs, rice hulls, wheat braw, groundnut shells, etc. are the rich sources of bioactive compounds and can be efficiently used for the production of antibiotics. The composition, quantity, and quality of developed antibiotics significantly depend on the starting material and the processing steps. Suitable fermentation methods, for example, SoSF, can be used to produce low cost antibiotics from agro-waste. Several antibiotics such as neomycin, oxytetracycline, and rifamycin have been developed using agro-waste as solid substrate. There are two major advantages of using agro-waste in production of antibiotics that are (a) cost-effective production process for antibiotics and (b) reduction in environmental pollution by agro-waste utilization. Therefore, it is necessary to further explore the potential of different agro-wastes in large-scale production of antibiotics.

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8 Valorization of Agri-Food Industry Waste for the Production of Microbial Pigments: An Eco-Friendly Approach

Prabhjot Kaur, Simranjeet Singh, Gargi Ghoshal, Praveen C. Ramamurthy, Parul Parihar, Joginder Singh, and Ashutosh Singh

Abstract

Globally, over one-third of total food production is wasted along the food value chain, which amounts to 1.7 billion tons per year that also includes agro-industrial residues such as fruits and vegetable waste in the form of peels, seeds, liquid, and molasses. A major portion of this waste is either anaerobically digested or utilized for animal feed and is dumped into landfills which contribute to greenhouse gas emissions that adversely affect the environment. The valorization of food waste can be achieved through the biorefinery processing of biomass into high-value components and energy. Microbial biocolors are the coloring agents that are derived from biological sources such as biomass and agricultural residues by microorganisms. Bicolor production from the microbial origin is beneficial in terms of nontoxic and superior quality, biodegradability, compatibility with the environment, and independence from seasonal variation. Thus, biotechnological production of natural colors with low-cost substrates such as agro-industrial residues is the cheapest source of natural color production. In addition to food

Prabhjot Kaur and Simranjeet Singh contributed equally with all other contributors.

P. Kaur · A. Singh (✉)
School of Engineering, University of Guelph, Guelph, ON, Canada
e-mail: asingh47@uoguelph.ca

S. Singh · P. C. Ramamurthy
Interdisciplinary Centre for Water Research, Indian Institute of Science, Bangalore, India

G. Ghoshal
Dr. S. S. Bhatnagar University Institute of Chemical Engineering and Technology, Panjab University, Chandigarh, India

P. Parihar · J. Singh (✉)
Department of Microbiology, Lovely Professional University, Phagwara, India
e-mail: joginder.15005@lpu.co.in

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color, natural colors also act as antimicrobial, antioxidant, antimutagenic, and precursor of vitamins, which help to reduce cancer, chronic diseases, macular degeneration, cataract and are used for the production of biopharmaceutics and cosmetic products. Natural colors have a very high market value; thus, the extraction of these pigments from waste can lead to high market revenue. This chapter covers a comprehensive review of the biotechnological production of microbial colorants from agro-industrial waste, discusses their physicochemical properties and applications in different industries.

Keywords

Agro-industrial waste · Food colorant · Microbial pigments · Fermentation · Novel technologies

8.1 Introduction

Biocolor is derived from a combination of two words "Bio" which means natural and "color" which provides a hue to the substance. Biocolorants or biopigments are the natural coloring agents obtained from the biological origin such as plants, animals, microorganisms such as bacteria, algae, fungi, yeasts, and insects (Parmar and Phutela 2015). Natural pigments obtained from biological origin are safe, renewable, environment friendly, and are biodegradable. Bio colorants are the coloring agents known for their safe usage in the food, nutraceutical, pharmaceutical, and cosmetic industries (Aruldass et al. 2018). These natural pigments are considered as potent green pigments to replace synthetic dyes. Nevertheless, artificial or chemically synthesized colorants are cheaper and more stable than the biocolorants, but excess usage of synthetic colorants in manufacturing of food products and drugs causes carcinogenic, toxicological, teratogenic, and allergenic problems (Heer and Sharma 2017).

On the other hand, synthetic color extraction depends on petroleum-based organic solvents and non-renewable sources. In addition to this, WHO and U.S. FDA had imposed the guidelines on usage or recommended daily intake of synthetic colorants in food products, nutraceuticals, cosmetics, and drug products (Tuli et al. 2015). Hence, to avoid the adverse effects of synthetic colorants, many research efforts have been made to produce natural colorants or biopigments.

Biocolorants can be synthesized from different renewable sources, i.e., plants, animals, and microbes. Plant pigments are produced by the photosynthesis process, which uses chlorophyll and carotenoids for their functioning. Examples of plant pigments are curcumin, lutein, lycopene, carotenoids, anthocyanin, betanin, chlorophyll, and bixin (Rodriguez-Amaya 2016). Most animals produce biological pigments or biochromes such as melanin in mammals, pterin, porphyrin, and flavonoids (Heer and Sharma 2017). Microbial production of pigments is the

potential source of biocolorant producers due to their benefits over plants and animals such as independence of geographical and environmental conditions, throughout the year availability, more stability, cost-effective, good yield, easy and solvent-free downstream processing (da Costa Cardoso et al. 2017). Microbial fermentation produces a variety of biopigments such as carotenoids, anthocyanin, quinines, flavins, monascins, violacein, prodigiosin, and indigoidine (Ganguly et al. 2019).

Microbial production of biopigments is considered the cheapest source of pigment production due to their effective growth on low-cost medium substrates. Hence a low-cost medium provides a thumb rule to produce low-cost pigments (Panesar et al. 2015). Among low-cost substrates, a wide range of agro-industrial residue or waste can be considered the ideal source providing carbon, nitrogen, and minerals in potent amounts (Lopes et al. 2013).

Agro-industrial waste or residue is the waste generated during the post-harvest or industrial processing of agricultural produce. The waste obtained during the agricultural practices in the field gives straw, stem, leaves, husk, stubbles, shell, and hulls produced throughout the year (Hernández-Alcántara et al. 2016). These agrowastes are rich source of nutrients which allows its use as raw material for solid-state fermentation. Hence, this waste provides a low-cost alternative substrate to produce high-value bioactive components and control environmental pollution (Zuin and Ramin 2018). On the other hand, waste or by-products from the agro-food processing industries produce bagasse, peels, brewer's spent grains, spent coffee grounds, etc. Liquid waste such as corn steep liquor and whey can be used as efficient medium component for carbon, nitrogen, and minerals in microbial pigment production processes (Lopes and Ligabue-Braun 2021).

The biocolors produced from microorganisms have Pro-Vitamin A and other medicinally important properties apart from being natural and safe to use. Microbial pigments provide other biological functions such as antimicrobials, antioxidants, antiproliferative, antiparasitic, and anticancer. Hence, the application of microbial pigment is not restricted to food industries but can be applied to the pharmaceutical, nutraceutical, cosmetic, and textile industries (Sen et al. 2019).

However, the microbial production of natural colorants is a cumbersome task with the fermentation conditions. Hence, with modern biotechnological methods, the extraction efficiency can be improved to meet the growing demand for natural colorants (Rymbai et al. 2011). This chapter will provide detailed information on sustainable microbial production of pigments from low-cost substrates such as agro-industrial waste, downstream processing to recover the pigments, and their application in different industries.

8.2 History of Microbial Pigments

Production of biocolorants from microbial sources is considered as a novel method for cheaper pigment production. The oldest use of natural colorants as the dye was recorded dated 2600 BC in China. In the Indus valley period (2500 BC), clothes of red color and madder dye traces were found in the destroyed sites of Mohenjodaro

and Harappa civilization (3500 BC), which gave the proof of dye or color invention at that period (Lopes and Ligabue-Braun 2021). Natural colorants were the primary or exclusive origin of colors prior to synthetic dyes. In 1856, Perkin prepared synthetic pigments, which was cheaper, easy to produce, and independent of the weather conditions (Joshi et al. 2003). Hence, with the advancement of technology and chemical methods, the production of synthetic pigments has become easy, cheaper, and higher production rate. Despite all these, excessive use of synthetic pigments in food, pharmaceuticals, and cosmetics can cause toxic effects to humans due to their carcinogenic nature. All these side effects from the synthetic colorants have shifted the interest of people towards natural and safe edible colorants from the last five decades (Venil et al. 2013).

8.3 Classification of Microbial Pigments

Microbial pigments can be majorly categorized based on color, microorganism type, and solubility of the pigments in different solvents (Table 8.1).

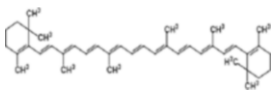
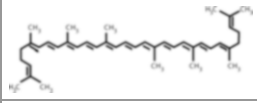
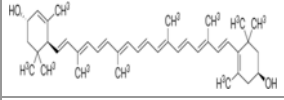
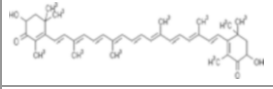
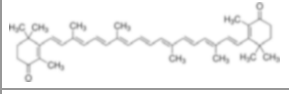
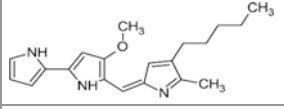
1. Based on the color of the pigment.
   (a) Yellow pigments (Carotenoids, Riboflavin).
   (b) Red pigments (Carotenoids, prodigiosin, porphyrin, arpink red).
   (c) Blue pigments (Anthocyanin, indigoidine, violacein, melanin).
2. Based on microbial sources.
   (a) Bacteria, algae, yeasts, fungus, protozoa, and molds.
3. Based on solubility.
   (a) Water and fat-soluble.
   (b) Polar and non-polar solvents solubility.

8.3.1 Carotenoids

Carotenoids are the major class of natural pigments containing isoprenoid structure and exhibit yellow to orange color. These red, orange, and yellow color pigments are mainly synthesized by bacteria, fungi, yeast, microalgae, and plants. Major carotenoids pigments of huge market interest, safe usage in the food industry, and synthesized by microorganisms are lycopene, β-carotene, lutein, astaxanthin, and canthaxanthin (Mussagy et al. 2021).

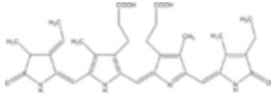
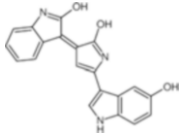
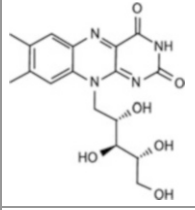
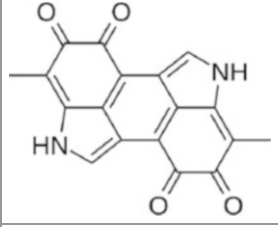
Agro-industrial waste such as grape must, carrot and other orange and yellow colored vegetable peels, corn steep liquor, beet molasses, glycerol, sugarcane molasses, glucose syrup, gram waste such as soybean flour, moong bean flour, and cereals waste can be considered as a low-cost carbon, nitrogen, and mineral sources for carotenoid production by microorganisms (Roukas et al. 2003).

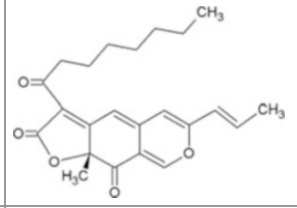
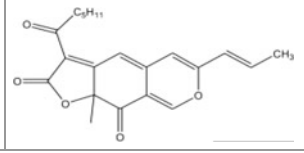
Table 8.1 Chemical structure and microbial sources of pigments

Microbial pigment	Color	Chemical Structure	Microbial sources	References
β-Carotene	Yellowish orange		• *Blakeslea trispora* • *Rhodotorula glutinis* • *Dunaliella salina* • *Rhodotorula rubra* • *Mucor circinelloides* • *Phycomycesblakes leeanus* • *Rhodotorula rubra*	Kaur et al. (2019)
Lycopene	Red		• *Blakeslea trispora* • *Fusarium sporotrichioides*	Zuorro et al. (2011)
Lutein	Yellowish-red		• *Chlorella* sp.	Lopes and Ligabue-Braun (2021)
Astaxanthin	Pink, Pink-red		• *Xanthophyllomyces dendrorhous* • *Agrobacterium aurantiacum* • *Paracoccus carotinifaciens*	Lopes and Ligabue-Braun (2021)
Canthaxanthin	Dark-red		• *Bradirhizobium* sp. • *Haloferax alexandrines* • *Gordonia jacobaea*	Dufossé (2006)
Prodigiosin	Red		• *Serratia marcescens* • *Pseudoalteromonas rubra* • *Streptomyces* sp. • *Vibrio gaogenes*	Kumar et al. (2015)
Phycocyanin	Light blue		• *Aphanizomenonflos-aquae* • *Spirulina* sp.	Dufossé (2016)

(continued)

Table 8.1 (continued)

Microbial pigment	Color	Chemical Structure	Microbial sources	References
			• *Pseudomonas* sp. • *Arthrospira* sp.	
Violacein	Purple		• *Chromobacterium violaceum* • *Janthinobacterium lividum* • *Pseudoalteromonas* sp.	Sigurdson et al. (2017)
Riboflavin	Bright yellow		• *Bacillus subtilis* • *Ashyba gossupi* • *Xanthophyllomyces dendrorhous* • *Candida guilliermondii* • *Debaryomyces subglobosus* • *Clostridium acetobutylicum*	Dufossé (2006)
Melanin	Black		• *Bacillus thuringiensis* • *Saccharomyces* sp. • *Neoformans* sp. • *Streptomyces virginae* • *Saccharomyces nigricans*	Venil et al. (2013)
Arpink red	Red		• *Penicillium oxalicum*	Kumar et al. (2015)

				
Monascus pigments	Red		• *Monascus pilosus* • *Monascus purpureus* • *Monascus ruber* • *Monascus froridanus*	Dufossé et al. (2005)

8.3.1.1 Carotenoid Synthesis from Bacteria, Yeast, and Fungi

Carotenoids are naturally occurring in bacteria, fungi, yeast, and microalgae. The colorants obtained from these microbial sources range from yellow to red. The synthesis of carotenoids from fungi and yeast can be improved by manipulating the media composition, substrates and light stimulation. As yeast and fungi are heterotrophic organisms, the culture medium needs to be optimized along with the fermentation conditions and medium composition. Agro-industrial waste serves as the cheapest nutrient source for microbial pigment production with higher yield and minimizes production costs. Different agro-industrial residues and by-products serve as the low-cost carbon source for microbes production (Papaioannou and Liakopoulou-Kyriakides 2012).

β-Carotene production by *Blakeslea trispora* (fungus) and the use of two mating species play a major role in the large-scale production of carotenoids (Ribeiro et al. 2011). Yeast species *Rhodotorula glutinis* plays a major role in the large-scale production of carotenoids such as β-carotene, astaxanthin, and zeaxanthin from agro-industrial waste such as fruit peels, cereals, raw stalks, bran and pulses husk, etc. (Malisorn and Suntornsuk 2008).

Lycopene is a dark red colored pigment and an acyclic isomer of conjugated carotenoid structure of β-carotene. It is more stable and has high antioxidant potential in comparison to other carotenoids such as trans-lycopene and β-carotene. Lycopene can be synthesized by fungus sp. *Fusarium sporotrichioides* on corn fibers and *Rhodotorula glutinis* yeast and *Blakeslea trispora* on agro-industrial residues such as tomato peels, etc. (Chandi et al. 2010). Zeaxanthin, a carotenoid alcohol, is yellowish-orange in color, majorly a bacterial pigment synthesized by *Staphylococcus aureus*, *Bacillus*, *Flavobacterium* sp., *Corynebacterium* sp. (Ganguly et al. 2019). On the other hand, astaxanthin, classified as a xanthophyll, is a reddish-orange lipid-soluble pigment mainly found in yeasts such as *Xanthophyllomyces dendrorhous*, and microalgae *Haematococcus pluvialis* (Bi et al. 2010). Bacterial sources for astaxanthin production may include *Paracoccus marcusii*, *Paracoccus carotinifaciens*, *Agrobacterium aurantiacum,* etc. (Dufossé 2006).

8.3.1.2 Carotenoids Synthesis from Microalgae

Astaxanthin, a yellowish-orange keto-carotenoid pigment, represents strong antioxidant activity compared to other carotenoids such as lycopene, lutein, β-carotene, and zeaxanthin. It has enormous demand in the pharmaceutical and nutraceutical industries (Honda et al. 2019). Astaxanthin can be produced in freshwater microalgae *Haematococcus pluvialis* by two-stage culture. Such intracellular production of pigment involves the morphological transformation, which turns green vegetative cells into dark-red astaxanthin-rich components (Chattopadhyay et al. 2008).

Agro-industrial wastewater can be treated with the production of well-nourished *Haematococcus* and *Dunaliella* sp. which further reduces the cost of algal biomass by valorizing it for the production of high-value bioactives and other bio-energy products (Spolaore et al. 2006). Agro-industrial by-products or waste materials such

as cassava processing wastewater, corn steep liquor, and ethanol effluent can be used as a growth media for the production of microalgae (Babitha et al. 2006).

During the treatment of wastewater with conventional biological processes, external carbon sources are incorporated to convert the excess nitrates into nitrogen gas and biomass. While the growth of microalgae in the wastewater assimilate nitrates present in it and convert this into nitrogen which helps in the production of astaxanthin pigment. Research reported by Kang et al. (2006) revealed that *Haematococcus* algae cultivation in the wastewater completely removed the inorganic wastes and helps in the conversion of green vegetative cells into red astaxanthin pigments (Brar et al. 2013).

Canthaxanthin, orange to dark pink colored microbial synthesized, is a ketocarotenoid pigment soluble in lipids. Microalgae species *Nannochloropsis gaditana* and *Chlorella zofingiensis* have been reported to produce canthaxanthin from agro-industrial residues such as corn steep liquor, glucose, and these carotenoids are considered as a natural antioxidant to prevent lipid oxidation (Rana et al. 2021).

8.3.2 Anthocyanin

Anthocyanin is the blue-purple color of natural pigments and belongs to the flavonoid group of polyphenols. Anthocyanin is a water-soluble rich intensity coloring pigments and has high antioxidant and antimicrobial activities (Rodriguez-Amaya 2019). Anthocyanin pigment is not stable at normal conditions, and its production is also not sustainable due to the variation in plant species. So metabolic engineered or engineered microorganisms such as *E. coli*, *Candida utilis*, and *Pichia pastoris* have been used to produce anthocyanin at an industrial scale using agro-industrial residue as carbon and nitrogen source (Ganguly et al. 2019).

8.3.3 Prodigiosin

Prodigiosin is a natural red colored pigment and secondary metabolite alkaloid mainly produced by bacteria. Prodigiosin is tetrapyrrole structured antibiotic pigments synthesized by *Serratia marcescens*, gram-negative bacteria such as *Pseudomonas magneslorubra*, *Rugamonas rubra*, *Hahella chejuensis*, *Vibrio psychroerythrus,* and *V. gazogenes* (Sánchez-Muñoz et al. 2020). This pigment represents biological functions such as antimicrobial, antiviral, anticancer, and antimalarial (Rana et al. 2021). This pigment is unstable at normal atmospheric conditions such as sensitivity to high temperature, poor solubility, and pH instability. Hence, to mitigate these limitations, prodigiosin is spray-dried and encapsulated in microcapsules to enhance stability (Darjily et al. 2016).

8.3.4 Violacein

Violacein is a naturally occurring di-indole-pyrrole violet-blue colored pigment that possesses numerous biological functions such as antimicrobial, antiviral, anticancer, antiulcerogenic, anti-leishmanial, and enzyme modulation properties (Narsing et al. 2017). Violacein is biosynthesized by bacterial species such as *Chromobacterium violaceum*, *Collimonas* sp., *Pseudoalteromonas* sp., *Pseudomonas aeruginosa*, and *Janthinobacterium* sp. This natural pigment is used extensively in the cosmetic, food, pharmaceutical, and textile industries (Baiano 2014).

8.3.5 Indigoidine

Indigoidine is a blue-violet organic pigment related to the Azaquinones group and synthesized by bacterial strains. It is biosynthesized by bacterial species such as *Streptomyces chromofuscus*, *E. coli*, and *Corynebacterium insidiosum* (Ganguly et al. 2019). It is used as a food colorant in cereal, baking, and ice-cream industries.

8.3.6 Phycocyanin

Phycocyanin is a distinct blue color photosynthetic and water-soluble pigment. This pigment is produced by photosynthetic microorganisms such as blue-green algae, *Spirulina platensis*, *Synechocystis* sp., and *Aphanizomenon flos-aquae* (Narsing et al. 2017). Phycocyanin provides various biological functions such as antibacterial, antiviral, antifungal, and anti-alzheimeric agents (Jayaseelan et al. 2014).

8.3.7 Melanin

Melanin is a nitrogenous indolic polymer known as eumelanins, allomelanins, and pheomelanins (Banerjee et al. 2011). Melanin provides photoprotection from UV radiations by absorbing radiations from the electromagnetic spectrum, also effective against chemical stress and high temperature. Due to these properties, it is used in cosmetics, eyeglasses, and pharmaceutical products. Melanin pigment is biosynthesized by several microorganisms such *as Magnoporthe grisea*, *Cryptococcus neoformans*, *Paracoccidioides brasiliensis*, *Vibrio cholera*, *Aspergillus fumigates*, *Alteromonas nigrifaciens*, and *Streptomyces* sp. (Sánchez-Muñoz et al. 2020).

8.3.8 Arpink Red

Arpink red is an anthraquinoid pigment majorly obtained from *Penicillium oxalicum*. Its structure is similar to cochineal caramine and a major alternative for

insect-derived pigment. It is used in food and pharmaceutical industries due to its nontoxic nature. It provides various biological functions such as antibacterial, antiviral, anticancer properties (Kumar et al. 2015).

8.3.9 Monascus

Monascus pigments are secondary metabolites mainly synthesized by filamentous fungi such as *Monascus* genus. *Monascus* sp. produce red, yellow, and orange colored pigments from various fungal species such as *Monascus pilosus*, *M. purpureus*, *M. ruber*, and *M. rubropunctatus*. This pigment is used as a natural food colorant in wines, yogurt, meat products (hams, sausages, and red meat). Apart from their usage in food industries, they also possess biological functions such as antimicrobial, anticancer, antioxidant, and anti-ulcerous agent (Malik et al. 2012).

8.3.10 Riboflavin

Riboflavin, also known as vitamin B_2, is a water-soluble vitamin and exhibits greenish-yellow in color. This natural pigment has been reported to be synthesized by *Candida guilliermondii*, *Eremothecium ashbyii*, and *Debaryomyces subglobosus*. This pigment is used as an additive in dairy products, fruit juices, baby foods, and canned fruits (Dufossé 2006).

8.4 Valorization of Agri-Food Industrial Waste for Production of Microbial Pigments

8.4.1 Fruits and Vegetable Processing Industry

Fruits and vegetable processing industries produce a huge amount of waste or by-products in pulp, peels, bagasse, seeds, stem, pulp, wastewater effluents, etc. These by-products contain number of nutrients that can be used for microbial growth and fermentation. Fruits and vegetable processing waste contain a high amount of carbohydrates, cellulose, dietary fibers, soluble sugars, minerals, and organic acids, which may be considered the best substrate for solid-state or submerged fermentation for microbial pigment production (Kaur et al. 2019).

In previous studies, carotenoids production from fruit processing by-products such as sugarcane molasses, sugarcane juice, corn syrup, and fruits and vegetable residues, i.e., papaya, carrots, tomato, watermelon, peaches, orange, and kinnow, was obtained with different microbes, i.e., *Blakeslea trispora*, *Rhodotorula glutinis*, and *Rhodotorula rubra* (Papaioannou and Liakopoulou-kyriakides 2012; Bhosale and Bernstein 2004; Buzzini 2001; Malisorn and Suntornsuk 2008).

The utilization of citrus fruit peels such as kinnow peel powder has been considered as an excellent low-cost substrate for the production of monascus

pigments (Dufossé 2006). Apple pomace, a rich source of sugars, minerals, and organic acids, has been used to produce carotenoids and violacein pigments. Wine industry waste also provides a cheap and affordable substrate for red, yellow, and blue pigments. Grape pomace waste was used to produce anthocyanin pigment by *Monascus purpureus* (Panesar et al. 2015). Tomato waste, a rich source of carbohydrates, proteins, and crude fat, serves as an excellent medium for the growth of yeast species such as *Rhodotorula* sp. for carotenoid production (Chandi et al. 2010).

8.4.2 Cereal Industry

The cereal processing industry produces by-products from three different processes, dry milling (for flour production), wet milling (for production of starch and glucose), and brewing industry. Thus, the by-products obtained from the cereal processing industry include germ meal, bran, gluten meal, husk, corn steep liquor (CSP), etc. (Charalampopoulos et al. 2002). The corn wet-milling industry produces corn steep liquor as a by-product that can be used to produce penicillin, β-galactosidase enzyme, and ethanol. They provide nitrogen, sugars, amino acids, and vitamins to the fermentation medium, which can further be used to produce microbial pigments. CSP obtained from corn industry contains a rich amount of nitrogen and salts, which helps in the production of red pigments by *Monascus ruber* (Papaioannou and Liakopoulou-Kyriakides 2012).

8.4.3 Dairy Industry

The dairy industry produces several by-products such as whey, skim milk, buttermilk, and residues during cream, butter, cheese, and ghee processing. The major waste of the dairy industry is whey, obtained during the processing of cheese after the removal of casein from milk. Whey is a rich source of milk protein and sugar (lactose) that serves as an excellent medium for the growth of microbes. Cheese whey is used to produce different fungal strains of filamentous fungus to produce yellow or carotenoid pigments (Lopes et al. 2013). Whey protein and coconut water have been used for submerged fermentation (SmF) of *Rhototorula rubra* to produce yellowish-pink pigments (Kaur et al. 2012).

8.4.4 Agricultural Residues and Agro-Industrial By-Products

The post-harvesting operations of crops give several by-products such as husk, bran, hulls, bagasse, cobs, molasses, germ meal, starch, corn steep liquor, soybean meal, and oil processing waste, etc. CSP and cassava liquid waste obtained from the corn and starch processing industry is considered as a low-cost substrate for the growth of *Serratia marcescens* for red pigments and prodigiosin production (De Araújo et al.

2010) (Table 8.2). Different nitrogen sources such as pea pod powder, green gram, okra waste, soy, and taro leaves have been reported to produce microbial pigments. Among all these, pea pod powder gave higher production of pigments with *Monascus purpureus* (Sehrawat et al. 2017). Corn cob powder has been reported as an excellent source of carbon in fermentation medium for the production of microbial pigment by *Monascus* sp. On the other hand, sugarcane bagasse and cornmeal have been reported as an excellent source of starch and carbon required for pigment synthesized by *Monascus* sp. (Moussa et al. 2018).

8.4.5 Poultry and Other Miscellaneous Waste

The fermentation medium constituents such as sugar, nitrogen, and minerals are expensive in their pure form, and hence enhance the cost of natural pigments produced using synthetic media culture. The most expensive component of the media composition is nitrogen provided by peptone and beef extract. To mitigate this high cost, experiments have been done to extract peptone from chicken feathers by acid hydrolysis. This was used as a substrate for carotenoids production by *Rhodotorula glutinis* (Taskin et al. 2011).

Waste obtained from the oil processing industries such as peanut, sesame, and coconut oil along with peanut seed powder and sesame seed powder has been tested for the production of prodigiosin by *Serratia marcescens*, which revealed that peanut seed powder gave a maximum yield of prodigiosin pigment than synthetic media (Shahitha and Poornima 2012).

8.5 Improvement of Quality of Microbial Pigments by Biotechnological Method

For biotechnological production of microbial colors, two approaches have been examined: to find out the source of natural pigment and further increase the disposition of color production. Hence, to increase the yield of microbial pigments, the primary step is to improve or develop the strain and optimize the fermentation or growth parameters. To enhance the yield of microbial pigments, a well-optimized process is needed for fermentation with a metabolic engineering approach (Negi 2019).

8.5.1 Strain Development

Conventionally, strain development was the major task achieved by mutagenesis and selection of the suitable strain. In the previous year's studies, the techniques related to gene deletion helped in the efficient inactivation of genome DNA that helps in metabolisms of bacteria. The industrial development of strain is economical as the wild strain of microorganisms produces a very low yield for economic processes.

Table 8.2 Bibliographic review of agro-industrial waste from different industries used for microbial pigment production

Agro-industrial by-products/ Waste	Pigment type	Microorganism sp.	Fermentation type	Fermentation/Optimization conditions	References
Cereal Industry					
Rice bran	β-carotene	*Rhodotorula glutinis*	SSF	Carbon: Nitrogen 4:1, pH = 5.0, moisture content: 70%	Roadjanakamolson and Suntornsuk (2010)
Bakery waste (Bread)	Orange and yellow pigments	*Monascus purpureus*	SSF, and submerged fermentation	Temp: 30 °C, time: 84–90 h, rpm: 250, moisture 60%	Haque et al. (2016)
Corn steep liquor	Carotenoids	*Sporodiobolus pararoseus*	Submerged fermentation	Temp: 25 °C, time: 84–90 h, rpm: 180, Corn steep liquor: 5.5%	Leite et al. (2013)
Bengal gram husk	Red colored pigment	*Talaromyces* sp.	Submerged fermentation	Temp: 30 °C, time: 10 days, rpm: 110, pH: 5.5	Pandit et al. (2019)
Corn meal	Red and yellow pigments	*Monascus purpureus*	SSF	Temp: 30 °C, time: 14 days, pH: 5.5, glucose conc: 8%	Nimnoi and Lumyong (2011)
Fruits and vegetable processing industry					
Onion peels/ mung bean husk	β-carotene, and phytoene	*Rhodotorula mucilaginosa*	Submerged fermentation in bioreactor	Temp: 26 °C, time: 84–90 h, rpm: 120	Sharma and Ghoshal (2020)
Papaya, orange, and carrot peels	β-carotene	*Blakeslea trispora*	SSF	Temp: 28 °C, time: 96 h, hours, rpm: 200	Kaur et al. (2019)
Orange peels	Carotenoids	*Monascus purpureus, Penicillium purpurogenum*	SSF and Submerged fermentation	Moisture 65%, Temp: 25 °C, time: 96 h, hours.	Kantifedaki et al. (2018)
Pineapple waste	Carotenoids	*Chryseobacterium artocarpi*	Submerged fermentation	Temp: 28 °C, pH: 7.0 rpm 200, time: 36 h	Aruldass et al. (2016)
Olive pomace	Astaxanthin	*Xanthophyllomyces dendrorhous*	SSF	Temp: 15 °C, pH: 4.5, time: 12 days, moisture: 90%	Eryilmaz et al. (2016)

Grape waste	Carotenoids	*Monascus purpureus*, *Penicillium chrysogenum*	Submerged fermentation	Temp: 30 °C, pH: 6.5, rpm: 125, time: 7 days, moisture: 90%	Lopes et al. (2013)
Sugarcane juice	Carotenoids	*Rhodotorula rubra*	Submerged fermentation	Temp: 30 °C, pH: 6.5, rpm: 200, time: 7 days	Bonadio et al. (2018)
Sugarcane bagasse	Red pigment	*Monascus ruber*	Submerged fermentation	Temp: 30 °C, pH: 6.5, rpm: 150, time: 12 days	Terán Hilares et al. (2018)
Cassava waste	Carotenoids	*Rhodotorula glutinis*	Submerged fermentation	Temp: 30 °C, pH: 6.5, rpm: 200, time: 120 h	Santos Ribeiro et al. (2019)
Dairy industry					
Cheese whey	Carotenoids	*Blakeslea trispora*	Submerged fermentation	Temp: 26 °C, pH: 6.5, rpm: 150, time: 4 days, β-ionene: 0.2%	Roukas et al. (2014)
Whey sugar	Yellowish pink	*Rhodotorula rubra*	Submerged fermentation	Temp: 25 °C, rpm 250, time: 3 days	Kaur et al. (2012)
Miscellaneous waste					
Peanut seed oil	Prodigiosin	*S. marcescens*	Submerged fermentation	Temp: 28 °C, rpm 160, time: 36 h	Hernández-Velasco et al. (2020)
Coffee husk	Carotenoids	*R. mucilaginosa*	Submerged fermentation	Temp: 28 °C, rpm 160, time: 120 h	Moreira et al. (2018)
Corn cob	Orange and red pigments, carotenoids	*Monascus purpureus*	Submerged fermentation	Temp: 30 °C, rpm 150, time: 10 days, pH: 4.5	Embaby et al. (2018)
Brewery wastewater	Carotenoids	*Rhodotorula glutinis*	Submerged fermentation	Temp: 25 °C, rpm 115, time: 7 days	Schneider et al. (2013)
Potato chips waste	Red, yellow, and orange pigments	*Monascus purpureus*	SSF	Temp: 30 °C, time: 15 days, pH: 6.5, moisture 60–70%	Ramadan and Mahmoud (2016)
Chicken feathers	Carotenoids	*Rhodotorula glutinis*	Submerged fermentation	Temp: 30 °C, rpm 200, time: 5 days, 0.8% peptone obtained from chicken feathers	Taskin et al. (2011)

Hence, pure strain isolation for pure pigment recovery is the major requirement for a cost-effective process. Thus, strains can be improved and purified by mutagens, i.e., EMS (ethyl methanesulfonate), NTG (1-methyl-3-nitro-1-nitrosoguanidine), and ultraviolet (UV), and further can be used for an increase in the production of pigments (Venil et al. 2013).

8.5.2 Fermentation

Fermentation is a metabolic process for producing a metabolite by the mass cultivation of microbial cells that convert complex substances into simpler ones. Production of microbial pigments by fermentation is a great interest that helps in the biotechnological production of natural pigments in the safest and pure form. The fermentation method depends on the type of organism and pigment produced, i.e., in solid-state fermentation (SSF) and submerged fermentation (SmF) (Joshi et al. 2003).

The solid-state fermentation (SSF) is defined as the phenomenon in which microbes grow on moist solid medium in the absence of free-flowing water. In SSF, the solid matrix or the dry material serves as both support and nutrient source to the fermentation medium. The solid matrix in the fermentation medium provides an inert substrate as a base material for the fermentation. Agricultural waste or food processing industries by-products such as rice bran, wheat bran, germ meal, gram husk, pea pods, etc., provide a complete nutritious medium for microbial growth. SSF technique is affected by different parameters like physical properties of the substrate (particle size, shape, porosity, consistency, etc.) and fermentation conditions (moisture content, relative humidity, temperature, pH, dissolved oxygen, and nutrient composition) (Vidyalakshmi and Mohan 2011). Hence, SSF is cost-effective, uses cheaper substrates from residues, saves wastewater, and gives a higher yield of the natural pigments. Several pigments have been produced using SSF, such as Monascus pigment by *Monascus purpureus* utilizing rice bran, red rice, and wheat by-products (Dufossé et al. 2005). Filamentous fungus such as *Blakeslea trispora*, *Monascus* sp., and *Penicillium* sp. have been reported to produce yellow to red color pigments using agro-industrial residues by SSF (Papaioannou and Liakopoulou-kyriakides 2012; Lopes et al. 2013).

While in SmF, microbes are cultivated and isolated aerobically in the presence of free-flowing water with pre-set agitation system for homogenous growth of cell mass and mixing of media components (Heer and Sharma 2017). López-Nieto et al. 2004 reported production of lycopene pigment by mated fermentation of *Blakeslea trispora* plus (+) and minus (−) strains in submerged media. Malisorn and Suntornsuk (2009) also reported the production of carotenoids in SmF medium by *Rhodotorula glutinis* using the waste generated from vegetable processing industry such as radish brine, carrots, and tomato.

Numerous bacterial strains have shown potential in the production of pigments through the utility of agro-industrial wastes; these include *Serratia marcescens*, *Serratia rubidaea*, *Vibrio psychroerythrous*, *Vibrio gazogenes*, *Rugamonas rubra*, *Pseudomonas magneslorubra,* and *Streptomyces longisporus* (Venil et al. 2020).

SmF is currently followed for microbial pigment extraction; however, solid-state fermentation (SSF) has been found to have more potential in pigment extraction (Kumar et al. 2015; Venil and Lakshmanaperumalsamy 2009). A comparative analysis was done by Sehrawat et al. (2017). In *Monascus purpureus* through solid-state fermentation, pigment accumulation up to 9.0 CVU/g was achieved on day 9 compared to SmF, where 5.1 CVU/g accumulation was achieved on day 15.

8.5.3 Downstream Processing of Pigments

The quality characteristics of the microbial pigments need to be improved for their usage in biological or food industries. The separation and purification processes for the production of pure microbial pigments still have many bottlenecks that need to be considered and constrain their large-scale implementation. The conventional method of separation and purification of microbial pigments was the extraction of pigments from the fermentation medium using organic solvents. Current strategies for pigment extraction include HHP (high hydrostatic pressure) and PEF (pulse electric field), membrane technology, sonication assisted extraction, and gamma irradiation enzymatic extraction; however, extraction is not limited to these techniques only (Parmar and Phutela 2015). Hence, during the extraction using organic solvents from the fermentation broth, many organic solvents were exhausted, which gives a very low yield of pigments due to the binding of pigments with the bacterial or fungal envelopes (Venil et al. 2013).

To mitigate the limitations of extraction using organic solvents, non-ionic resins have been used to extract and purify organic macromolecules such as proteins, peptides, nucleic acids, and other organic compounds. In this process, the target components can be adsorbed on the surface of non-ionic resin from the fermentation broth. This process will remove the cell disruption, separation, and extraction steps which further lowers the cost of operation by reducing the usage of organic solvents. In previous research, 86% of the recovery of prodigiosin pigment directly from the broth culture was observed using non-resin adsorbents (Wang et al. 2004). Hence, this process gave higher recovery compared to the conventional extraction methods and silica gel chromatography. In addition to this, extraction with vegetable oils can also be used to extract non-polar pigments, which could help prevent toxic reactions with the use of organic solvents. Sunflower oil is reported to be a green solvent for carotenoid pigment extraction from the fermentation broth (Dufossé 2006).

An economical method was developed to meet the demand for violacein pigment from the other species. A marine bacterium *Pseudoalteromonas* sp. gave thirteen times higher yield from the cell mass when the pigment was extracted from slurry with hot solvent such as methanol (Venil et al. 2013). Hence, several new technological advancements and developments are still required to efficiently recover microbial pigments from the cell culture by cost-effective and energy-efficient methods.

8.6 Metabolic or Genetic Engineering Approach for Industrial Production of Pigments

Natural pigment production by microbes is limited for industrial production as the wild strain of microbes gives the lower concentration of the pigment. Hence, genetic engineering or mutation approach is required to produce hyper-produced strains at an industrial scale. An easy method to produce mutant strains is mutagenesis, which gives a higher pigment yield with a shorter fermentation period. This technique is used to create genetic mutations by manipulating and altering the sequences of genes (Siddique et al. 2011). Production of metabolites by mutagenesis can be improved by creating genetic modulations with physical methods such as UV radiations, gamma radiations, and treatment to chemicals such as NTG, EMS, and antimycin A (Venil et al. 2013). Hence, the selection of suitable microorganisms is the foremost step for the biotechnological production of strain which can be improved by mutagenesis and increase the production of metabolites (Lopes and Ligabue-Braun 2021).

Several studies have been done on the application of metabolic engineering to increase the yield of microbial pigments. The modified strain of *Saccharomyces cerevisiae* yeast has been used to produce carotenoids—such as astaxanthin, cantha-xanthin, β-carotene, and lycopene due to inoculation of carotenogenic genes from the various microorganisms, i.e., *Xanthophyllomyces* sp., *Agrobacterium aurantiacum,* and *Erwinia uredovora,* into yeast (Ungureanu et al. 2012). Similarly, increased production of lycopene from the *Xanthophyllomyces dendrorhous* has been reported to incorporate carotenogenic genes into the organism (Verwaal et al. 2007). The use of non-carotenogenic yeast *Pichia pastoris* was also reported to increase the production of carotenoids by mutation. Hence, gene encoding of two different plasmids has also been reported to increase the yield of carotenoids (Araya-Garay et al. 2012).

Genetic engineering is being encouraged in the industrial production of pigments wherever strain development is required by the adoption of result-oriented strategies (Saini et al. 2020). CRISPR CAS9 has brought new trends in genetic engineering and is widely used nowadays. It can be used for metabolic engineering in bacteria, fungi, and yeast by injecting a colorant gene, leading to cost-effective production of natural colorants (Donohoue et al. 2018; Sen et al. 2019).

8.7 Application of Microbial Pigments in Pharmaceutical Industries

Microbial pigments possess important properties that include immune-suppressive, antimicrobial, and anticancer. These have shown potential in diagnosing several diseases such as leukemia, diabetes mellitus, cancer, etc. (Kumar et al. 2015). The red pigment from microbes has shown the highest antibacterial property, followed by orange and then green colored pigments (Soliev et al. 2011). Bacterial pigments are a potential source of anticancer and deserve further investigation (Srilekha et al.

Table 8.3 Biological activity and health-promoting benefits of microbial pigments

Microbial pigments	Biological activity/health benefits	References
Prodigiosin	Cytotoxic activity, Immunosuppressing activity, apoptosis in cell cancer lines in humans, used in the treatment of Diabetes mellitus	Furstner (2003), Kim et al. (2003)
Carotenoids	Treatment of disorders like erythropoietic protoporphyria, Cancer prevention- Breast, prostate, ovary, and liver cancer Prevent the risk of Cardiovascular diseases (CVD), blood pressure issues, and stroke Prevent the risk of neurodegenerative diseases such as Parkinson's, Alzheimer, and Dementia Helps in healthy fetal growth during pregnancy	Leong et al. (2018), Kirti et al. (2014)
Violacein	Antibacterial, Anticancer, Antiviral properties	Sanchez et al. (2006), Ferreira et al. (2004)
Marennine	Antiviral, Anticancer, Antimicrobial and Antioxidant	Gastineau et al. (2014)
Monascins	Effective against obesity-related inflammation	Fujimoto et al. (2012)
Canthaxanthin	Antioxidant, anticancer	Dufossé (2006), Ram et al. (2020)
Ankaflavin	Anti-allergic activity in mice lung cell line (A549) as well as lungs ovalbumin (OVA)	Lee et al. (2013)
Flexirubin	Used to treat chronic skin disease, gastric ulcers, eczema	Venil et al. (2015)
Fucoxanthin	Anticancer, Anti-obesity, Anti-inflammatory properties	Borowitzka et al. (2016)
Rubrolone	Antimicrobial	Venil et al. (2020)
Azaphenanthrne	Antibacterial, anticancer	Banerjee et al. (2011)

2018). Human skin is protected from harmful UV radiations by bacterial pigment melanin and hence is being used in sunscreens (Narsing et al. 2017). Similarly, adonirubin and astaxanthin (xanthophylls) play a role in heart attack, cancer, and stroke prevention (Kim et al. 2012). There are several other pigments with potential application in the pharmaceutical industries. Table 8.3 below enlists some of them.

8.8 Application of Microbial Pigments in the Food Industry

The word "organic" is being interchangeably used for "safe" in the current times, be it for food or any of the daily essentials of our lives. Due to increasing awareness about the environmental hazards and the side effects that have been observed over the years because of synthetic materials, efforts are being made to replace the synthetic materials with something organic that is friendly to our bodies as well as the whole environment (McCann et al. 2007; Potera 2010; Oplatowska-Stachowiak and Elliott 2017; Gebhardt et al. 2020). However, this shift is not easy since we have become habitual of the practices both at the commercial level and the domestic level.

Cancer, which is the second fatal disease in the world, and about ten million people die from it every year, has a genetic reason as well as epigenetic among which exposure to synthetic products is a major cause (Kim et al. 2019; Hofseth et al. 2020; Ahmed et al. 2021). When we talk of cancer through food, color is considered one of the researched reasons that cause it. Food coloration is a practice that goes decades back. By 1900, the food coloring industry has completely transformed as earlier used natural dyes were unstable and not as efficient as the synthetic ones but with time, the side effects that it posed became disastrous, and researches carried on made the government impose laws against their use and even now the synthetic color use is restricted to some countries while others use it freely due to economy depending on it and the inefficiency of natural colors production (according to the regulations by organizations like the United States FDA, World Health Organization (WHO), and the European Food Standards Authority (EFSA) (Wrolstad and Culver 2012; Galaffu et al. 2015; Oplatowska-Stachowiak and Elliott 2017; Coultate and Blackburn 2018; Shanmugasundaram and Rujaswini 2019). However, microbial products and plant pigments gain popularity due to increasing explorations and high-tech techniques for purification and stability. The demand for natural colors has increased so much that it is estimated to increase by 7% annually, and almost all the natural pigments are being used at this time for at least one department of the food industry (Clark 2011; Scotter 2011, 2015; Faustino et al. 2019).

Microbial pigments are the natural pigments that have quite extra advantages over the remaining classes of natural pigments like microbial handling is very easy and adequate without needing large spaces for their growth and care. Their environment can be easily regulated and are not hypersensitive to seasonal changes. Its exponential power of division would provide a sufficient amount in a limited time, a cheap practice relatively as their maintenance costs are less. The product yield is high, and thus, their commercial applications are promising (Panesar et al. 2015; Sen et al. 2019). To add to these advantages, these colors could also be beneficial to us by providing nutraceutical benefits in acting as antimicrobial, anticancer, or antioxidants and thus be added to food items as functional food ingredients additives manifesting the function of color along with various other benefits. A few examples include flavins, carotenoids [Lutein and Zeaxanthin (Lin et al. 2015); Sarcinaxanthin, Decaprenoxanthin—not synthesized by plants] (Dufossé 2018), Melanins, Azaphilones like *Monascus* Red, Anthraquinones like Fungal Natural Red, etc. (Downham and Collins 2000; Rajguru et al. 2016; Narsing et al. 2017; Heer and Sharma 2017). The present era of genomics and proteomics could take microbial pigment biosynthesis to an altogether new level where genes could be overexpressed or made stable through appropriate editing tools and in integration with nanotechnology is proving to be a success (Venil et al. 2013; Barnawal et al. 2017; Jixian et al. 2017; Lin et al. 2017; Pailliè-Jiménez et al. 2020).

At present, the challenge for microbial pigments to flourish in the commercial market is essential, and their competition is the synthetic colors. Their commercial success is dependent on their efficient generation, purification, stability, and approval by the food regulating authorities (Mapari et al. 2010; Tuli et al. 2015; Jurić et al. 2020). These factors that determine the success of microbial pigments are

few but have been worked upon by scientists for years. To build such qualities in organic products is practically very difficult as every organic product is prone to be affected by both biotic and abiotic factors very quickly, unlike synthetic colors. Use of synthetic color has been going on for years, and their stability, coloring effect, and shelf-life remain unchanged for months altogether, but microbial pigments need relatively large amounts of raw material, which further leads to their high-cost disparity (about 20 times more than synthetic pigments) (Sigurdson et al. 2017). The trials of their use are also not global, and hence their effects on different groups of populations remain unknown, along with what effects they create when used with different types of food items across the globe. The interactions between microbial pigments and the other organic biomolecules within the food items can vary, and uniformity is not possible as we observe that vitamin C is very compatible with microbial carotenoids but causes degradation of anthocyanins (Wrolstad and Culver 2012; Chaitanya Lakshmi 2014; Kirti et al. 2014; Rodriguez-Amaya 2016) and at the same time, both the pigments are destroyed under conditions of exposure to oxygen or light (Mayne 1996; Laos et al. 2007; Qiu et al. 2018). Similarly, authorities rarely permitted fungal pigments into commercial business because of the toxic effects due to mycotoxins (Frisvad et al. 2004). The *Monascus* species produce efficient red and yellow polyketide pigments, which have efficient commercial power in the coloration of sea foods like fish paste and surimi and also meats like hams and sausages. Due to the presence of mycotoxin, citrinin, they are not approved by European Union and the US food authorities (Dufossé 2006). Thus, a shift of synthetic to microbial pigments is essential and needed but making it possible requires great efforts. Such efforts have been going on from the past few years where nanotechnology (making nano-emulsions), biochemistry (making micro encapsulations), and metabolic engineering. Regulation has paved the way for giving microbial pigments a chance and make our lives better and our environments sustainable.

8.9 Application of Microbial Pigments in Nutraceutical Industries

Application of microbial pigments in food coloration imparts coloration of varied cuisines like processed meats, fish and their products, varied vegetable-based foods, improving wine quality, desserts, and even flavored milk (Dufossé 2006). Besides these diverse applications, microbial pigments, unlike synthetic pigments, prove beneficial not only to the environment due to their organic nature but also nutraceutical in nature. That is, in addition to making our foods colorful and attractive, they possess health benefits as well.

8.9.1 Antioxidant Activity

Various microbial pigments have been found to exhibit antioxidant activity (Chandra et al. 2020). For instance, violacein known to protect the lipid bio

membranes from the free radical activity is produced by *Pseudoalteromonas* sp. and *Chromobacter violaceum* (Konzen et al. 2006), and it also activates the mucosal defense mechanisms (De Azevedo et al. 2000; Antonisamy and Ignacimuthu 2010). Monascus pigments (Vandamme and Revuelta 2016), (rare C_{50} carotenoids like sarcinaxanthin and its derivatives from bacterium *Micrococcus yunnanensis*) (Osawa et al. 2010), phenolic carotenoids (3,30-dihydroxyisorenieratene from *Streptomyces mediolani*) (Martin et al. 2009), cyanobacterial pigments (lycopene, lutein, phycocyanins), and phycobiliproteins have all been reported to act against the oxidative damage and hence can be used as potential antioxidants (Sonani et al. 2016).

8.9.2 Antimicrobial Activity

Many microbial pigments show the property of antibiotics and even in some cases proved better than synthetic antibiotics like an endophytic fungus pigment proved more effective than Streptomycin (Visalakchi and Muthumary 2009). Similarly, violacein displays antifungal, antiprotozoal, and antiviral activities (Nakamura et al. 2003; Lopes et al. 2009; Sen et al. 2019). Gram-negative and gram-positive bacteria are effectively being attacked by prodiginine compounds produced by various strains of *Serratia marcescens* and have also been effective against several classes of fungi (Stankovic et al. 2014; Suryawanshi et al. 2017; Ji and Kim 2019). Marine bacteria like *Pseudoalteromonas tunicate* produce antibacterial and antifungal compounds called Tambjamines (Franks et al. 2005; Kim 2013). Phenazine compounds obtained from *Pseudomonas* and *Streptomyces* species have been observed to show antibacterial, antiviral, and antifungal properties (Schneemann et al. 2011; Saeed et al. 2019). Several quinones and anthraquinones are reported to show antibacterial and antiviral properties (Margalith 1999; Koyama 2006; Gessler et al. 2013).

8.9.3 Anticancer Activity

Various pigments have been shown to exhibit anticancer properties by causing apoptosis of the uncontrollably growing cell lines. A few examples are Prodigiosin pigments (Yip et al. 2019), violacein (Liu and Nizet 2009; Choi et al. 2021), bacterial phenazines (Chincholkar and Thomashow 2013; Hussain et al. 2019), *Monascus* pigments (Vandamme and Revuelta 2016), and phycobiliproteins (Sonani et al. 2016).

8.10 Conclusion

Consumer demand and perception have now increased towards the use of a healthy, safe, green, and eco-friendly nutritious diet that provides metabolic, physiological, and functional benefits. Natural pigments obtained from microbial sources are safe, eco-friendly, cheaper, and provide various biological benefits such as antioxidants, antimicrobial, anticancer, and anti-inflammatory agents. For the production of microbial pigments, agri-food industrial residue is considered as the safe and excellent medium for fermentation. These residues provide carbon, nitrogen, and minerals in potent amounts for fermentation and help in the sustainable management of food waste as well. Although in the past few decades, extensive research has been done on the production of microbial pigments using low-cost substrates, sustainable processing methods for strain improvement, or genetic modification of the strains for the synthesis of pure pigments. But the large-scale production and downstream processing of the pigments at the industrial level is still a challenge. Hence, there is a need to develop technologies to produce safe and clean microbial strains which can be used to synthesize colorants at a large scale to meet the increased market demand for natural colors.

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Commercial Production of Biohydrogen Using Microbes

9

Sumitha Banu Jamaldheen, Vijayanand S. Moholkar, and Arun Goyal

Abstract

Biohydrogen is a clean fuel, which can be produced through direct or indirect biophotolysis, photo-fermentation, and dark fermentation by microorganisms. In dark fermentation, organic waste degradation and hydrogen production go simultaneously. A variety of substrates from industrial wastewater to agricultural solid wastes have been used for biohydrogen production. Obligate anaerobes from genera *Clostridium* and *Desulfovibrio* species and facultative anaerobes from *Citrobacter*, *Enterobacter*, *Klebsiella,* and *Bacillus* species are known to utilize the organic wastes to produce hydrogen through dark fermentation. Biohydrogen production in batch and fed-batch reactors at lab-scale to pilot scale have been demonstrated by several researchers. The major bottlenecks for the large-scale production of biohydrogen are the costs of plant establishment and maintenance. This study gives an overview of the potential microbes and technology involved in the biohydrogen production from organic wastes through dark fermentation and the factors to be addressed for its commercial production.

Keywords

Biohydrogen · Bottlenecks · Commercialization · Dark fermentation

S. B. Jamaldheen
Centre for Energy, Indian Institute of Technology Guwahati, Guwahati, India

V. S. Moholkar
Department of Chemical Engineering, Indian Institute of Technology Guwahati, Guwahati, India

A. Goyal (✉)
Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, Guwahati, India
e-mail: arungoyl@iitg.ac.in

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9.1 Introduction

Biohydrogen is a reliable biofuel that has the potential to replace the fossil fuels. It is a green fuel as no harmful pollutants are released during its production. Another advantage of biohydrogen production is that it can be achieved using a wide range of feedstocks, especially, the organic wastes as substrates, thereby favoring the waste management (Hallenbeck 2011). Biohydrogen can be produced through various routes, as follows:

9.1.1 Direct Biophotolysis

The process of breakdown of water into hydrogen and oxygen in the presence of sunlight by phototrophic algae is termed as biophotolysis (Nath and Das 2004). The green algae, *Chlamydomonas reinhardtii,* produces hydrogen by direct biophotolysis. It splits water into hydrogen and oxygen through photosystem II (PSII) by using [Fe]-hydrogenase enzyme (Bolatkhan et al. 2019).

9.1.2 Indirect Biophotolysis

Indirect biophotolysis is the process of producing hydrogen through indirect utilization of sunlight by algae. It takes place in two steps, where the carbohydrates such as starch and glycogen are produced and stored inside the cell through photosynthesis in the first step. Then the stored carbohydrate is converted into hydrogen during the second step in the absence of oxygen (Anwar et al. 2019). The microorganisms such as *Chlorella* and *Ankistrodesmus* are capable of producing hydrogen through indirect biophotolysis (Jimenez-Llanos et al. 2020).

9.1.3 Photo-Fermentation

Certain phototrophic microorganisms can produce hydrogen from organic substrates by deriving energy from the sunlight. Purple non-sulfur (PNS) bacteria are the most studied microorganisms for producing and enhancing hydrogen production through photo-fermentation (Mishra et al. 2019). Photo-fermentation takes place in two steps, where in the first step, the bacteria assimilate organic substrate and accumulate as volatile fatty acids (VFAs) under dark and anaerobic conditions. In the second step, the accumulated VFAs are converted into hydrogen with the help of [Fe]-hydrogenase upon illumination (Show et al. 2018; Anwar et al. 2019).

9.1.4 Dark Fermentation

The dark fermentation, as the name indicates does not depend on light as the energy source for converting substrate to biohydrogen. It involves strict or facultative anaerobic bacteria to convert the waste organic substrates to hydrogen (Nath and Das 2004). Several species of *Clostridium* genus are known for their capability to produce hydrogen at high yields (Mishra et al. 2019). The process can be carried out with pure cultures or with microbial consortia. The microbes use acetate or butyrate pathway to ferment the organic matter into hydrogen and organic acids (Baeyens et al. 2020).

There are challenges in light-dependent hydrogen production. Light-dependent hydrogen production by phototrophic microorganisms suffers from the process stability in the product formation. The factors affecting these processes are the light source and weather conditions. Secondly, the area required for installation of photo-bioreactors is larger than the reactors required for dark fermentation (Show et al. 2019). In order to improve the efficiency of light-driven hydrogen production processes, genetic manipulation of the microalgae and phototrophic bacteria has been carried out by Show et al. (2018). Among the abovementioned processes, dark fermentation is the most efficient, as it works with variety of waste organic substrates and results in higher product yield than the other processes (Show et al. 2019).

9.2 Substrates for Dark Fermentation

Dark fermentation utilizes a variety of substrates like wastewater (Preethi et al. 2019), glycerol (Sarma et al. 2012), lignocellulosic and macroalgae biomass (Kumar et al. 2018), and starch-containing food waste (Das and Basak 2021). Biohydrogen research has fascinated the researchers to a great extent that the experiments have been carried out on any and every kind of organic waste available. One such interesting study involved cattle feeding with four different types of barley grain based diets (in the presence and absence of distiller's grains and condensed tannin) and the comparison of the respective manures for their potential to serve as substrates for biohydrogen production (Gilroyed et al. 2015). The study showed that the manure from barley diet without distiller's grains and tannin gave highest hydrogen production. The presence of tannin in the diet leads to the least hydrogen production. Table 9.1 shows the different substrates used for biohydrogen production.

9.2.1 Lignocellulose as Substrate

The lignocellulosic biomass comprising carbohydrate polymers (cellulose and hemicellulose) and non-carbohydrate polymer (lignin) serves as the second largest component after water on Earth. Lignocellulosic biomass is considered as the second-generation feedstock for biofuel production including biohydrogen. It is

Table 9.1 Various organic substrates studied for biohydrogen production

Substrate	Inoculum source	Conditions	Hydrogen yield	Hydrogen production rate	References
Duckweed from swine wastewater treatment	Mixed consortium (Anaerobic digester)	pH 5.5, 35 °C	75 mL H_2/g dry duckweed	NA	Xu and Deshusses (2015)
Brewery wastewater	*Klebsiella pneumoniae*	pH 5.5, 35 °C	1.67 mol H_2 /mol glucose	NA	Estevam et al. (2018)
Microbial fuel cell spent substrate	Mixed consortium (Anaerobic digestion plant)	pH 6.5–7.0, 37 °C	14.13 ml/g substrate	NA	Florio et al. (2019)
Tequila vinasse and nixtamalization wastewater	Mixed consortium (Biohydrogen producing reactor)	pH 6.5 followed by 5.8, 35 °C	2.7 NmL H_2/g volatile solids added	155 NmL/L/h	García-Depraect et al. (2019)
Agave bagasse enzymatic hydrolysates	Anaerobic granular sludge (Tequila distillery wastewater)	pH 7.5, 37 °C	3.81 mol H_2/mol hexose consumed	2.32 L H_2/L/d	Tapia-Rodriguez et al. (2019)
Corn stover hydrolysate	*Thermoanaerobacterium thermosaccharolyticum* W16 added to mixed consortium (Rotten corn stover/cow dung/ anaerobic sludge)	pH 7.0, 55 °C	9.90 mmol H_2/g sugar consumed	1.97 mmol H_2/L/h	Zhang et al. (2019)
Rice husk hydrolysate Rice straw hydrolysate	*Bacillus cereus* and *Rhodopseudomonas rutila*	pH 7.0, 37 °C	1.73 mol H_2/mol Glucose 1.82 mol H_2/mol glucose	NA	Dinesh et al. (2020)
Fruit waste hydrolysates	*Clostridium* strain BOH3	pH 6.8, 37 °C	2.51 mol/mol hexose	129.9 mL/L/h	Mahato et al. (2020)
Cassava processing wastewater	Mixed consortium (Chicken manure/vinasse effluent)	pH 6.0, 37 °C	4.12 mol H_2/mol maltose	21.82 mL H_2 /h/L	Martinez-Burgos et al. (2020)

NA Not available

the most investigated substrate for hydrogen production through dark fermentation, as it is naturally available in the plants and their wastes (Sivagurunathan et al. 2017). It contains high carbohydrate content available as cellulose and hemicellulose, but their complex structure complicates their utilization by the hydrogen-producing microorganisms. Hence, a preliminary pretreatment step becomes inevitable to fractionate the polymers into consumable monosaccharide for the microorganisms (Saratale et al. 2018). There are various types of pretreatments affecting the lignocellulosic structure in different ways. They can be grouped into physical, chemical, physico-chemical, and biological pretreatment methods (Monlau et al. 2013). Physical methods include mechanical disruption of the biomass using milling, extrusion, or irradiation. Chemical methods employ inorganic (acid/base) and organic (organosolvents) chemicals to break the lignocellulose and remove lignin (Jamaldheen et al. 2018). Physico-chemical methods involve the combined pretreatment by chemical and physical methods. Biological pretreatments involve lignocellulose degrading microbes like fungi (Sivagurunathan et al. 2017). Physico-chemical treatments are the most efficient methods as they consume lesser time to effectively break the lignocellulosic structure. The bottleneck related to the physico-chemical methods is the formation of inhibitors like furans and carbonic acids from carbohydrates and formation of phenolic compounds from lignin degradation (Saratale et al. 2018). Therefore, it requires an additional step called detoxification, involving evaporation, neutralization, overliming, or adsorption to remove the inhibitors (Sivagurunathan et al. 2017; Valdez-Guzmán et al. 2019).

9.2.2 Algae as Substrate

Algae are the third-generation feedstock for hydrogen production by dark fermentation. Marine algae species from *Codium*, *Gelidium*, *Ulva*, *Laminaria,* and *Garcilaria* genera are the reported feedstocks used for hydrogen production (Kumar et al. 2018). *Laminaria japonica* is a widely studied substrate and it contains ~50% carbon and the major carbohydrates are available as cellulose and hemicellulose (Liu et al. 2018). Therefore, the algal biomass needs pretreatment like the lignocellulosic feedstock to breakdown the complex carbohydrate into reducing sugars. Heat-shock, acid or alkali treatment, ultrasonication, and microwave treatment are the various methods used for pretreating macro-algal biomass for hydrogen production (Jung et al. 2011). The untreated and pretreated macro-algal substrate supports the growth of different hydrogen-producing microbes, when inoculated with a mixed inoculum. For instance, dark fermentation of untreated *Laminaria japonica* led to the dominance of *Enterococcus* species, while the microwave combined with acid pretreatment resulted in the dominance of *Clostridium* species in the fermentation medium (Yin and Wang 2018).

9.3 Microbiome Involved in Dark Fermentation

The hydrogen producers involved in dark fermentation are from *Clostridium*, *Enterobacter*, *Klebsiella*, *Citrobacter,* and *Bacillus* genera (Su et al. 2018). *Clostridium* sp. is predominantly present in most of the mixed consortia used for hydrogen production (Pugazhendhi et al. 2019a). The remarkable ability of *Clostridium* sp. to produce hydrogen has resulted in wide investigations using a variety of substrates (Sivagurunathan et al. 2017). The microbial diversity in the inoculum influences the biohydrogen production. For instance, a study comparing the potential of inocula of different sources (fruit bat feces, dairy farm liquid waste, and sugarcane-cultivated soil) in biohydrogen production in vinasse medium was carried out by Sydney et al. (2018). The experiments showed that the inocula from fruit bat feces and dairy farm waste contained higher microbial diversity than the inoculum from sugarcane-cultivated soil. This helped in the better performance of first two inocula to produce hydrogen than the third one. The first inoculum consisted of microorganisms from *Oxalobacteraceae*, *Lactobacillaceae,* and *Enterobacteriaceae*, whereas inoculum from dairy waste contained *Clostridiaceae* strains in addition to the abovementioned genera. The third inoculum contained 96% *Sporolactobacilaceae* and 3% *Clostridiaceae*. Other unidentified microbial population in the first two inocula was also higher than the third inoculum (Sydney et al. 2018). The presence of different microbial communities influences the formation of granules of hydrogen-producing microorganisms leading to enhanced hydrogen production. In the microbial community other hydrogen producers aid in the production of extracellular polymeric substances, which hold the hydrogen producers together as granules. This helps in reducing the washout of hydrogen producers during lower hydraulic retention times. The genera involved in granule formation are *Streptococcus*, *Bacilli*, *Selenomonas*, *Clostridium,* and *Desulfovibrio* (Pugazhendhi et al. 2019a).

9.4 Dark Fermentation Under Thermophilic Condition

Thermophilic condition favors the utilization of certain types of substrates for dark fermentation. For instance, sugarcane vinasse is one of the substrates used for biohydrogen production (Fuess et al. 2019). It is an organic waste of bioethanol production. Sugarcane vinasse is generated at the temperature between 90 and 100 °C and utilized for biohydrogen production under thermophilic condition at 70 °C. Microorganisms from the genus *Clostridium* and *Pectinatus* were found to be dominant during hydrogen production under thermophilic conditions, while using a mixed consortium as inoculum (Niz et al. 2019). Microorganisms classified under *Thermotogales* and *Thermoanaerobacterium* are extremophiles that are capable of producing biohydrogen at temperatures ranging between 55 and 80 °C (Koskinen et al. 2008). The often-investigated *Thermotoga* species are *T. maritima* and *T. neapolitana* (Dreschke et al. 2019; Shao et al. 2020). Under thermophilic conditions, the contamination by other microorganisms, especially methanogens,

is restricted, which enhances the hydrogen production. Another advantage in thermophilic process is the cell mass is highly suspended, thereby increasing the mass transfer for growth and the product synthesis (Niz et al. 2019).

9.5 Seed Pretreatment

The enrichment of hydrogen producers in the seed inoculum containing the mixed microflora is termed as seed pretreatment. Anaerobic sludge is one of the best inocula used for hydrogen production, as it contains a mixture of hydrogen producers, actively working in the organic waste environment. In addition to hydrogen producers, anaerobic sludge contains a handful of methanogens too (Pachapur et al. 2019). To enrich the hydrogen producers in the sludge, methanogens need to be eliminated from the inoculum and that is where the aid of seed pretreatment is required. There are various types of seed pretreatment methods: chemical treatment, heat-shock treatment, and organic load-shock treatment (O-Thong et al. 2009). Chemical treatments involve either acid or alkali to eliminate methanogens at extreme pH. Methanogens are heat sensitive, so the inoculum is treated at 100 °C during heat-shock pretreatment to kill them (Chen et al. 2002). During organic load-shock treatment, the inoculum is added to a medium with high concentration of organic substrate like sucrose (Voolapalli and Stuckey 2001). Upon assimilation of surplus organic substrate, the methanogens produce methanogenic products in excess, leading to product inhibition and decline in the growth of methanogenic microorganisms (Pachapur et al. 2019).

9.6 Dark Fermentation Using Bioreactors

Biohydrogen has been produced by using different types of reactors, e.g., continuous stirred tank reactor (CSTR), up-flow anaerobic sludge blanket (UASB) reactor, fixed bed reactor (FBR), anaerobic fluidized bed reactor (AFBR), and dynamic membrane bioreactor (DMBR) (Barros et al. 2011; Park et al. 2021). Fixed bed reactor can help in achieving higher hydrogen production rate (HPR) and hydrogen yield (HY) than CSTR, UASB, or DMBR (Park et al. 2021). This is due to the lesser washout of microorganisms favored by the immobilized material within the FBR. However, for longer runs, UASB provides more stable performance than FBR (Mota et al. 2018). The process cost of CSTR is lesser as compared with the other types of reactors. Advanced dynamic membrane bioreactor is capable of providing better HPR and HY while reducing the production cost too, but not lower than CSTR. More research on bioreactor design is yet to be carried out for large-scale operations to produce hydrogen through dark fermentation (Park et al. 2021).

9.7 Parameters

9.7.1 pH

pH plays a very crucial role in hydrogen production process. A steady pH maintenance helps in reducing negative effects of the varying organic composition of the organic waste (Fuess et al. 2019). When the pH changes, the different metabolic pathways get switched on, thereby resulting in different products in the hydrogen production reactor (Wu et al. 2017). For instance, the lactate production takes place below pH 5.0, biohydrogen and butyrate production is favored in the pH range 5.0 to 5.5, and biohydrogen production accompanied by sulfate reduction takes place at the pH above 6.0 (Fuess et al. 2019).

9.7.2 Hydraulic Retention Time

One of the most significant parameters during dark fermentation in a continuous reactor is hydraulic retention time (HRT). Kirli and Karapinar (2018) studied the effect of HRT (2–13 h) within an up-flow packed bed reactor (UPBR) immobilized with polyester fiber beads and fed with glucose. The study showed that the lowest studied HRT (2 h) resulted in higher hydrogen production. Another study showed that the hydrogen yield, hydrogen production volume, and volumetric hydrogen production rate in a similar UPBR filled with metal mesh covered plastic scouring sponge pad and fed with hydrolyzed wheat waste were highly dependent on HRT (Karapinar et al. 2020). The hydrogen yield increased from 0.49 mol/mol glucose to 0.89 mol/mol glucose when HRT was changed from 2 to 6 h. Therefore, HRT needs to be optimized while changing the substrate or immobilized material within same type of bioreactor.

9.7.3 Organic Loading Rate

High organic loading rate leads to restricted cell growth due to the substrate inhibition causing lower hydrogen production (Tawfik and El-Qelish 2012). On the other side when organic loading rate is low, the system favors the growth of homoacetogens. These utilize hydrogen and carbon-dioxide to produce cell biomass and acetate, thus reducing hydrogen yield. Therefore, it is important to optimize the organic loading rate during dark fermentation for higher hydrogen yield (Anzola-Rojas et al. 2014). Once the crucial parameters for biohydrogen production are optimized for a particular organic substrate feeding system at a small scale, the scaling up of the process becomes negligible with the help of reliable process models. This was proven from the experiment conducted by Sewsynker-Sukai and Kana (2017) at different process volumes such as 80, 800 mL and 8 L under the optimized conditions, derived from RSM (Response Surface Methodology) and ANN (Artificial Neural Network) models.

9.8 Additives

Supplementation of certain additives to the medium can enhance the biohydrogen production during the dark fermentation (Yang and Wang 2018). These additives improve microbial growth and enhance the metabolic activities, thereby increasing the hydrogen yield.

9.8.1 Metal Additives

Metal additives are used in the form of metal monomers, ions, or oxides. Iron is a highly significant metal additive in the biohydrogen production, as the fermentative microbes need iron as a co-factor for activating Ni–Fe hydrogenase and Fe–Fe hydrogenase enzymes. Nickel, palladium, magnesium, calcium, and sodium are other significant metal ions to achieve increased hydrogen production (Yang and Wang 2018). Although zinc is not often investigated for its importance in hydrogen production, Keskin et al. (2018) revealed that zinc is as significant as iron and nickel for the microbial growth during dark fermentation of organic substrates. Yang and Wang (2018) suggested the addition of metal rich wastes such as leachate from landfills and ashes coming from waste incineration in the fermentation medium to reduce the cost involved for metal additives.

9.8.2 Microbial Immobilization Additives

These additives are used in immobilized systems where the hydraulic retention time is lower to hold the cells inside the reactor. They act as carriers to provide higher density to the cells, thereby preventing them from washing out (Yang and Wang 2018). Activated carbon is the extensively used cell carrier for enhancing the hydrogen production during dark fermentation (Zhang et al. 2017). Its porous nature and large surface area favor the uniform distribution of microbial cells on the carrier leading to the better performance than in the system without the activated carbon (Park et al. 2019). Biochar, an additive produced during the thermal degradation of biomass called pyrolysis, has also been proven to improve the biofilm formation of the cells (Sharma and Melkania 2017) and act as a buffering agent (Sunyoto et al. 2017). Biochar contains trace elements, which serve as micronutrients for the microbial cell growth (Yang and Wang 2018). Biochar protects the cells from the inhibitory effects of VFAs and ammonia toxicity within the medium by acting as an effective absorbent (Sharma and Melkania 2017).

9.8.3 Bioaugmentation

Mixed microbial consortium such as anaerobic sludge is often used as the inoculum for dark fermentation of complex organic substrate (Goud et al. 2014). The process

of adding specific microbial strains along with the host inoculum is termed as bioaugmentation. These strains increase the performance of the overall process by accelerating the start-up of hydrogen production, while the mixed microflora getting adapted to the medium and protecting the consortium from the negative effect of organic overloading (Guo et al. 2010). *Ethanoligenens harbinense*, *Bacillus subtilis,* and *Lysinibacillus fusiformis* are some of the strains used for bioaugmentation during dark fermentation (Yang and Wang 2018).

9.8.4 Other Additives

L-Cysteine and hydrolytic enzymes are other additives reported to enhance the microbial growth and hydrogen production (Yang and Wang 2018). L-Cysteine has been proved to increase the mass transfer between the substrate and the microbial cells (Yuan et al. 2008). Direct addition of hydrolytic enzymes in the fermentation medium helps in fractionation of the organic load into simple nutrients for the fermenting microorganisms (Quemeneur et al. 2012).

9.8.5 Application of Nanomaterials

Additives in the form of nanoparticles (NP) increase the production of hydrogen. Inorganic nanoparticles such as Fe-NP and Ni-NP provide better efficiencies than conventional metal additives (Kumar et al. 2019). Co-addition of these inorganic nanoparticles gives higher hydrogen production than their individual addition (Patel et al. 2018). Organic nanoparticles include nano-activated carbon and carbon nanotubes (CNT). These nanoparticles are cost-effective against the normal activated carbon, due to their smaller size and larger surface area, which help in decreasing the additive dosage (Patel et al. 2018; Pugazhendhi et al. 2019b). It is important to optimize the nanoparticle additive dosage for different microorganisms to avoid toxicity to the microbial cells leading to the cell disruption (Kumar et al. 2019).

9.9 Multiple Process Integration

The economic feasibility of the biohydrogen process can be increased by integrating the dark fermentation process with other processes. This will result in production of multiple products, efficient substrate utilization, waste treatment, and a circular economy. Ethanol production plants generate large amount waste (vinasse) that gets collected at the bottom of the distillation column, which can be utilized as a potential substrate for biohydrogen production (Sydney et al. 2014). The integration of dark fermentation with ethanol production process can reduce the cost of substrate and improve waste management. Another process that can be integrated with the dark fermentation is the biogas production (Kaparaju et al. 2009). The waste

generated from dark fermentation can be carried over to anaerobic digestion process, thereby giving higher energy output, as compared with the single process (Pawar et al. 2013). Ferreira et al. (2018) investigated the possibility of integration of hydrogen production to the wastewater treatment process using microalgae. The microalgae (*Scenedesmus* sp.) grown in wastewaters from various sources were used as substrate for *Enterobacter aerogenes* in dark fermentation. The algal biomass grown in swine wastewater was found to be the best substrate among the tested candidates (Ferreira et al. 2018). Efforts to couple dark fermentation with the photo-fermentation was made by Redwood et al. (2012). The authors suggested that the sequential application of hydrothermal hydrolysis of food waste and dark fermentation with selective organic acid separation and photo-fermentation will enhance the gross energy generation from the waste. Other possible processes that can be integrated with dark fermentation are bioplastic production and power generation by microbial electrolysis to attain a sustainable circular economy (Chandrasekhar et al. 2020).

9.10 Commercialization of Biohydrogen

Dark fermentation has been operated at pilot scale level as listed in Table 9.2. The pilot studies show that biohydrogen production through dark fermentation by using complex organic wastes is a technically feasible process (Vatsala et al. 2008; Balachandar et al. 2020). Engineering economic analysis using Net Present Value (NPV) model is a method to calculate the economic feasibility of a technological process to attract the investors (Ilori et al. 1997). Such an analysis was carried out by Lee (2016a) and revealed that the production of biohydrogen and biobutanol is financially more feasible than that of biodiesel. Biohydrogen is more favorable for commercialization, as it can be produced from a variety of bioresources. Economic incentives can greatly support its commercialization by attracting the investors towards the industry (Lee 2016a). Among the cost of biomass, capital cost, and

Table 9.2 Pilot scale studies on dark fermentation

Substrate	Microorganism	Hydrogen yield	References
Sugarcane distillery effluent	Co-culture: *Citrobacter freundii* 01, *Rhodopseudomonas palustris* P2 and *Enterobacter aerogenes* E10	2.76 mol H_2/mol glucose	Vatsala et al. (2008)
Sucrose (synthetic wastewater)	Mixed microflora	1.74 mol H_2/mol sucrose	Lin et al. (2011)
Cane molasses and groundnut deoiled cake	*Enterobacter cloacae* IIT-BT 08	12.2 mol H_2/kg COD removed	Balachandar et al. (2020)
Palm oil mill effluent	Mixed microflora	0.5–1.1 L H_2/g COD consumed	Akhbari et al. (2021)

operating and maintenance cost, biomass cost is less significant than the latter costs for the biohydrogen production plant development. Focusing more on those two criteria (capital and operation costs) during decision-making process can help in the successful commercialization of biohydrogen in the future (Lee 2016b).

9.11 Conclusion

Extensive experimental and mathematical investigations show that the biohydrogen production through dark fermentation is technically and economically feasible. The biohydrogen production yields from pilot plant studies by using organic wastes are comparable to the lab scale yields. The integration of multiple processes with dark fermentation can bring down substantially the cost of the substrate and set a circular economy. However, this needs more exploration at the pilot scale level. A better decision-making by the government regarding the capital and operational costs combined with incentives will pave the way for successful commercialization of biohydrogen production through the dark fermentation.

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Microbial Synthesis of Polyhydroxyalkanoates (PHAs) and Their Applications

10

Aurelio Ortiz and Estibaliz Sansinenea

Abstract

Polyhydroxyalkanoates (PHAs) are polyesters produced in nature by numerous microorganisms, which are stored within the cells to be a source of energy and as a carbon store. PHA polymers are thermoplastic, and are, depending on their composition, ductile and more or less elastic. These plastics have the advantage that they are biodegradable and do not possess health effects when used in vivo; therefore they are used as a green solution in the production of bioplastics. In various industries, for instance in biomedical sector, PHAs have been widely applied. However, several disadvantages limit their use, such as their poor mechanical properties, high production cost, limited functionalities, incompatibility with conventional thermal processing techniques, and susceptibility to thermal degradation. The biosynthesis of PHAs has been improved changing certain conditions such as sources, bacterial strains, fermentation conditions, and improving the recovery techniques which cause an improvement on the yield and the purity. For example, recombinant *Bacillus subtilis* was used in production of polyhydroxyalkanoates (PHAs) using malt waste as carbon source for lower cost production. This chapter has focused on the production and applications of these interesting biopolymers.

Keywords

Polyhydroxyalkanoates (PHAs) · Polyesters · Bioplastics · PHAs copolymers · PHAs Blending

A. Ortiz · E. Sansinenea (✉)
Facultad de Ciencias Químicas, Benemérita Universidad Autónoma de Puebla, Puebla, Mexico

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10.1 Introduction

Plastics are synthetic polymers not produced by Mother Nature and widely used in almost every manufacturing industry. Plastics can have a wide range of strengths and shapes since they can be chemically manipulated. The synthetic plastics such as polyethylene and polystyrene are largely used in the world as they are easily molded into almost any desired shape (PlasticsEurope 2019). Therefore, they can be used in many durable, disposal goods and as packaging materials. The current status of the plastics shows an exponential increasing consumption in many countries, almost about 300 MT/year (PlasticsEurope 2019). However, their excessive use has caused crucial problems, many of them related with environment such as elevated CO_2 and toxin concentration in the atmosphere originated from plastic incineration. The plastic accumulation is an obvious problem which can be seen through ocean images full of plastics causing animal deaths. This severe problem causes a serious concern on consumers about the use of plastic packaging. The reason is that due to their excessive molecular size they are highly resistant to biodegradation persisting in soil or water for a long time (PlasticsEurope 2019).

To overcome this problem, manufacturers are looking for green solutions with all benefits of plastics (cost, marketing, and usages) but without the environmental contamination. Therefore, the plastics that have always been used need to be substituted by green others, for instance, biologically produced and/or biodegradable plastics, which are called bioplastics (Iwata 2015; Możejko-Ciesielska and Kiewisz 2016).

Polyhydroxyalkanoates (PHAs) are a class of bio-derived, biodegradable polymers which could be the most auspicious, that can be considered as valuable alternatives to commonly used plastics (Dietrich et al. 2017; Możejko-Ciesielska and Kiewisz 2016). These molecules appear inside bacterial cells in granules form (Koller et al. 2017) providing carbon storage and reducing equivalents for the bacteria. PHAs have several properties which are appropriate to substitute common plastics. The polymer industry can select PHAs because they are bio-based, biocompatible, and biodegradable ("green plastics"), are produced by living microorganisms, and finally PHAs can be processed to create marketable products for different applications, ranging from packaging to medical applications (Dietrich et al. 2017). In this chapter it is revised the key aspects of microbial PHAs properties and production focusing on the recent advances in the PHAs production on an industrial scale, making us familiar with the current state of enhancing the sustainability, economics, and product quality of PHA.

10.2 PHAs Chemical Structure

PHAs serve as water insoluble storage compounds which are synthesized by microorganisms as granules during times of environmental stress conditions. Different bacteria produce different type of PHAs. They are biosynthesized under certain conditions, such as the concentration of carbon source. If the concentration of the

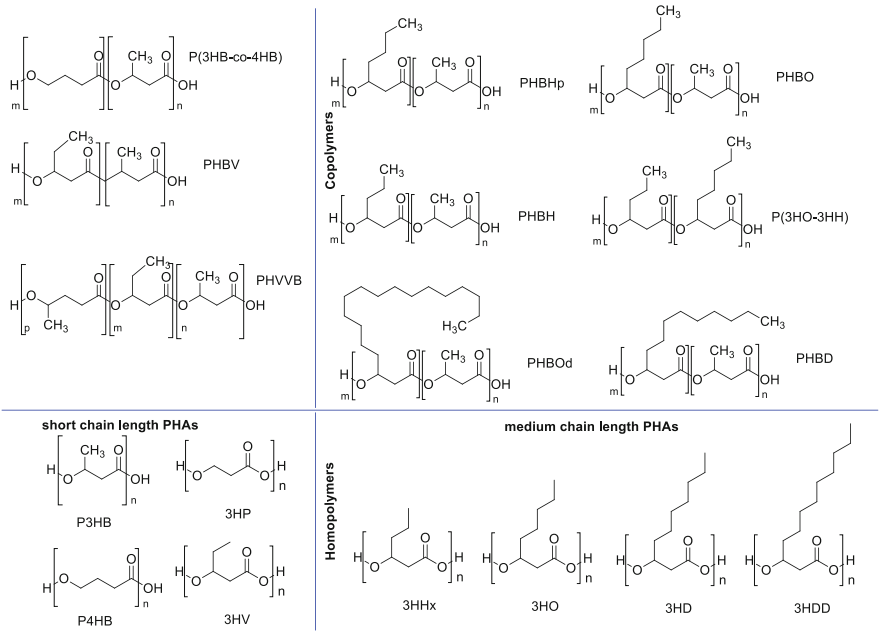

Fig. 10.1 PHAs family

carbon source decreases, the produced biopolymers can be degraded and can be used as carbon source again.

PHAs are linear polyesters, which are thermoplastics, consisting of hydroxy acid monomers (HA) connected by an ester bond which is the result for connecting the carboxylic group of a monomer with the hydroxyl group of a neighboring one (Fig. 10.1) (Philip et al. 2007). PHAs are classified mainly into two distinct groups depending on the number of carbon atoms in the monomers: scl-PHAs (short chain length PHAs which consist of 3–5 carbon atoms) and mcl-PHAs (medium chain length PHAs which are composed of monomers having 6–14 carbon atoms). PHAs can be classified either as homopolymers or copolymers. Homopolymers consist of one type of PHA such as pure P3HB, P4HB, 3-hydroxypropionate 3HP, 3-hydroxyvalerate 3 HV, and the middle-chain-length PHA monomers 3-hydroxyhexanoate 3HHx, 3-hydroxyoctanoate 3HO, 3-hydroxydecanoate 3HD, and 3-hydroxydodecanoate 3HDD. Copolymers on the other hand consist of two or more different PHAs, for instance, scl-copolymers such as poly (3-hydroxyalkanoate-3-hydroxyvalerate (PHBV) or poly(3-hydroxybutyrate-co-4-hydroxybutyrate) (P(3HB-co-4HB)) and mcl-copolymers such as poly (3hydroxyoctanoate-co-3-hydroxyhexanoate) (P3HO-3HH) (Fig. 10.1).

Changing the carbon source or bacterial strain the structural composition of PHAs polymers can be changed. *Pseudomonas putida* tends to synthesize PHAs by incorporating different functional groups. PHAs are produced from a wide variety

Table 10.1 Summary properties of some common PHAs

Polymer code and name	Material class	Properties
P3HB Poly(3-hydroxybutyrate)	Semi-crystalline thermoplastic	Strong Brittle Small thermal processing window High softening temperature
P4HB Poly(4-hydroxybutyrate)	Thermoplastic elastomer	Strong Flexible Ductile High melt viscosity
P(3HB-co-4HB) Poly(3-hydroxybutyrate-co-4-hydroxybutyrate)	Semi-crystalline thermoplastic/ thermoplastic elastomer	Strong Tough Large thermal processing window Ductile
PHBV Poly(3-hydroxyalkanoate-3-Hydroxyvalerate)	Semi-crystalline thermoplastic	Strong Brittle Large thermal processing window High softening temperature
PHBHH Poly (3-hydroxybutyratehexanoate)	Semi-crystalline thermoplastic	Flexible Ductile Easy to process Low softening and melting temperature

of substrates such as renewable resources, fossil resources, by-products, chemicals, and carbon dioxide (Abid et al. 2016; Raza et al. 2016).

These various PHAs have a wide range of properties having different applications. In general, PHAs are biodegradable, compostable thermoplastics. Other properties include their insolubility in water but solubility in chloroform and other chlorinated solvents and their resistance to hydrolytic attack and ultraviolet light. The degradation of PHAs depends mainly on their type and composition of the polymer, environmental conditions, and the type of microorganisms (Boyandin et al. 2013). Their melting temperature varies from 40 to 180 °C. Some common PHAs properties are shown in Table 10.1.

10.3 Biosynthesis: Genetic Basis

Current advancements in genetic and metabolic engineering have intensified the usage and application of different technologies to manipulate the biochemical processes such as for the improvement in bio-catalytic activity of PHA synthase, thus increasing the amount of biosynthesized PHAs.

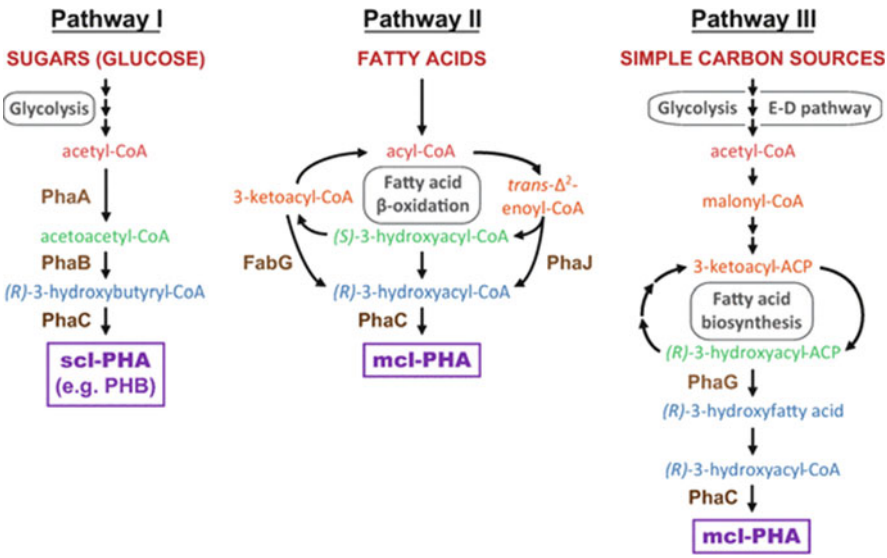

Fig. 10.2 Metabolic pathways involved in the synthesis of PHA adapted from Kniewel et al. (2019)

From a variety of microorganisms many genes encoding enzymes involved in PHA formation and degradation have been cloned and characterized. There are several different pathways for PHA formation, depending of PHA-producing microorganism. The regulation of PHA synthesis have been studied using genetic tools, developing that PHA synthase plays a crucial role in PHAs synthesis.

In Fig. 10.2 it can be seen that there are three metabolic pathways to generate precursors for PHA synthesis (Fig. 10.2) (Kniewel et al. 2019; Prieto et al. 2016). Through the glycolysis of sugars (Pathway I) scl-PHAs, such as PHB, are synthesized (Kniewel et al. 2019). In this pathway I there are three important key enzymes, β-ketothiolase, acetoacetyl-CoA reductase, and PHA synthase encoded by *phaA*, *phaB,* and *phaC* genes, respectively. In contrast, pathways II and III are commonly used for mcl-PHA synthesis in *Pseudomonas* sp. using other types of enzymes and precursors (Kniewel et al. 2019; Prieto et al. 2016).

The knowledge on the regulatory mechanisms at the molecular level is relatively limited especially in a case of medium-chain-length PHAs synthesis. Six proteins directly involved in the biosynthesis and degradation of mcl-PHAs have been already characterized at the molecular level (Możejko-Ciesielska and Kiewisz 2016).

One problem of the natural synthesis of PHAs is that the bacteria that synthetize PHAs have a long generation time and relatively low optimal growth temperature. There are bacteria with shorter generation times such as *E. coli*; however, they are incapable of synthesizing or degrading PHA. Therefore, with the help of molecular genetics, recombinant *E. coli* strains harboring the PHA biosynthesis genes, from producing bacteria, in a stable high-copy-number plasmid have been developed and used for high PHA productivity.

10.4 PHA Production

Although microbial plastics are more ecological than common plastics, the main problem is the high cost associated with microbial production based on fermentation process (Chaudhry et al. 2011). The cost of production is affected by the type of carbon source, fermentation and productivity process, yields of the production and downstream processing (Lee and Na 2013). The yield of production can be affected by the type of producer microorganism; therefore, a hyper productive microorganism is preferred to reduce the cost of production.

Another strategy in order to reduce the costs of production, the use of waste materials as carbon sources for microbial PHA production, has been proposed (Nielsen et al. 2017; Amaro et al. 2019). For instance, to produce PHAs with relative success several waste sources have been used (Koller et al. 2017; Koller 2017). Some of the waste sources that have been used are domestic wastewater (Carucci et al. 2001), food waste (Rhu et al. 2003), molasses (Carvalho et al. 2014), olive oil mill effluents (Dionisi et al. 2005), palm oil mill effluents (Din et al. 2012), lignocellulosic biomass (Bhatia et al. 2019), coffee waste (Bhatia et al. 2018), starch (Bhatia et al. 2015), biodiesel industry waste (Sathiyanarayanan et al. 2017), used cooking oil (Kourmentza et al. 2017), and cheese whey among others (Koller 2017; Raza et al. 2018). Unfortunately, some PHA-producing microbial species are unable to produce PHAs from whey. For instance, *Cupriavidus necator* is capable of accumulating PHAs when growing on glucose (Reinecke and Steinbüchel 2008), but it is unable to produce PHAs on lactose, the predominant carbon source of whey. The utilization of waste materials for the synthesis of PHAs has led to cost reduction; however, the presence of impurities and the waste composition variations results in varying final PHAs production yields. This is a problem for the use of PHAs in medical products, where high purity products are desirable. The products from wastes could have other microorganism contamination which could require further purification processes increasing the cost of production.

Considering the industrial production of several PHAs there are some strains and conditions that have been considered as optimal. For example, to produce PHB *Alcaligenes latus* is one of the strains that is most used, since the strain grows rapidly in several sources recovering PHB over 90% of the cell dry weight (Chen 2009). The PHB production and processing technology are now owned by Biomer, Germany. Different products, including combs, pens, and bullets, have been made from PHB produced by *A. latus*. In the same way, the Institute of Microbiology affiliated with the Chinese Academy of Sciences and Tianjin Northern Food, China, used *R. eutropha* strain to recover 80% PHB in their dry weight (Chen 2009). This strain also produced the copolymer PHBV, which is used to make shampoo bottles (trademarked as Biopol) being available in Europe supermarkets. To produce the copolymer P3HB-co-4HB, *R. eutropha* and recombinant *E. coli* are used by Tianjin Green Bioscience, China, and Metabolix, USA (Chen 2009).

Another strategy has been the creation of transgenic crop plants producing PHA which produces high product yields. These transgenic plants have normal plant phenotypes and transgenes that are stable overs everal generations

(Reddy et al. 2003). *Arabidopsis thaliana* was the first choice for transgenic studies since it is the model for heterologous expression studies in plants (Reddy et al. 2003). The other plants currently in use for PHA production are *Gossypium hirsutum* and *Zea mays*. In the UK, ZENECA Seeds is focusing its efforts on rapeseed, while in the USA, Monsanto is working on both rapeseed and soybean (Reddy et al. 2003).

The downstream processing also affects the final cost of PHAs production. Thermosetting aqueous two-phase extraction uses thermo-separating polymers like ethylene oxide or polyethylene oxide which separates into two layers (Leong et al. 2017). Therefore, the use of aqueous two-phase extraction can be considered as environmentally friendly downstream process for isolation and recovery of PHAs (Kit et al. 2017).

10.5 PHAs Modification

As it has been revised before, PHAs have some disadvantages such as poor mechanical properties, limited functionalities, high production cost, and susceptibility to thermal degradation. Therefore, its applications are limited due to its undesirable physical properties. For example, the PHB forms large crystals, which implies poor mechanical properties, and its melting point is like its thermal decomposition temperature. Some PHAs have elastomeric properties at room temperature and others at temperatures above or close to melting point. The polymers are completely amorphous and sticky. With the intention of changing their physical properties and finding better useful and biodegradable materials with better properties, PHAs have been exposed to modifications, using two important strategies, physical blends and chemical modifications (Li et al. 2016).

10.5.1 Modification of PHAs Via Blending

This strategy is based primarily on mixing two or more biodegradable polymers which can be chemically bonded. This mixture gives a new polymeric material with better physical and mechanical properties which retains its biodegradability. The PHAs are commonly blended with natural raw materials (biodegradable polymers) such as starch, cellulose derivatives, lignin, and different PHA types and synthetic biodegradable polymers such as poly(lactic acid) and polycaprolactone.

10.5.1.1 PHA Blending with Starch

Starch is a polymeric carbohydrate constituted by numerous glucose monomers joined by glycosidic bonds, which is abundant in staple foods like potatoes, wheat, corn, and cassava. Starch consists of the linear and helical amylose (20–25% in weight) and the branched amylopectin (75–80% in weight). The blending of PHB with starch has been widely researched, mainly its compatibility. Since starch is cheap and abundant, it can be mixed with PHB in a ratio 30:70 decreasing the cost of

PHB without changing its physical properties. The blending of both polymers in different proportions produces a crystalline material, with a single glass transition temperature (Tg) and increased tensile strength compared with the pure polymer PHB (Godbole et al. 2003).

10.5.1.2 PHB Blending with Starch Acetate (SA)

The blending of PHB with SA has been studied in order to analyze its thermal behavior and its phase morphology and it was founded that PHB/SA blends were immiscible and a shift of PHB melting point was observed increasing SA concentration. The presence of SA in the heating and cooling processes affects PHB crystallization. The blending of THB with starch or starch acetate reduces production cost; however, the physical and mechanical properties of blends have few significant changes with respect to pure PHB. Due to incompatibility between the S/SA and the PHA matrix, the blends were brittle and did not form intact films (Zhang et al. 1997a).

10.5.1.3 PHA Blending with Cellulose Derivatives

Cellulose derivatives are natural polymers widely employed as biomaterials; among them we can find ethyl cellulose (EC), cellulose propionate, and cellulose acetate butyrate (CAB). Cellulose derivatives have attracted the attention as blending components with PHA, due to their compatibility and their ability to enhance PHA degradation. The blending of PHB with ethyl cellulose (EC) provided a new material with a PHB composition depending-Tg. These blends display no crystallization when cool from a melt state (Zhang et al. 1997b). PHB blending with cellulose acetate butyrate (CAB) provides a new material with a constant Tg indicating that PHB/CAB blends were miscible.

10.5.1.4 PHA Blending with Lignin

Lignin phenolic polymers form important structural materials in the support tissues of vascular plants and some algae since they are part of cell walls of giving rigidity to them. It is the second most abundant organic polymer on Earth, after the cellulose. The presence of lignin reduced the crystallization half-time of PHB/lignin blends indicating that lignin fine powder can be employed as a new type of nucleating agent to modulate PHB crystallization (Weihua et al. 2004).

10.5.1.5 PHA Blending with Other PHA Types

PHB copolymerization with other MCL-HA monomers, such as 3HHx, 3HO, 3HD, and 3HDD, renders the resultant SCL-MCL-PHA copolymers more attractive since the blending of different types of PHAs is usually compatible, enhances co-crystallization, and changes PHAs properties, which facilitates expanded applications (Kai et al. 2003).

10.5.1.6 PHA Blending with Poly(Lactic Acid) (PLA)

The Poly(lactic acid) (PLA) is a linear aliphatic thermoplastic and biodegradable polymer, which can be synthesized from lactic acid or produced by fermentation of

different natural sources. PLA/PHBV blends serve to improve thermal stability and to show ductile plastic deformation (Gerard and Budtova 2012). The crystallization of PHB in the blends is affected by the amount of added PLA (Zhang et al. 1996).

10.5.1.7 PHA Blending with Polycaprolactone (PCL)

Polycaprolactone (PCL) is biodegradable and semi-crystalline aliphatic polyester with a low melting point of around 60 °C and a glass transition temperature (Tg) of around −60 °C, which is obtained from the polymerization of caprolactone. PCL is often used as an additive for other polymers. Since it has a low melting point, it is used as a plastic capable of being hand molded, useful for prototyping, repair of plastic parts and craftsmanship. It has also received great attention for its use as a biomaterial for implants in the human body. PCL can be obtained by open ring polymerization of ε-caprolactone, using a catalyst such as tin octanate. PCL is blended with PHBHHx, which is a flexible and soft polymer, and the blends are employed as a substrate for musculoskeletal tissue engineering since the blends exhibit enhanced toughness with substantial elasticity providing adequate matching of properties with human bone (Lim et al. 2013).

10.5.2 Modification of PHAs Via Chemistry

The functionalization of PHAs by chemical modification is carried out by two important synthesis approaches: graft copolymerization and block copolymerization. Chemical modification of PHAs allows easy and precise modification of the polymer structure with predictable functionalities, with significant impact of the expanded applications.

10.5.2.1 Chemical Modification by Graft Copolymerization

The graft copolymerization is the reaction of one PHA with a natural polymer, for instance the PHB-g-chitosan graft copolymers which are obtained by the reaction of PHB and chitosan. Chitosan is a biopolymer of amino-polysaccharides, composed of randomly distributed units of β-(1–4)-D-glucosamine (deacetylated units) and N-acetyl-D-glucosamine (acetylated unit). The amine groups of chitosan react with the carboxyl group-terminated PHB providing PHB-g-chitosan (Fig. 10.3). Other oligomers such as PHBV and PHO have also been grafted onto chitosan to yield either PHBV-g-chitosan or PHO-g-chitosan copolymers. Since these new grafted products have antimicrobial activity and biocompatibility, they have been applied in tissue engineering and drug delivery systems (Arslan et al. 2007).

The PHA/vinyl grafted copolymer and PHA/(meth)acrylate grafted copolymer are produced by radical polymerization from monomers that contain vinyl or (meth)-acrylate groups. The grafting chains that include polyethylene glycol (PEG) with acrylate groups are used to prepare PHA-grafted copolymer by free radical chemistry (Fig. 10.3). The resulting polymer can be used in blood-compatible biomedical applications (Chung et al. 2003).

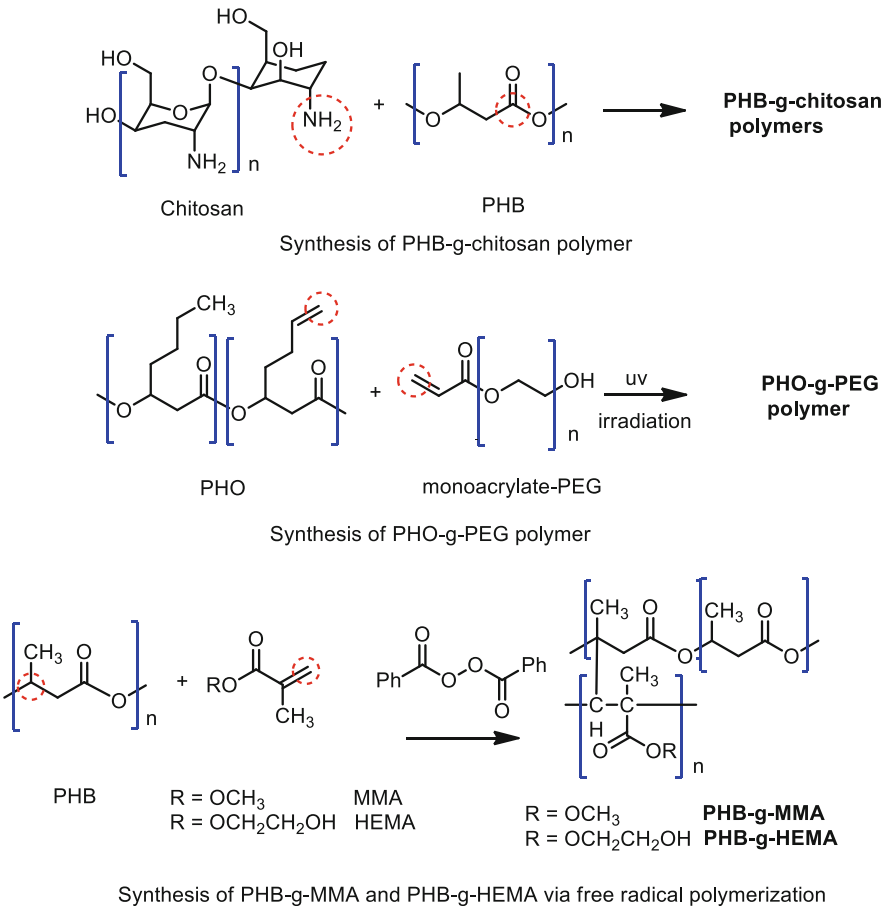

Fig. 10.3 Synthesis of some PHA-grafted copolymers

PHB-g-MMA and PHB-g-HEMA were synthesized by free radical polymerization. First the methyl-acrylate MMA was polymerized in the presence of benzoyl peroxide (BPO) as an initiator of free radicals. The methyl-acrylate polymerized branch PMMA was grafted on aqueous PHB suspension by the covalent bonding PMMA. The PHB-g-HEMA was also prepared from 2-hydroxyethylacrylate HEMA under the same reaction conditions, as shown in Fig. 10.3 (Lao et al. 2007).

Amphiphilic grafted copolymer (PHOU)-g-Jeffamine was synthesized via a thiol-ene reaction. The grafted copolymer is constituted of two parts: hydrophobic PHAs backbone and hydrophilic α-amino-ω-methoxy poly(oxyethylene-co-oxypropylene) (Jeffamine) branches (Fig. 10.4) (Le Fer et al. 2012).

The synthesis of PHA-grafted poly(ethyleneimine) (PEI) copolymer (PHA-g-bPEI) was achieved by Michael addition reaction between mP3/4HB-acrylated and branched PEI. The nitrogen atom attacks the double bond of the acrylated group,

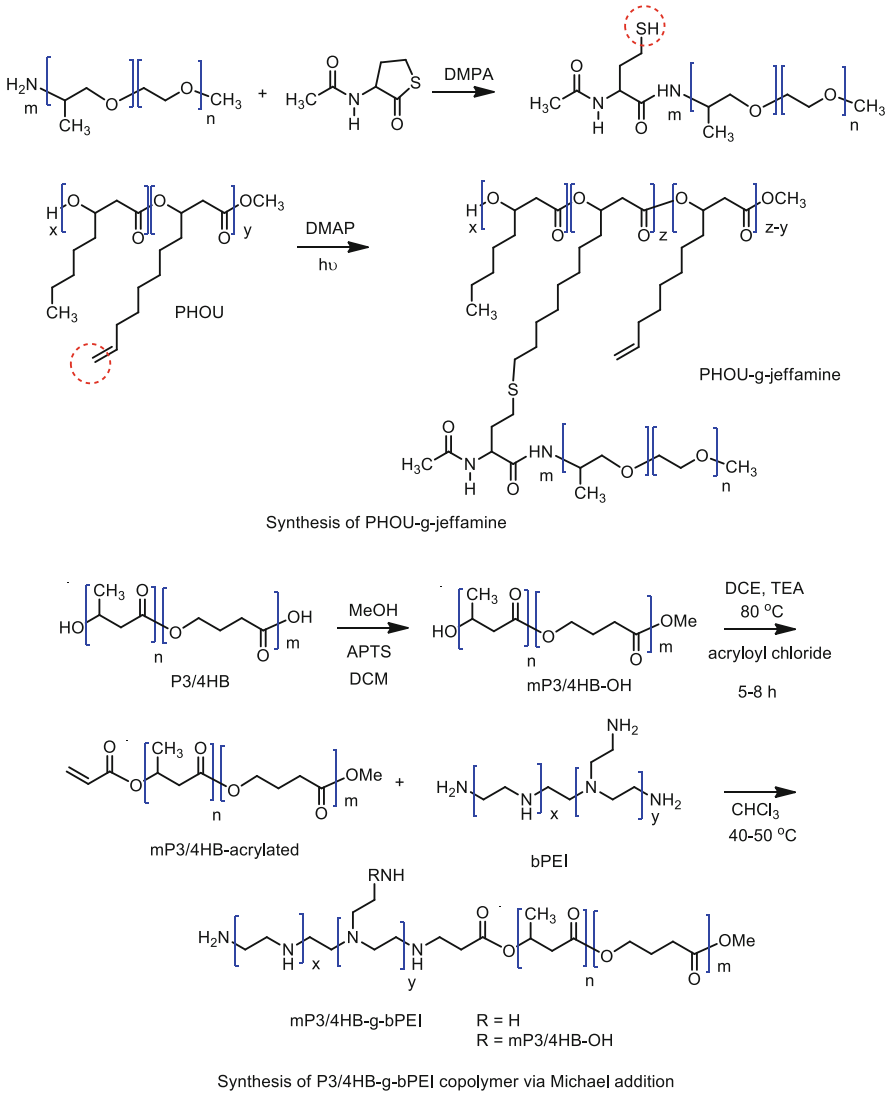

Fig. 10.4 Synthesis of some PHA-grafted copolymers

reaction carried out at 40–50 °C in chloroform, as shown in Fig. 10.4. The P3/4HB-g-bPEI copolymer was soluble in buffer solution (Zhou et al. 2012).

10.5.2.2 Chemical Modification by Block Copolymerization

Block copolymers consist of two or more blocks of different polymers chemically attached to each other that can exhibit properties that are very different from those of random copolymers. The A-B-C type of triblock copolymers are composed of a

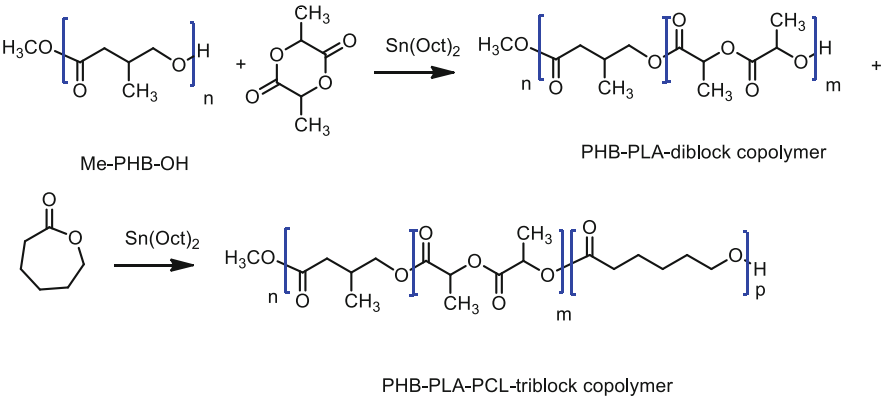

Fig. 10.5 Synthesis of triblock copolymer via a catalytic esterification reaction

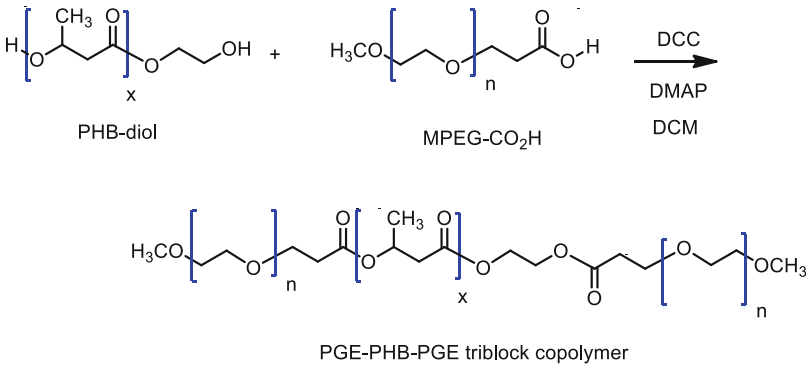

Fig. 10.6 Synthesis of PGE-PHB-PGE triblock copolymer via esterification reaction

PHB-PLA hard segment which serves as cross-linkers and a PCL soft segment that induces a microphase separation in the polymer film (Wu et al. 2008), as shown in Fig. 10.5.

The synthesis of amphiphilic triblock copolymer PEG-PHB-PEG was achieved by the coupling reaction between methoxy-PEG-monocarboxylic acid and PHB-diol, where the esterification reaction was catalyzed by dicyclohexylcarbodiimide (DCC) and DMAP in DCM. The water-soluble PEG-PHB-PEG copolymer was self-assembled into micelles, which are useful for potential drug delivery applications (Li et al. 2003), as shown in Fig. 10.6.

10.6 PHA Applications

The versatility of PHAs lends them to a wide range of potential market applications. In recent years companies have been interested in the use of PHAs in packaging, biomedical, and agricultural applications. In material industry the applications include packaging materials, daily consumables, paper coatings, and female hygiene products. In fuel industry PHAs are used as biofuels additives. They are used as chiral building blocks for organic synthesis of fine chemicals for therapeutic use. It is well known that PHAs were initially used for manufacturing cosmetic containers such as shampoo bottles, moisture barriers in sanitary products, or pure chemicals as raw materials to produce latex paints. Also, they can be used as carriers for long-term release of herbicides or insecticides. Ultra-high molecular weight of PHAs can be useful to produce ultra-strong fibers for fisheries industry (Możejko-Ciesielska and Kiewisz 2016).

As it can be seen due to their biocompatibility, biodegradability, and green credentials, PHAs are being extensively used in many fields. However, PHAs have special indicated properties as biomaterials that make them promising materials especially in a biomedical field. They are nontoxic, biodegradable products and they have a biocompatibility that able PHAS to support cell adhesion and growth. Currently, the most crucial aspect of the use of PHAs in medical application is their non-carcinogenic behavior (Ali and Jamil 2016). In recent years they are considered as materials in the fabrication of cardiovascular products, in drug delivery system, in wound management, and in orthopaedy among others.

High immunotolerance, low toxicity, and biodegradability are the benefits associated with PHAs in tissue engineering (Ali and Jamil 2016; Levine et al. 2015). PHB/hydroxyapatite composite materials used for bone tissues regeneration showed no chronic inflammation even after 1 year of exposure/use and bone formation occurred rapidly (Raza et al. 2018). PHB has also been reported for heart valve tissue engineering (Raza et al. 2018).

The absorbable sutures require some properties such as superficial texture, convenience to tie and grip, and biocompatibility, among others. These properties were exhibited by the P(3HB-co-3HH) strands, which showed excellent features for its use as biomedical sutures (He et al. 2013).

PHAs have also been successfully used as drug carrier systems based on micro-encapsulation technology (Shrivastav et al. 2013). Micro- and nanospheres of PHA are utilized as polymers. P(3HB), P(3HBV), P(3HB-co-4HB), and P(3HB-co-3HHx) have shown promising results for enhanced neural survival (Lizarraga-Valderrama et al. 2015).

As it can be seen PHAs have the necessary potential for being applied in diverse fields (Kalia 2019). However, the most promising application is focused on medical field, which perhaps gives the necessary push to PHA market to overcome economic barriers.

Table 10.2 Commercialized PHAs with their trademark and manufacturers and current stage

Manufacturer (location)	Trademark	PHA types	Development stage
Biomer (Germany)	Biomer	P3HB	Commercial
Kaneka Corporation (Japan, Belgium)	Kaneka	P(3HB-co-HH)	Commercial
Metabolix (USA)	Mirel	P(3HB-co-4HB)	Commercial
Tianan Biologic Material Co., (China)	Enmat	PHBV	Commercial
Bio-on SpA (Italy)	Minerv-PHA	PHB, PHBVV	Commercial
Newlight Technologies (USA)	AirCarbon	PHB, PHBV, PHBH	Commercial
Danimer Scientific (Meredian) (USA)	Nodax	PHBH, PHBO, PHBOd	Commercial
Tepha, Inc. (USA)	TephaFLEX	P3HB, P(3HB-co-4HB), P4HB, P (3HB-co-3HV), P(3HO-3HH)	Commercial
PHB Industrial (Brazil)	Biocycle	P3HB, PHBV	Pilot
Tianjin GreenBio Materials, (China)	BioGreen	P(3HB-co-4HB)	Pilot
Jiang Su Nan Tian, (China)	Jiangsu Nantian	P3HB	Pilot
PolyFerm Canada, Inc. (Canada)	VeraMer	PHBH, PHBHp, PHBO, PHBV PHBD, PHBDD	Research

10.7 PHA Market

Since some years ago many companies have been set up to commercialize PHAs as environmentally friendly bioplastics, fully independent from petroleum sources (Možejko-Ciesielska and Kiewisz 2016). The global PHA market is growing at a healthy rate owing to the increasing environmental awareness and changing preference of manufacturers from conventional to biodegradable plastic products. Packaging and food services and biomedical are the major applications of PHA as it has been seen before. It is estimated that demand for PHAs will grow tenfold by 2021 (Aeschelmann and Carus 2017). The global demand for PHA from various applications, including packaging and food services, biomedical, and agriculture, is expected to increase significantly during the forecast period. PHAs have been produced under the trade names of Nodax™, Biocycle™, Biomer™, and BioGreen™ which are the trademarks of some manufacturers, as it is shown in Table 10.2 (Joce 2018).

Europe has the most stringent policies and regulations regarding plastic consumption. This is the major driver of the growth of the PHA market in Europe. Bio-On SpA (Italy), Nafigate (Czech Republic), and Bochemie (Czech Republic) have

adopted capacity expansions as the key growth strategy in the last 5 years in the PHA market. For instance, Kaneka Corporation (Japan), which is a leading company, produces PHA in Japan and has a bioplastic plant in Belgium. The company can leverage its presence in Europe to produce PHA (Joce 2018; Research and Markets 2019). North America is second-largest market for PHA; it is projected that the market will also follow the same trends as Europe. Canada and the US are the two promising markets of PHA in the North American region, as most of the current productions and upcoming productions are concentrated in these two countries. Danimer Scientific and Newlight Technologies, two of the major producers, are present in the region (Joce 2018; Research and Markets 2019). Apart from the Chinese producers, Kaneka Corporation (Japan) is one of the major players present in the region, while CJ CheilJedang BIO (South Korea) is a major emerging player, as the company has acquired all the necessary IP and technology from Metabolix Inc., now known as Yeild10 Bioscience (Joce 2018; Research and Markets 2019).

The market for short chain length PHA is relatively large as most of the work and development since the 1980s have been done in this segment. All the larger companies produce short chain length PHA, except Danimer Scientific. Initially, ICL and Metabolix Inc. developed short chain length chain polymers, and various applications were identified. The most common short chain length PHA is Poly (HydroxyButyrate-co-HydroxyValerate), which is manufactured by Tianan Biologic Materials China. However, the most important factor that can be attributed to the larger share and higher growth of short chain length PHA is the low cost of these polymers as compared to medium chain length PHAs (Joce 2018; Research and Markets 2019).

In terms of value, the medium chain length PHA accounts for almost half of the market because this PHA type is costlier than the short chain length PHA. The current industry leader, Danimer Scientific, produces Nodax PHA, which is of medium chain length type (Joce 2018; Research and Markets 2019). The market for medium chain length PHA is huge in Europe as most of its applications are being explored in the region. Furthermore, Bio-On and Nafigate are the two companies, which have already tested PHA for the cosmetics application, and Bio-On has already launched its cosmetic product into retail partnering with Unilever.

10.8 Conclusions and Future Perspectives

Considering the wide use of plastics worldwide, they have caused environmental damage such as contamination and health problems. Therefore, there is a growing demand for eco-friendly plastics, namely bio-based plastics and biodegradable plastics, to establish a more sustainable society and to solve global environmental and waste management problems. PHAs are valuable materials to replace petrochemical plastics due to their biodegradability. PHAs materials have already been utilized as food packaging materials or in miscellaneous disposable goods with daily usage. Other applications of PHAs are as agricultural engineering materials, as

materials for fisheries, in a medical context as bioabsorbable materials, and as sanitary goods.

However, their high production cost has been the major problem to apply. For reducing the cost of synthesis and obtaining effective recovery and downstream processing methods of PHAs more research and development are still needed. On the other hand, since these biodegradable polymers have been applied in the medical related field, economical extraction and recovery methods are needed.

The maintaining of the optimal bacterial growth conditions is one of the major limitations of the industrial production of PHAs. To date, most fermentations processes do not allow maximum synthesis of PHAs granules at the end of the cultivation.

From an environmental point of view, another problem arises when PHAs are blending with other polymers to obtain the desired properties. PHAs are biodegradable but if they are blended with non-biodegradable polymer, only PHAS components will be degraded in the environment and non-biodegradable plastics could cause more serious pollution because they will be broken into smaller particles which diffuse into the environment causing great contamination problem. In the same way is desired a good biodegradation rate, therefore with this propose some starters or triggers of biodegradation are added (Iwata 2015).

Despite having some problems like the ones mentioned above, the use of biodegradable plastics is promoted positively in Europe, where it was decided in 2010 that all disposable shopping bags should be either reusable or produced from biodegradable plastics. The global PHA market is growing at a healthy rate owing to the increasing environmental awareness and changing preference of manufacturers from conventional to biodegradable plastic products. Packaging is the most promising application, where PHA can replace conventional plastics; however, as it has been described above medical field is a very promising to use PHAs materials. PHAS polymers are the strongest candidates to replace the conventional plastics in the future.

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Biosynthesis of Polyunsaturated Fatty Acids from Microalgae for Nutraceuticals

11

Pritikrishna Majhi, Mahendra Kumar Mohanty, and Saubhagya Manjari Samantaray

Abstract

Fatty acids are the organic compounds comprised of carboxylic groups and hydrocarbons. These are the responsible factors for the hydrophobic properties of lipids and are mostly involved in signal transduction pathways, energy storage in the form of triacylglycerols (TAGs), protein modifications, source of fuels and constituent of hormones and lipids. Basing upon the nature of their hydrocarbon chain they may be classified into unsaturated and saturated fatty acids. These may be long- or short-chained components. Especially the TAG form of fatty acids is the most important composites of commercial attention (both food and feed). In order to fulfil this commercial interest, the characterization of lipid content, its composition and quantification are foremost requirements. Subsequently, we need a better source for the high production of fatty acids and regarding the above-mentioned circumstances microalgae can be considered as one of the most attractive and economical options. Since they are single-celled photosynthetic microorganisms, their distribution is pervasive and can propagate at any extreme conditions, so they are supposed to contain various forms of fatty acids. Hence exploration of microalgae with the intention of fatty acid diversifications having importance in nutraceuticals is the most important aim of this review.

Keywords

Microalgae · Polyunsaturated fatty acid (PUFA) · Biosynthetic pathway · Nutraceuticals · Disease

P. Majhi · S. M. Samantaray (✉)
Department of Microbiology, College of Basic Science and Humanities, Bhubaneswar, India

M. K. Mohanty
College of Agriculture and Engineering Technology, Bhubaneswar, India

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11.1 Introduction

Fatty acids are the combined group of carboxylic acids and long aliphatic chains that may be saturated or unsaturated, straight or branched. They may also contain some keto, halogens, hydroxyl, epoxy groups. Polyunsaturated fatty acid (PUFA) like eicosapentaenoic acid (EPA, $C_{20}H_{30}O_2$), arachidonic acid (ARA, $C_{20}H_{32}O_2$), linolenic acid (ALA, $C_{18}H_{30}O_2$) and docosahexaenoic acid (DHA, $C_{22}H_{32}O_2$) are indispensable source of nourishment for human health which cannot be synthesized itself in human body. They are the major constituents of storage energy, biological membranes, hormones, vitamins and bile acids (Paik et al. 2009). Hence they are mostly useful in case of infant growth, brain and retina development, antidote for heart diseases, arrhythmia, high blood pressure, atherosclerosis, asthma, rheumatoid, cancer and depressions (Patil et al. 2007). So additional uptake of PUFA is essential, in order to beat the adverse effects of fatty acid deficiency. Commercially, fish oil collected from marine fish such as mackerel, salmon and mullet are served as the major fatty acid components for decades but currently these are losing attraction due to their unpleasant odour, taste, meagre oxidative stability and occurrence of chemical contaminants like mercury (Adarme-Vega et al. 2012; Abdo et al. 2015). Besides, fish, plants, bacteria, fungi and algae are also being used for production of PUFA, among which microalgae are the finest deliberated springs till date because they possess some unique characters like high growth rate, higher biomass production, effective accumulation of lipids, shorter incubation period, lower degree of unsaturation and minimum requirement of nutrients (Ramachandra et al. 2009; Supriya and Ramachandra 2012). They contain about 12–35% proteins, 2–23% fats, and 6–23% carbohydrates of their dry weight on an average (Majhi and Samantaray 2021). So microalgae are not only the major source of food diversity but also can be easily grown in extreme worst conditions which lead them to produce several value-added compounds. Microalgae-based components are available in the form of capsules, powder, concentrates and tablets (Handayania et al. 2011). Furthermore, about 10–70% PUFAs are produced from algae. *Spirullina* sp., *Nanochloropsis* sp., *Chlorella* sp., *Chlamydomonas reinhardtii* and *Dunaliella* sp. are the most important microalgae for the production of PUFA (Kumari et al. 2013). Both pharmaceuticals and nutraceuticals are considered for superior health benefits. Some most common diseases like Alzheimer, major depressions, cardiac arrest, inflammation, liver failure, high cholesterol, cirrhosis, acute hepatitis, ageing, etc. are caused due to the deficiency of unsaturated fatty acids in the body. For the treatment of such diseases EPA, DHA, etc. are the mostly needed fatty acid compounds. This review deals with a brief collection of synthesis, structure, nature and benefits of several fatty acids generating from the huge algal diversity.

11.2 Fatty Acids

Fatty acids are broadly classified into saturated and unsaturated fatty acids. Saturated fatty acids contain only single bonds between them and here the carbon atoms are fully saturated with the hydrogen atoms and are solid in nature when kept in room temperature. Some examples are propionic acid ($C_3H_6O_2$), butyric acids ($C_4H_8O_2$), valeric acid ($C_5H_{10}O_2$), caproic acid ($C_6H_{12}O_2$), lauric acid ($C_{12}H_{24}O_2$), palmitic acid ($C_{16}H_{32}O_2$), etc. but the unsaturated fatty acids are those fatty acid chains which contain at least one double bond in them and present in liquid state when stored in room temperature. The most common examples are arachidonic acid ($C_{20}H_{32}O_2$), linoleic acid ($C_{18}H_{32}O_2$), oleic acid ($C_{18}H_{34}O_2$), docosahexaenoic acid, etc. When the number of double bonds between these chains is more than one, it is termed as polyunsaturated fatty acids (PUFAs), and the fatty acid chain containing only one double bond is called as mono-unsaturated fatty acid (MUFA). Foods like walnuts, sunflower seeds, flax seeds, fish, safflower, soybean oil, corn oil, etc. are rich in unsaturated fatty acids. If we compare then unsaturated fatty acids will be proved to be the best one because they not highly saturated being used to reduce the cholesterol levels and the risk of heart diseases by replacing the trans form or saturated fats. So it is the high urge of the time to get more and more amount of unsaturated fatty acids for our health benefits. Some of the most important unsaturated fatty acids (such as Eicosapentaenoic acids, Docosahexaenoic acids, Arachidonic acids, and ϒ-linolenic acids) are listed below.

11.2.1 Eicosapentaenoic Acids (EPA)

This is an omega-3-fatty acid where the last double bond is present at the third C-atom from the methyl terminal (20:5 ω 3, 6, 9, 12, 15) and the configuration of all the double bonds is in cis form. EPA is the precursors for the production of eicosanoids (Wen and Chen 2003). The main functions of EPA are:

1. Regulates the level of fibrinogen and hence reduces thrombosis.
2. Inhibits rheumatoid arthritis.
3. Plays an important role in anti-inflammatory and anti-cachexia (weight loss and muscle wasting) activity.
4. Prevents arteriosclerosis by lowering the low-density lipoprotein level.
5. Prevents arrhythmias (problem in heartbeats).
6. Reduces the chances of heart attack by regulating the rhythms, chemical responses and electrical behaviours of heart.

11.2.2 Docosahexaenoic Acids (DHAs)

This is also an omega-3-fatty acid which is required about 0.2–0.3 g/day (Handayani et al. 2011) as a daily intake of diet. It is naturally present in breast milk but absent in

cow's milk. Its structure is 22:6 ω 3, 6, 9, 12, 15, 18. According to Spolaore et al. 2006, it is the major component of the grey matter and the retina of eye. Tanon et al. (2002) reviewed that docosahexaenoic acid is mostly important for:

1. Prevention of hypertension, arthritis, Type-II diabetes, coronary heart diseases, etc.
2. Brain and eye development in infants.
3. Preparation of food additives and nutritional supplements.
4. Reduces cystic fibrosis and ocular diseases.
5. Also acts as a key component for aquaculture.
6. Reduces Parkinson and Alzheimer's disease.

11.2.3 Arachidonic Acids (ARAs)

Arachidonic acids are the long poly unsaturated fatty acids of omega 6 group (Shanab et al. 2018). Its chemical formula is $C_{20}H_{32}O_2$. Some important functions that are carried out by the arachidonic acids are as follows:

1. Important for the regulation of skeletal muscle and nervous system.
2. Helps in cell signalling, stress and inflammatory responses, emotions, pain and blood clotting.
3. Plays a vital role in immune system by acting as an immune suppressant and also resistant to allergies and parasites.
4. Acts as a precursor of prostaglandins and eicosanoids.
5. Important for the growth and development of infant's brain and retina.
6. It is an important constituent of phospholipid membrane of brain.
7. It acts as a natural anti-freezing compound in case of arctic animals.
8. Hair fall, anaemia, fatty liver degeneration, reduced fertility, etc. are some other symptoms of arachidonic acid deficiency.

11.2.4 ϒ-Linolenic Acids (GLAs)

ϒ-Linolenic acid is an omega-6 fatty acid having most powerful implications for human health. Its configuration is 20:4 ω 6, 9, 12, 15 (Spolaore et al. 2006). Its chemical formula is $C_{18}H_{30}O_2$. According to Sajilata et al. (2008) the major health benefits of ϒ-linolenic acids are:

1. Alleviate the symptoms of pre-menstrual syndrome.
2. Reduce the chances of inflammatory diseases (atopic eczema, rheumatoid arthritis, asthma, etc.)
3. Show some kinds of antiviral activity.
4. Act as precursor of prostaglandin E_1.
5. They have the capacity to kill or destroy the tumour cells without causing harm to the normal healthy cells.

11.3 Biosynthetic Pathway Involved for the Production of PUFA

Lipid biosynthesis is one of the most accepted pathways for the production of fatty acids and triacylglycerols (TAGs). The presence of numerous genes or enzymes is responsible for the production inside the algal cells. The complete process of biosynthesis starting from carbon dioxide fixation to synthesis of TAG and their sequestration, all take place in a specific cell. Then accumulation of TAGs found in the form of lipid bodies (densely packed) are located in algal cytoplasm (exception found in case of some green algae where the lipid bodies are present in inter-thylakoid membrane of chloroplast). This activity increases when the organism continues to move in a stress condition (Hu et al. 2008). TAG synthesis in algal cell mostly occurred by Kennedy Pathway (Ratledge 1988). The major stress conditions for TAG accumulation in algae are pH, nutrient starvation, salinity (chemical stimuli) and light intensity and temperature (physical stimuli). Besides these factors, growth phase of the algal culture also affects the content and composition of TAG compounds (Cagliari et al. 2011). It has been observed that when the algal cells get optimal environmental growth conditions, they produce lipid content only 5–10% of their dry cell weight, while if the growth takes place in severely harsh condition, then the lipid content leads to become 20 to 50% of total dry cell weight (Tonon et al. 2002; Yongmanitchai and Ward 1991).

The biosynthesis of fatty acid requires Acetyl-CoA which is known as a common carbon donor (Baba and Shiraiwa 2013). Acetyl-CoA is received through numerous sources and then consequently converted into malonyl-acyl carrier protein (ACP) following a sequence of reactions in the presence of sunlight and carbon dioxide (Fig. 11.1). A molecule of acetyl-CoA carboxylation utilizes one ATP molecule to yield one malonyl molecule. Hence, malonyl-ACP (C3) and acetyl-CoA (C2) are the primary substrates for fatty acid biosynthesis. Decarboxylation and condensation reaction of both the substrates and the reduction of keto unit (derivative of non-malonyl-ACP) produce butyryl-ACP (C4). Two NADPH molecules are responsible for the reduction of the keto group. Consequently, two NADPH and one ATP are spent for the extension of fatty acid chain. Hence, acyl (C16 or C18)-ACP is generated by the elongation of Acyl-ACP. In this way the molecules with C2n-1-carbon chain are synthesized by the loss of carbon from C2n-compounds (Řezanka and Sigler 2009). Fatty acid (C16 or 18) is produced from acyl (C16 or 18)-ACP by subsequent removal of ACP. According to Joyard et al. (2010) the fatty acids synthesized on the plastid envelopes are transferred into cytosol probably due to the binding of CoA.

Again there are four major enzymes found to involve in the synthesis of fatty acids. They are the enzymes responsible for the reverse catalysis of β-oxidation, type-I fatty acid synthase (FAS), type-II FAS and elongases. Type-II FAS gene is possessed by *Chalmydomonas reinhardtii* (Riekhof et al. 2005). Mostly the type-I fatty acid synthase (FAS) was occupied by fungi and animals (Joyard et al. 2010; Chan and Vogel 2010) and the type-II FAS was found in bacterial plastid and mitochondria (Hiltunen et al. 2010). The de novo synthesis of fatty acids through reverse β-oxidation was observed in *Euglena gracilis* during anaerobic conditions

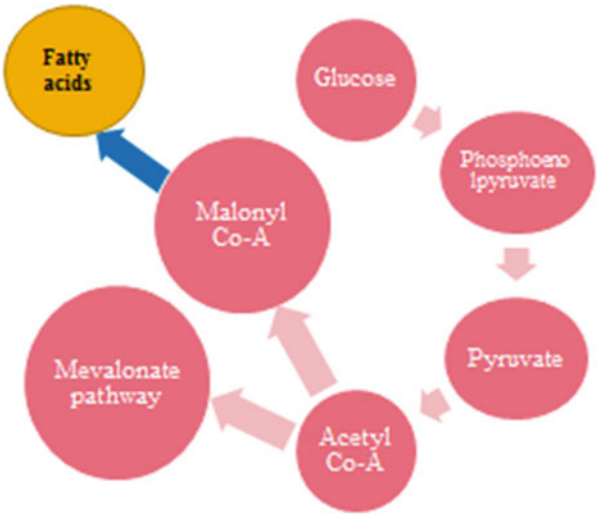

Fig. 11.1 Biosynthetic pathway of fatty acid production

(Hoffmeister et al. 2005; Inui et al. 1984). Most often it was observed that haptophytes (such as *Isochrysis galbana*, *Emiliania huxleyi*, *Pavlova salina*), diatoms (such as *Thalassiosira pseudonana*, *Phaeodactylum tricornutum*) and some euglenophytes have elongases/desaturase which convert C18 fatty acid and their derivatives into PUFA (EPA and DHA) (Fig. 11.2) (Venegas-Calerón et al. 2010).

11.4 Possible Applications of Algae

Algae represent the most diversified microbial group which is considered as a key component in the base of food chain (Cagliari et al. 2011; Harwood and Guschina 2009). As per the reports of few researchers algae have many possible applications for the betterment towards the environment, human health and plants (Kothari et al. 2017; Bilal et al. 2017). Due to greater diversity in their morphology and habitation they become capable of containing high amount of lipids and fatty acids (Harwood and Guschina 2009). The major solicitations of these beneficial microbes are listed below.

1. They contain staple foods and vitamins (Phycobiliproteins as food dye, carbohydrate, protein, β-carotene, phycocyanin and phycoerythrin and vitamins like A, B1, B2, B6, B12, C, E, nictitate, biotin, folic acid and pantothenic acid).
2. Algae also contain major sources of PUFA (Arachidonic acid, eicosapentaenoic acid, linolenic acid, docosahexaenoic acid, etc.)
3. They also involve in bioenergy (biodiesel, methanol, biogas, biohydrogen, oil) production.
4. They are the major source of bio-fertilizer.

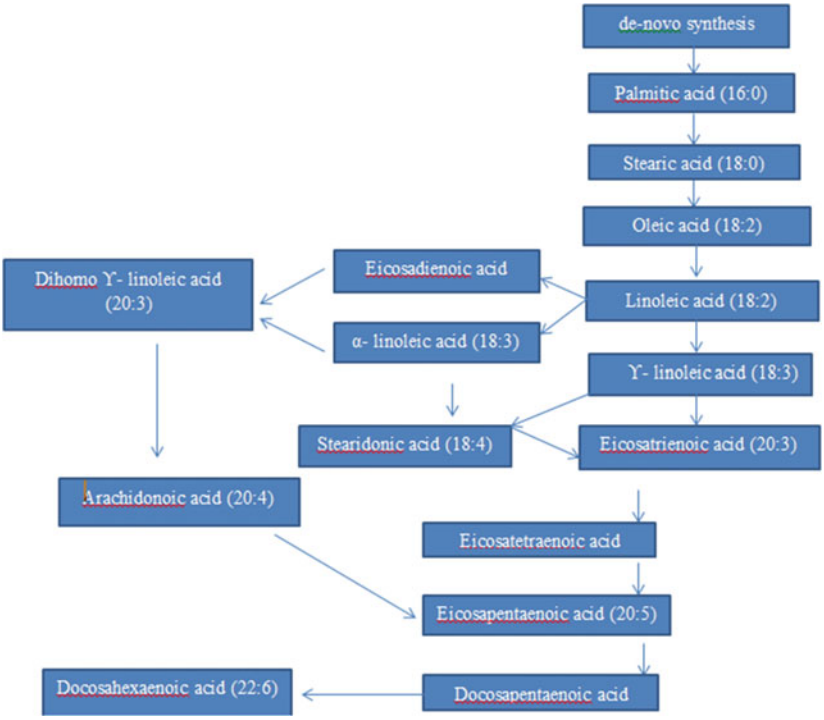

Fig. 11.2 Pathway for biosynthesis of EPA and DHA polyunsaturated fatty acids

5. They are involved in wastewater treatment.
6. They are helpful in pigment production.
7. Some algal compounds are useful in the production of cosmeceuticals.
8. They are used as clinical and diagnosis research reagent.

A report was summarized by Richardson et al. (2012) showing the oil production from different plant source also compared with algal production of oil (Table 11.1).

11.4.1 Bioprocessing of Algal Biomass for Production of Fatty Acids

Microalgae utilizes light and carbon dioxide (CO_2) as the sources for energy and carbon (Chisti 2007). Factors like 15–30 °C temperature, specific growth medium containing inorganic elements (phosphorous, nitrogen, iron, sodium, potassium, etc.), neutral pH, specific incubation and photo period are maintained for the optimum growth of algae. Mass culture is the major step after optimizing the growth, mostly done by using photobioreactors and open pond methods (Harun et al. 2010). Following the optimum incubation period the biomass is harvested by dewatering the cultures from their growth media (Fig. 11.3). Dewatering is done by

Table 11.1 Oil production by different crops and algae

Crop	Oil yield (L/acre)
Microalgae	19,000–57,000
Jatropha	788.33
Oil palm	2403.47
Sunflower	386.07
Corn	68.13
Canola	495.83
Rapeseed	480.69
Soybean	181.68

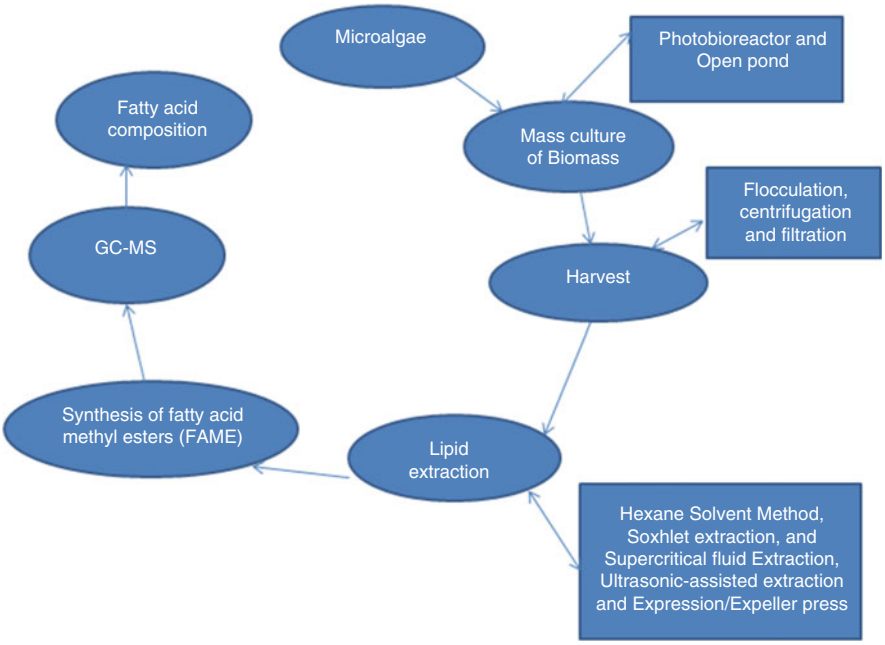

Fig. 11.3 Methods for fatty acid production

centrifugation, flocculation and filtration process (Borowitzka 1999). Then the microalgal lipid extraction is carried out either by chemical methods (Soxhlet extraction, Hexane Solvent Method or Supercritical fluid Extraction) or physical methods (Ultrasonic-assisted extraction and Expression/Expeller press) (Mata et al. 2010). Methylation is taking place for the preparation of fatty acids. The lipid samples are methylated using 5% of methanolic HCl and heated at 60 °C under reflux process. Petroleum ether is used for the extraction of fatty acids at 40–60 °C. Then the ether extract is washed with distilled water thrice, dried over anhydrous sodium sulphate and filtered (Abdo et al. 2015). Now GC-MS is performed for FAME analysis which shows the fatty acid composition of the extracted sample (Abdo et al. 2015; Ichihara and Fukubayashi 2010).

11.4.2 Algal Strains Responsible for Production of PUFA

Normally the foods containing fatty acids are very costly and not easily available in all kinds of environment. So some alternatives of these foods are the need of the present time. From some reports it has been found that microbes like bacteria, fungi, yeast and algae are also capable of producing unsaturated fatty acids (Pereira et al. 2006). Among all these, microalgae are proved to be a potent source of fatty acids because they are capable of existing in very harsh environment, low requirement of nutrients, fast growth and easily available (Table 11.2). Besides these the major cause of producing higher amount of fatty acids is due to the utilization of sun light and atmospheric carbon-dioxide for photosynthesis. By this microalgae can convert the light and CO_2 into large amount of carbohydrates, lipids and protein which are major components of algal cell wall. During sufficient nutrient conditions they use all the nutrients and their biomass become larger and a starvation in nutrition starts. In order to overcome this nutrient scarcity they produce more and more lipids and

Table 11.2 Major algal sources of PUFA and their fatty acid contents

Name of the PUFA	Name of the algal source	% of total fatty acid content	References
Arachidonoic acid	*Phormidium pseudopristleyi* strain 79S11 *Phormidium pseudopristleyi* strain 64S01 *Porphyridium purpureum* *Euglena gracilis* *Parietochlorosis incise* *Phaeodactylum tricornutum* *Thalassiosira pseudonana*	24 32 40 77	Shanab et al. (2018) Guschina and Harwood (2006)
Docosahexaenoic acid	*Crypthecodinium cohnii* *Schizochitrium mangrovei* *Schizochitrium aggregatum* *Phaeodactylum tricornutum* *Porphyridium cruentum* *Isochrysis galbana*	0.8–1.3 32.29–39.14 0.3 0.8 0.2 8.4	Spalaore et al. (2006) Handayani et al. (2011)
Eicosapentaenoic acid	*Schizochitrium aggregatum* *Phaeodactylum tricornutum* *Porphyridium cruentum* *Isochrysis galbana* *Nannochloropsis occulata* *Nitzschia laevis*	1.0–1.2 29.8 23.9 22.6 39.9 75.9	Handayani et al. (2011) Renaud et al. (2002)
Linoleic acid	*Phaeodactylum tricornutum* *Porphyridium cruentum* *Isochrysis galbana*	2.2 6.2 0.9	Handayani et al. (2011)
ϒ-linolenic acids	*Arthrospira* sp. *Spirulina maxima* *Spirulina plantesis*	18.5	Collinius (2016)

carbohydrates by using the light which accumulated in their body as storage products (Adarme-Vega et al. 2012). The major PUFA producing countries are the USA, Canada, Germany, the UK, Italy, Spain, China, Japan, India and Latin America.

11.5 Conclusion

In the recent years algal biotechnology has developed many folds. A wide range of applications like production of antioxidant, antimicrobials, lipids, biofuel, single-cell protein, animal feed, etc. were started up by many entrepreneurs in the global market. Besides this the left over biomass after the extraction of desired product can be utilized by their anaerobic digestion and pyrolysis in terms of carbon sequestration and nutrient recycling for the production of bio-char. It has been observed that among a huge diversity of alga only a few were collected and examined for their content and industrial production as nutraceuticals. So a continuous study on the cultivation, isolation, screening, identification and applications of those algal strains along with algal biochemistry, genetics and physiology are required in this mean time as a result of which microalgae are not only regarded as the third generation of energy production but also will be well known as game changer for the production of value-added products.

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Microbial Polyhydroxyalkanoates (PHAs): A Brief Overview of Their Features, Synthesis, and Agro-Industrial Applications

12

Lavanya Addagada, Pankaj Pathak, Muhammad Kashif Shahid, and Prangya Ranjan Rout

Abstract

Polyhydroxyalkanoates (PHAs) are biopolymeric intracellular inclusions that serve as carbon and energy storage compounds for diversified microorganisms. PHAs are synthesized by a variety of bacterial strains such as *Alcaligenes latus*, *Azotobacter vinelandii*, *Pseudomonas* sp., and *Escherichia coli* under limited oxygen but sufficient availability of carbon source. Rubber-like nature along with biocompatibility, biodegradability, eco-friendly, renewable, and biological production features make the PHAs as promising alternatives to synthetic plastics which can mitigate plastic-waste disposal mediated environmental pollutions. However, carbon source requirement driven high production cost and low yield limit the large-scale production of bioplastics and so as to its wider applications. For minimization of production cost, many researchers focused on utilization of waste/by-product based carbon sources for PHA biosynthesis. On the other hand, several other researchers emphasized on the exploitation of genetically engineered microbes and plants to address the low yield issues. Consequently, these attempts of improvements will be helpful in making the bioplastics more

L. Addagada
National Environmental Engineering Research Institute (NEERI), Council of Scientific & Industrial Research (CSIR), Nagpur, India

P. Pathak
School of Environmental Science, SRM University, Amaravati, India

M. K. Shahid
Research Institute of Environment and Biosystem, Chungnam National University, Daejeon, Republic of Korea

P. R. Rout (✉)
Department of Biotechnology, Thapar Institute of Engineering and Technology, Patiala, India
e-mail: prr10@iitbbs.ac.in

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competitive than the conventional ones in long run. In addition to their common use as bioplastic, PHAs are widely used in various fields, including medical, pharmaceutical, agro-industries, textiles, households, etc. This chapter provides an overview of classification, structural components and properties of microbial PHAs, progresses in PHA biosynthesis, highlighting different biosynthetic pathways, role of various substrates, microorganisms, and experimental parameters on PHA biosynthesis. Recent advancements in enhancing the physico-chemical properties of PHAs and trends in agro-industrial applications of PHAs have also been discussed. Futuristic approaches to overcome the challenges associated with the yield and improved mechanical properties of PHA are also recommended in this chapter.

Keywords

Bioplastics · Biosynthesis · Environmental pollution · Genetically engineered microbes · Plastic waste disposal · Polyhydroxyalkanoates (PHAs)

12.1 Introduction

Conventional plastics that are derived from petroleum and oil are commonly used for various domestic, industrial, and environmental applications for the past century due to their unique features such as light-weight, stability, durability, and immune to degradation (Anjum et al. 2016). Moreover, these synthetic plastics replaced the glass and paper in packaging worldwide and their consumption estimated to be 150 million tonnes per year throughout the world. The dependence on conventional plastics has resulted in serious global environmental issues. There are several ways to manage plastic wastes that includes recycling, incineration, source reduction, and photo-degradation. Though these are the commonly practiced alternatives, there are some inherent issues linked with these approaches. Incineration is highly expensive and leads to the entry of noxious substances into the environment. Similarly, recycling is time-consuming and sorting of waste materials is very tedious process. Furthermore, existing natural petroleum reserves would run dry within the near future. Therefore, it is imperative to replace conventional petroleum-derived plastics with biodegradable plastics to mitigate the environmental hazards (Castilho et al. 2009).

Biodegradable plastics are the ones in which degradation takes place by naturally available microorganisms such as fungi, algae, and bacteria. Biodegradable plastics are mainly classified into three categories.

1. *Photodegradable*: These plastics consists of light-sensitive groups that are easily disintegrated by UV irradiation and make them vulnerable to bacterial degradation.
2. *Semi-biodegradable*: These plastics mainly contain starch, proteins, and cellulose and are partially biodegradable in nature.

3. *Completely biodegradable*: Polyhydroxyalkanoates (PHAs) are completely biodegradable (100%) in nature. Under aerobic conditions, they are completely degraded to H_2O and CO_2 and into CH_4 under anaerobic conditions by microorganisms (Reddy et al. 2003).

Generally, PHAs are the most widely used bioplastics; they have high biodegradability, sustainability, and eco-friendly properties. Moreover, PHAs are biocompatible, which makes them utilize in medical and pharmaceutical sectors. Furthermore, wider application of PHAs in day-to-day activities reduces the dependency on fossil fuels and makes them be applicable in agricultural, industrial, and marine fields. Therefore, PHAs are considered as a promising alternative to conventional plastics. PHAs including poly (3-hydroxybutyrate) (P(3HB)), poly (3-hydroxyvalerate) (P (3 HV)), etc., are reported in the literature to possess numerous structures and properties. PHAs are accumulated within the microorganisms (gram-positive and gram-negative bacteria) as an energy reserve (Rout et al. 2017). The first PHA discovered was Polyhydroxybutyrate (PHB) in the gram-positive bacterium *Bacillus megaterium* by Maurice Lemoige in 1926. PHB is the best characterized and the most extensively studied PHA. The monomers, namely 3HB, 3-hydroxyhexanoate (3HHx), and 3 HV, are majorly responsible for the formation of PHA (Anjum et al. 2016).

12.2 Classifications and Structures of PHAs

PHAs are elastomeric polyesters or thermoplastics of hydroxyl alkanoic acid monomers, which are synthesized by a large variety of bacterial species, including gram-positive and gram-negative bacterial strains as their energy reserves, especially when other vital nutrients such as phosphorus or nitrogen are limited and there is excess availability of carbon. The monomer unit that is responsible for the formation of PHAs is presented in Fig. 12.1. The molecular weight of PHAs ranges from 0.2×10^6 to 3×10^6 Da based on the growing condition such as pH, carbon source, concentration, mode of operation (batch or continuous), and type of microorganism. PHAs are formed in cell cytoplasm with range of 0.2–0.5 mm diameter and can be observed using light microscopes or staining dyes, such as oxazine dye, Sudan Black B, Nile red or Nile blue A, etc.

PHAs are mainly classified into short and medium chain length PHAs according to the number of carbon atoms present in the chain. PHAs consisting of 3–5 carbon atoms are classified as short chain length, while PHAs with 6–14 or more than 14 carbon atoms are categorized as medium chain PHAs (Anjum et al. 2016). Examples for short chain length PHAs are P(3HB), poly (4-hydroxybutyrate) (P

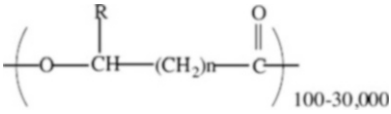

Fig. 12.1 Monomer unit for PHA formation

(4HB)), P(3 HV), and a copolymer of P(3 HV) and P(3HB). Likewise, poly (3-hydroxyhexanoate) (P(3HHx)), poly(3-hydroxyoctanoate) (P(3HO)), and copolymers of P(3HHx) and P(3HO) are considered as examples for medium chain length PHAs. The ability of the PHA synthase enzyme to accept specific substrates that is 3-hydroxyalkanoates (3HAs) of a certain length of carbon chain becomes the basis of the differences between short and medium chain length PHAs (Khanna and Srivastava 2005).

12.3 Properties of PHAs

The properties of PHAs such as melting point, hydrophobicity, degree of crystallinity, and glass transition temperature depend upon the monomer composition (Crank et al. 2004).

12.3.1 Short Chain Length and Medium Chain Length PHAs Properties

Short chain length PHAs are brittle, less flexible, and have high crystallinity (60–80%). On the other hand, the medium chain length PHAs have low tensile strength, high flexibility, and low crystallinity (25%) (Reddy et al. 2003).

12.3.2 P(3HB) Properties

PHB is the extensively investigated and characterized PHA. The P(3HB) is insoluble in water; hence it has resistance to hydrolytic degradation. The O_2 permeability and elongation to break of P(3HB) are lower than the conventional plastics whereas the tensile strength and stiffness (young's modulus) are higher as compared to the conventional plastics (i.e. polypropylene) (Sudesh et al. 2000). Also, P(3HB) possesses good thermoplastics properties when compared to polypropylene. P (3HB) is stiff and brittle in nature. The molecular weight of P(3HB) varies from 1×10^4 to 300×10^4 Da based on the type of bacteria that produces PHAs (Philip et al. 2007).

12.3.3 P(4HB) Properties

Poly (4-hydroxybutyrate) P(4HB) is a tough and malleable thermoplastic material. The tensile strength of P(4HB) is similar to that of polyethylene. Moreover, it consists of 100% elastic properties. The properties of P(4HB) vary when it is combined with other hydroxy-acids (Martin and Williams 2003).

12.4 Synthesis of PHAs

PHAs synthesis was mainly achieved by using chemical and biological methods. PHAs that are synthesized using chemical methods have low biodegradability when compared to PHAs synthesized by biological methods. PHA synthesis by chemical method requires mixing of both R and S—stereoisomers within the polymer chain, which reduces the biodegradability. Hence, the chemical synthesis of PHAs has not gained attention. However the biological synthesis of PHAs contains only R-stereoisomers and is completely biodegradable. Therefore, the biological synthesis of PHAs is more advantageous when compared to chemical synthesis.

Furthermore, PHAs synthesis can also be achieved by incorporating PHA synthase gene in plants. But the main drawback of plant-based PHAs synthesis is the low yield (i.e. $<10\%$ dry weight). Also, the growth of the plant is reduced after incorporation of PHA synthase gene. On the other hand, microbial synthesis results in significantly higher yield of PHA (i.e. 90% of cell dry weight). Thus, microbial synthesis of PHAs is more advantageous as compared to the other ways of PHA synthesis.

12.5 Microbial Synthesis of PHAs

Several gram-negative and gram-positive bacteria produce PHAs in their cell cytoplasm as an energy source (Meur et al. 2012). More than 300 varieties of PHA-synthesizing bacterial species are reported in the literature. In most of the bacterial species PHAs are synthesized under unfavourable growth conditions like nutrient (nitrogen, phosphorous, sulphur, magnesium, etc.) limitations. All the PHA-synthesizing bacteria are broadly categorized into two classes based on the stress conditions under which they synthesize PHA. The first kind of bacterial species synthesize PHA under nutrient limitation condition in the presence of excess carbon source. However, in case of the second class of bacterial species nutrient limitation is not a desirable condition for PHA synthesis. The examples for the first kind of bacteria are *Protomonasoleovorans* and *A. eutrophus*. Similarly, *Alcaligenes latus* belongs to the second kind of bacteria (Ojumu et al. 2004).

12.5.1 Microorganisms Utilized for PHAs Production

Various microbial species either wild or recombinant are employed to produce PHA. Among those, *Bacillus* species (Kanjanachumpol et al. 2013), *Aeromonas* species (Shen et al. 2009), *Pseudomonas* species (Costa et al. 2009), *A. eutrophus* (Kamilah et al. 2013), *Cyanobacteria* (Balaji et al. 2013), recombinant *E. coli* (Andreeßen et al. 2010), Activated sludge (Liu et al. 2011), *Azotobacter* species (García et al. 2014), *A. latus*, *Thermus* thermophiles (Pantazaki et al. 2009), *Halomonas* species (Kawata and Aiba 2010), *Methylobacterium* species (Nath et al. 2008) and

Chromobacterium species (Bhubalan et al. 2010) are some of the most commonly employed species for PHA production.

12.5.2 Different Substrates Used for PHAs Production

A wide variety of substrates have been utilized for PHAs production. The selection of substrate is one of the most essential factors that is responsible for high production costs. Hence to minimize the production cost, now-a-days researchers have started using low-cost substrates like industrial by-products, agricultural waste materials, sugars, domestic waste materials, fats, oils, and wastewater. The wastewaters that are used for PHAs production are municipal wastewater, biodiesel wastewater (Dobroth et al. 2011), brewery wastewater (Liu et al. 2011), paper mill wastewater (Bengtsson et al. 2008), food processing wastewater (Venkateswar Reddy and Venkata Mohan 2012), olive oil mill effluents (Ntaikou et al. 2009), swine wastewater (Cho et al. 1997), kraft mill wastewater (Pozo et al. 2011), and palm-oil mill wastewater (Wu et al. 2009). Oils and fats that are employed for PHA production are cooking oil (Kamilah et al. 2013), frying oil (Verlinden et al. 2011), coconut oil (Ashby and Foglia 1998), olive oil, palm oil, sesame oil, soybean oil (Akiyama et al. 2003), butter oil (Solaiman et al. 2001), linseed oil (Bassas et al. 2008), jatropha oil (Ng et al. 2011), hazel-nut oil, and edible and non-edible oil cakes (Mohanty et al. 2021). Broad research has been conducted on PHA production using fish peptone, sugar cane (Bengtsson et al. 2010) and soy molasses (Full et al. 2006), cheese whey (Koller et al. 2011), starch (Halami 2008), galactose (Munoz and Riley 2008), sucrose (Nonato et al. 2001), glucose (Li et al. 2007), mannose (Munoz and Riley 2008), acetic acid (Wang and Yu 2001), lactic acid (Tsuge et al. 2001), and oleic acid (Eggink et al. 1992). Overview of the various studies that employed microbial species and different substrates used for the production of PHA are given in Table 12.1.

12.5.3 Biosynthetic Pathways for PHAs Production

Mainly, three biosynthetic pathways are responsible for PHAs production within the microorganisms as shown in Fig. 12.2. In the pathway I, PHA formation occurs in three steps. In the first step, enzyme β-ketothiolase present in the microorganism condensate two molecules of acetyl-CoA to acetoacetyl-CoA, then the formed acetoacetyl-CoA would be converted to 3-hydroxybutyryl-CoA by reductase enzyme in the second step. In the third step, polymerization through esterification of 3-hydroxybutyryl-CoA to P(3HB) takes place with the help of PHA synthase enzyme. In the pathway II, the second step differs from pathway I, where acetoacetyl-CoA reduced to S-(+)-3-hydroxybutyryl-CoA by reductase enzyme and then converted to R-(−)-3-hydroxybutyryl-CoA by 2-anoyl-CoA hydratase. Pathway III helps to produce PHA from cheap carbon sources like fructose, sucrose, and glucose. The R-(−)3-hydroxyacyl intermediate formed in the pathway III are

Table 12.1 Overview of PHAs production using different substrates and microbial species

Sl. No.	Substrate	Microorganism	References
1.	Fluorophenoxyalkanoic acids	*Pseudomonas putida* 27 N01	Takagi et al. (2004)
2.	Glucose, acetate	*P. guezennei*	Simon-Colin et al. (2008)
3.	Soybean oil, fructose	Recombinant *R. eutropha*	Tsuge et al. (2009)
4.	Dextrose	*Bacillus circulans* MTCC 8167	Phukon et al. (2012)
5.	Glucose	*Bacillus cereus* FA11	Masood et al. (2012)
6.	Canola oil	*W. Eutropha*	López-Cuellar et al. (2011)
7.	Lactose, glucose	*Pseudomonas hydrogenovora*	Koller et al. (2008)
8.	Paddy straw	*R. eutropha* MTCC 1472	Sandhya et al. (2013)
9.	Castor seed oil, coconut oil,	*Comamonas testosteroni*	Thakor et al. (2005)
10.	Sugarcane molasses	*B. megaterium*	Gouda et al. (2001)
11.	Acetic acid, propionic acid	*Comamonas* sp. EB172	Zakaria et al. (2010)
12.	Glucose, sodium octanoate	*Pseudomonas guezennei*	Simon-Colin et al. (2012)
13.	Jatropha oil	Recombinant *C. necator*	Wong et al. (2012)
14.	Glycerol	*C. necator* JMP 134	Mothes et al. (2007)
15.	Sugarcane liquor	*P. fluorescens* A2a5	Jiang et al. (2008)
16.	Spent palm oil	*C. necator*	Rao et al. (2010)

converted from their acyl carrier protein (ACP) form to CoA form by phaG (acyl-ACP-CoA transacylase) enzyme.

12.6 Biocompatibility and Biodegradability of PHAs

The major characteristics that make PHAs unique are biodegradability and biocompatibility. The biodegradability of PHA is comparatively higher than the petroleum-derived conventional plastics. Conventional plastics are inert in nature, insoluble in water, stable in air, and possess resistance to moisture (Ojumu et al. 2004). Under the aerobic conditions, PHAs would completely degrade to carbon dioxide and water; likewise, under anaerobic conditions carbon dioxide and methane would be produced due to microbial degradation of PHAs (Khanna and Srivastava 2005). The microorganisms generate extracellular PHA de-polymerase enzymes that transform the polyesters into monomers and oligomers, which are water soluble in nature and can be utilized by the microorganisms as a carbon source (Reddy et al. 2003). Correspondingly, PHA-producing microorganisms also possess capacity to degrade polymers intracellularly. During the intracellular degradation, de-polymerase

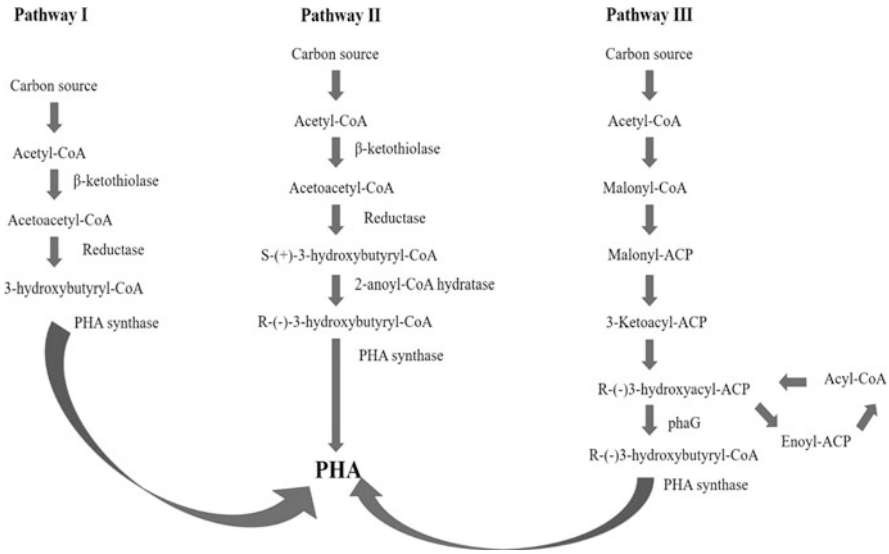

Fig. 12.2 Biosynthetic pathways for PHA production

enzyme present in the cell degrades P(3HB) to 3-hydroxybutyric acid. Then the dehydrogenase enzyme present in the microorganism oxidizes the 3-hydroxybutyric acid to acetylacetate. Furthermore, acetylacetate breaks down to acetyl-CoA by the action of β-ketothiolase enzyme. In the presence of oxygen, acetyl-CoA undergoes oxidation and converts to CO_2. This β-ketothiolase plays a major role in biodegradation and biosynthetic pathways.

PHAs can also be degraded using enzymatic hydrolysis and thermal techniques. Biodegradation of PHAs is not only influenced by their own physico-chemical properties but also influenced by other factors such as structure, accessibility to depolymerizing enzymes, and crystallinity. Along with these factors, pH of the system, moisture content, availability of nutrients, and temperature also play a key role in the biodegradation of PHAs (Sudesh et al. 2000). Melting temperature is also another important aspect from biodegradation point of view. As the melting temperature rises, the biodegradability of PHAs reduces due to low enzymatic activity (Philip et al. 2007). Studies reported that under the aquatic environmental conditions and at a temperature below 60 °C PHAs were degraded in 254 days. Similarly, within 7 weeks 85% of PHA degradation was achieved. Thus, PHAs can be utilized instead of conventional plastics.

Biocompatibility is defined as non-production of toxic substances during the degradation process. P(3HB) is a biocompatible polymer, as 3HB is a component of blood in most of the eukaryotes (Reusch 2000). Therefore, PHAs are considered as potential alternatives to conventional plastics and can be utilized in various biomedical applications due to their biocompatibility nature.

12.7 Parameters Influencing PHAs Production and Composition

There are several factors that are influencing PHA formation. Among those factors, pH, feeding strategy, nutrient availability, and organic loading rate are greatly influencing the PHA composition and production.

12.7.1 pH

pH plays a major role in monomer composition and in PHA production. Initial pH of 9 would help for the generation of the required amount of volatile fatty acids (Chen et al. 2013). The PHA formed by mixed microbial cultures was greater in hydroxyvalerate (HV) contents when shifted from the sequential batch reactor (SBR) at pH 8.5 to another batch reactor at pH 9.5 without any reduction in PHA quantity. Even though, pH within the SBR changed, microorganisms have the potency to maintain constant cytoplasmic pH. Therefore, pH variation in the system does not have a significant impact on PHA quantity. Dionisi et al. (2005) reported that, as the pH increases from 5.5 to 9.5, the HV content of PHA increased from 10 to 30%, respectively. The PHB accumulation at a neutral pH (i.e. 7.0) is 25% of cell dry weight (CDW) and is greater when compared with PHB content at basic pH (i.e. 9.0) of 8.5% CDW and at acidic pH (i.e. 6.0) of 15% of CDW. From pH 7.7 to 9.5 and at an organic loading rate of 8.5 g chemical oxygen demand (COD) per litre, the growth of the culture was enhanced using acetic acid and propionic acid mixture as a carbon substrate. This enriched culture showed higher PHA yield in SBR at pH 7.5 and *Lampropedia hyalina* being the dominant species.

12.7.2 Nutrient Availability

Limited nutrient content would enrich the PHA accumulation whereas higher nutrient conditions enhance the biomass growth rather than PHA production. Low nutrient (nitrogen and phosphorous) content favours the formation of PHA. Under the low concentration of phosphorous and nitrogen condition, the PHA production was reported to be 54.2% of CDW and 45.1% CDW, respectively.

The carbon-to-nitrogen (C/N) ratio is another important factor that influences the PHA production and also C/N ratio of 25 is sufficient to achieve maximum PHA production (Mokhtarani et al. 2012). A C/N ratio of 28.3 enriched the PHA production by 1.8 times at a pH of 6.5 and at a temperature of 33 °C using *Alcaligenes latus* ATCC 29713 species and sucrose as carbon source (Grothe et al. 1999).

The composition of the polymer depends upon the type of substrate utilized. If propionate is used as a substrate it leads to the formation of P(3HB-co-3 HV) with higher HV content whereas for acetate substrate the content of hydroxybutyrate (HB) would be high. Similarly, butyrate also results in higher HB content. If the mixture of acids were used as the substrate, it would result in copolymer formation

with greater HV content. Acetate and butyrate are considered as effective substrates when compared to propionate for the polymer composition.

12.7.3 Organic Loading Rate

The organic loading rate (OLR) is in direct relation to the PHB accumulation. The PHB accumulation increases with an increase in the OLR. Greater substrate availability requires a longer time to store PHB. Campanari et al. (2014) reported the effect of OLR on PHA production. The authors observed that OLR has significant influence of PHA production. The PHA content corresponding to the organic load in the range of 2.4–8.4 g COD/L per day was 150–339 mg COD/g COD. The optimum condition for achieving the highest storage (i.e. 339 mg COD/g COD) and the yield (0.56 mg COD/g COD) was 8.4 g COD/L per day using mixed microbial cultures. Venkata Mohan et al. (2010) reported that at different OLRs of 2.91, 3.54, 4.58, and 7.53 kg COD/m^3 per day, the PHA production reported was 25%, 15%, 8.5%, and 6%, respectively. These results indicated that maximum PHA content was achieved at low substrate concentration due to feast conditions existed in the system, which increase the production of PHA rather than biomass.

12.7.4 Feeding Strategy

The feeding strategy also influences the composition and production of PHA. Feeding affects the monomer composition of PHA formed. NMR spectroscopy results revealed that the microstructure and composition of P(3HB-co-3 HV) were impacted by the feeding regime (Ivanova et al. 2009). SBR with aerobic condition and acetate as a carbon source does not have a significant influence on microbial composition whereas the utilization of substrate is higher with pulse feeding than that of the continuous feeding mode. Furthermore, the HV contents of PHAs are greater in continuous feeding mode rather than pulse feeding mode. P(3HB-co-3 HV) is produced with 18% of HV using periodic feeding at a feeding frequency of 2 h. Similarly, *Cupriavidus necator* DSM 545 species using pulse feed with soybean oil as substrate and under the limited conditions almost 81% of PHB accumulation was obtained (Pradella et al. 2012).

12.8 Recent Advances in Improving the Properties of PHAs

12.8.1 Nanocomposites of PHAs

The biodegradability and biocompatibility properties of PHAs made them utilize in various applications such as packaging, agriculture, and biomedical fields (Gumel et al. 2013). In spite of their promising applications, PHAs that are produced with greater 3-hydroxybutyric acid content possess brittleness, low ductility, poor

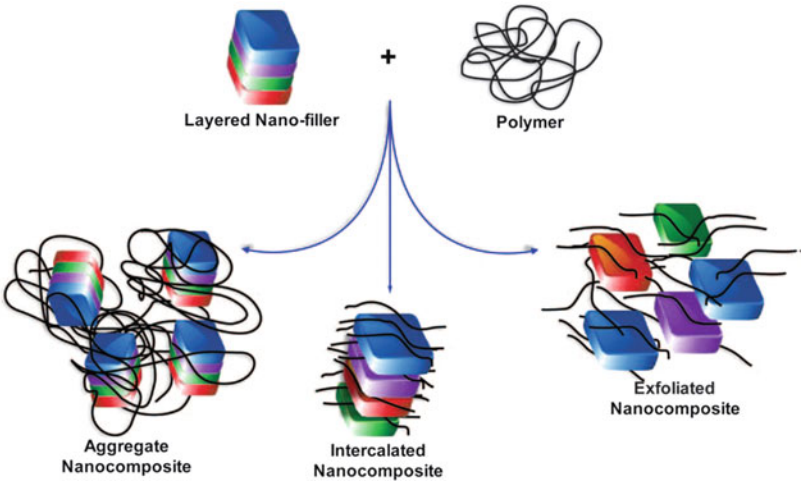

Fig. 12.3 Schematic representation of nanocomposite PHA synthesis

gas-barrier properties, and low malleability properties (Ray and Bousmina 2005). Hence, to improve the properties and quality of PHAs, nanocomposites of PHAs come into play. Therefore, nano reinforcement of PHAs with nanofillers (nanoparticles) increases polymer crystallization, physico-chemical properties when compared to native PHAs (Ray and Bousmina 2006). The most universally used nanofillers are kaolinite (Zhang et al. 2009), montmorillonite (Wang et al. 2005), cellulose nanocrystals (Yu et al. 2011), etc. Among all those nanofillers, clays such as layered silicates of hectorite and saponite are considered as the most appropriate materials due to the presence of vander waals gap in the clay particles, which effectively intercalate polymeric molecules (Ray and Bousmina 2006). Different techniques are available to synthesize nanocomposite PHAs. Most widely used techniques include polymerization (Zhang et al. 2005), deposition (He et al. 1999), ultrasonication (Lee et al. 2004), intercalation (Zhang et al. 2009), sol-gel (Chiang and Ma 2002), and melt intercalation (Shen et al. 2002). Among these techniques, intercalative polymerization is an emerging and promising method. The performance of nanocomposite PHA depends upon the dispersion and blending of nanofillers in the polymer matrix. Schematic representation of nanocomposite polymer is shown in Fig. 12.3.

Several nanocomposites reported in the literature are PHBV/C-30B, kenaf reinforced PHB, (PHB)/m-LDH, etc. Wu and Liao (2014) synthesized chestnut shell fibre incorporated PHA and the properties such as biocompatibility and mechanical properties are improved when compared to PHA. Wei et al. (2015) prepared green composites using potato peel and also observed that thermo-physical properties of the composite were enhanced.

12.8.2 Blends of PHAs

Blending is also another technique that helps to reduce the production cost and also improve the performance of polymers (PHAs). There are several polymers that can be blended together to enhance the physico-chemical properties of polymers. The characteristics of the blend are impacted by the nature of the dispersion medium, nature of the dispersed phase, size of the particles of the dispersed phase, and the ratio of the dispersion medium and the dispersed phase. Various blends reported by different researchers include blends of PHA with poly (vinyl phenol) (Cai et al. 2012), poly (ethylene glycol) (PEG) (Kim et al. 2012), poly (vinyl acetate) (PVAc) (An et al. 1997), poly (ethylene oxide) (PEO) (Bianco et al. 2013), rubber (Bhatt et al. 2008), and poly (vinyl alcohol) (PVA) (Yoshie et al. 1995). Similarly, naturally, biodegradable materials such as cellulose, polysaccharides, and starch are also blended with PHA. Reis et al. (2008) reported the morphological properties of maize starch blended with polyhydroxybutyrate-hydroxyvalerate. Similarly, Khasanah et al. (2015) reported the crystallization behaviour of chitin-blended PHB. Furthermore, poly (3-hydroxybutyrate-co-hydroxyvalerate) is blended with chitosan using electrospinning technique and biocompatibility and biodegradability of the developed polymer are enhanced and successfully applied in the biomedical field (Veleirinho et al. 2011).

12.9 Application of PHAs in Agricultural Sector

The properties of PHAs similar to the conventional plastics have made them to be utilized in various industrial applications. However, the present study highlights the application of PHAs in agricultural sectors. In the agricultural sector, PHAs are mainly used for the manufacturing of agricultural nets, seeds and fertilizer encapsulation and making agricultural grow bags.

12.9.1 Mulch Films

Mulch films are employed in agricultural sectors to enhance crop yield. These films are useful in maintaining moisture content and good soil structure, and reduce weed growth and contamination (Rydz et al. 2015). In general, mulch films are of two types, namely natural and synthetic. Synthetic mulch gained greater attention than natural mulch due to its effective weed control property. Production of mulch using PHAs is more advantageous over the conventional plastics. The commercially available agricultural mulch was made using copolymer of poly (3-hydroxybutyrate-co-3-hydroxyhexanoate), which is one of the PHAs. The mulch produced from PHA is not photodegradable and compostable in nature. Additionally, Mirel resin (PHA base polymer) is also used for manufacturing mulch films. P(3HB-3 HV) is used in the agricultural field to control the release of

insecticide. Furthermore, bacterial inoculants are helpful for fixing nitrogen in plants.

12.9.2 Agricultural Nets

Agricultural nets are also essential to protect the crops from birds, winds, hailstone, and overheating and minimize chemical input to the crops. In addition to that, nets are also used to protect the shade crops from sunlight. Moreover, usage of nets also minimizes the evaporative loss of agricultural water resources. Agricultural nets manufactured using biodegradable PHAs are more effective than conventional plastics. The major advantage of using PHAs net is its compostability, which permits the disposal of net in the soil along with organic and food residues. The PHAs such as P(4HB) and PLA/PHA blends are utilized for manufacturing agricultural nets (Williams et al. 2013). The properties of PHAs like high tensile strength (800 MPa) and greater elongation at break (1000%) made them to be utilized for producing agricultural nets.

12.9.3 Agricultural Grow Bags

In addition to the above-mentioned applications, PHAs are also used for manufacturing agricultural grow bags. Grow bags are also called as planter bags and seedling bags. The main purpose of the grow bags is to stabilize the soil temperature and to maintain the moisture content in the soil. Moreover, grow bags are also used to isolate the plants individually to avoid root disturbance, thereby enhancing the crop survivability. Bilck et al. (2014) found that the root deformation was not observed in plants growing with PHAs, whereas the deformations in roots were observed in plants grown with conventional plastics due to lack of space. This ultimately influenced the pathogenic immunity, growth, and stress resistance of crops. PHAs are considered as a source of reducing power and microbial growth matrix for water denitrification due to its insoluble nature in water. Furthermore, PHA is effective in removing nitrogen from water; thus, usage of PHA as grow bag would significantly minimize the eutrophication of water bodies. On the other side, usage of non-biodegradable polyethylene bags indirectly enters into the waterways and blocks the sewerage system as well. Therefore all the mentioned are the applications of PHAs in agriculture.

In addition to the above-mentioned agriculture applications, PHAs are also widely used in packaging, medical, and tissue engineering industries. The gas-barrier property of PHAs is helpful to employ them in manufacturing plastic bottles and food packaging sectors. PHAs can also be used as bioindicators in estimating the pollution levels of environmental health. In medical, particularly PHAs are utilized in making biodegradable carriers such as surgical needles, bone tissue replacement, wound management, and suture materials.

12.10 Industrial PHAs and Their Applications

12.10.1 Nodax

Nodax is developed by Procter and Gamble and has 3HB and less quantity of MCL monomers. The MCL units include 3-hydroxyoctanoate, 3-hydroxydecanoate and 3-hydroxyhexanoate. Nodax is mostly used for making flush in septic systems, tampon applicators, and hygienic wipes. Furthermore, it is also used in manufacturing surgical garments, bags, and carpet. Nodax is available in several forms including films, fibres, and foams.

12.10.2 Biopol

Biopol is manufactured by Imperial Chemical Industries in 1980. It is obtained by copolymerisation of P(3HB) and P(3 HV). It is used for making coat papers and paperboards. Biopol has a wide range of applications such as packaging material, making of disposable knives, cups, forks, and razors.

12.10.3 Degra Pol

Degra pol consists of two different kinds of polymers polyhydroxybutyrate-diol and α, ω-dihydroxy-poly-caprolactone. The applications of degra pol are in packaging, manufacturing disposable goods such as utensils, carpets, bags, and cups.

12.11 Conclusions

Biodegradable polymers are considered as the most effective and efficient alternative to conventional plastics. Biodegradable polymers, particularly PHAs, have gained attention in research and in industry. Even through the production cost of biodegradable polymers is high compared to conventional plastics, research is continuing towards bringing down the production cost. The economics of PHAs can be minimized by employing low-cost substrates and using mixed microbial cultures. PHAs production using industrial by-products, wastewater, and agricultural feedstock not only helps to reduce the cost but also helpful for eco-friendly PHAs production. This chapter summarizes the various substrates utilized, factors influencing the PHAs production, and applications of PHAs in different industrial sectors. Furthermore, the recent advances such as blends and nanocomposites of PHAs are also explained in this chapter. In future, PHAs would play a major role in the plastic field due to their eco-friendly features.

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Trends in Probiotics on Human Health and Industrial Application

13

Rahul Arora, Jyoti Trivedi, Swati Mohapatra, and Prashant Kumar

Abstract

Probiotics are living microorganisms present in gastrointestinal tract of human. They prevent the host from certain diseases and thus are beneficial for their hosts. This chapter will provide a comprehensive overview of probiotics, including their description, categorization, mode of action, therapeutic, and harmful effects. Moreover, different activities of probiotics, industrial processing, preservation, along with their dosage will be notified. The chapter also mentions various clinical trials to evaluate efficacy of probiotics. The nutritional properties, activities and applications of probiotics on the health of the host are going to be discussed. The benefits of taking probiotics on a daily basis will be discussed, as well as the importance of monitoring new adverse responses.

Keywords

Antibiotics · Nutrition · Prebiotics · Probiotics · Probiotic applications

R. Arora
Department of Pharmacology, Teerthanker Mahaveer College of Pharmacy, TMU, Moradabad, India

J. Trivedi
Department of Biochemistry, Teerthanker Mahaveer Medical College and Research Centre, TMU, Moradabad, India

S. Mohapatra
Department of Infection Biology, School of Medicine, Wonkwang University, Iksan, South Korea

School of Science, Gujarat State Fertilizers and Chemicals University, Vadodara, India

P. Kumar (✉)
Department of Biotechnology, Motilal Nehru National Institute of Technology, Allahabad, India

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13.1 Introduction

The term "probiotic" comes from the Greek words "pro" and "bios", which mean "for life". The precise definition of probiotics by World Health Organization and Food and Agriculture Organization of the United Nations is "live microorganisms which when administered in adequate amounts confer a health benefit to the host". In general, probiotics are live microbial agents, consumed as a food supplement and sympathetically affect the microbial system of the host. Probiotic organisms require specific activities in order to provide maximal therapeutic impact by specifically encouraging the growth of specific bacterial species present in the host's colon (Mack et al. 1999; Sanders 2008). Microbiota is the collection of microorganisms, their genomes and metabolites, as well as the environment in which they reside in the human body. The gastrointestinal tract contains the greatest number of microorganisms that make up the microbiome. The oldest proposed probiotic benefit is microbiota balance. According to Metchnikoff it is "seeding" of the intestinal tract with innocuous lactic acid bacteria that destroy the growth of harmful proteolytic bacteria (Fuller 1991; Guarner 1998). Prebiotics, probiotics, synbiotics, and postbiotics are different forms of microbiota. Prebiotics are used as food by microorganisms, which has a positive influence on the host's health. Human milk oligosaccharides, lactulose, and inulin derivatives are examples of prebiotics that are currently available. Probiotics, on the other hand, affect the gut microbiota directly by specifically delivering beneficial microbes to the gastrointestinal system (Fuller and Gibson 1997). Bacteria from the genera *Lactobacillus*, *Bifidobacterium*, and *Streptococcus*, as well as yeast *Saccharomyces cerevisiae*, are the most commonly utilized probiotics. The notion of postbiotics is based on the knowledge that the microbiota's beneficial effects are mediated by metabolite secretion. Postbiotics are not regarded synbiotics in the literature, despite the fact that synbiotics are a combination of prebiotics and probiotics that have a favourable effect on the gut flora (Dunne et al. 2001).

The human body provides a nutrient-rich and stable home for live microbes in exchange for numerous benefits. The immune system is stimulated, food is better absorbed, digestion is enhanced, and germs are less likely to proliferate (Kesarcodi-Watson et al. 2008). The positive effects of the interaction between the microbiota and the gastrointestinal system can be seen locally as well as in distant organs. The "gut-organ axis" is the name given to this phenomenon (Juven et al. 1991).

It is the need of the hour to maintain a good health of probiotics because of its nutritional demand for all age group. Hence to maintain the health of the probiotics industrial processing plays vital role starting from its formulation to delivery and preservation.

13.2 Mechanism of Action

Despite the fact that the particular processes by which probiotics acquire their therapeutic effects are unknown. One of these ways explains why probiotics compete for cellular attachments by competing for adhesion sites. To colonize the

gastrointestinal system efficiently, many pathogenic organisms generally interact by forming a bond with the epithelium (Mishra and Lambert 1996). On the other hand, some strains of *Bifidobacteria* and *Lactobacilli* adhere to the epithelium and act as "colonization barriers", preventing pathogens from sticking to the mucosa. *Lactobacillus rhamnosus* strain GG and *Lactobacillus plantarum* 299v both inhibited the attachment of *Escherichia coli* to human colon cells (Schiffrin et al. 1995).

The "modification of the microbial flora through the creation of antimicrobial chemicals" is another possible method (Perdigon et al. 1995). Bacteriocins and other antimicrobial substances are produced by *lactobacilli* and bifidobacteria. Bacteriocins are the "compounds that are bacterially generated chemicals and comprise a bactericidal action along with biologically active protein moiety" (Vijayaram and Kannan 2018). The release of these chemicals by probiotic organisms alters the microflora in a positive way (Sütas et al. 1996). However, not all "*lactobacilli"* or "bifidobacteria" strains have antibacterial properties. The researchers observed that immune response can be stimulated by probiotics (Schiffrin et al. 1995). This immune reaction can be observed as a result of an increase in the secretion of Immunoglobulin-A (IgA), increased numbers of natural killer cells or enhanced macrophage phagocytic activity (Goldin 1998), if the IgA secretion increases, which reduces the number of pathogens in the stomach and improves the microflora composition (Pelto 1998). Because of their immunomodulatory properties, probiotics may aid with inflammatory bowel disease, food allergies, and vaccine adjuvants in addition to fighting intestinal and urogenital infections (Miele et al. 2009). Probiotics can compete for nutrients that infectioning microflora would eat (Vanderhoof and Young 1998). This condition arises with *Clostridium difficile*, a potential pathogen which depends upon monosaccharides for its growth (Fig. 13.1). The available monosaccharides are utilized by probiotic organisms, which inhibits *Clostridium difficile* (Wilson and Perini 1988).

Probiotic bacteria break down organic materials and enhance water quality in the "aquatic ecosystem". Exoenzymes produced by microbial cultures, including as amylase, protease, and lipase, aid in the degradation of unconsumed feed faeces in the pond. The ability of these enzymes to promote feed digestibility and utilization is a prospective application for them in animal nutrition. The mode of action of probiotics include (1) by producing bacteriocin-like chemicals (BLC), a disease can be inhibited, (2) in order to compete for attachment sites, (3) nutritional competition (4) pathogen enzymatic activity, immunostimulatory function, and nutritional benefits have all been altered i.e. improvement of feed digestibility and utilization (Gupta et al. 1998; Macfarlane and Cummings 1999).

13.3 Classification

The *Lactobacillus* and *Bifidobacterium* genera are the most common probiotic bacteria. Other bacteria and yeast, however, have probiotic characteristics. Different microorganisms used as probiotics currently are briefly described below.

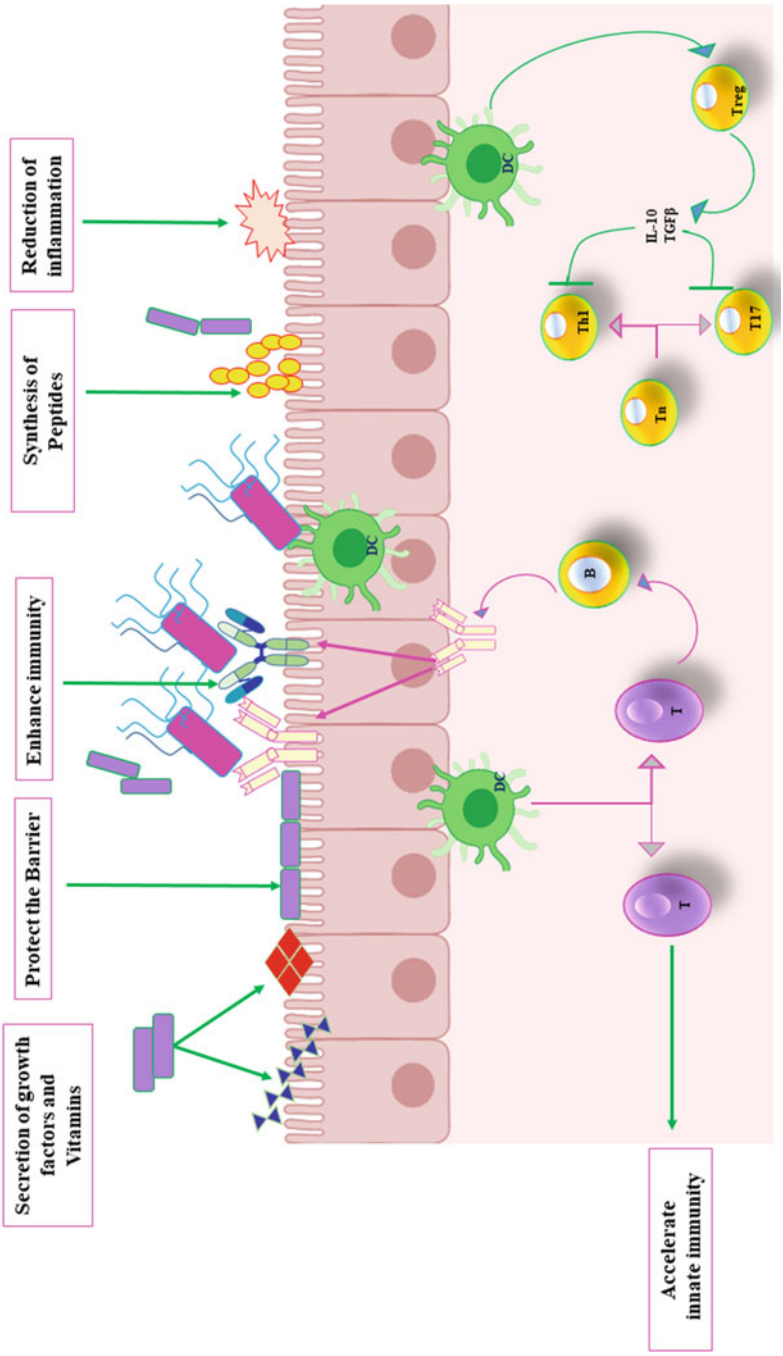

Fig. 13.1 Schematic diagram of different mechanisms includes the way of protection provided by probiotics to protect the epithelium and to enhance the immunity. Note: IEC: intraepithelial cells, DC: dendritic cells, T: T cells, B: B cell, Th: T helper cell, IL: Interleukins, TGFβ: Transforming growth factor beta. Treg: Regulatory T cells

13.3.1 *Lactobacillus* Species

Lactobacillus is a Gram-positive bacteria that produces lactic acid and drives anaerobes out of the human gastrointestinal tract (Vanderhoof and Young 2004). The *lactobacillus* denotes the ability of bacterium to produce lactic acid. *Lactobacilli* are used as probiotics therapeutically. They're also known as "friendly bacteria", and they're utilized to recolonize parts of the body in order to provide nutritional benefits including promoting growth factors and increasing mineral absorption (Bruce and Reid 1988; McGroarty 1993). It also aids in the regulation of the mucosal barrier as well as the reduction of intestinal permeability (Madsen et al. 1999).

Changes in the usual flora allow pathogenic organisms to colonize, causing symptoms such as diarrhoea, cramps, and, in rare cases, pseudomembranous colitis (PMC), which is caused by *C. difficile* (Shornikova et al. 1997). Most of the research studies depicted that combination of probiotics and *lactobacillus* cannot be prescribed at a time as this combination could be harmful by reducing the normal flora of the human body (Reid et al. 1990; Sanders and Klaenhammer 2001). *Lactobacilli* that create hydrogen peroxide have antibacterial effects against the vaginal pathogen *Gardnerella vaginalis*, and their presence in the vagina has been linked to lower rates of bacterial vaginosis and trichomoniasis (Sullivan 2003). *Lactobacilli* create lactic acid, which lowers vaginal pH and prevents pathogen growth (El-Nezami et al. 1998; McIntosh et al. 1999).

Lactobacilli and other probiotics have been shown to be effective in the fight against cancer in some trials. *Lactobacilli*, particularly *Lactobacillus plantarum*, have also been shown to reduce the severity of chemotherapy-induced enterocolitis in previous studies (Goldin et al. 1996). *Lactobacillus bulgaricus* and *Lactobacillus sporogenes*, according to other studies, show hypolipidemic and anti-atherosclerotic properties. According to some clinical evidence, it lowers total and low-density lipoprotein cholesterol while having no effect on high-density lipoprotein cholesterol (HDL) (Mastromarino et al. 2009). Cholesterol is reduced by fermented dairy products like yoghurt and acidophilus milk. *Lactobacilli* and other probiotic bacteria bind bile acids to cholesterol and enhance fatty acid production in the gut, lowering circulatory fatty acid concentrations by blocking hepatic cholesterol synthesis or transferring cholesterol from the plasma to the liver (Mao et al. 1996).

13.3.2 *Bifidobacterium* Species

Bifidobacterium is an anaerobic, Gram-positive, non-spore-forming, pleomorphic bacteria. Lactic and acetic acids are produced by the bacteria in the *Bifidobacterium* genus, as by-products of utilization of glucose (Losada and Olleros 2002). *Bifidobacterium longum subsp. longum* BB536 was the first probiotic bacteria isolated from the intestinal system of healthy newborns, according to reports *Bifidobacteria*, in combination with *Lactobacillus* species and the probiotic yeast *Saccharomyces boulardii*, appear to reduce the negative impact of *Helicobacter* infection but not compliance. Furthermore, combining *Bifidobacterium infantis* with

Lactobacillus acidophilus appears to reduce Necrotising enterocolitis (NEC)-related mortality in critically unwell neonates (Oberhelman et al. 1999).

13.3.3 *Bacillus* Species

Due to the property of lactic acid production, *Bacillus coagulans,* a Gram-positive rod, is often misclassified as lactic acid bacteria, i.e. *lactobacillus*. In fact, commercial items containing *B. coagulans* are promoted as "spore-forming lactic acid bacteria" or *Lactobacillus sporogenes*. The property of forming spores differentiate these species from *lactobacillus*. *B. coagulans*, on the other hand, is not found in the typical human flora, but it is utilized therapeutically in the same way as *Lactobacillus* and *Bifidobacterium* are. Every probiotic must be capable to persist and colonize in the intestinal mucosa, in order to be effective for restoring normal flora and prevent pathogenic colonization. After ingesting the spores by human, it is unknown what happens to the spore and Bacillus spore is capable of germinating in the intestinal tract or if colonization occurs (Cremonini et al. 2002).

13.3.4 *Saccharomyces* Species

Saccharomyces cerevisiae, often known as *S. boulardii,* is a nonpathogenic yeast strain. It is a diarrhoea medication and used to treat and prevent diarrhoea. In Indochina, *S. boulardii* was isolated from the skins of tropical fruits. Since ancient times, the indigenous people of Indochina have employed these fruit skins to prevent and treat diarrhoea (Hoyos 1999).

13.4 Probiotics Activities

13.4.1 Probiotics in Antibiotics

Probiotics aid in the stimulation of the immune system, the prevention of allergies, and the reduction of cholesterol levels (Duc et al. 2004). Microbial drugs are used as chemotherapeutic agents.

The discovery of actinomycin as an anticancer agent led to a foray into the world of microbes. The medications that demand special attention are actinomycin D, anthracycline, bleomycin (mithramycin, streptozotocin, and pentostatin), calicheamicin, and taxol epothilones. Actinomycin, a Streptomyces antibiotic, was found to be effective in treating children with Wilms tumour (Szajewska et al. 2001).

13.4.2 Probiotics in Antibacterial Activity

Probiotics' therapeutic benefits have increased their ability to boost the gut's immunological and nonimmunological defence barrier, improved intestinal penetrability, and altered gut microbiota. From black tiger shrimp (*Penaeus monodon*), 12 diverse intestinal bacterial colonies were isolated. Among these, the bacterium *Bacillus subtilis* was investigated and classified as having antagonistic qualities against three pathogenic bacterial strains: *Vibrio alginolyticus*, *Vibrio harveyi*, and *Vibrio vulnificus* (Buts 2005; Ringø et al. 2018).

A number of microorganisms have been identified as being pathogenic to aquatic animals. Six Gram-negative rods (*Proteus*, *Citrobacter*, *Aeromonas*, *Pseudomonas*, *Flavobacterium*, *Chromobacterium*) and three Gram-positive cocci (*Micrococcus*, *Streptococcus*, and *Staphylococcus*) have been identified (Rengpipat et al. 1998).

13.4.3 Dosage

The amount of living organisms contained in a probiotic product determines the dosage. Clinical investigations have shown that utilizing between 10^7 and 10^{11} sustainable bacteria per day can produce effective results (Chauhan and Singh 2019). Remarkably, it appears that a dairy product requires 100 times less sustainable bacteria than a freeze-dried supplement to achieve comparable amounts of live bacteria in the lower colon (Mahajan et al. 2013). Dairy products operate as an ideal transportation medium for bacteria, allowing them to survive longer in the upper gastrointestinal tract (Vijayaram and Kannan 2018).

13.4.4 Therapeutic and Adverse Actions

Probiotics are living bacteria that provide health advantages in addition to providing needed nourishment. Probiotic bacteria have a wide range of beneficial effects, including improved lactose intolerance symptoms, lower blood cholesterol, anticancer properties, constipation relief, and relief from vaginitis. Many strains of *Lactobacillus* such as *L. rhamnosus*, *L. acidophilus*, and *L. casei* and strains of Bifidobacterium like *Bifidobacterium longum*, *B. infantis*, *B. adolescentis*, and *B. breve* exhibited noteworthy suppression of colon tumour (Posteraro et al. 2005).

Although probiotics are extensively used and side effects are uncommon, certain studies have found that *Lactobacillus* GG causes liver abscess, sepsis, and endocarditis in people with severe disease (Zocco et al. 2006).

13.4.5 Drug Interactions with Probiotics

Antibiotics and alcohol do not affect Lactobacilli and *Bifidobacteria* (Hatakka 2001). Despite the fact that studies suggesting the organism has no effect on

antibiotic activity, *L. acidophilus* strains can impact the metabolism of sulfasalazine and chloramphenicol palmitate (Khalighi et al. 2016).

13.5 Applications

Probiotics control pathogens through different mechanisms and are used as an alternative to antibiotics (Segarra-Newnham 2007). Probiotics were used for nutritional purpose in the human and animals (Elahi et al. 2008) but now they are being used in aquaculture also. Probiotics provide a number of advantages, including a moderate rejection of harmful bacteria as a source of nutrition, enzymatic engagement in digestion, and direct application of dissolved organic material assisted by the bacteria. Probiotics also help to strengthen the immune system's response to harmful bacteria (Whelan and Myers 2010).

13.6 Probiotics and COVID 19

Evidences supported the role of probiotics' in immune system regulation, also proposed its decisive role in viral infections. Probiotics taken as supplements might decrease the severity of COVID-19 and also reduced its morbidity and rate of mortality. Probiotics can prevent cytokine storms by boosting innate immunity and preventing adaptive immunity from overreacting. Effective treatment will reduce the pandemic's impact on people's lives and economies around the world. Thus, probiotic supplementation in high-risk and critically ill patients, as well as frontline health professionals, may help to bind the pathogen and flatten the COVID-19 curve.

13.6.1 Updates on Industrial Probiotics

These days application of the probiotics have been accelerating a lot, research and development of the probiotics industry now focusing towards using multiple strain of the probiotics such as *S. thermophilus*, *E. faecium*, *B. breve*, *B. infantis*, *B. longum*, *L. acidophilus*, *L. plantarum*, *L. casei*, and *L. delbrueckii* subsp. *bulgaricus* for enhancing the body immunity and protecting the body from various pathogenic virus, bacteria, fungus, etc. by converting the protein to bioactive peptides and other powerful metabolites.

Apart from the normal probiotic yoghurt, most of the industries are now working on making flavoured probiotics. Some probiotics brands are also focusing to enhance the nutrient of the probiotics by adding some prebiotics. Some industries such as Truebasics, Bifilac, Lee-Biotic, Biovir, Hmf Forte, Yakult, and Lactobact are adding the medicinal plant extracts, fruits, and flower extract also to the probiotics, for example, to the milk and yoghurt as mentioned in the below flow chart (Fig. 13.2).

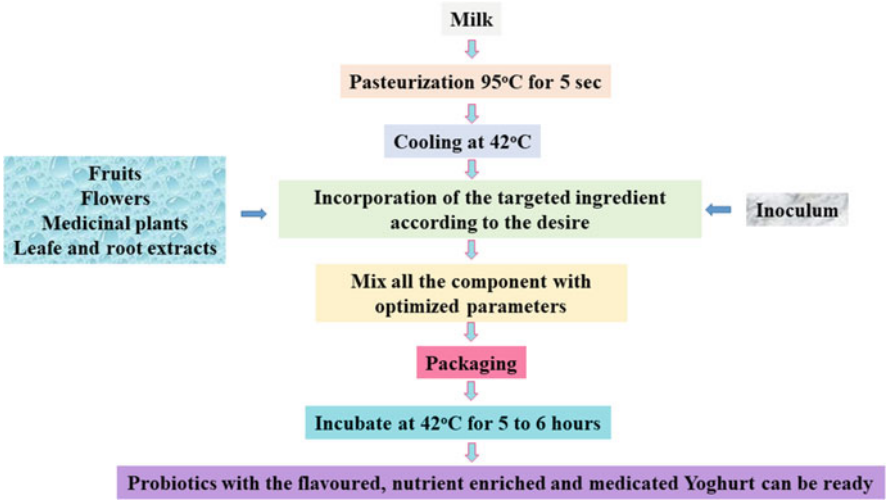

Fig. 13.2 A probiotic dairy product with added medicinal plant extracts

Nowadays some experiments proven the fruitful journey from home probiotics to industrial probiotics was proven as a best candidate for the treatment of ulcerative colitis, cancer, COVID-19, irritable bowel syndrome and many more.

13.7 Conclusion

Probiotics is a governing body of our digestive system which leads to every metabolic activity very smooth. As probiotics are very helpful for human health and curing most of the disease, now the markets are demanding more probiotics in different form of food. Hence more research is highly essential for formulation of novel probiotics and enhancement of shelf life period of the probiotics in the food.

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Plant Secondary Metabolites: A Biosensing Approach

14

Saipriya Ramalingam, Simranjeet Singh, Praveen C. Ramamurthy, Daljeet Singh Dhanjal, Jayashankar Subramanian, Joginder Singh, and Ashutosh Singh

Abstract

Plant secondary metabolites are essential, biologically active compounds that play an important role in the plant's defense mechanism. While primary metabolites often showcase the plant's metabolism through synthetic pathways, secondary metabolites are unique to the species they belong. Terpenes, phenolics, and nitrogen-containing compounds have been studied over the years for their application in human health and nutrition. These plant bioactive compounds enrich the food we consume, but reports of its undesirable effects have also been recorded. Hence, it bears on the global issue of food safety and security which requires accurate analytical techniques to determine its levels in food. A biosensor is an analytical device that is able to respond to an external chemical stimulus and convert it into readable data. The data observed can be in the form of an electrical signal or a change in absorbance. Different biosensors have been developed to determine the presence of secondary metabolites such as polyphenols, vital for nutraceutical analysis of food. Compared to conventional

Saipriya Ramalingam, Simranjeet Singh and Praveen C. Ramamurthy contributed equally with all other contributors.

S. Ramalingam · A. Singh (✉)
School of Engineering, University of Guelph, Guelph, ON, Canada
e-mail: asingh47@uoguelph.ca

S. Singh · P. C. Ramamurthy
Interdisciplinary Centre for Water Research, Indian Institute of Science, Bangalore, India

D. S. Dhanjal · J. Singh (✉)
Deaprtment of Microbiology, Lovely Professional University, Phagwara, Punjab, India
e-mail: joginder.15005@lpu.co.in

J. Subramanian
Department of Plant Agriculture, University of Guelph, Guelph, ON, Canada

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detection techniques, biosensors offer a wide range of advantages, including better accuracy, higher precision, and low occurrence of false positives. Being quick and versatile allows these biosensors to be used in an industrial setting or even available at the consumer level. This chapter highlights the different types of biosensors and recent advancements in the biosensing of plant secondary metabolites

Keywords

Biosensors · Nutraceuticals · Secondary metabolites · Transducers · Receptors

14.1 Introduction

Metabolites are a class of small organic compounds produced during an enzymatic reaction in a metabolic pathway. They can be broadly classified into two main categories: (1) primary metabolites and (2) secondary metabolites. With the advent of modern biochemistry, the role of primary metabolites was outlined to be those involved in plant life sustenance, such as respiration, food storage, reproduction, and growth (Guerriero et al. 2018). In other words, they are stable by-products of photosynthesis, glycolysis, Krebs-cycle, and other similar pathways essential in the functioning of any living cell.

Secondary metabolites (PSMs), on the other hand, are structurally diverse phytochemicals that are known to possess a biological value that benefits both plants and humans (Singh et al. 2020c,e,j). Although their production is non-essential to the general functioning of the organism, PSMs have been known to perform other vital roles in plants such as attracting pollinators, defense from herbivores, chemical compounds that promote plant germination and survival and providing UV-protection, to name a few (Kumar et al. 2015b,2020a; Singh et al. 2020a, d, f, h; Singh et al. 2021a; Yang et al. 2018). In addition to performing internal functions, PSMs can also be harvested for the production of oils, dyes, glues, natural flavors, and waxes, which are further applied in the production of nutraceuticals, natural drugs, and other by-products. Certain PSMs, more than others, have earned research focus due to their harvestable compounds that promote human well-being by reducing susceptibility to certain cancers and cardiovascular diseases (Kumar et al. 2015a; Singh et al. 2016, 2017; Singh et al. 2020e,k). Hence, these PSMs are of high economic and commercial value, and several techniques have been devised to maximize their yield. This book chapter highlights the importance of detecting secondary metabolites, gauging their availability and quantifying them. It also touches upon the functioning of a biosensor while encompassing recent advances in the field of biosensing systems.

14.2 Classification of Plant Secondary Metabolites

The PSMs can be broadly classified on the basis of their chemical structure and attached functional groups: (a) polyphenols, allied phenols, and flavanols, (b) nitrogen-containing alkaloids, and (c) terpenes. The main classes PSMs have been pictorially represented in Fig. 14.1. Phenolic compounds are structurally identified by the attachment of one or more hydroxyl groups to the base aromatic ring (Jones and Kinghorn 2006). Of over 8000 phenolic structures that have been discovered in the plant kingdom, the number of carbon atoms in their structure contributes to their classification. They range from a simple 7-carbon low-molecular-weight structure to a complex 15-carbon long-chain polyphenol. Phenols can be further sectioned into (1) flavonoids and (2) non-flavonoids, where flavonoids further branch into flavanols, flavones, isoflavones, flavan-3-ols, flavanones, and

Fig. 14.1 Graphical representation of chief classes of secondary metabolites obtained from plants

anthocyanins. Flavonoids are the most extensively available polyphenolic compounds comprising 3-carbon bridge structures connecting two large aromatic rings (Kumar et al. 2020b). Several fruits and vegetables are an important and rich source of antioxidants that release flavonoids as a consequence of various biotic and abiotic stress conditions of the environment (Panche et al. 2016). Flavonoids, in specific, play a functional role in the plant's sustenance, such as UV-protection, drought resistance, antimicrobial defense mechanism, and heat-cold regulation, to name a few. Research on flavonoids has garnered particular interest among the scientific community due to their excellent antioxidant properties and health-promoting effects in both human and animal applications. An average of 6000 flavonoids have been identified from vegetables and fruits that add to their color. Non-flavonoid phenolics, on the other hand, can be classified into phenolic acids, hydroxycinnamates, and stilbenes. They are generally smaller in size and less complex in their structure as compared to their flavonoid counterparts (Rasouli et al. 2017).

On the contrary, lignans, stilbenes, coumarins, and phenolic acids do have up to 15 carbons and have an equally complex structure as flavonoids. One of the most common examples of dietary phytochemical sources rich in flavonoids and non-flavonoid compounds is wine. Resveratrol is an example of stilbene which has been proven to possess antitumor properties and is widely available in grapes (Frémont 2000).

Derived from amino acids, alkaloids are an assorted group of low-molecular weight nitrogen-rich compounds. Alkaloids are an important sub-classification of PSMs that play a pivotal role in the plant's defense mechanism. Additionally, they have also been widely explored for their pharmaceutical importance. Some of the best known alkaloids have derivatives of clinical importance, such as codeine and morphine. Morphine is a widely recognized example of benzylisoquinoline which is a class of alkaloids having a benzyl group at the C1 position of the isoquinoline heterocycle. While it is used to provide relief to patients suffering from neuropathic conditions and pain during the treatment of cancers, they are also sometimes used to synthesize illicit drugs such as heroin. Tropane alkaloids are another noteworthy class of alkaloids with a unique 8-azabicyclo [3.2.1] octane known for its pharmacological activity as both a medicine and poison (Makkar et al. 2007). For example, atropine, a vasodilator, can be harvested from deadly nightshade (*Atropa belladonna*), known to treat cases of poisoning while consumed in moderation. Harvested nicotine has been administered in several forms, such as chewing, cigarettes, pipes, and cigars (Sheen 1988). Smoking is the most common form known to disrupt the oral mucosal lining, eventually resulting in long-term deadlier effects such as stroke, heart disease, chronic obstructive pulmonary disease (COPD), infertility, and cancer (Sharma et al. 2019). Terpenoid indole alkaloids are another class of alkaloid compound that contains an indole moiety and a terpenoid component. Like other alkaloids, they act on the central nervous system by exhibiting tranquilizing and anti-neoplastic properties, e.g., passion flower (*Passiflora incarnata*).

Also known as isoprenoids, terpenes are another important classification of PSMs that encompass about 30,000 compounds of plant origin. They are responsible for

providing a wide range of characteristic pigments and fragrances in plants and are also found in lipids, antibiotics, and hormones. Some of the prominent examples of these naturally occurring bioactive compounds include the most common pigment "chlorophyll", and the Taxol component used in the treatment of various cancers. Terpenes come from a family of compounds that have a 5-carbon chain structure, with various attached functional groups, that determine their sub-classification (Bach 1995). For instance, carotenoids, the pigment in plants and algae, are a classic example of tetraterpenes that carry a characteristic bright yellow/orange color. They are known to have strong antioxidant properties, aside from their role in human nutrition i.e., their conversion to vitamin A (retinol). PSMs are not limited to the above-discussed examples and classifications. Extensive reviews and book chapters on the above classifications of PSMs are available (Erb and Kliebenstein 2020; Suwal and Marciniak 2019; Theis and Lerdau 2003). The further length of the introduction will touch upon some of the conventional detection techniques used to detect some important PSMs.

14.3 Conventional Detection Techniques

Plant secondary metabolites have been extensively studied for their medicinal value since the early 1900s. With the advent of biotechnology and molecular sciences, multistep screening devices have been developed over the years to study and further understand these complex yet small structures. Once isolated, the bioactive compound must be tested for their composition and purity to be deemed useful for industrial application. The schematic flow of steps involved in phytochemical assessment is illustrated in Fig. 14.2. Some of the earliest separation techniques included chromatographic techniques such as TLC (thin-layer chromatography) and HPLC (high-performance liquid chromatography) in addition to an LC/MS (mass spectrometer) (Kumar et al. 2013a,b; Kumar et al. 2020; Mehta et al. 2020; Singh et al. 2021c). Their principle of the operation depended on the ability of the sample to separate into its constituent compounds resulting in a characteristic peak. The

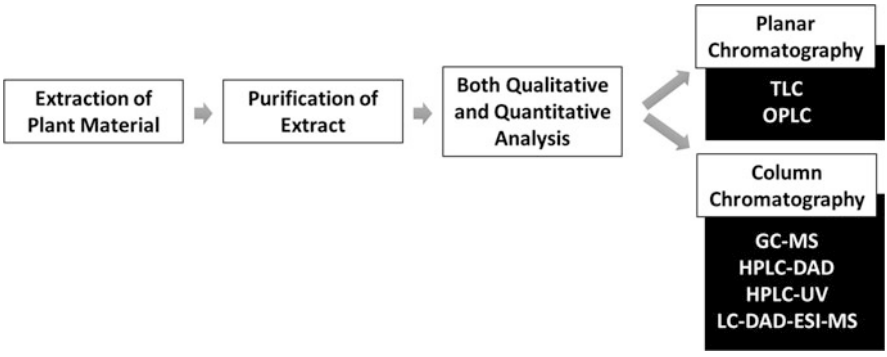

Fig. 14.2 Schematic of steps involved in phytochemical assessment

integration of the LC with the mass spectrometry device improved the sensitivity, selectivity, dynamic range of separation, and quantification for the sample (Fæste et al. 2011). The condition of separation largely depended on the choice of the stationary, mobile phase and ionization of analyte.

Capillary electrophoretic (CE) technique is a unique system of separation and identification of unknown compounds. It was based on the principle of migration of charged particles to oppositely charged electrodes on the application of an electric field. Inert columns are used to dispense the extract at the anode, which is then subject to high voltage while their migration is followed to the cathode. The pH of the buffer and electrolyte concentrations are important parameters in determining the migration time and efficiency (Tomás-Barberán 1995). Flavonoids, being weak acids, have an acid dissociation constant (pKa) of 9–10. This high pKa value results in the compounds getting charged, yielding inconclusive results. Hence optimization of pH within the working range of 6.5–8 (around neutral) is preferred for this technique.

Besides CE, some spectral studies have also garnered interest among potential detection techniques, especially for organic compounds. For instance, Nuclear Magnetic Resonance (NMR) works on the spin orientation principle when subject to a strong magnetic field. As different molecules absorb electromagnetic radiations differently to produce resonance, the pattern of NMR signals obtained provides the user with insight into the chemical structure of the material (Wüthrich 1986). PSMs are generally very complex and require multiple purification steps to determine the compound of interest from the extract. For example, a ^{13}C NMR was performed on African birch extract resulting in the identification of seven secondary metabolites (Hubert et al. 2014). Although the above techniques provide accurate information on the structural composition of the PSMs, they did come with some significant challenges. The sample preparation and assay time were significantly longer, in addition to high operational cost. It also required skilled personnel to perform and interpret the results (obtained peaks) as they were not easily comprehendible.

To combat the above challenges, a lab-based assay was developed known as ELISA (Enzyme linked immunosorbent assay). After being perfected over the years, it is now considered as the "gold standard" of sample quantification. As the name suggests, Enzyme Linked Immunosorbent assay is an immunological detection technique that involves the unique binding of epitopes present on the PSM with the antibody specific to the target. They are broadly classified into two: sandwich and competitive ELISA. This affinity-based biosensor uses the color change that develops on the interaction of immobilized antibody with the incoming antigen (Lequin 2005; Paulie et al. 2006). Conducting the ELISA process requires time, expertise, and equipment, which sometimes may be inaccessible in resource deficient areas (Jordan 2005). Some of the weaknesses of ELISA-based detection include: (1) the use of expensive antibodies per assay, which are generally harvested from animal sources, (2) cross-reactivity among species due to the lack of interaction specificity, (3) multiple washing steps and labor-intensiveness, and (4) low stability of antibodies. Hence, all of the aforementioned drawbacks create a significant

knowledge-gap, which can be bridged by introducing a user-friendly, portable, efficient, and accurate device called a "biosensor" for the detection of PSMs.

14.4 Types of Biosensors and Their Working

Detection of plant secondary metabolites has assumed a new form in the past few decades with the introduction of the "biosensor." The definition of a biosensor given by the International Union of Pure and Applied Chemistry (IUPAC) (IUPAC 2014) is: "A device that uses specific biochemical reactions mediated by isolated enzymes, immunosystems, tissues, organelles or whole cells to detect chemical compounds usually by electrical, thermal or optical signals." In simple terms, a biosensor is a compact, analytical device that responds to an input stimulus, which is biological (Mehrotra 2016). It comprises the following major components: (a) substrate, (b) transducer, and (c) signal amplifier. A schematic diagram of the building blocks of a biosensor has been illustrated in Fig. 14.3. The substrate or a detection element is the one that directly comes in contact with the analyte/target substance. A transducer converts any observable physical or chemical change in the analyte to a measurable signal. The amplifier is responsible to improve the quality of the obtained electrical signal, by increasing the signal-to-noise ratio (SNR). This is finally displayed in the form of a graph with the help of a user interface of choice. Furthermore, the choice of the transducer will majorly depend on the type of sensing output we expect to build.

Although conventional detection systems remain the gold standard for analyte detection, in recent times, biosensors are preferred for the following reasons:

(a) Short analysis time: The time frame between analyte contacts and the response is to the tune of minutes.
(b) Easy-handling and user-friendliness: Apart from enzymatic reactions, many label-free systems have emerged, reducing cost and improving the overall specificity of the system.

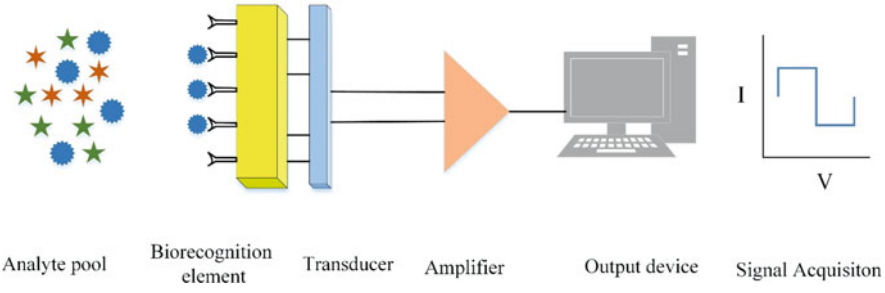

Fig. 14.3 Schematic representation of the building blocks of a biosensor

(c) Continuous monitoring feature: Biosensors are deployed on a wearable basis keeping track of the person's biological data, which was deemed impossible in conventional detection techniques.
(d) Minimum sample requirement: The amount of analyte required for the assay to be carried out is scaled down to a few microliters.
(e) Precision and Accuracy: Improved sensitivity and reduced the risk of interference or cross-reactivity when multiple biomolecules are involved.

Biosensors can be broadly classified based on the basis of their (1) biorecognition element (BRE) and (2) transduction element. Following are the most common types of transducers.

14.4.1 Optical Transducer

Optical detection has been widely applied in various areas of food science due to their simple detection technique, reasonably low cost, and portability (Cush et al. 1993). They rely on the combined principles of analytical chemistry and the opto-electronic properties of the system being used, the latest of which is the use of nanomaterials that change color in the visible range. The operating principle of an optical biosensor is based on the interaction of light with the conjugated ligand and analyte. In other words, the optical sensing system, works on the interaction of vibrational or rotational energy states of the atoms or molecules in the sample, when light of a certain wavelength interacts with it. This interaction can be (a) absorbance, (b) luminescence, or (c) reflectance (Narayanaswamy 1993). The absorbance feature forms the basis of Beer Lambert law which provides the relationship between the intensity of the light incident on the sample against the intensity of light absorbed by it. Luminescence, on the other hand, is when a beam of monochromatic light is absorbed by the sample that promotes its vibrating electrons to a higher energy state. The relaxation mode releases a photon whose frequency is generally lower than that of incident light, causing this phenomenon (Roda et al. 2016). The reflectance phenomenon, however, is when light of a certain wavelength hits the boundary surface and bounces back, resulting in no transmission through the sample itself. A standard optical setup would include an illuminating source (of fiber optic origin), an appropriate photodetector, modulator, amplifying circuitry, and readout. The interaction between the analyte and light occurs at one end of the fiber optic source, such as light emitting diodes (LED) and lasers. The photon intensity from the sample is accumulated and counted by the detector, which further processes the wavelength at which the peak intensity was observed. In addition, filters such as Band Pass filters and Notch filters are applied to improve the SNR before a spectral scan is displayed (Borisov and Wolfbeis 2008).

Surface plasmon resonance (SPR) is a key feature that is observed on the surface of conducting materials when illuminated by the light of a specific wavelength at the junction of media with varying refractive indices. This SPR effect has been deployed to promote interaction between analyte and biorecognition system causing a change

in the angle of incidence (Homola et al. 1999). Compared to the bare surface, this reduction in reflected light is identified by the detector and depicted as a change in the output signal.

With the application of nanomaterials in the field of biosensing, localized surface plasmon resonance (LSPR) property was majorly observed in metal nanoparticles (MNPs) such as Au and Ag (Kumar et al. 2019). This optical phenomenon results from collective electron beam oscillations that occur on the interaction of photons with the electron cloud surrounding an MNP. Some contributing parameters to observing LSPR phenomenon include size, shape, ionic balance, and inter-molecular distance of the synthesized metal nanoparticle. Being a "label-free" approach, they have been applied in the state-of-the-art analytical devices, both in the field of food safety and clinical diagnostics. Metal nanoparticles such as Au and Ag have been conjugated with biorecognition molecules such as proteins, antibodies, and aptamers to improve specificity, sensitivity to the target molecule, and sensor recovery.

Another type of label-free biosensor is an Evanescent Wave biosensor that works on Total Internal Reflection (TIR). The light from the waveguide is internally reflected at the junction between two surfaces having different refractive indices. The rate at which the evanescent wave (EW) decays from incident to target determines the sensing mechanism (Chocarro-Ruiz et al. 2017). The analyte concentration in the sample is detected by the EW created and the affinity the biomarker exhibits with the target. Changes to the physical properties of the waveguide, such as the material, thickness, etc., have been done over time to improve its sensitivity.

Labelled detection systems, in contrast, can be prepared by tagging a fluorescent molecule to the epitope of a biomarker or employing dyes such as Methylene Blue or a quantum dots to the ligand. A donor chromophore is initially in the electronically excited state in a FRET (fluorescence resonance energy transfer) system. The acceptor molecule derives energy from the donor forming a non-radiative dipole-dipole couple (Narsaiah et al. 2012). The "FRET pair" exhibit their luminous intensity on quenching to determine decay-time and energy transfer kinetics of the system.

14.4.2 Electrochemical Transducer

Electrochemical biosensors are based on the oxidation and reduction reaction of the electroactive substance to varying voltage sources. IUPAC defines an electrochemical biosensing system as "a self-contained integrated device, which is capable of providing quantitative or semi-quantitative results" (Dudok de Wit 1987). A general electrochemical setup includes three electrodes: (1) working electrode, (2) reference electrode, and (3) counter electrode. The working electrode generally is immobilized with an enzyme or receptor molecule which can bind to the analyte of interest. The reference electrode ensures the 3-electrode system is provided with a constant potential difference to maintain its functioning. Ag/AgCl electrodes are renowned for their stable electrode potential that is able to buffer and block stray currents into the working electrode. The counter electrode, also known as the "auxiliary

electrode," is used to balance the current generated at the working electrode and close the circuit. A wide range of electrode materials from the class of noble metals (Au, Ag, Pt) are good choices for counter electrode materials owing to their inertness. Conducting polymers (polyaniline, polypyrrole) to carbon electrodes are available as working electrodes for various applications (Campuzano et al. 2017). Electrodes made of gold, copper, nickel, silver, etc., are known for their excellent electron transfer kinetics and conductivity (~10^{710} Scm^{-1}) but are more expensive than their carbon or doped counterparts. Semiconducting materials such as indium-tin-oxide (ITO) have shown promise due to their ease in fabrication, despite lower conductivity. Glassy carbon has found its niche as an electrode material due to its availability and wide working potential range (−0.4–1.7 V). Organic redox molecules work best on glassy-carbon surfaces, which further have been developed into screen-printed carbon electrode chips. Contrary to noble metals and carbon materials, conducting polymers have much lower conductivity (~10^{317} Scm^{-1}). These electrode materials have been widely applied in various electrochemical detection in three main strategies, namely: voltammetric, impedimetric, and conductometric techniques (Kounaves 2007).

14.4.2.1 Voltammetry Technique

It requires the application of a potential difference between the working electrode (WE) and the reference electrode (RE). The oxidation/reduction cycle happening on the surface of the electrode is mapped in the form of the current flowing between the working electrode (WE) and the counter electrode (CE). One of the most common settings of voltammetry is Cyclical voltammetry (CV), where a varying potential difference is applied within the potential working window. Following this, the same is applied in the opposite direction until the initial potential is reached. Another mode of voltammetry is differential pulse voltammetry (DPV), where an amplitude potential is applied to a linearly increasing potential. The difference between the current generated before and after the application of pulse is calculated and plotted.

14.4.2.2 Impedance Spectroscopy

Particularly it measures the resistance and capacitance property of the material after the application of a frequency-dependent electrical potential in AC mode. An equivalent electric circuit can be derived from the impedance values after fitting the data. This technique provides a comprehensive idea of the electrical conductivity and mass transport kinetics of the system.

14.4.2.3 Conductometry

It involves the measurement of change in conductivity of the sample in a bulk setup. Broadly termed as chemiresistors, it gives a change in electrical resistance in response to the chemical changes in and around the sensor environment.

14.4.3 Acoustic Wave Transducer

Piezoelectric crystals exhibit the property of piezoelectricity, where an electrical dipole is generated on the application of a mechanical strain to the surface of a piezoelectric material. These crystals have garnered acclaim in biosensing due to their functionality in contemporary electronic applications (Alassi et al. 2017). The Quartz Crystal Microbalance (QCM) arrangement of piezoelectric material is achieved by sandwiching the crystal between two conducting electrodes. The Sauerbrey equation gives the relationship between change in the crystal base frequency (f_0) with respect to mass variations on its surface (Δm). Further, the shift in resonance frequency also depends on the crystal's effective surface area (Ae), the crystal's loaded resonant frequency (f_M), its odd harmonic overtone ($N = 1,3,\ldots$), shear modulus of the crystal material (μ_q), and its density (ρ_q).

$$\frac{\Delta f_M}{f_0} = -\frac{2Nf_0\Delta m}{A_e\sqrt{\mu_q\rho_q}} \tag{14.1}$$

In a conventional mass balance QCM setup, an AT-Cut quartz crystal is preferred due to its near-zero temperature co-efficient around room temperature. An AT-Cut QCM operating within a temperature range from 10 to 50°C provides a 1 ppm/°C change in its resonant frequency. While operating in liquid media, the overall shift in resonant frequency is a combination of multiple factors such as temperature (Δf_T), viscosity (Δf_L), and mass (Δf_M).

$$\Delta f \approx \Delta f_T + \Delta f_M + \Delta f_L \tag{14.2}$$

QCMs have now been widely applied to the field of biosensing, in addition to its existing application of chemical, temperature, humidity, and pressure sensing (Wang 2020). The low thickness of the QCM results in higher sensitivity and resonant frequency. The most commonly used QCM crystals are 1.27 cm or half an inch in diameter. For liquid phase QCM, optically polished crystals are used as they were having less non-specificity. Variation in the viscous nature of liquids pose a challenging situation for liquid phase QCM. This is due to the signal attenuation and changes in frequency which the proper algorithm will overcome to disperse out the effects. This sensor can be automated for continuous and real-time monitoring, while the sensor itself is cheap and has a quick response rate.

14.5 Biosensors Used in the Detection of Polyphenols

Biosensors have established as accurate and specific analytical tools, used to assess antioxidant compounds. In general, antioxidants are those compounds which restrict/inhibit oxidation and are available in low concentrations in fruits and vegetables. In the human body, antioxidants serve as the defense system against reactive chemical species such as like free radicals formed during cellular metabolic processes (Kapoor et al. 2019; Sidhu et al. 2019, Singh et al. 2020i, 2021b). The potential of flavonoids,

polyphenols, and other chemical compounds to scavenge free radicals is associated with the specificity of chemical structure, owing to aromatic rings. During the interaction of a chemical compounds with a free radical, the electron (unpaired) reducing from the free radical is neutralized by delocalizing over the aromatic ring. The seized electron is further stabilized through resonance effects of the aromatic compound, causing the termination of the free radical chain. Thus, it should be noted that polyphenolic compounds impede the oxidation process through the application of different mechanisms (Cuvelier et al. 1992; Kähkönen et al. 2001; Steinmetz and Potter 1991; Stich and Rosin 1984). The redox potential of polyphenols enables them to act on free radicals by serving as both hydrogen donors and electron donors (Tsao and Deng 2004). This could be explained by the fact that polyphenols can oxidize themselves to phenoxyl radicals, either by losing the hydrogen atom from the –OH group or by releasing an electron. Moreover, –H intramolecular bonds also play an imperative role in stabilizing the phenoxyl radical (Halliwell 1990). Here, the donation of –H occurs due to the requirement of low dissociation energy to complete the reaction (Laguerre et al. 2007).

To date, high antioxidant efficiency has been recorded for polyphenols 1,2 dihydroxy substituted on the aromatic ring. Polyphenols encompass different sub-groups of flavonoids and their derivatives, as well as phenolic acid. The extraction of secondary metabolites from raw material is one of the prerequisites to the assessment of polyphenol content. Different parts of plants (dried or fresh) like berries, seeds, citrus fruits, grapes, leaves, and roots have been used for the extraction of secondary metabolites. In the last few decades, in vitro cultivation methods have been extensively used to improve the plant's ability to synthesize polyphenols. Especially, it has been recorded that under oxidative stress induced by high temperature or light illumination, plant elevates the biosynthesis of polyphenols. This progress has enabled researchers to regulate and improve the biosynthesis of antioxidants present in vitro with the help of molecular techniques for industrial-scale production and utilization as food additives (Halliwell 1990). The chief sub-class of flavonoids are flavanols (myricetin, kaempferol, and quercetin), flavones (apigenin, tangeritin, and luteolin), flavanones (catechins, hesperetin, and naringenin), and isoflavones (daidzen, genistein, and glycitein) (Laguerre et al. 2007).

Although quantitative assessment of polyphenols is a tedious task, there remains a significant requirement to develop sensitive, precise, and specific methods. To enhance the performance and simplicity in the detection of polyphenols, biosensors could potentially replace the traditional methods like HPLC due to their simplicity of operation and selectivity (Larson 1997). Detection using biosensors could be semi-quantitative or quantitative, depending on the biochemical receptor or biological recognition element, which is in spatial and direct contact with the transductor element (Singh et al. 2020g, 2020a). Moreover, biosensors are further categorized on the basis of the active biological entity involved in the mechanism, the mode of signal transduction, or blend of these two attributes. Furthermore, the transducer and biological material selection depend on the properties of the sample and the physical variable type, which is to be measured. Since the type of biocomponent determines

the degree of specificity and selectivity of the biosensor, (Litescu et al. 2010) the three main groups of recognition elements, i.e., biocatalytic, bio-affinity, and hybrid receptors have been discussed below.

14.5.1 Biocatalytic Receptors

These receptors can be whole-cell system (involving microbes like bacteria, eukaryotic cells, fungi, and yeast), animal or plant tissue slice system, cell organelles and mono- or multi-enzyme system. Additionally, the biosensors that use animal or plant tissue, microorganism as biocomponents, display a unique advantage, as it does not involve the extraction and purification steps that are often laborious and time-consuming due to the specificity of biological constituent (Ogawa et al. 1999).

14.5.2 Bio-Affinity Receptors

Affinity-based biosensors either use nucleic acids or antibodies as chemoreceptors which provide them high selectivity and specificity to form a stable complex. As observed in earlier studies, antigen-antibody complexes easily bind to the transducer of interest, which in turn intensify the signal. Among them are the electrochemically active substances, avidin-biotin complexes, enzymes, radionuclides, and fluorescent compounds (Fitzpatrick et al. 2000; Thévenot et al. 2001).

14.5.3 Hybrid Receptors

In general, hybrid receptors are nucleic acid-based chemoreceptors. The deoxyribonucleic acid (DNA) has a double helix structure composed of two polynucleotide strands consisting of nucleobases, i.e., adenine, cytosine, guanine, and thymine. These hybrid receptors are formed using a unique well-known nucleic acid base sequence, making it a highly selective and specific recognition system. Generally, biosensors that estimate the content of phenolic metabolites use electrochemical transducers and biocatalytic receptors, where its evaluation is conducted by amperometry (Blasco et al. 2005).

In general, the term "total phenolics" is used for all the phenols that are accountable for exhibiting antioxidant potential against the particular sample. In amperometric detection, the electrochemical reaction is used for detecting polyphenols involving two main steps: The first step involves the presence of the substrate on the surface of the electrode, which gets oxidized via the action of an enzyme, in the presence of oxygen. The second step involves enzyme regeneration to the original state, which is carried out by electron transfer from an appropriate compound. Peroxidases and phenoloxidases are the two most extensively used enzyme biocatalysts used to determine the phenolic content in samples. As derivatives of phenols are suitable substrates for oxidases, electrodes have been modified with

cellobiose dehydrogenase, tyrosinase, laccase, and peroxidase to detect phenolic compounds. In this, tyrosinase and laccase are the most extensively used biological recognition elements in most biosensors designed to detect polyphenols (Jarosz-Wilkołazka et al. 2004).

14.5.3.1 Laccase-Based Biosensors

Laccase is a cuproprotein and member of a small enzyme group named blue oxidase, which has the potential to catalyze the oxidation reaction of numerous aromatic compounds (Thurston 1994; Xu 1996). Besides, this enzyme also oxidizes various other non-phenolic compounds (Arregui et al. 2019). Additionally, it aids in catalyzing the removal of hydrogen atom from the hydroxyl group of para- or ortho-substituted mono- or polyphenolic substrates (Decker and Tuczek 2000; Leonowicz et al. 2001). The main reason for selecting laccase as a biomediator is attributed to its high specificity and sensitivity towards phenolic compounds. The published literature has unveiled that laccase obtained from fungal species have high catalytic potential (Singh et al. 2020b). Moreover, various attempts have been made to immobilize laccase on various solid surfaces like carbon fibers, carbon paste, graphite, redox hydrogel on glassy carbon, platinum and polyether sulfone membrane (Decker et al. 2000; Freire et al. 2002; Gomes and Rebelo 2003; Leech 1998; Leite et al. 2003; Yaropolov et al. 1995). A biosensor was fabricated using laccase obtained from *Coriolus versicolor*. This was immobilized onto a polyether sulfone membrane stabilized by Pt–Ag support. This biosensor was developed to determine anthocyanidins, flavonoids, and polyphenols from complex matrices. This biosensor had linear range between 2–14 $\times$ 10^{-6} mol L^{-1}, sensitivity around 0.0566 mA/mol L^{-1}, and the limit of detection about 1 $\times$ 10^{-6} mol L^{-1} (Gomes and Rebelo 2003). Jarosz-Wilkołazka and his colleagues also reported a laccase-based biosensor developed to detect caffeic acid, catechin hydrate, epicatechin gallate, epicatechin, and prodelphinidin. The developed biosensor was sensitive in the range of 7.81–57.92 nA/μmol L^{-1} (depending on the immobilized enzyme that was adhered to the substrate) and detection limit in the range of 0.56–2.44 μmol L^{-1} (Jarosz-Wilkołazka et al. 2004). Gamella et al. (2006) fabricated a laccase-based biosensor to determine the polyphenol index of wines. This enzyme was immobilized on a glassy carbon electrode cross-linked with glutaraldehyde, while gallic acid and caffeic acid were used as standards in this study. Moreover, the polyphenol index was estimated in both flow injection and batch conditions.

14.5.3.2 Tyrosinase-Based Biosensors

Tyrosinase biosensors have been proclaimed to have limited ability to monitor phenolic compounds that have at least one ortho position available. Tyrosinase catalyzes two specific oxygen-dependent reactions which occur sequentially, o-hydroxylation of monophenols to o-diphenols and successive oxidation of o-diphenols to o-quinones (ElKaoutit et al. 2008; Quan et al. 2004). Carralero Sanz et al. (2005) developed a tyrosinase biosensor immobilized onto a glassy carbon electrode (GCE) amended with electrodeposited gold nanoparticles. The developed biosensor was used to estimate the phenolic content in beverages.

Abhijith et al. also fabricated a tyrosinase biosensor on the Clark-oxygen electrode membrane cross-linked with glutaraldehyde. This biosensor worked on the principle of enzymatic transformation of polyphenols in the presence of oxygen and used this biosensor for assessing the polyphenols in tea (Abhijith et al. 2007). Schmidt and Schuhmann (1996) developed a multilayer tyrosinase-based biosensor. Redox dye was bound covalently to the electrogenerated poly-ω-carboxyalkylpyrrole layer, which was further enclosed by a second layer of polypyrrole encapsulating the enzyme. This multilayer setup protected the electrode from fouling due to the polymerization of quinone derivatives. Li et al. (2006) developed a mediator-free phenol biosensor in which tyrosinase was adsorbed on the ZnO nanoparticles surface via electrostatic interactions and successively immobilized on glassy carbon electrode with the help of a chitosan film. This biosensor was used to determine phenolic compounds like catechol, phenol, and p-cresol. An amperometric carbon biosensor was developed to assess polyphenols in complex matrices. Herein, the enzymatic solution was blended with glutaraldehyde and amended by adding 0.5% bovine serum albumin (BSA). The linear range of this biosensor was determined to be in the range of 0.04–2 μmol L^{-1}, and the detection limit was determined to be 0.06 μmol L^{-1} (ElKaoutit et al. 2007).

14.6 Conclusion

Plant secondary metabolites are essential compounds that find their application in the nutraceutical and pharmaceutical realm. Concentrates of these bioactive compounds have been used as food additives to impart human health and well-being. As elaborated in this chapter, some of the most widely harvested, low molecular weight compounds are phenols which are known to counteract the effects of reactive oxygen species. Although, extensive research has already been conducted in the extraction of such compounds, its estimation and quantification still uses equipment which is bulky, time-consuming, and nonspecific. Therefore, this chapter was an initiative to introduce the advancements that have been made in the field of biosensing towards bioactive compounds. While evaluating the performance of the fabricated biosensors against conventional detection techniques, it was found that biosensors were more sensitive and selective to the species of interest. Although biosensors are a futuristic approach to reduce effective time and reagent cost per sample run, further research in the field of PSM biosensing is necessary.

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