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Sikandar I. Mulla
R. N. Bharagava *Editors*

Enzymes for Pollutant Degradation

Microorganisms for Sustainability

Volume 30

Series Editor

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Microorganisms perform diverse roles on our planet most of which are important to make earth a habitable and sustainable ecosystem. Many properties of microorganisms are being utilized as low input biotechnology to solve various problems related to the environment, food security, nutrition, biodegradation, bioremediation, sustainable agriculture, bioenergy and biofuel, bio-based industries including microbial enzymes/ extremozymes, probiotics etc. The book series covers all the wider aspects and unravels the role of microbes towards achieving a sustainable world. It focuses on various microbial technologies related to sustenance of ecosystems and achieving targets of Sustainable Development Goals. Series brings together content on microbe based technologies for replacing harmful chemicals in agriculture, green alternatives to fossil fuels, use of microorganisms for reclamation of wastelands/ stress affected regions, bioremediation of contaminated habitats, biodegradation purposes. Volumes in the series also focus on the use of microbes for various industrial purposes including enzymes, extremophilic microbes and enzymes, effluent treatment, food products.

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Enzymes for Pollutant Degradation

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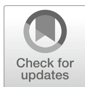
Chapter 1
Oxidoreductases for Removal of Environmental Pollutants



Ahmad Reza Bagheri, Nahal Aramesh, Hira Munir, Zaheer Ahmed, Abdulrazaq Yahaya, Muhammad Bilal, and Hafiz M. N. Iqbal

Abstract In recent years, environmental pollutants have been dramatically increased primarily due to anthropogenic activities (human-made processes). Therefore, people are now more concerned around the globe. Although various types of organic contaminants, including pharmaceuticals, pesticides, dyes, and heavy metals, have been clarified in our water systems, the routine methods cannot ensure their complete removal. All these contaminants are a considerable threat for humans and animals, which is due to a lack of effective strategies for mitigation. It has become meaningful to track alternative technology that is essentially smart, greener, and environmentally competent. Enzyme holds a promising role to mitigate any kinds of contaminating agents in the environment in a specific, easy-to-monitor, and highly controllable manner. Enzyme-based processes have unique properties, such as nontoxicity, low energy input, the capability of operating under mild aqueous conditions, reduced sludge generation, and use for different types of contaminations. This chapter focuses on using oxidoreductases, including laccases and peroxidases,

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as an applicable method for the abatement of environmental pollutants to ensure a safe environment.

Keywords Biocatalysis · Biocatalytic platform · Enzyme immobilization · Multifunctional entities · Environmental sustainability

1.1 Introduction

In recent years, the entrance of different pollutants and especially emerging pollutants (EPs) to the environment and water sources has become a considerable concern and threat for humans and animals. These concerns are because these pollutants and contaminations are too toxic, which can produce different diseases like various kinds of cancer and different mutations and can accumulate in the body of animals and other spheres (Ahmed et al. 2017a; Bilal et al. 2018a; Bilal et al. 2018c; Rasheed et al. 2018). Figure 1.1 shows the different ways for the entrance of EPs to the environment. One of the main pollutants is dye molecules, which can enter the water sources and change the physical and chemical properties of water. Dyes also can inhibit the photosynthesis process, which can be hazardous for the organism. To this end, the removal and treatment of these pollutants seem to be vital and essential. Up-to-dately, many efforts have been done for the removal of contaminants that have various physical and chemical properties (Chávez et al. 2020; He et al. 2020; Lin et al. 2020; Liu et al. 2020a; Pazos et al. 2020; Taoufik et al. 2020; Xu et al. 2020). For example, Sahu and co-workers fabricated CuO-ZnO nanohybrids and used them to remove various dyes (Sahu et al. 2020). The proposed CuO-ZnO was fabricated using a simple chemical process and characterized and identified using different techniques. The prepared CuO-ZnO was also applied for the photodegradation of 4-nitrophenol (4-NP) as a toxic material. The synthesized catalyst not only improved the degradation process but also decreased electron-hole recombination. In the other study, La(III) supported carboxymethylcellulose-clay composite was prepared and applied as a sorbent to treat different pollutants (Sirajudheen et al. 2020). The constructed sorbent was applied to remove different dyes from water. The effect of different factors on the removal process was investigated. Also, different isotherm and kinetic models were assessed. Despite the application of different methods for the removal of pollutants, the application of effective removal and degradation approaches for the removal of these contaminations has become a main challenge and concern in terms of their effects on the environment.

The applied methods can interfere with the other systems called EDs. The properties of EDs make them important materials for removal from different areas (Preda et al. 2012). Although EDs and their treatment are vital, the lack of available and important information about these materials makes it hard to remove the environment. Moreover, most of the applied methods are expensive, have insufficient affectivity, produce high amounts of sludge, and form toxic materials and products (Bilal and Iqbal 2019c). To address these problems, the design and

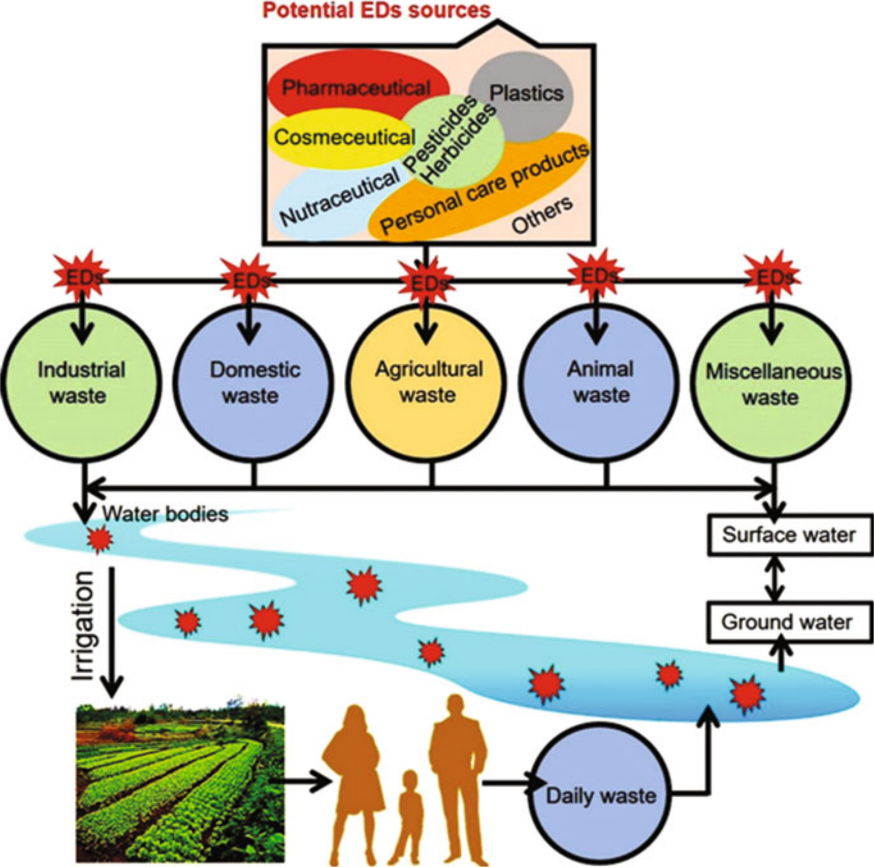

Fig. 1.1 The different ways for the entrance of EPs to the environment. (Reproduced with permission (Bilal et al. 2018b) from Elsevier. Copyright © 2018 Elsevier B.V. License Number: 4978590929487)

development of new approaches seem to be vital. One of the most promising methods for the degradation of different kinds of EPs is biological systems, especially systems based on the enzymes (Ahmed et al. 2017b; Bilal and Iqbal 2019a; Mahmoodi et al. 2020; Qayyum et al. 2020; Zhang et al. 2020a; Zhou et al. 2020). The application of biosystems for the treatment of pollutants can be an ideal choice. In these systems, microorganisms such as enzymes are applied. In the biodegradation process, the microorganisms use the pollutant as a substrate, which can induce enzymes; then, contaminations are transformed into materials with low toxicity. The biosystems have unique properties compared to traditional removal methods. For example, bio-based systems are simple, easy, cheap, safe, and considered a green method (Rauf and Salman Ashraf 2012; Al-Maqdi et al. 2018; Čvančarová et al. 2020; Zhang et al. 2020b). Although bio-based systems are applicable, they also have some limitations. For example, these processes are long, and some bio-based

materials are not stable in different conditions. To address these limitations, many efforts have been done. This review focuses on applying different bio-based systems, especially systems based on the application of enzymes in the removal of different pollutants.

1.2 Enzymatic Biodegradation

Enzymes are the tools of nature, and modern technologies and industries have been used in different areas, which is due to their unique properties (Jun et al. 2019; Ran et al. 2019). The enzyme-based methods are simple, easy, safe, and are applicable to different types of pollutants (Chatterjee et al. 2019; Mahmoodi and Abdi 2019). But they also have some limitations. For instance, some of these systems have insufficient stability and cannot be applicable for several cycles. To address these drawbacks, immobilization of the enzymes on various supports is an alternative method. Moreover, up-to-dately, many efforts have been done for immobilization of enzymes, which can improve their stability and application for several cycles. Laccases and peroxidases are two of the main enzymes that have been broadly applied to remove pollutants (Alneyadi et al. 2018; Bilal et al. 2019c). The main property of these enzymes is that they can catalyze the reactions which are based on the oxidation-reduction process (Sheldon 2011; Zdarta et al. 2019). Laccases and peroxidases also have high ability for the degradation of various contaminants. The mechanism of action of these enzymes is that they form radicals which can degrade pollutants (Zdarta et al. 2018; Bilal et al. 2019a). In the following, we will discuss different types of laccases and peroxidases, their properties, as well as their application in the removal of pollutants.

1.2.1 Laccase—Biocatalytic Features and Removal of Pollutants

Laccases (Lac) as a kind of oxidases that have different numbers of coppers are achieved from different sources. Laccases are the most useful and suitable enzymes for large and industrial scales, which is due to their unique properties for application in different biotechnological processes. The most important property of laccases is that they do not need any nutrient supply while they can work with just the presence of oxygen as a cofactor (Albarrán-Velo et al. 2020). The processes based on the application of laccases are fast, and the kinetics of the reaction depends on the substrate's affinity. The obtained laccases from wood-decaying fungi are in the hotspot, which is because it has a unique ability for oxidation (Ouzounis and Sander 1991). Generally, enzymes can be classified into three main categories, which are based on the copper centers (type 1 (blue), type 2 (normal), and type 3 or coupled

binuclear). Each type 1 and type 2 have only one Cu atom, while type 3 has two Cu atoms. Compared to enzymes that are based on the plant, the laccases have a higher potential for redox, which shows the higher catalytic efficiency of laccases. Laccases mediate the single-electron oxidation of hydrogen donating substrates accompanied by the concomitant molecular oxygen reduction to water. Atmospheric oxygen can be applied as a source of electron acceptor and can be used for consumption. Laccases have the main property in which they are able to cleave different substrates while they don't need any H_2O_2 sources. Laccases have been broadly used for the removal of different EPs. For instance, Lloret et al. (2010) investigated the application of laccase to remove different hormones.

1.2.2 Soybean Peroxidase (SBP)—Biocatalytic Features and Removal of Pollutants

Soybean peroxidase is an enzyme that has been broadly utilized in the elimination of pollutants (Sadraei et al. 2019; Wang et al. 2020). SBP can be found in soybean hulls, which is a byproduct of the soybean processing industry. Between different peroxidases explored, SBP has unique properties in terms of high thermal stability, high chemical stability in different conditions like different pH, and easy availability (Yang et al. 2019; Tummino et al. 2020). Previous studies showed that SBP could remove dye molecules in the presence of hydrogen peroxide (H_2O_2). The mechanism of dye removal is based on the cleavage of azo bonds. In this regard, Kaur and co-workers used SBP for the degradation and removal of hazardous materials (Kaur et al. 2020). The applied method was used to remove dye molecules in water. Based on the experimental results, DY12 was not detected to be a substrate of SBP. In the other work, SBP was used to remove various contaminations (Mukherjee et al. 2020). In their study, the influence of different factors on the removal process was evaluated, and the optimum conditions were achieved. Figure 1.2 represents the possible oligomer products for SBP-catalyzed treatment on 4-COT. The applied method is simple and cheap and can be an alternative method for removing different pollutants. In the other study, the application of SBP for bioconversion of 2,4-dichlorophenol was investigated (Fernandes et al. 2020).

In this work, soybean peroxidase was achieved from a transgenic cultivar. The 1- and 24-h reaction times were enough to remove 2,4- dichlorophenol 80% and 96%, respectively, using SBP, while soybean hulls converted only 30% 2,4-dichlorophenol in 24 h. In the other study, pentachlorophenol (PCP) was removed from water using SBP (Tummino et al. 2020). In this work, PCP was removed in the pH range of 5–7. Results proved that the addition of Fe (II) to the applied system can improve removal percentage. This is due to a synergetic effect of the enzymatic process and Fenton reaction. In the other work, the catalytic activity of SBP as a wastewater treatment was investigated by Mashhadi and co-workers (Mashhadi et al. 2019). The effect of different effective factors on the removal

Fig. 1.2 Possible oligomer products for SBP-catalyzed treatment on 4-COT. Loss of NH and Cl was observed as fragmentation products of the standard in (**a**) and (**b**), respectively. There was no direct evidence of oxidative dimer and trimer (shown in square brackets in (**c**) and (**e**), respectively). However, the structures shown in (**d**) and (**f**), oxidative dimers and trimers which have been dechlorinated, deaminated, and oxygenated, must have arisen from the structures in (**c**) and (**e**), respectively. Reproduced with permission (Mukherjee et al. 2020) from Elsevier. Copyright © 2020 Elsevier Ltd. License Number: 4978591434930

process was evaluated. Mass spectrometry analysis was used to investigate the obtained products. Recently, the recalcitrant atom-bridged bis-anilino compounds, 4,4′-methylenedianiline (MDA), and 4,4′-thiodianiline (TDA) were removed from water using SBP (Mukherjee et al. 2019). The targeted SBP was obtained from soybean seed hulls (coats), which can act in the presence of H_2O_2. In this work, the effect of different factors like H, H_2O_2, and SBP concentrations on the removal process was investigated. In the other work, SBP was immobilized on silica-coated magnetic particles and used for pollutant removal (Silva et al. 2016). The synthesized sorbent was used to degrade ferulic acid. After the synthesis of magnetic nanoparticles, they were functionalized with amino groups as support. In the next step, SBP was immobilized on this support.

1.2.3 Horseradish Peroxidase (HRP)—Biocatalytic Features and Removal of Pollutants

Horseradish peroxidase is another important enzyme that is widely used for the treatment of contaminations. It can be found in the root of the horseradish (*Armoracia rusticana*) herb in which HRP-C is the most abundant isoenzyme (Bilal et al. 2019b; Shi et al. 2019; Bian et al. 2020; El-Shishtawy et al. 2020). Recently, the application of HRP for the degradation of diclofenac was evaluated using stopped-flow spectrometry. Results showed that using HRP and addition of H_2O_2 can change diclofenac to the dark orange-colored product in which the colored product is indeed highly reactive diclofenac-2,5-iminoquinone which can be used as a precursor for different bio-conjugates and fragmented products in plants (Huber et al. 2016). In the other work, HRP was immobilized on electrospun magnetic nanofibers and used for the removal of phenols (Li et al. 2019a). The electrospinning method was used for the preparation of Fe_3O_4/polyacrylonitrile (PAN) magnetic nanofibers and subsequently immobilization of the immobilization of HRP. The diameter of the fabricated nanofibers was 200–400 nm, while their magnetic strength was 19.03 emu g^{-1}. The modified HRP represented high catalytic activity. In the other study, immobilized HRP on multifunctional hybrid microspheres was applied to eliminate Aflatoxin B1 (AFB1) (Zhou et al. 2019). This study was used as the first report in which HRP has immobilized on an alginate/chitosan/montmorillonite (SA/CS/MON) hybrid microsphere. Figure 1.3 shows the fabrication process of (a) and AFB1 immobilization (b).

The HRP was immobilized using covalent interaction. The negative charges of MON improve the adsorption of AFB1 and the adsorption of CS chains. Nanoencapsulation of HRP is another method for HRP application in the elimination of pollutants (Liu et al. 2020b). This method was used recently by Liu and co-workers. The most important property of the nanoencapsulation of HRP is that it can enhance the performance of the enzyme for pollutants removal. Using this method represented high thermostability and environmental tolerance. In this work, the individual enzymes were encapsulated within a nanogel that has been employed. In the other study, HRP was immobilized on hydrous-titanium and used for the removal of phenol (Ai et al. 2016). The applied immobilization method improved stability. The effect of different factors on the removal process was investigated. Based on the results, the immobilization process had not only any adverse effects but also slightly increased enzymatic catalytic kinetic. In the other study, lignin was removed from pre-hydrolysis liquor using HRP catalyzed polymerization (Li et al. 2019b). The effect of different factors on the removal process was investigated, and the optimum conditions were achieved.

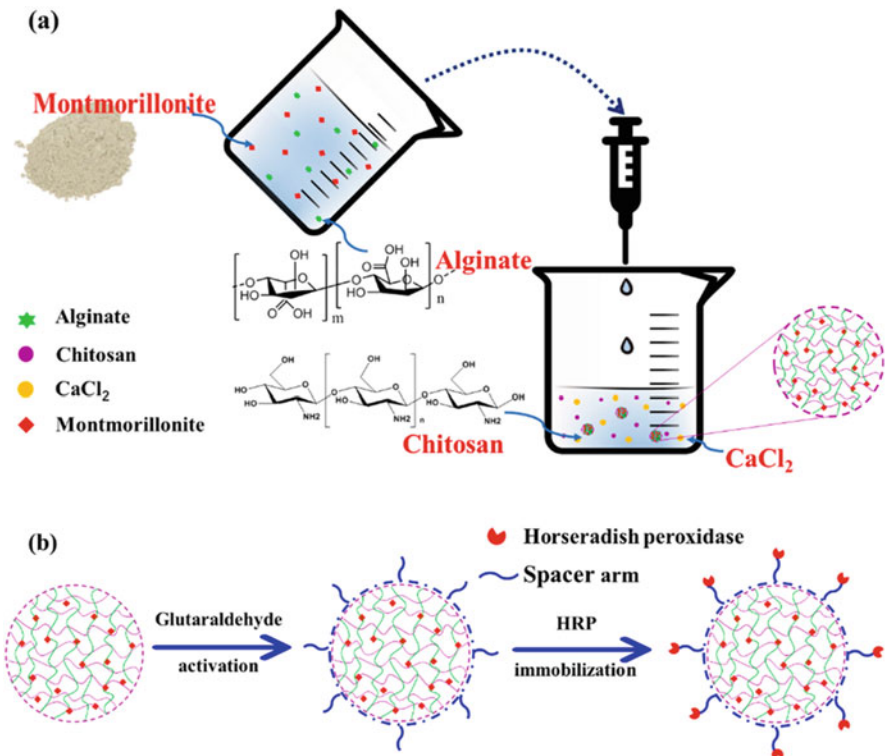

Fig. 1.3 Fabrication process of (SA/CS/MON) hybrid microsphere (**a**) and AFB1 immobilization (**b**). (Reproduced with permission (Zhou et al. 2019) from the American Chemical Society. Copyright © 2019 American Chemical Society)

1.2.4 Lignin Peroxidase (LiP)—Biocatalytic Features and Removal of Pollutants

Lignin peroxidase is an enzyme that contains heme, which can catalyze the H_2O_2-dependent oxidative depolymerization of lignin (Đurđić et al. 2020; Shi et al. 2020). From the structure aspect, LiP is considered a hemoprotein, which can function at acidic pH (around pH 3.0) by application veratryl alcohol as the catalytic substrate (Bilal and Iqbal 2020; Morsi et al. 2020). The most important property of LiPs is that they have high redox ability. In this regard, it is possible for them to oxide various compounds like phenolic families. One of the main pollutants which can produce different diseases is dye molecules. These compounds have stable and complex structures and subsequently are stable towards routine removal methods. To this end, removal of them from the environment and especially from water sources is vital. Up-to-date, different studies have been focused on the synthesis of different materials and their application in the removal of dye molecules. One of the popular

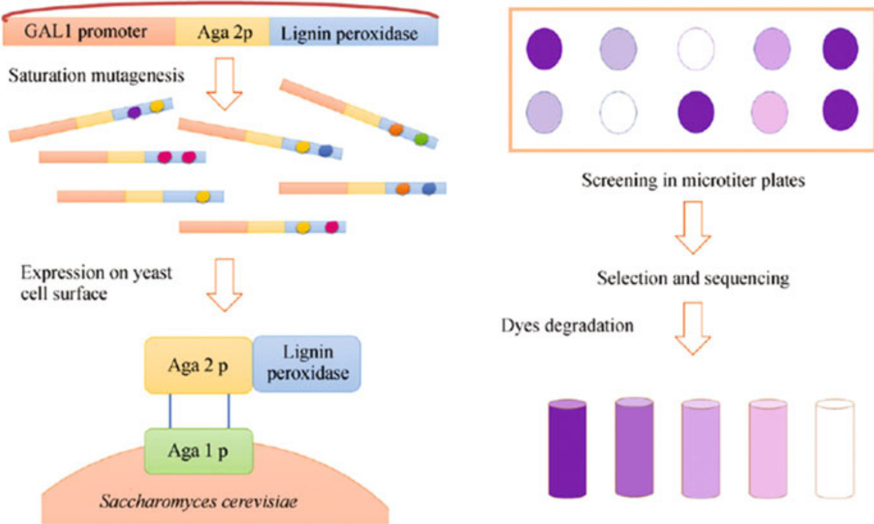

Fig. 1.4 The application of LiP for degradation of azo dyes. (Reproduced with permission (Ilić Đurđić et al. 2020) from Springer Nature. Copyright © 2020, Higher Education Press. License Number: 4978600170848)

methods for the removal of dye molecules is the application of the enzyme's immobilization method. In this regard, Kiran and co-workers immobilized LiP on graphene oxide functionalized $MnFe_2O_4$ superparamagnetic nanoparticles and used it for the removal of dye molecules (Kiran Rathour et al. 2020). The applied sorbent was a thermostable and reusable nanobiocatalyst, which makes it an excellent candidate for removing pollutants. In this work, LiP was obtained from *Pseudomonas fluorescens* LiP-RL5. The magnetic nanoparticles were prepared using the sol-gel auto-combustion method. The thermal stability of the immobilized LiP was 50 °C, while its half-life was 14 h. It is interesting to say that immobilized LiP kept 50% of its enzyme activity even after nine successive reactions cycles. The immobilized LiP removed dye molecules more than 80% within 1 h of incubation at 30 °C. In the other work, LiP was used for the degradation of azo dyes (Ilić Đurđić et al. 2020). The proposed LiP was achieved from *Phanerochaete chrysosporium* is a heme-containing. The applied yeast is applicable for cleavage the structure of proposed dye molecules. Figure 1.4 shows the application of LiP for the elimination of azo dyes.

Bilal and Iqbal immobilized LiP on Ca-alginate beads and evaluated its degradation ability in a packed bed reactor system (Bilal and Iqbal 2019b). The immobilization of LiP onto high-quality Ca-alginate beads was carried out using covalent interactions. In this regard, glutaraldehyde was applied as a cross-linking agent. To investigate the effects of influential factors, their interaction, and the ability to obtain optimization conditions, response surface methodology (RSM) was a powerful mathematical tool. Figure 1.5 represents the immobilization process of LiP on

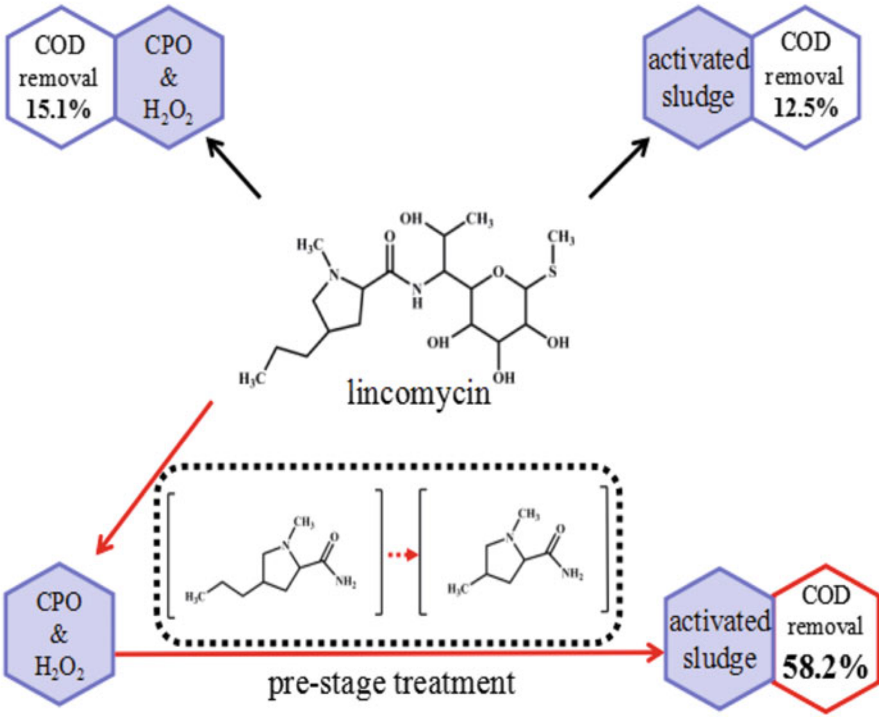

Fig. 1.5 The treatment process of lincomycin using CPO. (Reproduced with permission (Zhu et al. 2020b) from Elsevier. Copyright © 2020 Elsevier B.V. License Number: 4978600516629)

Ca-alginate beads. The effect of different factors on immobilization efficiency was evaluated. The immobilized LiP was used for the removal of different dyes. The most important thing about the applied system was its high stability, which made it able for five consecutive batch operations. The unique properties of Ca-alginate immobilized LiP make it a fantastic candidate for application on large and industrial scales. In the other work, LiP was immobilized on Fe_3O_4@SiO_2@polydopamine nanoparticles and applied for the degradation of organic pollutants (Guo et al. 2019). LiP was achieved from *Pichia methanolica* using the heterologous expression. After extraction and purification, LiP was immobilized on Fe_3O_4@SiO_2@polydopamine. In this study, the effect of different conditions on immobilization efficiency was evaluated, and different parameters were optimized. Results proved that the immobilization of LiP could improve its thermal stability, storage stability, and stability in different pH compared to free LiP. The other main property of immobilized LiP was its high ability in the application for eight cycles.

1.2.5 Manganese Peroxidase (MnP)—Biocatalytic Features and Removal of Pollutants

Manganese peroxidase is a heme glycoprotein that has a catalytic cycle like other peroxidases like LiP and HRP, which have heme while MnP uses Mn^{2+} (Hofrichter 2002). MnP catalyzes phenolic compounds to phenoxy radicals. For this purpose, MnP oxides Mn^{2+} to Mn^{3+} with the help of H_2O_2. This reaction can cleave an array of phenolic substrates. Using these properties, MnP has been widely applied for the removal of different compounds. For example, Siddeeg and co-workers extracted MnP from *Anthracophyllum discolor* fungi and then immobilized it on the surface of the magnetic nanocomposite Fe_3O_4/chitosan (Siddeeg et al. 2020). The main reasons for the selection of Fe_3O_4/chitosan are that it can produce a high surface area for immobilization of MnP and also can produce an increased number of reaction sites. In this work, the effect of different factors including temperature, pH, as well as storage duration on the activity of MnP was investigated, and optimum conditions were obtained. The fabricated sorbent was applied for the removal of dyes that have been used in different industries. The constructed nanocomposite removed MB and RO 16 more than 96% ± 2% and 98% ± 2%, respectively. The other main advantage of this nanocomposite was its high ability for application for five cycles. In the other work, MnP was achieved from *Irpex lacteus* F17 and then was combined with graphene oxide (GO) to introduce an efficient enzyme system (GO-MnP) (Yang et al. 2020). The fabricated system had high stability in a different pH range and had a high ability to oxidate aromatic compounds and remove dyes. The most important thing about the fabricated system was that MnP had high activity. In the other study, Bilal et al. immobilized MnP onto chitosan beads and used it to remove dyes (Bilal et al. 2016a). The immobilization of MnP was performed by cross-linking. The used system was able to remove the target compound up to 97.31%. Bilal et al. immobilized MnP in Ca-alginate beads at optimum conditions (Bilal et al. 2016c). Under the optimum conditions of H_2O_2 (1 mmol l^{-1}), 1-hydroxy benzotriazole (1 mmol l^{-1}), pH (5.0), and temperature (40 °C), the maximum removal percentage of 87.4% was obtained. In the other study, Bilal and co-workers obtained MnP from *Ganoderma lucidum* and then immobilized it on agar-agar support using the entrapment technique (Bilal et al. 2016b). At 4.0% agar-agar gel, maximum immobilization yield was achieved. Compared to free MnP, immobilized MnP showed higher stability in different pH and temperatures. Also, immobilized MnP had higher thermal stability compared to free MnP. These fantastic properties made immobilized MnP an exceptional candidate for application in removing different pollutants.

1.2.6 Chloroperoxidase (CPO)—Biocatalytic Features and Removal of Pollutants

Chloroperoxidase is considered oxidative biocatalytic and has the ability for application in different fields. The main ability of CPO is that it can transform different compounds (Vázquez-Duhalt et al. 2001; C.-Basurto et al. 2007; Guerrero et al. 2013; García-Zamora et al. 2018). The main point about CPO is that its biocatalytic performance is based on the presence of activating salts such as chloride (or bromide) ions. Lincomycin is found in different areas like wastewater treatment plants and groundwater and can produce many hazardous diseases. In this regard, the removal of lincomycin is important. Recently, Zhu removed lincomycin using CPO (Zhu et al. 2020b). The applied CPO was able to remove lincomycin about 90.16% at optimum conditions of 20 min and pH of 3.0. For the investigation of the degradation products, HPLC analysis was applied. Also, *Chlorella pyrenoidosa* was used as an indicator to evaluate the eco-toxicity of CPO. Figure 1.5 shows the treatment process of lincomycin using CPO. In the other work, Halloysite nanotube (HNT) as a natural, low-cost, biocompatible, and stable nanomaterial was modified and then used as an appropriate support matrix for immobilizing CPO (Zhu et al. 2020a). To this end, at first, Fe_3O_4 nanoparticles were deposited on HNT, which can reduce the separation of HNT due to magnetic property (Fig. 1.6).

The immobilized CPO showed higher stability, enzyme activity, thermal resistance, and recyclability than free CPO. The immobilized CPO was applicable for at least ten cycles. Diclofenac and naproxen are two of the main non-steroidal anti-inflammatory drugs that have been extensively applied in different areas, like treating various pains. Despite applicability, they are usually not removed and can adsorb by the human body. The routines methods cannot remove these drugs, which

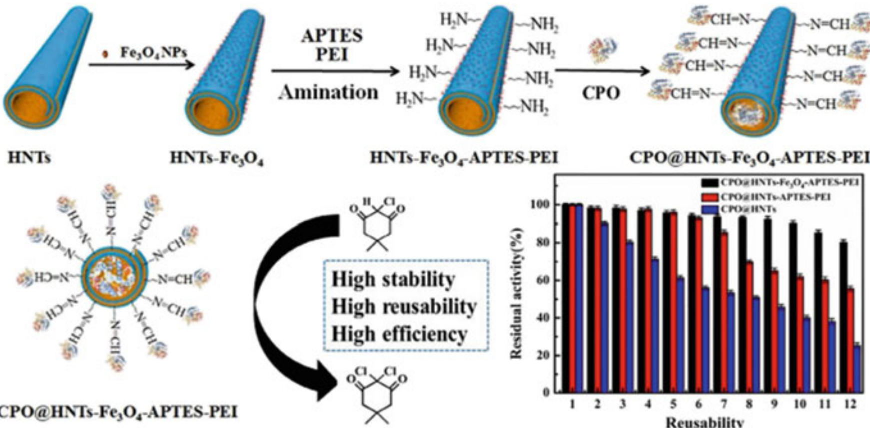

Fig. 1.6 The modification of HNT and its immobilization with CPO. (Reproduced with permission (Zhu et al. 2020a) from Springer Nature. Copyright © 2020, Springer-Verlag GmbH Germany, part of Springer Nature. License Number: 4978600712637)

can introduce hazardous effects on humans and the environment. In this regard, the introduction of effective methods for removing these drugs seems to be vital. As an effective method, CPO was obtained from *Caldarimyces fumago* and used to convert diclofenac and naproxen (Li et al. 2017). Only 9 and 7 min was enough for the complete conversion of targeted pollutants. To assess the converted products, HPLC-MS was applied. In this work, eco-toxicity was also investigated.

1.3 Conclusion

Environmental pollutions have become a global concern during the last decades. This fact is due to side effects and, subsequently, hazardous effects of environmental pollutants on both humans and animals. To this end, the elimination of these contaminants from the environment and especially water sources is vital. Up-to-dately, various methods have been employed for this purpose. Although these methods are applicable, they are also expensive and have low efficiency. To address these limitations, the introduction and application of alternatives methods are in a hotspot. The application of enzyme-based methods is very interesting and popular due to the amazing properties of enzymes like simplicity, ease of operation, low cost, and eco- and environmentally friendly properties. In spite of these unique features, enzymes like laccases and peroxidases have the main limitation in terms of low stability. To address this drawback, the immobilization of these enzymes is an ideal choice. This method enhances the stability of these enzymes and increases their recycling and makes them applicable for using large and industrial scales. To this end, this review article is focused on the recent development and application of enzyme-based methods for the removal and treatment of different pollutants from the environment. This review article will open new doors for researchers to understand better the excellent properties of different enzymes and their application in treating various pollutants.

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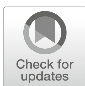
Chapter 2
Synthesis of Industrial Enzymes from Lignocellulosic Fractions



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Abstract The lignocellulosic material consists of three subunits, hemicellulose, lignin, and cellulose, that are fractionated to extract and produce value-added compounds, such as food additives, organic acids, ethanol, and enzymes. Various biotechnological applications such as effluent bioremediation, hydrolysis and paper bleaching, and construction of biosensors require large quantities of low-cost enzymes. Thus, an appropriate choice for the production of low-cost enzymes is the use of lignocellulosic residues, which contain soluble carbohydrates that can be used as inducers of enzymatic synthesis. However, the depolymerization of lignocellulosic components through various treatments (chemical, physical, physicochemicals, and biological) is necessary for the efficient production of enzymes. Therefore, this book chapter addresses the chemical composition of lignocellulosic residues and their various treatments that allow obtaining value-added products, mainly enzymes. The main types of industrial enzymes, their application in several technological areas, and their market in the world are also addressed.

Keywords Biomass · Lignocellulosic treatment · Industrial enzymes

2.1 Introduction

Different sources of lignocellulosic biomass are produced worldwide due to high agricultural activity, which favors the availability, diversity, and use of agro-industrial waste. The chemical composition of lignocellulosic residues (cellulose, hemicellulose, and lignin) is fractionated and extracted to produce compounds such as organic acids, ethanol, and enzymes (Canilha et al. 2010; Maitan-Alfenas et al. 2015). In addition, the use of these wastes could help to reduce the environmental impact caused by the agricultural and agro-industrial sectors (Ferreira et al. 2011). However, for waste to be used more efficiently, it is necessary to subject them to treatment processes (Ruzene et al. 2008).

Biomass treatments can be grouped into four types: chemical, physical, physicochemical, and biological. From the individual or conjugate use of some of these treatment categories, fractions rich in phenolic compounds, lignin, pentoses, and hexoses, can be used as substrates in bioprocesses using microorganisms mainly as bacteria and fungi. Among the variety of fungi, the white-rot fungi have the ability to degrade lignocellulose components due to their metabolic system that produces lignocellulolytic enzymes such as β-glycosidase, cellulase, xylanase, lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase (Barakat et al. 2014; Inácio et al. 2015).

In addition to fungi, some groups of bacteria have also been used in an attempt to potentiate the process. Although there are many ligninolytic bacteria, most of them have not been exploited in degradation processes (Davis and Sello 2009). Among those that are already well described regarding the action potential on lignocellulose, phenols, and xenobiotic compounds, the bacteria belonging to the group of

Sphingomonas, *Pseudomonas*, *Rhodococcus*, *Nocardia*, and *Streptomyces* (Bugg and Rahmanpour 2015; Bugg et al. 2016) can be mentioned.

As mentioned previously, several enzymes with function in the degradation of the lignocellulosic residue are released by the metabolic systems of the involved microorganisms. From this, it is possible to highlight the action of the enzymes through the direct application of the microorganisms or to perform extraction and purification steps. In general, these enzymes can be applied in processes of different industrial processes such as food additives, cosmetics, pharmaceuticals, biosensors, and bioremediation.

2.2 Literature Review

2.2.1 Lignocellulosic Residues

Lignocellulose is the main component of cell walls of plants and consequently of the woody plants and dead plant materials and constitutes the most abundant biomass on the planet (Knežević et al. 2013; Barakat et al. 2014), reaching an estimated production of 1.3 billion liters per year worldwide (Silva et al. 2009; Chiu and Lo 2016). The lignocellulosic residue has a complex structure of three main components, lignin cellulose, and hemicellulose, cited by the prevalence of composition (Fig. 2.1) (Song and Ni 2013; Ciolacu 2018). Much of this lignocellulosic waste is produced through forests, agricultural practices, and many agro-industries, causing serious problems of environmental pollution, among them the dispersion of pollutants in the atmosphere caused by the burning of the lignocellulosic material, thus causing the increase of the greenhouse effect and problems which are still evaluated superficially due to the non-identification of many of their potential polluters (Martinelli et al. 2010; Ferreira et al. 2011).

Faced with a decline in nonrenewable resources combined with environmental and economic concerns, the development of new raw materials for the production of

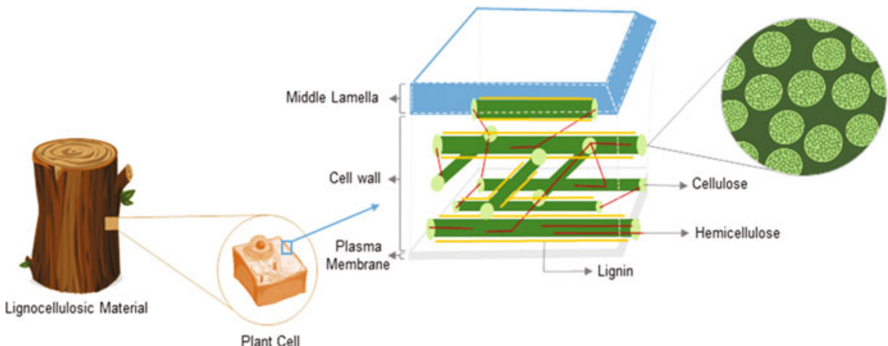

Fig. 2.1 Representation of the source and main structural components of lignocellulosic biomass

Table 2.1 The composition of biomass obtained from different agricultural sources

Lignocellulose	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Hardwood	40–55	24–40	18–25
Softwood	45–50	25–35	25–35
Corncobs	40–45	35–45	10–20
Corn Stover	38–42	22–28	7–22
Barley straw	31–45	20–38	8–19
Wheat straw	37–41	27–32	13–15
Rice straw	22–35	10–24	18–22
Bagasse	32–48	19–24	23–32
Soy stalk	40–48	10–20	10–18
Cotton stalk	42–45	5–12	0–15

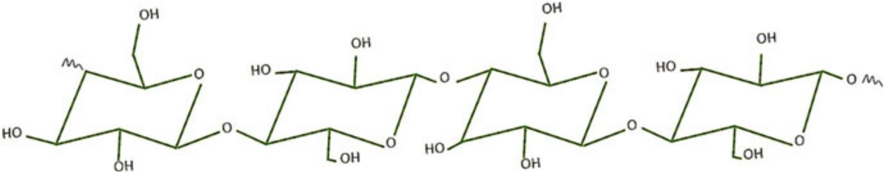

Fig. 2.2 Representative structure of cellulose polymerization from glycosidic bonds

fuels and chemicals from renewable sources as lignocellulose is necessary. In addition, the use of lignocellulose is a potential raw material for obtaining value-added compounds, such as enzymes, ethanol, organic acids, food additives, and others (Canilha et al. 2010; Maitan-Alfenas et al. 2015; Menezes et al. 2017). This fact has attracted the interest of many researchers due to the high potential of lignocellulose residues to obtain new products (Ruqayyah et al. 2013; Zhou et al. 2013).

According to the ECN database Phyllis2 (www.phyllis.nl), which provides reports on biomass and waste composition, it is possible to measure the chemical composition of different lignocellulosic raw materials based on publications between 2016 and 2018 (Table 2.1). Usually, the biomass dry weight contains cellulose (50%), hemicellulose (10–30% in wood or 20–40% in herbaceous plants), and lignin (20–40% in wood or 10–40% in herbaceous plants) (Sharma et al. 2015). However, the composition and proportions of these compounds vary among plants, depending on factors such as age, harvesting season, and growing conditions (McKendry 2002; Prasad et al. 2007).

2.2.1.1 Cellulose

Cellulose, the main component of plant cell walls and algae, is a β-1,4 linker glucose polymer (Fig. 2.2), making it the most abundant carbohydrate in nature, accounting for about 40–45% of the dry weight of lignocellulosic matter. Due to these

characteristics, this matter has attracted the interest of researchers in its use for the development of renewable sources (Deswal et al. 2011; Cheroni et al. 2012; Krogell et al. 2013).

Structurally, cellulose is composed of highly crystallized microfibrils between amorphous matrices, making it difficult to access hydrolysis by enzymes. The use of cellulose as a source of nutrients requires its depolymerization by the cellulase enzymes that promote the cleavage of β-1,4-glycosidic bonds releasing the glucose units (Liu and Cao 2013). The biotechnological potential of cellulolytic enzymes has been demonstrated from basic and applied studies, focusing on several industries, including food, animal feed, beverages, agriculture, biomass refining, pulp and paper, textiles, and laundry. As an example, in recent years, the use of cellulases has become one of the most important measures to improve the performance of food digestion by cattle and poultry and is used as an additive in food (Zhou et al. 2013). In addition, the action of cellulases favors the conversion of cellulose into simple sugars that can be fermented in ethanol (Deswal et al. 2011; Saini et al. 2015).

2.2.1.2 Hemicellulose

Hemicellulose is the second most abundant polysaccharide in plants and may vary depending on the plant species. This polysaccharide presents a flexible character that allows to bind to the surface of the cellulose, forming chains between the microfibrils of cellulose, resulting in a cohesive network of low molar mass, with a degree of polymerization (GP) between 80 and 425 monomeric units (Ayoub et al. 2013). Hemicellulose is associated with other cell wall constituents such as proteins, lignin, and other phenolic compounds formed by covalent bonds and hydrogen interactions. The hemicellulose consists of monosaccharides such as xylose, mannose, arabinose, and galactose (Fig. 2.3), which serve as raw material for the conversion of value-added chemicals such as furfural, 5-hydroxymethylfurfural (HMF), levothyroxine, xylitol, and ethanol (Yi et al. 2013; Zhou et al. 2013; Behera et al. 2014).

The main difference of hemicellulose in relation to cellulose is given by its ramifications composed of chains of several sugars, whereas cellulose has oligomers easily hydrolyzed (Sipos 2010).

Due to the trend towards sustainability in the green industry and a growing interest in the concept of biorefinery, it is possible to use hemicelluloses as a resource to obtain higher added value products such as pectins, ionic polymers, and hydrogels for release (Krogell et al. 2013; Sun et al. 2013; García et al. 2016). There are two determinants for the practical use of hemicellulose isolates: the first one considers chemical composition, and the second suggests that hemicellulose isolation methods influence their structures and, consequently, the possible domains of their applications (Jiang et al. 2014).

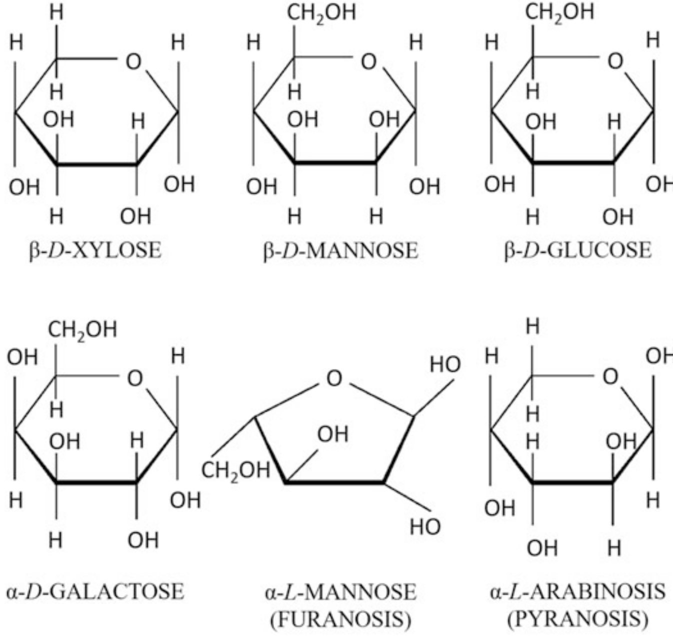

Fig. 2.3 Molecular structure of some monomers commonly found in hemicellulose

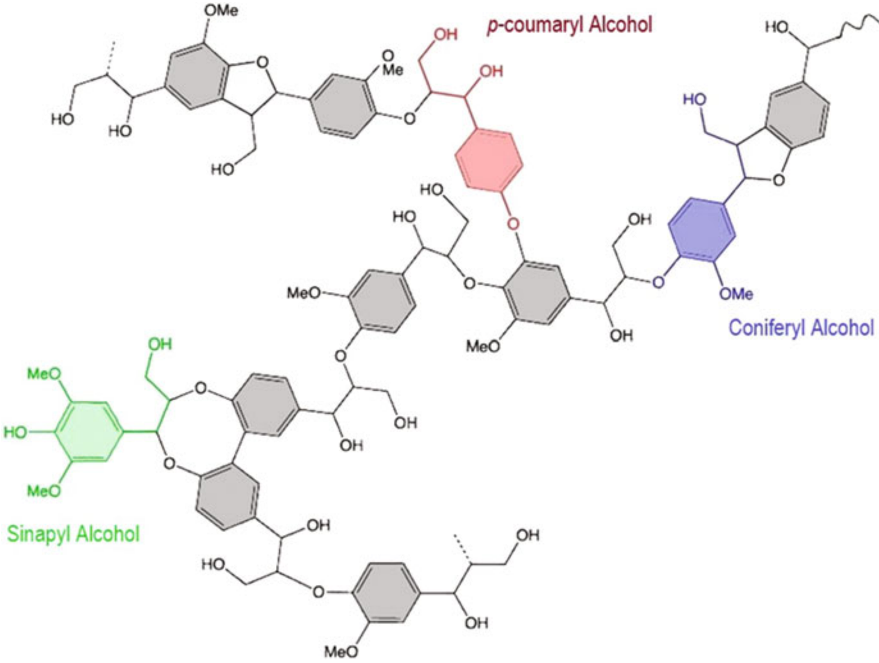

Fig. 2.4 Main connections and functional groups of lignin

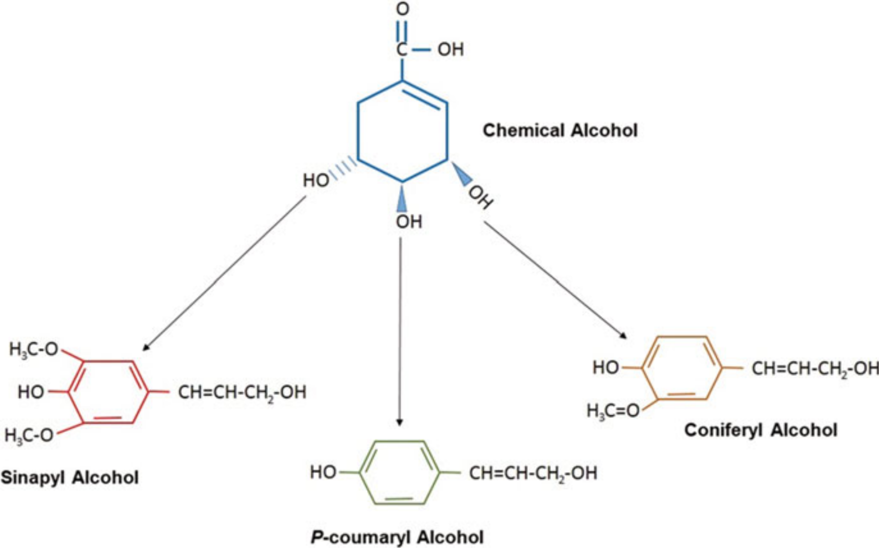

Fig. 2.5 Molecular structure of lignin precursor phenols

2.2.1.3 Lignin

According to Eudes et al. (2014), lignin is a heterogeneous and irregular arrangement of the phenylpropanoid polymer (Fig. 2.4) formed by the polymerization of three monomers: coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol, formed by a common precursor (Fig. 2.5), in addition, which represents about 20–30% of the mass total lignocellulosic matter. This composition confers resistance to chemical or enzymatic changes, protecting cellulose from degradation (Krogell et al. 2013). In this way, it is possible to identify that the degradation of lignin is a limiting step for the rate of carbon recycling (Sánchez 2009).

This polymer can be classified as native lignin present in biomass and technical or industrial lignin isolated from biomass by a variety of processes (Bozell et al. 2007).

Due to lignin having a complex polymer structure, the process of mineralization of the structure may take a long time, despite the bacterial and fungi involvement in this process (Bugg et al. 2011; Janusz et al. 2013). These microorganisms have the potential to degrade lignin from agro-industrial and agricultural residues such as corn, converting biomass and synthesizing enzymes with potential use in the paper industry, for example, and in bioremediation of residues (Janusz et al. 2013). In addition to being the fuel for boilers, lignin can be used as polymer additives, surfactants, coloring agents, and as source material for the production of chemicals, fertilizers, adhesives, and dyes. However, lignin is not yet used to its full potential (Sahoo et al. 2011; Ghaffar and Fan 2014).

The fungi present suitable enzymatic mechanisms for the transformation of complex molecules into simple compounds by their ability of selective delignification, which adds value to the bioconversion process. Thus, the microbial

conversion of "unused" residues such as corn results in new sources of energy (Elisashvili et al. 2008).

2.2.2 *Pretreatment of Waste*

Considering the high productivity and economic conversion power, several biotechnological processes were developed to use agricultural residues in the production of different products such as alcohol, enzymes, and organic acids. For this to be possible, these residues need to undergo a pretreatment stage, which will allow the fractionation of its components (cellulose, hemicellulose, and lignin), favoring its use more effectively (Phitsuwan et al. 2013; Azelee et al. 2014). In addition, these processes may involve techniques that aim to reduce the recalcitrant character of lignocellulose, making them more reactive and digestible, so that they can be applied in hydrolysis processes (Santos et al. 2012).

In general, the processes of treatment of lignocellulosic materials are classified into four categories: chemical, physical, physicochemical, and biological. Organic solvents (acetone, butanol, toluene, and dichloromethane), mineral acids (HCl, H_2SO_4), and also some bases are generally used within the chemical method. In the physical methods, the parameters temperature, pressure, and mechanical action are applied in a primary way, whereas physicochemical processes involving electrochemistry stand out (Yi et al. 2013; Lee et al. 2014). In addition, there are still biological methods, which make use of microorganisms such as fungi and bacteria, which have metabolisms capable of producing specific enzymes to degrade organic matter. In Fig. 2.6 it is possible to observe a flowchart with the main treatment groups for lignocellulose materials, as well as some of the most used methods in each one of them.

2.2.2.1 Chemical Methods

There are several chemical pretreatment alternatives that can be applied to lignocellulosic materials; however, the main chemical treatment routes used are acids, alkalis, gases, oxidizing agents, and solvents. Although it is possible to obtain high efficiency, in this process, it is necessary to calculate the potential effects against the matter used, since drastic reaction conditions, especially in acidic processes, can modify and/or destroy the components (Zheng et al. 2014; Shirkavand et al. 2016).

Alkaline Extraction

Alkaline pretreatment is often used to increase the digestibility of lignocellulosic materials because of its high capacity to de-structure the lignin bonds and to hydrolyze the hemicelluloses, since this type of treatment promotes the de-

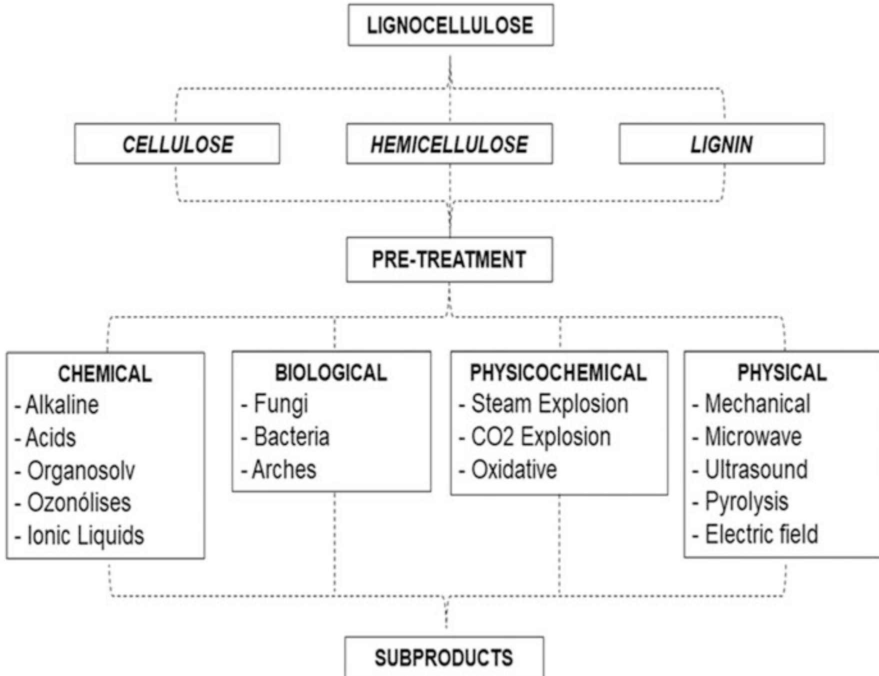

Fig. 2.6 Flowchart for the treatment of lignocellulosic biomass, showing the types of processes that can be applied

structuring of the intermolecular hydrogen bonds between cellulose and hemicellulose and the total or partial cleavage of the ester bonds between ferulic acid and arabinane or galactan in the cell wall, resulting in the dissolution of hemicellulose (Egues et al. 2012; Zhang et al. 2013; Ayoub et al. 2013). In addition, this treatment causes fiber swelling, increasing the internal surface area, and decreasing the crystallinity of the cellulose (Chen et al. 2011). The most bases used in the alkaline treatment are potassium, sodium, calcium, and ammonium hydroxides.

Alkaline processes use lower temperatures and pressures compared to other technologies. They can be performed under environmental conditions, but the reaction time is higher. Compared with acidic processes, this method causes lower sugar degradation, is less corrosive, therefore has a lower reactor load, and allows recovery or regeneration of the salts (Kim et al. 2016). In contrast, the compounds employed are more expensive and are used in higher concentrations. Another problem is related to possible environmental problems, which can increase waste treatment costs and recycling is necessary to make them viable (Prasad et al. 2007). There may also be problems with the biomass itself, which can absorb the bases and hinder fermentation (Hamelinck et al. 2005). The degradation of lignin generates by-products in the form of monomers and phenolic oligomers that negatively affect the fermentation process.

The hydroxide ions present in NaOH provide the release of a portion of the hemicellulosic material in solution. In addition, partial hydrolysis, oxidation or esterification of the hydroxyl groups, and cross-linking can modify the properties of hemicellulose, providing a polymeric raw material for new industrial applications, among them starch products for biodegradable plastics (Ayoub et al. 2013).

Organic Solvents

Pretreatment by organic solvents can be efficient in removing lignin and hemicellulose through the break of the internal bonds. However, disadvantages as solvent recovery, high cost, as well as flammability, and volatility should be considered (Brum et al. 2012). Among the parameters that directly affect the efficiency of this process, temperature, retention time, and solvent concentration can be cited. Organic solvents such as methanol, ethanol, acetone, and ethylene have been used for the pretreatment of different lignocellulosic materials (Kumari and Singh 2018).

In this treatment group, the technique called organosolv, an alternative treatment using a lower concentration of reagents using a mixture of water and organic solvents (Koo et al. 2011; Mood et al. 2013), may be highlighted. This type of delignification process allows the total utilization of the constituents of the vegetal biomass, allowing the recovery of the solvent used (Mesa et al. 2011). Thus, this method reduces the environmental impact of conventional delignification processes, allowing the greater viability for the integral use of the lignocellulosic components, using low investment capital.

Other advantages of organosolv application are the absence of strong odors during the process, ease of recovery of hemicelluloses and lignin that are less degraded, and ease of adjustment of bleaching steps in pulps using non-chlorinated reagents (Mesa et al. 2011). However, the organosolv process does not allow rapid washing of the lignocellulosic residue in water as in conventional processes, due to the precipitation of the lignin on the residue and the volatility of the solvent requiring that the process be well controlled (Sun and Chen 2008).

Acids

Currently, acids have been of interest in the treatment of lignocellulosic materials for the production of bioethanol, since they promote satisfactory results in the hydrolysis of hemicelluloses and the attack on lignin bonds. The most used acids in the pretreatment of biomass are sulfuric acid, nitric acid, hydrochloric acid, and phosphoric acid (Mosier et al. 2005; Tian et al. 2018). The acid pretreatments have advantages over alkaline processes because they are highly reactive, have high efficiency in converting most of the hemicellulose into soluble and fermentable sugars, significantly improve the hydrolysis of cellulose, and are relatively cheaper. The yield in sugars for the fermentation increases significantly. However, the process generates chemicals (such as furfural and phenolic components), which

are undesirable in the subsequent processes (Pereira Jr. et al. 2008; Kumar et al. 2009).

For acid treatments of the biomass, usually are used concentrated acids or diluted acids such as H_2SO_4 and HCl. However, despite their efficiency in degrading biomass, these compounds are corrosive, toxic, and require corrosion-resistant reactors in industrial plants, as well as, a recovering process for the concentrated acid, which makes the pretreatment process very expensive (Kumar et al. 2009). In the diluted acid hydrolysis method, further to using a low concentration acid, high temperatures are also used to dissolve hemicelluloses from the cell walls of the biomass, making the cellulose more accessible for subsequent hydrolysis steps. The processing conditions can be adjusted according to the type of raw material, temperature, and reaction time (Alvira et al. 2010).

Ionic Liquids

Ionic liquids (LIs) have shown to be very promising for the treatment of lignocellulosic residues, especially those that have at least one organic ion (cation) and with melting points below or not much above room temperature, which would favor industrial use by reducing energy costs (Seoud et al. 2007; Aung et al. 2018). LIs are referred to as "green" solvents due to exhibit thermal and chemical stability, extremely low vapor pressure, and are non-flammable, allowing the solvent to recycle during the treatment process. These compounds also have the ability to separate organic and inorganic material, as well as variable miscibility in water and inorganic solvents, favoring their use in the treatment of biomass (Seoud et al. 2007; Yoo et al. 2017). The LIs present physical and chemical properties that can be controlled by the combination of cations and anions that compose them, thus favoring the connections between the LI molecules and the residue to be treated. Moreover, it is possible to highlight that the less lipophilic is an ionic liquid, the more efficient its performance in applications, such as the dissolution of cellulose (Wang and Sain 2007; Boujemaoui et al. 2015).

The mechanism of depolymerization of the lignocellulosic material in ionic liquid involves the hydroxyl atoms of the cellulose that forms a donor/receptor electron system, which interacts with the LIs. Within this system, oxygen atoms act as electron donors, while hydrogen acts as a receptor. In this way, the free ions of the LIs associate with the protons of the hydroxyl of the cellulose, whereas the cations complex with the oxygen of the hydroxyl, thus interrupting the hydrogen bonding in the cellulose, promoting its fragmentation (Cao et al. 2009). In addition, it is possible to highlight that imidazolium-based lithium-based lithium may contribute to the dissolution of cellulose, due to its relative acidity and oxygen association capacity of the OH groups of cellulose (Fei et al. 2007).

2.2.2.2 Physical Methods

The physical pretreatment methods can be characterized by the use of techniques that promote the increase of the surface area of the residue as the granulometry decreases, as well as the reduction of the degree of polymerization of part of its components, that can favor the action of treatments. There are several physical treatments that are usually described in the literature, such as mechanical treatment, extrusion, irradiation (ultrasound, microwave), and pyrolysis (Behera et al. 2014).

Mechanical Treatment

Mechanical treatment of biomass is used to reduce particle size and is typically applied primarily to other treatment methods. The reduction of biomass particle size increases the accessible surface area for subsequent hydrolysis processes, reduces the degree of cellulose crystallinity, and decreases the degree of polymerization of cellulose to improve the degradation (Behera et al. 2014). The mechanical action on the biomass can be accomplished using milling machines or grinding machines, including balls, vibro, hammer mills, knife, two rollers, colloids, and friction (Barakat et al. 2014; Bhutto et al. 2017).

The moisture content of the lignocellulose raw material is an important factor in choosing a grinding machine. Biomass with moisture below 15% (wet basis) are usually treated with rolls, hammer, friction, and knife mills, while materials with a moisture content higher than 15% are treated with colloid mills and extruders, already for dry or wet materials are used ball mills and vibro (Miao et al. 2012).

Irradiation

Irradiation, a physical biomass pretreatment, includes several procedures such as microwaves, ultrasound, gamma rays, and electron beam. Among them, microwave pretreatment is widely studied, through which the energy from an electromagnetic field acts directly on the material, providing rapid heating and reduced thermal gradients (Pellera and Gidarakos 2017; Kumari and Singh 2018). The microwave field and material dielectric response are decisive for its heating ability by microwave energy, representing an effective alternative compared to conventional heating. This allows for quick heating besides a reduction in treatment time and energy consumption (Hassan et al. 2018).

Extrusion

Extrusion is a thermo-physical procedure through which the residue is subjected to an association of conditions, such as heating, shearing, pressure, etc. The process can

promote the material abrasive wear as a function of the pressure, which promotes the biomass de-structuring, realizing cellulose, hemicellulose, lignin and protein, and sugar and amino acid degradation. This process depends closely on operational parameters including pressure, reaction time, and dry biomass (Chen et al. 2014a; Rodríguez et al. 2017).

As advantages of this process, high shear rate, fast mixing, short residence time, moderate temperature, formation of secondary compounds such as HMF, hydroxymethylfurfural, process adaptability, and easy scaling can be cited. However, the technique has some negative points, among them, the generation of effluents and the increase in costs, due to the additional steps required for the treatment of formed toxic by-products (Gatt et al. 2018).

2.2.2.3 Physicochemical Methods

Steam Explosion

In this process, there is disintegration, and disintegration of the fibers of the material may occur until its rupture. There is also the breakdown of some chemical bonds of the biomass components, which have their activation energies overcome due to the elevated temperature (Duque et al. 2016). What happens in this pretreatment is that the vapor that penetrates the lignocellulosic material condenses and becomes liquid water at high temperatures. If the pressure is withdrawn, the water comes into contact with the vapor and evaporates so rapidly that the explosion takes place (Biswas and Ahring 2016; Lorenzo-Hernando et al. 2018).

Steam blasting acts both chemically and physically in the transformation of the material, involving the treatment with saturated steam at temperatures of 160–240 °C for a reaction time ranging from 2 to 30 min, with or without an acid catalyst. The most commonly used catalysts are SO_2, CO_2, H_2SO_4, and H_3PO_4 (the most commonly used H_2SO_4), and their use leads to more complete removal of hemicelluloses. The process requires low energy consumption, compared to other physical processes, and has lower environmental impact and capital investment (Chen et al. 2014b; Martín et al. 2018).

Explosion of Carbon Dioxide (ECD)

This method applies the supercritical CO_2 (SC-CO_2), which under pressure conditions increases the lignocellulosic biomass digestibility which is subjected at high pressure (1000–4000 psi) and maintained under controlled temperature and time conditions (Carneiro et al. 2016). The CO_2 interacts with the biomass under high pressure, which in contact with water promotes carbonic acid formation, which helps in the hemicellulose hydrolysis. The release of pressurized gas generates the biomass structure disruption, increasing the specific surface area required for the solvent access to the matrix (Dong and Walker 2008). The hydrolytic yield efficiency by

ECD pretreatment requires a certain moisture content in the biomass. As a positive aspect of the process, highlighted can be the low cost of carbon dioxide, the nontoxic by-product generation, and low operating temperatures. However, the high cost of equipment, capable of withstanding high-pressure conditions, restricts its industrial application (Santos et al. 2011; Benazzi et al. 2013).

2.2.2.4 Biological Methods

Based on the complex and heterogeneous structure of lignocellulosic matrices, the biological mechanism of pretreatment is closely associated with the microbial capacity to produce enzymes, whose action can release the biomass components (Behera et al. 2014; Bhutto et al. 2017). Some bacteria, mainly found in soil, present the degradation capacity of the structural components of lignocellulose. Frequently, due to their rapid growth, bacteria are applied in processes for the polymeric molecule degradation, whose products can be recovered to obtain value-added products, including enzymes (Hatakka 2005).

In addition, some bacteria also have the ability to degrade the polymeric structures present in the residue, facilitated mainly by the extracellular xylanases that induce the degradation of the hemicellulose and release simultaneously interconnected lignin components (Vasco et al. 2016). Notwithstanding, bacteria are less applied in processes of lignocellulosic biomass degradation and efficacy when compared to the fungi, due to their inefficiency in producing ligninolytic enzymes, compared to fungi (Akhtar et al. 2015; Xu et al. 2018). Furthermore, some limitations restrict the use of bacteria under environmental stress conditions, such as low nutrient levels, low pH, high contaminant concentrations, and low efficiency in the degradation of water-soluble or soil-bound compounds, in addition to the induction of its enzymatic system to occur only in the presence of the contaminant. Thus, certain contaminant levels could be insufficient to induce the enzyme expression or activity of the enzymes required for the biodegradation process (Moreno et al. 2004).

According to (Kirk et al. 2008), there are approximately 29,914 known species of basidiomycetes, ranging from the popular mushrooms and wooden ears to the coals, rust, gastropods, and jellies. Most species are saprobic, but many obligate or facultative parasites occur as well as mycorrhizal parasites. Basidiomycetes play a crucial role in the nutrient cyclings in nature, especially in the carbon cycle, as efficient lignin degraders, the second most abundant biopolymer on the planet. These fungi also contribute to maintaining the nitrogen, phosphorus, and potassium cycle, incorporated into cell wall insoluble components (Carlile et al. 2002).

Fungi capable of degrading woods and pollutants are divided into three groups: soft, brown, and white fungi. The mild degradation fungi are deuteromycetes and ascomycetes, which have great capacity for degradation of polysaccharides, but they lose performance when they are used for the degradation of lignin. Brown-rot fungi are efficient cellulose and hemicellulose degraders (Sánchez 2009; Sindhu et al. 2016), whereas white-rot fungi degrade lignin. Among fungi, lignolytic enzyme

producers, the most studied for the degradation of compounds with lignin is *Phanerochaeta chrysosporium* and the genera *Pleurotus*, *Trametes,* and *Ceriporiopsis*, because they present an enzymatic complex with nonspecific extracellular release (Aguiar and Ferraz 2012).

Studies on lignin degradation by white-rot fungi have revealed three types of extracellular enzymes responsible for its initiating depolymerization, identified as lignin peroxidase (LIP), manganese peroxidase (MnP), and laccase (Lac) (Menezes and Barreto 2015). The enzyme expression is closely related to microorganisms since some secrete LiP and MnP (without Lac), while others can secrete only MnP and Lac. Further, white-rot fungi display efficiency in degrading a range of persistent environmental pollutants such as heterocyclic aromatic hydrocarbons, chlorinated aromatic compounds, dyes, and synthetic polymers (e.g., carbamazepine, diclofenac) (Jayasinghe et al. 2008; Rubilar et al. 2008; Si et al. 2013; Golan-Rozen et al. 2015). This degradation is probably due to a strong oxidative activity and low specificity of ligninolytic enzymes to the substrate. Thereby, white-rot fungi and their enzymes display a wide application in some industrial processes to synthesize bioproducts and in bioremediation procedures (Janusz et al. 2013).

Fermentation

After the initial selection process of the microorganisms with enzymatic potential for degradation, they can be applied in fermentation processes, where they will be inoculated in culture media containing the residue to be treated, associated with controlled conditions of pH, temperature, agitation, and aeration (Farinas 2015). From this, it is possible to obtain the fragmentation of the residue infractions or to promote the removal of toxicity from contaminants. However, this process is generally used as a tool to obtain by-products, such as biofuels and enzymes, which have numerous applications in the most varied industrial sectors (Najafpour 2015).

Fermentation methods include submerged fermentation (SmF) and solid-state fermentation (SSF). SSF can be defined as that developed using solid substrates with low or no water content. Nevertheless, substrates require sufficient moisture to promote microbial growth and metabolic sustainability. This type of fermentation is probably the oldest used by man, with references dating from 1000 BC, when food was already being produced, using agricultural products (wheat, barley, soy) as a substrate (Soccol et al. 2017).

The SSF has advantages such as the use of substrates with low added value, reduced medium volume, reduced investment in bioreactors, direct inoculation, reduced contamination, and facilitated aeration. On the other hand, this type of fermentation presents restrictions regarding its application, which may be associated with the inefficiency of the growth of some microorganisms in systems with low humidity and difficulty in controlling the parameters. In addition, the difficulty of homogenization and diffusion in the reaction medium also present as process problems (Singhania et al. 2009; Martins et al. 2011).

The SmF consists of inoculating a microorganism (inoculum) in a liquid medium (must). This process uses fermenters supplied with several devices to monitor variables such as agitation and aeration, pH, temperature, and dissolved oxygen. Furthermore, in this type of medium the nutrients are dissolved in the liquid medium and are easily accessible for use by microorganisms. Compared with FMS, the submerged processes offer several advantages such as ease of handling and higher volumes of a medium; nutrient absorption and metabolic excretion are performed more efficiently, resulting in shorter fermentation time and, consequently, higher productivity (Fazenda et al. 2008; Hansen et al. 2015).

2.3 Enzymes (Definitions)

Industrial processes involving chemical reactions with polluting potential are present mostly in the manufacture of products or consumer goods by man. Most of these reactions are catalyzed by chemical agents that can be replaced by enzymes. The enzymes are capable of accelerating chemical processes, displaying great advantages over chemical catalysts since enzymes are (i) natural biological and biodegradable products, (ii) feature reaction specificities, (iii) implement nonconsumption during the process, (iv) catalyze chemical reactions by decreasing activation energy, (v) are stereoselective, and (vi) act at mild pH and temperatures. Therefore, enzymes become an environmentally viable solution (Sarrouh et al. 2012). Nowadays, several industries use enzymes as catalysts with emphasis on food, health, and environmental areas (Monteiro and Silva 2009).

Thereby, currently, there is a biotechnological challenge regarding the prospect of new enzyme sources and in improving the performance of those enzymes already known. Concomitantly, the study with enzyme-producing microorganisms these enzymes is constant by the application of molecular biology and genetic engineering tools aimed at microbial prospecting and development of genetically modified organisms. Notwithstanding, several problems impede this development, such as the difficulty of reproducing environmental conditions and the transfer condition from ab-scale to pilot plant-scale or industrial scale, associated with the low research-based industry (Monteiro and Silva 2009; Zhang et al. 2012; Fasim et al. 2021).

According to the International Union of Biochemistry and Molecular Biology, enzymes are classified into six classes based on the type of catalyzed reaction: (i) oxiredutases: catalyze oxidation-reduction or electron transfer reactions; (ii) transferases: transfer functional groups such as amine, phosphate, acyl, carboxyl, between molecules; (iii) hydrolases: catalyze covalent bond hydrolysis reactions; (iv) liases: catalyze addition and elimination reaction; (v) Isomerases: interconversion reactions between optical or geometric isomers; and (vi) ligases: condensation of two molecules, always with the aid of energy, usually ATP (Sarrouh et al. 2012).

2.3.1 World Market

Global enzyme trade is divided into two categories: special enzymes (therapeutic enzymes, diagnostic enzymes, enzymes for chiral chemistry, and enzymes for research) and industrial enzymes (mainly for food and animal feed industries). According to Business Standard, global enzyme demand has grown steadily at an annual rate of 4.5% and is expected to reach a value of US$ 7.2 billion in 2020, referring to the two categories of industrial application enzymes such as amylases, followed by cellulases and lipases. The Latin American market accounts for 3.4% of the enzyme worldwide demand, of which 60% of this consumption corresponds to Brazil. Nevertheless, Brazil represents a small part of the international market, importing 86% of enzymes while exporting only 14% of this input, which reveals technological and strategic backwardness (Valencia and Chambergo 2013; Daiha et al. 2016; Rodrigues et al. 2020).

The global enzyme market was valued at US$ 9.9 billion in 2019 with a compound annual growth rate (CAGR) of 7.1% for the period 2020 to 2027 (Arnau et al. 2020; Sharma and Upadhyay 2020). According to Research and Markets report, "Industrial Enzymes—Global Market Overview," the growing enzyme global market is by industrial demand from the food and beverage, biofuels, animal feed, and household cleaning sectors (Arnau et al. 2020; Market Analysis Report 2020).

The industrial enzymes applied in the area of food and beverages correspond to the largest segment of the market, absorbing a quarter of the total produced. It was estimated that the market would reach US$ 2.1 billion in 2016, a CAGR of 10.4%. However, it was observed that the projections did not occur as expected and had a 26% share equivalent to US$ 1.4 billion in 2017, evidencing a significant drop, which may be linked to the growth of other segments. In the context of industrial enzymes, it is also possible to highlight the production of biofuels and detergents with 18 and 14%, corresponding to US$ 969.3 and 754.4 million, respectively. However, the enzyme demand for the biofuel sector is growing rapidly compared to other segments with a compound annual growth rate of approximately 7.3% up to 2024.

Nowadays, the demand for enzymes grows rapidly due to the industrial environment transformation promoted by trends in the development of differentiated food and beverages to meet the consumer demand and acceptance attributed to changes in the population's lifestyles. Furthermore, world governments have encouraged biofuel use as a clean energy-based alternative, which has boosted the industrial enzyme markets. Thereby, biotechnological advances, particularly in the protein engineering area, have driven the biocatalyst sector to meet the enzyme demand for industrial application. Regarding technical enzymes, this category was responsible for the revenue of approximately US$ 1.2 billion in 2011, which corresponds to an annual growth rate of 8.2% (Comyns 2012; Arnau et al. 2020). This explains prospecting studies for enzymes more tolerant to pH and temperature variations to develop industrial processes since these variations directly influence the enzymatic activity

Table 2.2 Enzymes and their applications in several industrial processes

Industry	Enzyme	Application
Cleaning	Amylase	Removal of starch stain
	Cellulase	Cleaning, bleaching, anti-cotton deposition
	Lipases	Fat stain removal
	Protease	Removal of protein stains
	Ligninases	Removal of synthetic dyes
Foods and beverages	Amylase	Production of syrups, reduction of calories from beer
	Cellulase	Lightening of furrows, concentrates of coffee
	Lipases	Flavor to cheeses
	Protease	Milk coagulation, meat sweetening, brightening beer
	Ligninases	Degradation of waste from production
Cosmetics	Amylase	Anti-signs, free radical fighting
	Cellulase	Auxiliary digestion/carbohydrate
	Lipases	Deep cleansing of the skin, treatment of acne and dandruff
	Protease	Peeling, stretch marks, oil control, and seborrhea
Therapies	Amylase	Control of pancreatic juice deficiency
	Cellulase	Support digestion
	Lipases	Restore pancreatic enzymes and control digestion
	Protease	Auxiliary of digestion; debridement of ulcers/protease
	Ligninases	Antimicrobial

Adapted from Ole et al. (2002), Monteiro and Silva (2009)

efficiency and, consequently, its application (Shakeel and Husain 2012). Table 2.2 shows some enzymes of industrial interest and their applications.

The use of enzymatic engineering coupled with recombinant DNA technology and the heterologous expression of enzymes will also represent a new leap forward for enzyme production in the next decade and will be one of the basic points in the development of new enzyme-produced industrial products. Another very promising area for enzyme technology is environmental control to gradually replace chemical processes with enzymatic processes, so-called green technology, playing an important role mainly in industries that apply lignocellulosic raw material to transform it into higher value-added products (Ravindran et al. 2012; Sharma et al. 2018; Leite et al. 2021).

2.3.2 *Enzymes in the Food and Beverage Industry*

As previously mentioned, the food and beverage enzyme market is the largest industrial segment, with an estimated revenue of approximately US$ 2.0 billion up to 2020. Enzymes used in the food industry can be classified into food additives and auxiliary agents during processing which have a significant application in bakery, beverages, and cheeses sectors (Liu and Kokare 2017).

In the beverage industry, it is possible to highlight the use of enzymes in juices, wines, and beer production in order to reduce production costs, energy consumption, besides improving sensorial aspects of the product (Quang et al. 2014). Regarding juice production, a serious problem is the high pectin and starch concentration in ripened fruits, which generates turbidity and higher viscosity in the final product (Danalache et al. 2018). Thus, pectinases and amylases, naturally occurring enzymes, are responsible for the clarification step as well as enhancement of taste and texture, as well as xylanase and cellulase, which are applied to facilitate the release of the juice (Ephrem et al. 2018; Mushtaq 2018; Souza et al. 2020). Enzymes are also applied in wine and brewing processes to improve the raw material component extraction and yields. In addition, enzymes hydrolyze high-molecular-weight substances. This allows solving problems during the filtration process caused by the presence of β-glucans in the malt and controlling the maturation and storage process (Llaubères 2010; Du et al. 2013; Gibson and Newsham 2018).

Concerning the bakery industry, the focus is on the lipolytic enzyme application since studies have shown their use to replace or supplement conventional emulsifiers due to the enzyme's ability to degrade wheat lipids and favor their solubilization (Brites et al. 2018). In addition, there are enzymes such as α-amylases and xylanases that prevent the hardening of loaves, while lipases are related to the degradation of fats, oils, and related compounds, being applied in taste and fragrance control (Bock 2015; Fallahi et al. 2018).

In the dairy sector, enzymes such as proteases, lipases, and lactase are applied. The first stage of the milk coagulation process for cheese production is carried out with rennin, whereas lipases and proteases are added to accelerate cheese maturation (Sobrevilla et al. 2015). Furthermore, lactase hydrolyzes lactose into glucose and galactose, enhancing its solubility and sweetness in several dairy products and reducing the milk allergenic potential (Patel et al. 2017).

2.3.3 Bioremediation

The worldwide population associated with industrial activities has promoted waste accumulation whose polluting capacity has reached critical levels. We are currently interacting more and more with numerous persistent pollutants that are released from various manufacturers, such as industrial solvents, pesticides, heavy metals, petroleum products, dyes, fertilizers, and food additives. The accumulation and recalcitrance of most pollutants are responsible for a severe environmental impact, causing deleterious effects on human and animal health (Das and Dash 2014; Ghosh et al. 2017). Industrial and urban wastes are deposited in landfills or subjected to chemical and/or physical treatments, whose limited efficiency can generate secondary contaminants due to the complex nature of the compounds. Thus, bioremediation is the focus of studies as an ecological and economically sustainable technique to treat contaminated environments (Dzionek et al. 2016; Sharma et al. 2018).

Bioremediation is a strategy that applies the microbial metabolic capacity; therefore, their enzymes play an important role in the maintenance and sustainability of any ecosystem due to microbial ability to degrade or dissimilar toxic compounds transforming them into less toxic molecules to the environment. Thus, bioremediation represents an economical and ecologically friendly tool (Rao et al. 2010, 2014; Pande et al. 2020). In this context, bacteria, algae, fungi, and even plants have been studied to identify species that display xenobiotic-degrading capacity (Sutherland et al. 2004; Liu and Kokare 2017). The research contributes to the development of bioprocess to reduce pollutant accumulation and also obtain added-value substances. The bioremediation effectiveness depends on environmental conditions for the microorganism growth with a metabolic capacity to use the organic pollutant as a carbon source to transform it into CO_2, H_2O, ATP, and simpler molecules, which implies availability or accessibility of the contaminant for its microbial degradation (Khatoon et al. 2017; Sun et al. 2020).

Nonetheless, the use of microorganisms to degrade contaminants with the help is a lengthy process, which, in practice, can affect the bioremediation feasibility. Therefore, isolated enzymes have been used more for bioremediation compared to the direct application of microorganisms (Sharma et al. 2018). Overall, an enzymatic process basically depends on the catalytic activity of the enzyme, while microbial degradation requires an inductive environmental condition. However, microbial adaptability, versatility, and mutability represent an advantage for degrading different compounds (Vallero 2010).

The main classes of enzymes applied directly applied to degrade transform pollutants are oxidoreductases and hydrolases due to their dual function in degrading and protecting (Manubolu et al. 2018). Hydrolases have the ability to reduce complex substrates by transferring electrons from reducers to oxidants, facilitating their microbial metabolism. These enzymes work in cooperation with oxidoreductases which catalyze the breakdown of organic compounds into simpler molecules available for the transformation of the pollutant into CO_2, water, and ATP (Sharma et al. 2018).

2.3.4 Biosensors

A biosensor is an analytical device which may contain biological structures origin, such as antibodies, cells, or enzymes, which are associated with a transducer to convert a biological signal into an electrical signal directly proportional to the target analyte to be monitored (Gianfreda et al. 2016). A biosensor consists of three elements, a bioreactor, a transducer, which can be electrochemical, optical, piezoelectric, and thermometric, as well as a signal processor (Muguruma 2017). Regarding enzymatic biosensors, their initial construction applied immobilization methods by adsorption of enzymes to the support through van der Waals forces, ionic or covalent binding (Mehrotra 2016).

Considering their purpose, biosensors can be highly selective due to the interaction or affinity of the target compound with the sensor substrate. In addition, they offer advantages such as reagent-free tests, quick and preferably reversible responses. To design a biosensor, stability, analysis time, selectivity, and analytical signal are parameters that must be considered (Davis and Higson 2012).

Transducer surfaces are often modified to increase enzyme stability and analytical sensitivity, while this change in electrochemical transducers aims to reduce the sensor's interaction with other analytes (Pacheco et al. 2017). In addition, enzyme immobilization on the surface of the transducer is a factor that ensures long-term performance and stability improvement (Rao et al. 2014). The biosensor manufacture requires the production of its materials, transduction devices, and immobilization methods, representing a multidisciplinary area, involving chemistry, biology, and engineering. The biosensor materials are categorized based on their mechanisms: biocatalytic group, bio-affinity group, and microorganisms. Therefore, biosensors are extensively researched and developed as an application tool in the medical, environmental, food, and pharmaceutical fields (Muguruma 2017).

In the medical field, the biosensor application is growing rapidly, mainly for diabetes mellitus diagnosis and blood glycemic control, which represents 85% of the world market in this segment. Furthermore, biosensors are widely used in the diagnosis of infectious diseases, quantitative determination of cardiac markers, and rapid and accurate detection of cancer markers as well as immunosensor for clinical identification of acute leukemias (Karunakaran et al. 2015).

Concerning the food industry, one of the main requirements is related to quality, safety, maintenance, and processing of the raw material to obtain the final product. This evaluation is performed by chemical and physical traditional techniques, although displaying due to human failures, cost, and long response time. Therefore, the development of biosensors for the food sector emerges as a promising alternative to meet commercial demand since sensors provide simple, selective, and real-time responses (Gao and Lu 2015; Bahadır and Sezgintürk 2017). The use of biosensors allows you to monitor the aging of foods and detect pathogens and pesticides (Pacheco et al. 2017). It is also possible to develop sensors to control parameters such as appearance, taste, smell, texture, and even nutrients during the manufacturing process (Amine et al. 2016).

In the environmental area, the application of biosensors is interesting, since they present important characteristics when environmental monitoring is desired, such as portability, low cost, minimal sample preparation, and stability of the instrument in front of the numerous obstacles in this field. Biosensors in this segment are generally applied to soil and water monitoring and control, identifying the presence of pesticides and heavy metals, proven to be toxic and carcinogenic compounds, which are linked to agribusiness, mining, chemical, cosmetics, and paint industries (Amine et al. 2016).

2.4 Conclusion

The chemical composition of lignocellulosic residues, formed by cellulose, hemicellulose, and lignin, as well as its renewable nature, show the potential of this material to produce value-added compounds as low-cost enzymes for use in various industrial sectors. Hydrolases and oxidoreductases are obtained from fermentation using lignocellulosic biomass as input and, during the previous fermentation process, the choice of the biomass treatment type is extremely important for obtaining these enzymes. These enzymes are required in biotechnological processes applied mainly in the areas of bioremediation, food and beverage industry, paper bleaching, and biosensor construction. Thus, the use of lignocellulosic residues as a source of convertible molecules is promising not only for obtaining enzymes but also for other compounds of industrial interest.

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Chapter 3
Pleurotus-Derived Laccases, Immobilization, and Bioremediation Applications

Sadia Aslam and Muhammad Bilal

Abstract Enzymes offer a huge potential in improving existing industrial bioprocesses and establishing new procedures for achieving high-value products. They contribute to environmental sustainability by providing more efficient, cleaner, and greener industrial processes. Laccases are a copper-containing versatile class of oxidases that possess multifaceted applications in environmental remediation and many other biotechnological and industrial areas. Increasing research efforts have been directed in the last decade to exploit these fascinating biocatalysts in various fields, such as lignocellulose biotechnology, biotransformation of dye pollutants, pharmaceutical compounds, enzyme-mediated biosensors, or biofuel cells. This chapter outlines comprehensive information on lignocellulosic biomass, white-rot fungi (*Pleurotus* strains), and their enzyme extracts, purification, immobilization, and their applications for bioremediation of textile dyes and bio-stoning of dyed jeans. It focuses on an overview of the distinguished attributes and mechanism of action of the laccase enzyme. Besides, their applications in dye degradation and denim stone are revealed.

Keywords Laccase · *Pleurotus* · Immobilization · Textile dyes · Degradation

3.1 Introduction

The existence of enzymes in different processes is associated with the ancient work of Greece who used microbial origin enzymes for cheese making, brewing, baking, and alcohol production, which have been well-known since ancient times (Haki and Rakshit 2003; Sarrouh et al. 2012). Fermentation technology is in trend to produce a variety of enzymes by the involvement of different microbes followed by the use of

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different bacteria and fungi under stringent circumstances. In order to produce a variety of different enzymes criteria used involves the different strategic points followed by excerption of relevant enzyme, best strain, and innovative technology, which involve the overproduction by genetic engineering as well as the selection of optimized conditions for relevant culture media and its recovery (Leisola et al. 2002; Sarrouh et al. 2012).

All ligninolytic enzymes have enormous environmental, textile, biotechnological, and pharmaceutical applications due to their hidden biotechnical potential (Morsi et al. 2020; Bilal et al. 2019; Bilal et al. 2020a, 2020b). Laccases are glycoproteins in nature having molecular mass (MW) from 60 to 80 KDa with isoelectric point 3–6 (pI) (Isroi et al. 2011). Laccases enhance the conversion of phenoxyl radicals to the ketone, demethoxylation, cleavage of carbon-carbon in phenolic structures. Oxidation of non-phenolic structures of lignin is also carried out by concomitant action of laccases and exotic mediators. Enzymatic degradation of plant biomass is afflicted with different architectural features of biomass including complex constituents of celluloses, lignin, hemicelluloses, and acetyl groups.

Laccase was first revealed by Yoshida in 1883 during his work on latex from the Japanese lacquer tree *Rhus Vernicifera*, and it was named as a fungal enzyme in 1896 by Bertrand and Laborde (Viswanath et al. 2014). Laccases were discovered in some higher plant species, insects, some bacteria, and fungi (Dittmer et al. 2004). WRFs are the highest laccase enzyme inducers, but these are also secreted by ectomycorrhizal and litter decomposing fungi (Chaurasia et al. 2013). Fungal laccases have a great contribution to the development of fruiting bodies, sporulation, plant pathogenesis, and lignin degradation as well as pigment production (Mayer and Staples 2002). Laccases are responsible for the degradation of organic pollutants including organochlorines, 2, polyaromatic hydrocarbons, and different wood parts (Pointing 2001).

Laccase enzyme used in paper and pulp, food and textile industry where high redox potential of fungal laccases contributes to their extensive application in biotechnology (Maciel and Ribeiro 2010; Mohammadian et al. 2010) as well as their special role in food processing including the stabilization of fruit juices (Asad et al. 2012; Ramachandran et al. 2013). There is still a huge margin of finding different research strategies to produce laccase enzymes through a biotechnological perspective due to its extensive and unique applications in many fields as well as laccase enzyme purification and immobilization (Azevedo et al. 2012).

3.2 Lignocellulosic Biomass and Compositional Aspects

Organic material from stems of plants, agricultural crops, shrubs, and trees is generally referred to as lignocellulosic plant biomass. Biomass is an excellent renewable resource used to generate many beneficial products (Yang et al. 2015; Asgher et al. 2016). A sufficient amount of organic waste material with suitable composition is supplied by agro-industrial, urban, and industrial waste residues

(Weland 2006; Prasad et al. 2007; Liguori et al. 2013). A rapidly growing interest in lignocellulosic biomass is because of its minimal cost, renewable and nontoxic nature (Asgher et al. 2013; Kalia et al. 2014). A large amount of lignocellulosic material can be molded into many useful bioproducts (Isroi et al. 2011; Iqbal et al. 2013; Bilal and Iqbal 2020a). Green biotechnology associated with lignocellulosic biomass has gone through many improvements over the many years (Asgher et al. 2013; Anwar et al. 2014).

Pretreatment of lignocellulosic biomass is a crucial step before its conversion into useful products. Methods of pretreatment include chemical, biological methods, and enzymatic degradation (Sills and Gossett 2011; Xiao et al. 2012; Abdulkhani et al. 2013). The biorefinery concept to make biochemicals from renewable energy sources is good to overcome the increasing demand for value-added products. The main purpose of the biorefinery concept is to generate energy and useful chemicals from lignocellulosic biomass by different industrial sectors (Iqbal et al. 2013; Bilal and Iqbal 2020b; Xia et al. 2019).

Lignin, cellulose, and hemicelluloses collectively comprise 75% of lignocellulosic material including high-molecular-weight polymers too (Abril and Abril 2009). Complex polysaccharides can be converted into simple sugars by hydrolysis, but their digestibility is limited by many factors including the complex composition of lignin and its degree of polymerization (Fig. 3.1). Moreover, acetyl groups bonded to hemicelluloses, surface area, pore-volume, and particle size of biomass also create hurdles in their conversion into the simplest sugars (Alvira et al. 2010; Isroi et al. 2011).

Hemicelluloses are polymers made up of pentoses and hexoses with repeated patterns, and cellulose is a structural component which provides mechanical strength to plant cell walls (Calvo-Flores and Dobado 2010). The composition of all these lignocellulosic components varies due to genetic variations in different sources (Monsalve et al. 2006; Bertero et al. 2012; Iqbal et al. 2013). Being a renewable source of energy lignocellulosic biomass utilization for its potential biotechnical exploitation involves different pretreatment methods including mechanical, physical, biological, and chemical (Mosier et al. 2005; Hendriks and Zeeman 2009; Isroi et al. 2011; Iqbal et al. 2013; Anwar et al. 2014). The most attention has been diverted towards biological methods despite physical and chemical pretreatment methods because of several advantages including high substrate specificity, high yield, and low pollution (Kirk and Chang 1981; Isroi et al. 2011).

Interest has been developed towards the investigation of biological methods for effective utilization of biomass and the development of environment-friendly processes (Yu et al. 2009; Dias et al. 2010; Taniguchi et al. 2010; Isroi et al. 2011). Lignocellulosic waste materials are being used for enzyme production because they are most attractive to be used as economical renewable and natural resources crucial to the functioning of the industrial society. These agricultural waste materials are mostly generated as byproducts of crop production (Pérez et al. 2002). Lignocellulosic biomass has hidden potential to be convertible into useful products. Its renewable nature has drawn attention towards its constructive utilization instead of its

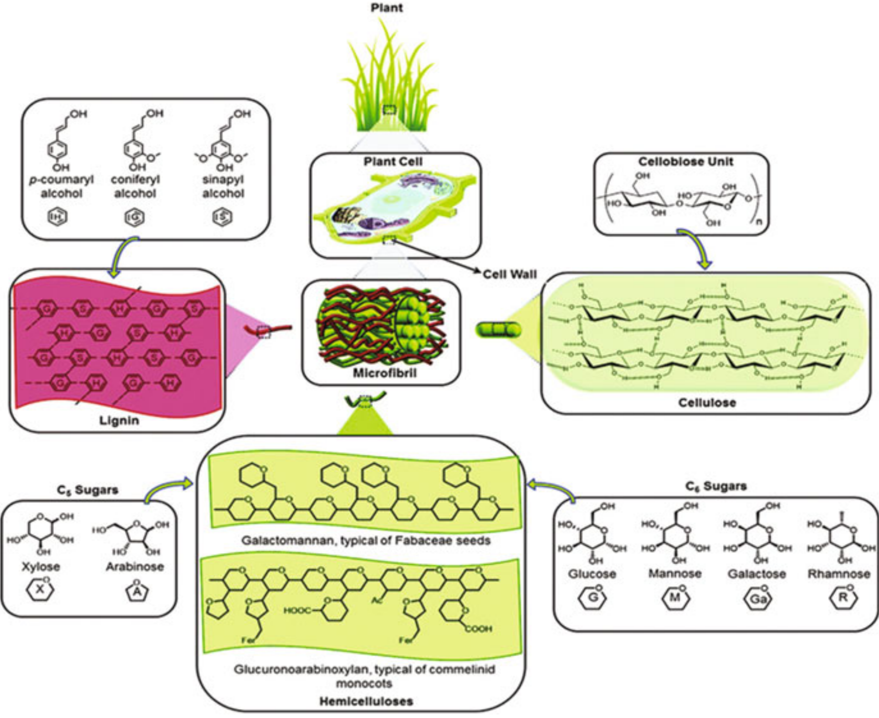

Fig. 3.1 The main components and structure of lignocellulose. (Isikgor and Becer 2015)

disposal by biomass burning and landfilling that is not limited to developing countries as well (Iqbal et al. 2013; Ofori-Boateng and Lee 2013; Anwar et al. 2014).

3.3 WRFs and Enzyme System

Fungi are eukaryotic, spore-forming microorganisms having the ability to degrade lignin and cellulose by forming special fruiting bodies on dead trees by the attractive developmental process (Batool et al. 2013). White-rot fungi have the ability to decompose all complex components of plant biomass like lignin, cellulose, and hemicelluloses by leaving the wood with a white powdery appearance. The ability of white rot basidiomycetes to degrade lignin depends on the fungal species and environmental conditions (Isroi et al. 2011; More et al. 2011; Batool et al. 2013).

White rot fungi alter the chemical and physical properties of lignocellulosic biomass by a nonspecific oxidative process. The exotic enzymatic pattern of white-rot fungi is also responsible for the bioremediation of industrial dyes (Songulashvili et al. 2007). Many species of white-rot fungi including *Pleurotus ostreatus, Shyzophyllum commune, Pleurotus eryngii, Phanerochaete*

Chrysosporium, Ganoderma lucidum, Trametes versicolor, Iprex lacteus, Fomes fomentarius, phelebia radiate, and *Dichomitus* have been reported as best lignocellulose degraders (Hatakka 2001; Patel et al. 2009a, b; Isroi et al. 2011). White rot fungi use hardwood including aspen and birch for their growth. While other species including *Heterobasidium* and *Phellimus pini* prefer softwoods like pine and spruce (Blanchette 1995; Isroi et al. 2011). Selective and nonselective decay regarding degradation of lignin by white-rot fungi is dependent on a specific source of lignocellulosic species. White rot fungi possess a selective and a nonselective mode of action. The large polymer of lignin, its complex bonding arrangement, and stereo irregularity pattern affect the white-rot fungi potential to degrade lignin (Wong 2009).

Research is still in progress to explore new fungal strains for their exquisite potential to produce ligninolytic enzymes. White rot fungi make use of their enzymatic and nonenzymatic mechanisms to degrade all lignocellulosic components of plant biomass (Fackler et al. 2007; Singh and Singh 2014). *Pleurotus* mushrooms are healthy foods rich in vitamins, chitin, minerals, and proteins and lower in fat. The extracts of *Pleurotus* species have been found effective in the protection of the brain, heart, and liver of aged rats. Antioxidant properties of *Pleurotus sajor caju* have also been investigated (Chirinang and Intarapichet 2009).

3.4 The Genus *Pleurotus*: A Universal Group of Ligninolytic Fungi

Pleurotus is a genus of gilled mushrooms that belongs to phylum Basidiomycota which includes many species of edible mushrooms like *Pleurotus ostreatus*. Species of *Pleurotus* are also known as oyster mushrooms (OM) because they produce oyster-shaped basidiocarps (Patel et al. 2012; Abdulmalk 2013). *Pleurotus* species may be stalked or sessile, white to variously colored, above or underground. *Pleurotus* species easily grow on different types of lignocellulosic substrates by producing oyster mushrooms rich in minerals, vitamins, and proteins with a low amount of sugar and extremely low cholesterol (Patel et al. 2012). The genus *Pleurotus* is a universal group of ligninolytic fungi with high nutritional value and many biotechnological applications (Cohen 2002; Bettin et al. 2011).

3.4.1 Pleurotus Nebrodensis

Pleurotus nebrodensis (Fig. 3.2a) was first discovered in 1866 by an Italian Botanist Inzenga. The older name of this mushroom was *Agaricus nembrodensis*, and it was well-known for its delicious taste (Estrada and Roysa 2008). It was declared as a critically endangered species because of its less production due to the lack of its

Fig. 3.2 Representation of (**a**) *Pleurotus nebrodensis*, (**b**) Pleurotus eryngii, (**c**) Pleurotus sajor, caju, and (**d**) Pleurotus sapidus

natural habitat (Venturella 2006). The cultivated forms of this fungus are known for their good flavor and aroma. Research is still in progress on *Pleurotus nebrodensis* for the exploration of its medicinal potential and properties. The antioxidant potential of *P. nebrodensis* has also been evaluated by using fungal fruiting bodies (Alam et al. 2011). Up till now, about 70 species of *Pleurotus* have been reported. While work on the discovery of new species has been in progress (Kong 2004). *Pleurotus nebrodensis* has the ability to produce ligninolytic enzymes. Cotton seeds, maize cobs, and sawdust are mostly used for their cultivation. It also improves the physiological activity of *Pleurotus nebrodensis* (Hu et al. 2014). *Pleurotus nebrodensis* produce laccase as well as peroxidase enzymes by utilizing the agro-industrial waste products in the fermentation process (Tellez-Tellez et al. 2013).

3.4.2 Pleurotus Eryngii

It is also known as king oyster mushroom (Fig. 3.2b), which is edible and saprophytic (Moonmoon et al. 2010). The phylogenetic relation of *Pleurotus eryngii* species with different taxa was studied by complex molecular surveys followed by gene isolation, cloning, and DNA sequencing (Rana et al. 2013). The yield and growth of *Pleurotus eryngii* are affected due to differences in the genotype of

different strains and the varying nature of the substrates (Moonmoon et al. 2010). The cultivation of *Pleurotus eryngii* at the commercial level has gained interest because of its exotic texture and excellent flavor. Versatile peroxidase was first discovered in *Pleurotus eryngii* on a straw-based culture medium. The first versatile peroxidase was isolated from *Pleurotus eryngii* by the cloning method (Camarero et al. 2001). The *Pleurotus eryngii* has also the potential to utilize lignocellulosic waste material by secretion of extracellular enzymes. Moreover, mushroom production by using lignocellulosic biomass LB is an economical way for its better accommodation to avoid environmental pollution (Carlile et al. 2001). *Pleurotus eryngii* have been investigated for their potential to produce ligninolytic enzymes through solid-state fermentation (Akpinar and Urek 2014). Toxic chemicals are being degraded by *Pleurotus eryngii* using different fermentation types.

3.4.3 Pleurotus Sajor Caju

Mushrooms are a great source of protein and medicine, and it is also used in many dishes including pizza. *Pleurotus sajor caju* (Fig. 3.2c) is an edible mushroom. It can be grown on agricultural waste material because of its ability to work under diversified climatic conditions (Asghar et al. 2007). *Pleurotus sajor caju* potentially converts the banana stalk substrates into useful products (de Siqueira et al. 2011). Amino acid production from *Pleurotus sajor caju* has also been reported. *Pleurotus sajor caju* has been grown on various agro-industrial wastes including wheat straw, sunflower stalk to determine their ability for yield enhancement and biological effectiveness (Patil 2012; Pala et al. 2012; Survase 2012). The antimicrobial activity of *Pleurotus sajor caju* has been investigated followed by the formation of silver nanoparticles (Nithya and Ragunathan 2009).

3.4.4 Pleurotus Sapidus

Pleurotus sapidus is also known as phoenix mushroom (Fig. 3.2d), and it resembles some extent with *Pleurotus ostreatus*. It is widely distributed in tropical and subtropical regions (Kong 2004). *Pleurotus sapidus* can degrade lignin to produce biotechnologically important products. Lignin degrading enzymes have been reported by the cultivation of *Pleurotus sapidus* on different lignocellulosic substrates during submerged fermentation (Salem 2014). It has the ability to degrade β-carotene by producing a versatile peroxidase enzyme (Hammel and Cullen 2008). The heterologous expression of versatile peroxidase enzyme by *Pleurotus sapidus* has been studied recently (Schüttmann et al. 2014). The studies on different characteristics of *Pleurotus ostreatus* and *Pleurotus sapidus* are still in progress using polymerase chain reaction techniques (PCR) which serve as molecular identification tools (Abdulmalk 2013).

3.5 Ligninolytic Enzymes of *Pleurotus* Species

Ligninolytic enzymes belong to a group of extracellular enzymes that are oxidative in nature and produced as a result of fungal secondary metabolism. The products produced during secondary metabolism are species-specific (Patrick et al. 2014). Ligninolytic enzyme production is a rapidly growing field of biotechnology. These enzymes change the whole composition of the lignocellulosic substrate resulting in the good growth and development of mushrooms followed by conversion of high-molecular-weight polymers which are then used as energy and nutrient sources essential for the enlargement of mushroom fruiting bodies (Kurt and Buyukalaca 2010).

Ligninolytic enzymes are crucial to the development and growth of mushrooms (Kuforiji and Fasidi 2008; Patrick et al. 2014). A new group of heme-containing versatile peroxidase has also been discovered and first isolated from *Pleurotus ostreatus* and *Pleurotus eryngii* (Cohen 2002). Lignin degrading enzymes are oxidative in nature responsible for the breakdown of the complex aromatic structure of lignin. The ligninolytic enzymes use H_2O_2 and different mediators for their catalytic activities.

3.6 Laccase

Laccases are the most efficient group of multicopper blue oxidase responsible for monoelectronic oxidation of phenolic subcomponents of lignin and aromatic amines. Laccases are N-glycosylated heme-containing glycoproteins having a molecular mass of ~50–95 KDa (Bagewadi et al. 2017) distinguished by the presence of three cupredoxin domains similar to that of ascorbate oxidase (Hatakka 2001). For several decades, the function and activity of laccase have been extensively investigated. Many processes relevant to the formation of fruiting bodies, sporulation, pigments synthesis, and lignin degradation are efficiently contributed by fungal laccases (Mayer and Staples 2002). Phenoxyl radicals formation is accompanied by laccases catalytic mechanism (Fig. 3.3) which leads to alkyl aryl cleavage, demethylation, carbon α hydroxyl oxides to the ketone, and cleavage of Cα- Cβ in phenolic subcomponents of lignin. Non-phenolic components of lignin are also degraded by laccases.

3.7 Laccase Enzyme Production by Different Strains of *Pleurotus*

The constitutive production of laccase by many fungi has been reported (Lomascolo et al. 2003). *Pleurotus* species can be grown on various lignocellulosic substrates which generate as a result of agricultural practices. Lignocellulosic biomass

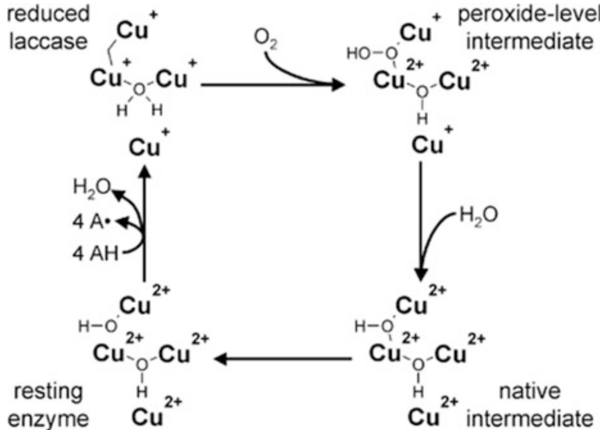

Fig. 3.3 The catalytic cycle of laccases. (Reproduced with permission from Wesenberg et al. 2003)

degradation is affected by many factors including different fungal strains, sources, and concentration of carbon and nitrogen, moisture content, pH, temperature, aeration, and the addition of different metal ions including Cu^{2+} and Mn^{2+}. All three factors regulate the activity of white-rot fungi. All the physical factors control the mechanism of lignin degradation simultaneously or selectively during the fermentation process (Isroi et al. 2011). These factors should be essentially considered to get maximum production of ligninolytic enzymes and for enhanced degradation of lignin.

Pleurotus sajor caju P1–27 has been reported best MnP and laccase enzyme producer when cultivated on a defined medium supplemented with glucose (Rivela et al. 2000). To select the appropriate and right isolate with maximum ligninolytic enzyme activity, screening is a must among a large variety of fungal isolates. Moreover, substrate selection and nitrogen concentration are also important factors. The nitrogen concentration effect also varies according to species and strains of white-rot fungi (Dorado et al. 2001).

The variation in nitrogen metabolism is the main cause of variable responses among the different strains. The variation in behavior of different *Pleurotus* species has been reported with the supplementation of different nitrogen sources. The maximum peroxidase activity was found in the case of *Pleurotus ostreatus* culture supplemented with peptone (Stajić et al. 2006). Moisture is also an important factor that affects the degradation of lignin by affecting fungal growth (Shi et al. 2008). Increased laccase production by *Pleurotus pulmonarius* was observed with the initial increase in moisture level (Patel et al. 2009a, 2009b). Fungal physiology is also affected by temperature (Patel et al. 2009a, 2009b).

SSF is preferred on SmF due to the use of cheaper raw materials and easily available agricultural waste materials. SSF gives a greater yield and good quality as compared to submerged fermentation SmF. The use of lower water content in SSF makes it economical because of small fermenter size, lessened downstream processing, and minimum sterilization costs (Raghavarao et al. 2003). Many

Table 3.1 Comparison of solid-state and liquid state fermentation for enzyme production

Solid-state fermentation (SSF)	Liquid-state fermentation (LSF)
The culture media are simple. Some substrates can be used directly as solid media or enriched with nutrients	Composition of medium is slightly expensive and additional nutrients are required for enhanced growth
The used inoculum is the natural flora of the substrates, spores, or cells. The inoculation with spores facilitates its uniform dispersion through the medium	Less inoculum is required and it may be in the form of aqueous spore suspension to generate free water culture
The low humidity content and greater inoculum used in SSF reduce vastly the possibility of microbial contamination. This allows working in aseptic conditions	A large amount of water is required which increases the chances of contamination
Higher levels of aeration are required, especially adequate in those processes demanding an intensive oxidative metabolism	No problem with aeration due to continuous shaker and use of probes
Solid-state fermentation is characterized by low energy requirement which reduces the production cost at an industrial level as autoclaving, or vapor treatment, mechanical agitation, and aeration are not often necessary in some cases	SmF is costly due to higher energy requirements and expensive chemicals. Moreover, mechanical shaking varies and a high amount of moisture is necessary
The products obtained in SSF are more thermotolerant than their counterparts obtained in SmF	–

disadvantages are associated with solid-state fermentation like difficulty during the scale-up process but the work is in progress to overcome these problems by the improvement of bioreactors. SSF has a unique potential to produce many compounds from natural sources. Solid-state fermentation (SSF) provides an environment-friendly substitute for the usage of agro-industrial wastes. Table 3.1 illustrates the comparison of solid-state and liquid-state fermentation for enzyme production.

The optimization of fermentation parameters using response surface methodology results in an enhanced yield of enzymes (Zhang et al. 2012) and is a better strategy as compared to the classical method. Pratheebaa et al. (2013) observed enhanced laccase enzyme production by *Pleurotus ostreatus* using response surface methodology. Response surface methodology has also been applied for the optimization of different physical parameters (Niladevi and Prema 2007).

Solid-state fermentation and submerged fermentation are biotechnologically important processes mainly concerned with the production of valuable products including organic acids, vitamins, biofuels, and biopesticides. Lower production of laccase enzyme was observed in submerged fermentation of *Streptomyces chartreusis* due to the presence of strong proteolytic activity (Chhaya and Modi 2013). The metabolic divergence among these two fermentation technologies has a blunt effect on fungal growth and physiology (Hölker et al. 2004).

3.8 Purification of Laccase Enzyme

A number of laccase isozymes have been reported from different *Pleurotus* species. Mansur et al. (2003) purified the four laccase isozymes from the strain of *Pleurotus ostreatus* and observed the difference in their elution properties and their mobilities on SDS- PAGE. Laccase I and laccase II were reported to have a molecular mass of 60 and 65 KDa, while laccase 3 and laccase 4 having molecular weights around about 80 and 82 KDa. Purification of laccase enzyme from culture filtrates is carried out by series of multiple steps including ultrafiltration, precipitation by using organic solvents (Baldrain 2006). Laccase enzyme has also been produced and purified from *Ganoderma* species (Sivakumar et al. 2010). Purification of laccase enzyme from *Neurospora crassa* by celite chromatography showed an increase in specific activity (Shekher et al. 2011). Various methods of purification including ethanol precipitation, Sephadex G 100, phenyl sepharose, and DEAE- sepharose have also been adopted for purification of laccase enzyme by *Trametes versicolor* (Hess et al. 2002). Purification of laccase from fruiting bodies of different fungal strains has also been reported (Khammuang and Sarnthima 2009). The laccase enzyme produced from marine fungus *Trematosphaeria mangrovei* showed improved enzyme characteristics after purification. The laccase enzyme isolated from a mushroom *Hypsizygus ulmarius* exhibited a molecular mass of 63 KDa followed by different purification steps (Ravikumar et al. 2012). Laccase enzyme isolated from *Funalia trogii* purified chromatographically showed a molecular weight of 58KDa on SDS-PAGE (Patrick et al. 2014).

3.9 Enzyme Immobilization

The enzymes are immobilized to avoid thermal instability, high sensitivity of the free enzyme to denaturing agents, the susceptibility of the free enzyme to attack by proteases, activity inhibition, and difficulty in separation and reuse of free catalyst at the end of reaction from its reaction mixture (Khan et al. 2006; Bilal et al. 2015; Zhang et al. 2020; Gan et al. 2020). Enzymes can be immobilized by physical methods as well as by chemical methods that involve covalent bond formation with the enzyme (Kirk and Christensen 2002; Sheldon 2007). There are many methods of enzyme immobilization like adsorption, inclusion or entrapment, and microencapsulation. The physical method belongs to the containment of enzymes in a membrane reactor. Adsorption of the enzyme may be physical or ionic on a water-insoluble matrix. New designs and strategies regarding immobilization support material are under research as well as surface characteristics and structure of target enzymes have been worked out (Krajewska 2009; Bilal et al. 2018a; Adeel et al. 2018).

Immobilized enzymes are thermostable and reusable. New immobilization techniques are also under consideration like microwave irradiation technology, single

enzyme nanoparticles, photo immobilization technology, enzymatic immobilization of enzyme, and multistep immobilization (Parmar et al. 2000; Kumar and Nahar 2007; Zhou 2009; Hegedűs and Nagy 2009). The benefit of laccase immobilization is an increase in its thermostability and resistivity to chemical reagents and extreme conditions. Entrapment is an easy method without altering the enzyme structure (Brady and Jordaan 2009). While microencapsulation is the confinement of bioactive agents to core micro-size spheres made up of semipermeable material. Different semipermeable membranes like polyethyleneimine and inorganic material like silica surround the microencapsulated laccase enzyme (Rochefort et al. 2008). The covalent binding method of immobilization has been the most commonly used method. Currently, a new variety of self-immobilization methods have been developed recognized as spherical catalytic macroparticles or sphere enzymes.

3.10 Immobilization of Laccase

Different techniques are used for enzyme immobilization including adsorption, covalent binding, entrapment, and affinity immobilization. Different materials are also used for immobilization including natural polymers, inorganic materials, and organic supports (Datta et al. 2013; Bilal et al. 2020c, 2020d). Moreover, the immobilization of laccase from *Pleurotus ostreatus* using the sol-gel entrapment method showed good decolorization ability (Asgher et al. 2012). The improved stability and reusability of laccase immobilized on silica SiO_2 nanoparticles have been reported by Patel et al. 2014. Studies on the preparation of cross-linked enzyme crystals of laccase showed good thermal stability than the native enzyme in the organic solvents (Roy and Abraham 2006). Immobilization by using chitosan as support material has been performed to immobilize laccase on chitosan beads (Biró et al. 2008). Surface adsorption is not as effective as the adsorption entrapment technique (Otero et al. 2008). Improvement in thermal and operational stability of laccase has been observed by its adsorption on porous supports (Wang et al. 2008). A number of studies on sodium alginate beads reported that it is an ideal matrix for the immobilization of laccase enzyme (Duran and Esposito 2000). Novel biocatalytic attributes of enzymes with an industrial employment perspective are portrayed in Fig. 3.4 (Bilal et al. 2018b).

3.11 Bioremediation of Dyes

Dyes are made up of carcinogens like aromatic compounds and benzidine, so the recent concern is growing towards effective and economic removal of these carcinogenic compounds from wastewater generated by textile industries. So effective solutions regarding the use of laccases seem an excellent strategy for degradation of complex structure dyes including synthetic dyes too (Rodriguez et al. 2006).

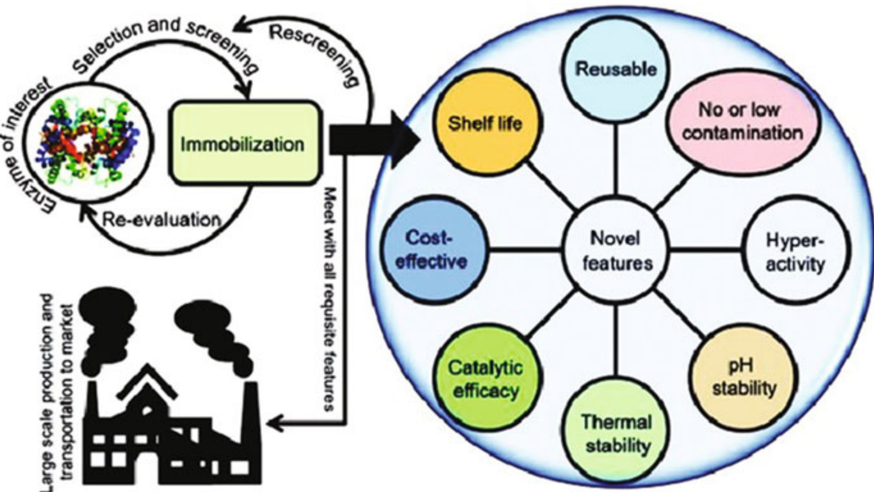

Fig. 3.4 A schematic representation of enzyme screening to immobilization with novel biocatalytic attributes. (Bilal et al. 2018b)

Biological decolorization of dyes by using different microorganisms is cost-effective. About 10% of dyes solution enter the environment by different water channels. The capability of laccase to degrade chromophore compounds like triaryl methane, indigo dyes, anthraquinone compounds, and azo dyes suggests its application for the industrial dye decolorization process (Subramanian et al. 2014).

One of the major environmental problems includes the contamination of water, soil, and air by toxic chemicals mostly generated through textile dyeing industries which produces wastewater in large volumes by consuming a large amount of water from the different steps of dyeing and finishing processes, and this wastewater contains color, residues of reactive dyes, chemicals, and different complex compounds including alkaline and acidic contaminants. These dyes consist of heterocyclic and aromatic compounds which are difficult to degrade and a continuous source of pollution (Shaolan et al. 2010). Decolorization of different synthetic dyes including Congo red, methyl green, methylene blue, pink, and toluidine blue by using laccase from different bacterial strains have been reported also (Romero et al. 2006).

Laccases are also being used for dechlorination processes (Kolankaya and Unal 2001). A laccase obtained from *Pleurotus ostreatus* showed less degradation of 3-hydroxy biphenyl as compared to 4-hydroxy analogues (Keum and Li 2004). Laccase of *Trametes villosa* proved efficient for remediation of soil by the degradation of 2,4, dichlorophenol (Ahn et al. 2002). Removal of aromatic and phenolic amines from water has been reported by the application of laccase enzyme followed by enzymatic oxidation of pollutants to free radical and quinones (Niku-Paavola and Viikari 2000). Laccases from *Clavariopsis aquatica* efficiently degrade xenoestrogen nonylphenol (Viswanath et al. 2014).

3.12 Bio-Stoning of Denim Jeans

Laccase enzymes are also under consideration for their use as an effective agent for producing stone washing effects on denim fabric in the presence or absence of mediators (Pazarlıoğlu et al. 2005). Denim is heavy grade cotton with adsorbed dye on the surface of fibers and due to this reason, a fading effect can be produced without loss of its strength. Cellulases can be used to reduce the load of pumice stones which have a corrosive effect on the surface of fiber by producing a stone-washed look. Laccases are also used to bleach indigo-dyed denim fabric to shade for bio-stoning (Campos et al. 2001). A large amount of back staining, the excessive requirement of pumice stones, and the wear and tear effect of the machine provide a stimulus for the utilization of laccase and cellulase enzymes instead of pumice stones (Pazarlıoğlu et al. 2005; Mojsov 2011). A small amount of enzyme is enough to replace kilograms of pumice stones. Bio-stoning was first introduced in 1984 in Europe; then it was adopted in the USA. Moreover, bio-stoning is an environment- and eco-friendly and most friendly process.

Laccases are also being used for the bleaching of dyed denim fabric to produce high shades without any harm to fabric strength and weight (Chatha et al. 2011). Cleaner production of denim jeans has also been reported by applying one-step enzymatic treatment with different enzymes from different microorganisms including laccases, amylases, and cellulases (Maryan and Montazer 2013). Degradation of indigo on fabric has been reported from laccases of *Sclerotium roflsii* and *Polyporus sp*. (Campos et al. 2001; Pazarlıoğlu et al. 2005). Figure 3.5 shows the laccase-catalyzed mechanism for denim bio-stoning.

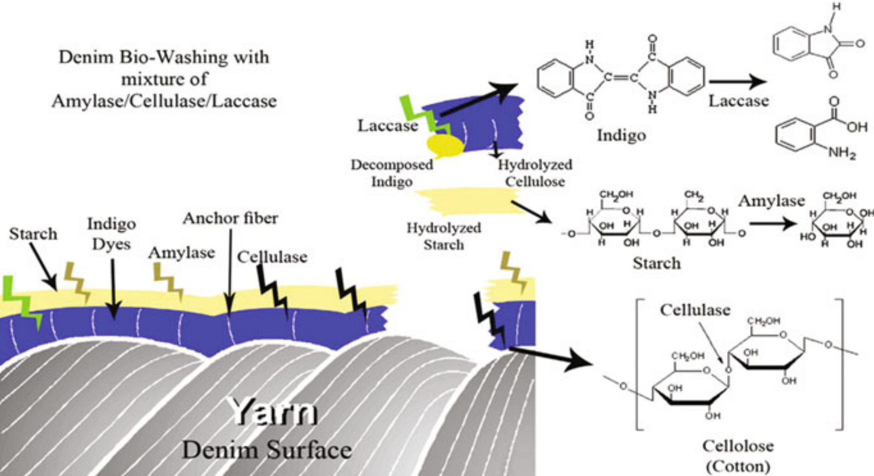

Fig. 3.5 Mechanism of denim bio-stoning. (Maryan and Montazer 2013)

3.13 Conclusion and Future Perspectives

Biotechnological advancement and crucial implication of the laccase enzyme have led to increased demand for this enzyme. The benefit of laccase immobilization is an increase in its thermostability and resistivity to chemical reagents and extreme conditions. Moreover, immobilized laccases can be easily obtained from reaction products. New designs and strategies regarding immobilization support material are still under research, and there is a tremendous amount of work yet to be done before industrial application. Easy separation of the enzyme from product and reusability of the enzyme are these two most considerable benefits which stimulated the interest in synthesis and preparation of immobilized enzymes.

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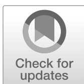
Chapter 4
Microbial Lipases for Polyester Degradation



Misbah Amin, Haq Nawaz Bhatti, and Muhammad Bilal

Abstract In the past century, plastic-derived products have facilitated our daily life but with time, their harmful effects have also affected our environment in the form of massive plastic waste materials. The gross production of synthetic polymers per annum that cannot be decomposed has reached 300 million tons and continues to increase, creating a worldwide environmental concern. These facts have fascinated the scientific attention to overcoming the buildup of undesirable plastics in the ecosystem. Conventional chemical or thermal treatment for polymer degradation typically involves harsh conditions and the release of toxic gases. Microbial degradation has been employed to decompose plastic waste materials, but unfortunately, all the conventionally used plastics remain unaffected by the microbial attack. Over time, a number of methods have been investigated for the degradation of polyesters. Owing to biocompatibility and mild conditions, lipases-assisted technology exhibits a wide substrate specificity and incredible perspective to catalyze the hydrolysis and depolymerization of polyesters. In this chapter, a comprehensive review of the production, purification, and characterization of microbial lipolytic enzymes, and their use in the biocatalytic degradation of polymers has been given. A high potential of lipase as a green and robust biocatalyst for the degradation of polyester ensures its promise for environmental safety.

Keywords Lipase · Biocatalysis · Polyester · Biodegradation · Environmental sustainability

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4.1 Introduction

A healthy environment plays a key role in a sustainable development context. For the betterment of the environment, we should seriously think about those factors which affect our environment in either positive or negative ways. The fossil reserves need to be preserved, and the industries should consume less energy to reduce pollution and find ways to use raw materials as natural resources (Lucas et al. 2008). Plastics serve us in every field of our day-to- life, but meanwhile, nonbiodegradable plastics are creating a significant environmental threat as there are no waste management facilities (Ahmed et al. 2018).

In both aqueous and nonaqueous reaction media, lipases (triacylglycerol acyl hydrolases, EC 3.1.1.3) are the most imperative biocatalysts for an array of industrially pertinent catalytic reactions (Fig. 4.1). Lipases ideally catalyze the hydrolysis and transesterification of the lipids (Fig. 4.2) (Prasad 2014; Lima et al. 2019; Li et al. 2020). There are various industrial applications of lipases like the synthesis of pharmaceuticals, cosmetics, detergents, flavor enhancers in the food industry, and

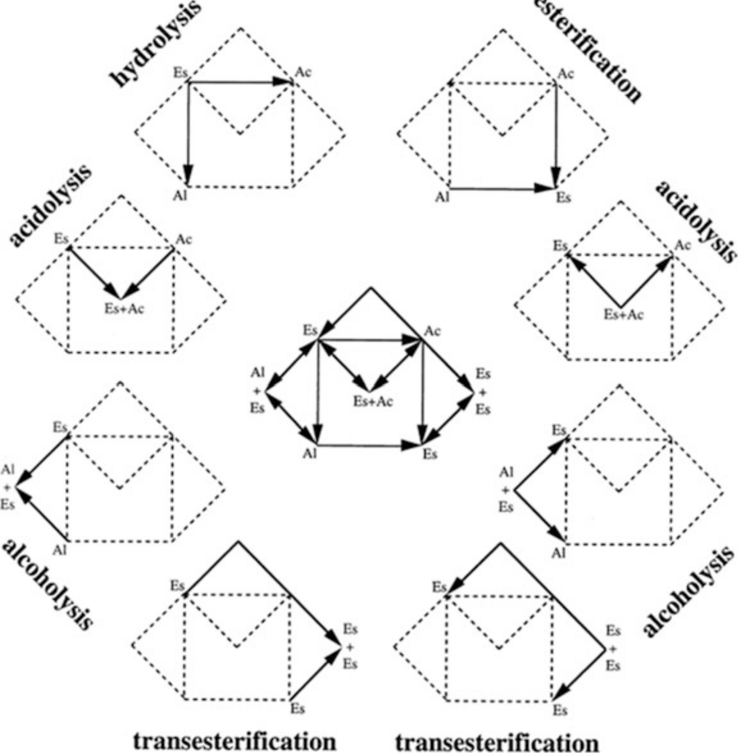

Fig. 4.1 Wireframe of the lipase-catalyzed reaction domain. (Reproduced with permission from Paiva et al. (2000). Copyright 2000, Elsevier)

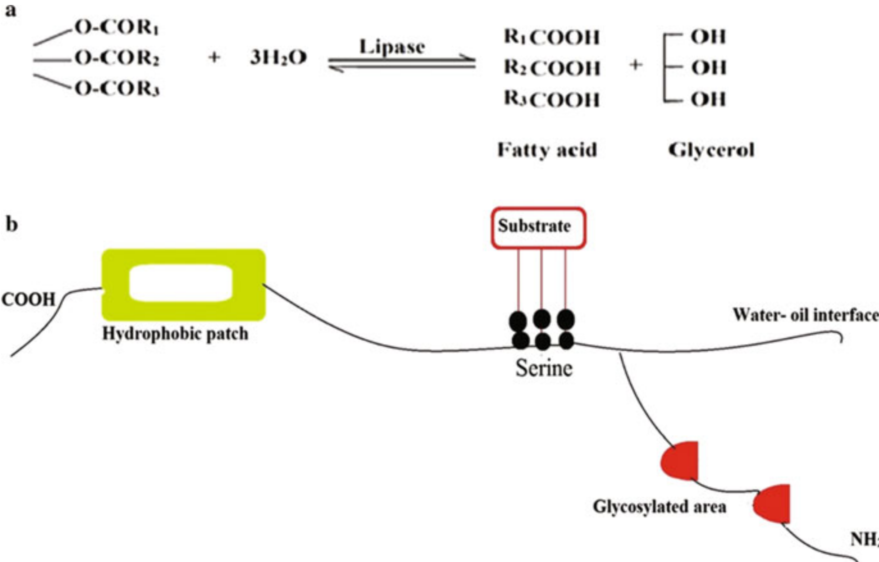

Fig. 4.2 (**a**) Hydrolysis conversion of triglyceride into glycerol and fatty acid. (**b**) Depiction of a lipase molecule with its characteristic features. (Chandra et al. 2020; an open-access article licensed under a Creative Commons Attribution 4.0 International License)

removal of oil in wastewater (Veerapagu et al. 2013; Das et al. 2016; Chandra et al. 2020; Contesini et al. 2020; Mehta et al. 2020). Other than these, bioremediation for solid waste is an interesting horizon and lipases have a vital role in the bioconversion of polyester waste into useful products.

Lipases are worldwide in nature and are synthesized by various microorganisms, animals, and plants. Microbial lipases do not require cofactors for their action as well as due to their faster production and higher yields, they are a better choice than those produced from plants or animals (Dey et al. 2014). Owing to their stability, selectivity, and extensive specification for substrate, they have been the center of interest for special industrial applications. Fungal strains are considered as better lipase producers since they produce enzymes, which can be simply removed from the media used for enzyme production (Maia et al. 1999; Sonkar and Singh 2020; Rehman et al. 2019). Fungi have numerous other advantages in the colonization of solid substrates as compared to unicellular microorganisms (Yu et al. 2007; Treichel et al. 2010). Filamentous fungi are the most excellent and perfect tailored microorganisms for solid-state fermentation (Swetha et al. 2014). Enzyme production can be augmented by the selection of effective strains and optimization of their processing conditions. The biosynthesis of fungal lipase is controlled by both nutritional and physicochemical parameters, and it is compulsory to include various factors, such as nutrients (sources of carbon and nitrogen), temperature, metal ions, inoculum volume, and pH.

Lipases can be synthesized by solid-state fermentation (SSF), as well as, submerged fermentation (Barberis et al. 2008; Balaji and Ebenezer 2008). Fungi are ideally cultured in SSF to produce lipases, while submerged fermentation is preferred for yeasts and bacteria (Dutra et al. 2007). In contrast to the submerged system, SSF possesses many advantages, including higher yields, concentrated products, simpler extraction techniques, simpler growth, and production media (Malilas et al. 2013). The final outlay of the enzyme may be decreased using by-products as growth substrates for enzyme production, which provide low-cost substrates with high value (Rodriguez et al. 2006; Menoncin et al. 2010). The solid wastes of vegetable oil processing are extensively used to produce industrial enzymes because they offer wonderful aid for the development of microorganisms as well as good sources of nutrients that need no supplementations (Ramachandran et al. 2004). Moreover, they are economical and plentiful in agricultural countries like Pakistan, but their utilization is limited to animal feed or just as landfills (Graminha et al. 2008). While using fungal species for enzyme production, oil cakes have been reported to be better substrates (Ramachandran et al. 2007).

4.2 Lipase Production Under Optimal Processing Parameters

4.2.1 Moisture Content

Moisture level is a critical experimental factor for increasing the effectiveness of the fermentation process. It varies in various microbial strains because of different environmental factors, metabolic activities, and kinds of cultivation substrates as well as microorganisms. It is demonstrated that the substrate utilized during the SSF should have an adequate humidity level to fulfill the microbial supplies for liberating valuable bioproducts, i.e., enzymes (Asgher et al. 2016a; Vaseghi et al. 2013). Water, which exists as such in the solid substrate contributes to the fungal development that might be ascribed to the efficient transportation of oxygen. A high level of moisture can decrease the porosity and oxygen transfer in the substrate, promote the initiation of stickiness, and likely enhance contamination. On the other hand, low humidity contents diminish the solubility of nutrients in the substrate (Contesini et al. 2010; Mahanta et al. 2008).

Kamini et al. (1998) used SSF on gingelly oil cake substrate, and standardized experimental conditions for the production of lipase by *A. niger* MTCC 2594. They found favorable moisture content of 60%. Among different fungal strains, which were separated for lipase production, significant enzyme production was recorded from *R. arrhizus* and *A. niger* when cultured in submerged conditions using a synthetic oil medium (Mahadik et al. 2002). In SSF, the maximum lipase was produced with a synthetic oil-based medium at a 1:2.5 ratio (71%) by *A. niger*. Balaji and Ebenezer (2008) investigated the optimized lipase synthesis using SSF. It

was detected that 1:1.5 was suitable moisture content for the maximum lipase production. Through the process of physical adsorption and covalent bonding on the sol-gel matrix, Santos et al. (2014) estimated the immobilization of lipase from *A. niger* and characterized both soluble and supported enzymes. The maximum quantities of lipase were obtained when pumpkin seed flour was moistened at 30% and the temperature was kept at 30 °C for 120 h.

4.2.2 Incubation Time

Various reports have detected the maximum enzyme activity of fungal species at 48–72 h. After this optimal time, the enzyme yield was decreased (Abdullah et al. 2018a, b) that might be ascribed to the depletion of vital nutrients and buildup of growth-inhibiting metabolites in the medium (Sudeep et al. 2020). A reduction in lipase production after extended cultivation periods is presumably attributed to the nutrients exhaustion and poor oxygen or moisture supply because of the fungal mycelia compaction and proteases production resulting in enzyme inactivation (Sánchez et al. 1999). In the beginning, microorganisms are likely to adjust to environmental circumstances. The highest lipase activity was achieved in an exponential growth phase and slowly reduced at the end of the log phase due to citric acid production in the medium (Kamzolova et al. 2005).

Imandi et al. (2010) employed SSF for lipase synthesis using *Yarrowia lipolytica* NCIM 3589. Lipase activity began to increase after 24 h, reaching the maximum after 96 h, and diminished at further incubation periods. The cause behind this reduction was supposed as the exhaustion of nutrients, assemblage of toxic products, or loss of moisture, as well as, any alteration in the medium pH. Pradeep et al. investigated the characterization of lipase produced by *Serratia marcescens* MBB05. The optimum time of incubation was determined by incubating the selected medium for varied time durations and then analyzed for lipase activity. The highest lipase titer (0.5 U/mL) was attained by incubating for 20 h, but further increase in incubation time indicated a sharp decline in lipase activity. Selvam and Vishnupriya (2013) cultivated *S. variabilis* NGP3 for various time durations (1–10 days) and observed the utmost mycelial growth on the seventh day. However, the rate of lipase production reached its full extent (61.2 U/mL) on the fifth day. In addition, a gradual decrease was observed in enzyme productions after an optimum period. In vegetable oil processing factories, Veerapagu et al. (2013) separated bacterial lipase producers from oil spilled soil. One isolated strain, among twenty others, showcased comparatively higher lipase activity. After 8 and 16 h of experimental time, lipase was not produced by *Pseudomonas gessardii.* Only after 24 h of incubation, a reasonable quantity of lipase was produced, but it was declined after 48 h.

4.2.3 pH

The lipase production is considerably affected by the pH of the growth medium. PH plays a key role to determine the variety of microorganisms that can take over a specific substrate. It has been described that each microorganism flourishes and acts at a specific optimum pH since its growth and metabolic activity are affected by pH alterations. Filamentous fungi are capable of flourishing in a range of pH values due to a high pH buffering capacity of solid substrates (Amin et al. 2008).

Lipase production by *A. niger* was examined by Mahadik et al. (2002) under SSF. The optimal pH for lipase production was reported as 2.5. Moreover, it was noted that at quite an acidic pH of 1.5, the enzyme showed high activity (75%). Anbu et al. (2011) chose a couple of bacterial strains (BK43 and BK44) that were quite similar in their attributes to *Acinetobacter junii*. For lipase production, the optimal pH was 6.0 at 30 °C after 24 h of incubation using BK43. At the same time as for BK44 optimum pH was found to be 6.0 with 12 h of experimental time at 25 °C. The study showed that under acidic conditions, the genus *Acinetobacter* is an effective choice for lipase synthesis. Padmapriya et al. (2011) conducted some experiments on *Lactobacillus* sp. with pH of the medium ranging from 6 to 10. Both lipase production and microbial growth were stopped at pH 10, but maximum quantities of lipase (39.6 U/ml) were produced at pH 9.

Lipase from *Serratia marcescens* MBB05 was synthesized at different levels of pH ranging from 5.5 to 8.0. The highest lipase production of 1.3 U/mL was attained at pH 6.0. Refinement and bioremediation of wastewater by lipase were studied by Selvam and Vishnupriya (2013). Lipase was produced from *Streptomyces variabilis* NGP 3 at pH values from 5.0 to 12.5. The highest titer (105 U/mL) was attained at pH 9.0 along with the utmost growth (26.5 mg at dry weight) at pH 9.0. Veerapagu et al. (2013) performed experiments on bacterial lipase producers and BLP2 *Pseudomonas gessardii* was discovered as the best lipase producer. It was observed that the bacteria can produce lipase in the medium of pH range 4.0–10. *Pseudomonas gessardii* produced the highest quantity (114.0 UmL^{-1}) of lipase at pH 7.0. However, it was also reported that with the increment in pH from 7.0 to 10.0, lipase production went downwards. Santos et al. (2014) employed SSF on pumpkin seed flour to produce *A. niger* lipase. At an acidic pH of 4.0, free lipase demonstrated comparatively the highest activity. The enzyme was found almost inactive at higher acidic pH as the lowest enzyme activity was observed at pH 2.0 due to excess H^+ ions. Adio et al. assessed lipase activity by *A. niger* at different medium pH (6.0–8.0) with moisture level 60% (w/v), 72 h incubation period, and inoculums level 1.0%. The highest lipase activity was observed with pH 7.0.

4.2.4 Inoculum Size

In order to good microorganisms' growth, inoculum should be properly and sufficiently distributed in SSF. First, the fungal spores attach to the substrate particles, gradually develop, reproduce, and then penetrate the substrate for action. Hence, an appropriate level of inoculum size is necessary for the production of lipase. Inoculum size is an imperative factor in SSF as higher inoculum size increases the water level of the substrate, thus hindering fungal development and induction/secretion of enzymes. On the contrary, a low level of inoculum requires a longer duration for substrate fermentation in SSF. Therefore, the homogenous distribution of a sufficient inoculum level is important for optimal microbial growth (Ramachandran et al. 2004).

Yu et al. (2009) depicted the production and characterization of lipase by *Pseudomonas* Lip35 under different conditions. The highest enzyme was produced with 6% inoculum concentration of *Pseudomonas* Lip35. Pradeep et al. evaluated the outcome of varying concentrations (1.5–3.5%) of *Serratia marcescens* MBB05 inoculum on lipase titer. The higher lipase activity (0.67 U/mL) resulted from *S. marcescens* MBB05 at 2.5%. The lowest activity was obtained at 1.5% of *S. marcescens* MBB05. Adio et al. reported lipase production by *A. niger* F7–02. The effect of diverse inoculum concentrations was observed on lipase production after 72 h of incubation. The lipase activity was increased by increasing inoculum concentration and was at its maximum level (75.4 U/mL) at a 1.0% level.

4.2.5 Temperature

Temperature is a critical factor that fluctuates from organism to organism growth. A rise in temperature causes the increase in the number of useful collisions between the substrate and enzyme molecules to develop activated complex and ultimately the reaction rate is amplified. However, this process is limited to some extent and a certain temperature. At higher temperatures, a large amount of metabolic heat is produced that is responsible to shoot up the substrate temperature, thereby retarding the growth of microorganisms and enzyme synthesis (Murray et al. 2003; Bhatti et al. 2007).

The temperature must have to be monitored as its optimum value varies among different organisms. Balaji and Ebenezer (2008) investigated the optimization study of lipase production by *C. gloeosporioides* under SSF. They observed that the optimum temperature was different with varied substrates. Results depicted that the highest enzyme activity was achieved at 25 °C while COC yielded the maximum enzyme units at 35 °C. Anbu et al. (2011) isolated and screened bacteria using Rhodamine-B and spirit blue agar media. Two of the isolated strains (BK43 and BK44) showed a better clear zone indicating the higher lipase activity as compared to others. These selected strains resemble *Acinetobacter junii*. The optimal

temperature by BK43 for lipase synthesis was observed to be 30 °C while that of BK44 was 25 °C. Padmapriya et al. (2011) reported lipase production by *Lactobacillus* sp. at varying temperature levels from 30 to 70 °C and pH 6–10. The titer of lipase was improved by increasing temperature up to 40 °C, reaching the utmost lipase activity (39 U/mL) at 40 °C. A further rise in temperature triggered a sharp decline in lipase activity.

Pradeep et al. depicted the impact of growth temperature on lipolytic activity by *Serratia marcescens* MBB05, incubating the selected medium at varying temperature levels. It was conducted at 20 to 30 °C maintaining all other experimental factors at their optimum levels. The lipase production (1 U/mL) was highest at 25 °C. Selvam and Vishnupriya (2013) assessed the influence of temperature on *S. variabilis* NGP 3 mycelia growth and lipase production between 25 and 70 °C. It was reported that the growth (30.0 mg) was superior at 30–35 °C. The lipase production was observed to be the maximum (39.4 U/mL) at 35 °C.

4.2.6 Lipase Inducer

Lipidic carbon sources and olive oil are recognized as potential inducers for lipases production (Wang et al. 2008). Though a few studies have achieved appreciable lipase activity without the inclusion of oils and fats, an elevated lipase activity yield has been recorded in several studies using olive oil as an inducer (Anbu 2014; Gupta et al. 2004; Mohan et al. 2008). Therefore, the concentration of olive oil is necessary to optimize for better lipase production.

Mahadik et al. (2002) revealed the reports of lipase production under SSF by *A. niger*. Biosynthesis of lipase occurred only when lipid substrate was present in the medium. The highest yields of lipases were attained employing wheat bran as a growth substrate combined with olive oil. Tan et al. (2004) observed the usability of the Tween series for enhanced lipase production by *P. camembertii*. Using Pongamia oil cake, Tween-60 produced 1170 U/g DM lipase while olive oil synthesized 2560 U/g DM. It was interesting to know that using COC as the substrate, none of the lipid substrates improved lipase activity. Balaji and Ebenezer (2008) investigated different lipid sources as inducer substrates to produce extracellular lipase. Tween 60 was found the best lipid source among many other substances. Different inducers were considered to enhance the enzyme production by BK43 and BK44. Among the tested inducers, both strains produced a great quantity of extracellular lipase utilizing Tween 80 as a lipid source (Anbu et al. 2011). Oliveira et al. (2017) conducted the optimization studies of lipase synthesis by *A. ibericus* MUM 03.49 using olive pomace as a substrate. It is ascribed that olive pomace holds residual olive oil that performs as an inducer for lipase synthesis. To estimate its effect on lipase production, a number of mixtures of WB: olive oil and OP: WB was studied. The maximum lipase activity was attained with lipids level from 10.2 to 13.7%.

4.2.7 Supplemental Carbon Sources

The carbon source has always been reported as the key feature for the illustration of lipase activity (Gupta et al. 2004). Carbon sources mainly provide help in energy production for microorganisms. Although carbon sources are obligatory for fungal growth and production of enzymes, their effectiveness can vary among different microbial strains and cultivation conditions. The fungi are usually able to produce lipases using carbohydrates as carbon sources.

Tan et al. (2004) observed the higher lipase yield with peptone as an organic nitrogen source compared to the control by *P. camembertii*. Balaji and Ebenezer (2008) produced lipase under SSF by *C. gloeosporioides*. Among various carbon and nitrogen sources, peptone, and xylose with POC, have been the best enzyme yielding sources. The effect of diverse sources of carbon on lipase synthesis by two bacteria strains was considered. Lipase yield was improved when the bacterial strains were grown in the presence of sucrose (1%) (Anbu et al. 2011). Pradeep et al. evaluated the influence of different sources of nitrogen, such as peptone and yeast extract one by one and then in the form of a mixture (1:1). They were supplemented in the medium to a final concentration of 5 g/L. Similarly, different carbon sources like glucose, olive oil, tween 80, and glycerol were supplemented separately (0.5% final concentration) using the same medium. Both mycelial growth and lipase activity were significantly affected. The lipase activity was low by *S. marcescens* MBB05 on yeast extract, but it exhibited higher activity at peptone alone. In the case of carbon source, good lipase yield was obtained in the presence of glycerol.

Veerapagu et al. (2013) carried out enzyme production by BLP2 *Pseudomonas gessardii* using different carbon sources. Maltose, galactose, and sucrose inhibited lipase production presumably due to catabolite suppression by easily accessible carbon contents in the culture medium. Amongst the different organic nitrogen sources, peptone and protease improved lipase production, whereas soy peptone, casein, and soybean meal have a negative effect on lipase productivity. Oliveira et al. (2017) investigated the effect of urea, NH_4Cl, $NaNO_3$, and $(NH_4)_2SO_4$ on lipase synthesis. All these sources of nitrogen exhibited a considerable positive effect on the production of lipase. Results revealed the highest lipase yield using ammonium salts (2%).

4.3 Statistical Optimization for Lipase Production

The statistical optimization of experimental factors has advantages over the classical method of considering one experimental factor at a time, such as evaluation of the interaction effects among variables while performing the lower number of experiments (Amin et al. 2017). A well-organized and extensively used approach is the application of response surface methodology (Abdullah et al. 2018a, b). RSM can

effectively demonstrate the correlation among different factors and responses along with pinpointing the ideal levels for each factor. The production of lipase has been meaningfully improved using RSM in various microbial cultures, including *Bacillus pumillus*, *Pseudomonas aeruginosa,* and *Cadida* sp. 99–125. Kumari et al. (2009) conducted experiments on the production of lipase by *Enterobacter aerogenes* using RSM with CCD. The optimization of experimental conditions led to a 1.4-fold enhancement in lipase activity. Those optimum conditions were pH 7.0, inoculum level 7%, oil concentration 3%, and incubation time 60 h at 34 °C to achieve the maximum lipase activity. Faisal et al. (2014) synthesized lipase by *Pseudomonas* sp. using RSM for optimizing the experimental conditions. Four important parameters were chosen for statistical optimization by using the software Minitab 14. Results revealed the 0.7-fold enhancement in enzyme production under the optimum experimental conditions i.e., pH 5.9, temperature 28 °C, incubation time 2 days, and moisture content 33%.

RSM was applied to optimize lipase synthesis by *Halobacillus trueperi* with a substrate of marine waste. By using CCD, optimum medium components for maximum lipase synthesis were found to be olive oil (5.05 mL/L), NaCl (72.42 g/L), pH 9.0, and 45 °C (Sathishkumar et al. 2015). A comparative model was developed to produce lipase by *Bacillus subterraneus* TNUS15 employing RSM and Artificial Neural Network (ANN) (Thyagarajan et al. 2017). A better lipase production, 57.61 IU/g was obtained by the application of second order polynomial model. A close agreement of the experimental value (57.61 IU/g) with that of the predicted one (56.3 IU/g) depicted that a statistically optimized plan can be employed to get better lipase production to fulfill the growing demand. Bernal et al. (2017) used RSM to assess the experimental conditions that increase the mycelia growth of *Acidocella facilis* and ultimately improve lipase titer. RSM results demonstrated that agitation and yeast extract were the main experimental factors, which enhanced microbial growth and enzyme production by 4.5-fold.

4.4 Purification of Lipases

Lipases are the enzyme of great industrial importance. These enzymes are not required in a purified form for many industrial applications; however, some industries like fine chemicals, pharmaceuticals, and cosmetics necessitate the requirement of the purified enzyme up to homogeneity level (Bilal et al. 2015; Asgher et al. 2016b). The extent of purification is always dependent on the final application. Moreover, the three-dimensional architecture of enzymes and structure-function relationships can be better studied in the purified form (Gupta et al. 2004). Low yields and a long time taken for the purification are the main issues related to the conventional purification techniques. As the microbial lipases are mostly extracellular, the separation of cells from fermentation broth by filtration or centrifugation is the first step after the fermentation process. After obtaining the cell-free crude enzyme extract, it is passed through different purification steps, including

concentration, chromatographic steps, membrane processes, and immuno-purification. Uttatree et al. (2010) isolated *Acinetobacter baylyi* which could produce lipolytic enzymes naturally. Lipase was purified effectively (21.89-fold) to uniformity by ammonium sulfate fractionation and molecular sieving chromatography having a molecular mass of 30 kDa. Padmapriya et al. (2011) purified lipase produced by *Lactobacillus* sp. two-fold purification was resulted in 75% recovery by ammonium sulfate precipitation. The crude enzyme after dialysis showed 36.5-folds of purification with 66.6% recovery of lipase from *Lactobacillus* sp. Lipase produced by *Serratia marcescens* MBB05 was subjected to ammonium sulfate precipitation and dialyzed against buffer overnight. The dialyzed solution having some impurities passed through a column that equilibrated with Tris-HCl buffer. The lipase-loaded fractions were collected and kept aside as the purified lipase. Tripathi and Singh (2014) purified *Microbacterium* sp. lipase by ammonium sulfate fractionation and column chromatography for salting out the proteins. Desalting was carried out to increase the enzymatic activity. 2.1-fold of purification resulted in a total yield of 20.8%. This little enzyme yield might be due to having trouble removing high contents of lipopolysaccharide present in the microbial species.

4.5 Characterization of Lipases

4.5.1 Effect of pH on Lipase Activity

It is observed that microbial lipolytic enzyme form strains display distinct optimum pH, but generally, their pH optima are found to be near neutral. A significant change in the medium pH results in enzyme denaturation, and thus markedly dropping its catalytic activity (Priyanka et al. 2019). Uttatree et al. (2010) isolated *Acinetobacter baylyi*, which could produce lipolytic enzymes. The enzyme articulated the maximum activity at pH 8.0 and was observed to be highly resistant in the pH range of 6.0–9.0. Pradeep et al. examined the lipase activity with olive oil as a substrate using the pH range of 3.5–9.0. Olive oil was hydrolyzed by purified lipase considerably over a comparatively wide pH range. *S. marcescens* MBB05 lipase showed maximum activity at7.0 pH. At alkaline and acidic pH, the enzyme activity was decreased steadily. Moreover, lipase was observed stable at pH 7.0 for 40 min. Dey et al. (2014) determined the pH tolerance ability of lipase. The pH of the mixture used to find enzyme activity was varied with different buffer systems of pH 2–11. Two optima of pH for lipase activity were noticed at 3.5 and 8.5, respectively. The pH stability profile made this lipase valuable for industrial applications. Colla et al. (2015) demonstrated that lipases produced from *Aspergillus* species by submerged fermentation showed optimum pH as 7.2 having 80% stability in acidic range of pH. On contrary, lipases obtained by SSF presented optimum pH 6 and had almost 60% stability in the alkaline range of pH.

4.5.2 Effect of Temperature on Lipase Activity

Temperature exerts a critical role to find out the activity of enzymes. A significant reduction in enzymatic activity is usually observed above optimum levels; this is most likely due to the instability of the lipase at elevated temperatures (He et al. 2019). The possible explanation for this instability might be the cause of the disturbance of the enzyme tertiary structure. It is then changed the configuration of the active site, deteriorating the enzyme-substrate contact (Das et al. 2016). *A. baylyi* was isolated from marine sludge that could synthesize lipase. The enzyme showed the maximum activity at 60 °C with pNPP substrate and was observed to be stable between 60 to 80 °C (Uttatree et al. 2010). Padmapriya et al. (2011) studied the thermal attributes of *Lactobacillus* sp. lipase. The residual activity was calculated after every hour of incubation at different temperature levels from 30–70 °C. High enzyme stability was noted between 30 and 50 °C. In another study, the lipase activity was investigated at a temperature range of 20–80 °C. The lipolytic activity was increased steadily by increasing temperature and was reached at its maximum level at 40 °C. Still, 85% of activity was achieved at 50–60 °C. Colla et al. (2015) reported that lipases produced from *Aspergillus* species by submerged fermentation showed optimum temperature as 37 °C and those produced by SSF presented 35 °C as the optimum temperature. It was also more thermostable presenting 72% of residual activity at 90 °C.

4.5.3 Effect of Metal Ions, Inhibitors, and Organic Solvents

No cofactor is usually required for the activity of lipases. Still, the effects of metal ions either stimulatory or repressive, on lipase activities have been well established. The stability of lipases in the presence of any foreign substance whether it is a metal ion or any organic solvent represents numerous benefits for their applicability in organic synthesis (Amin et al. 2020a). Kumar et al. (2012) investigated the solvent stability of lipase and the enzyme exhibited good stability (>75%) against petroleum ether, hexane, cyclohexane, chloroform, acetone, ethanol, 1-propanol, and 2-propanol, while the relative activity was 68–10.1% in the presence of ethyl acetate, diethyl ether, methanol, benzene butanol, and DMSO. Among the seven lipase-producing bacterial strains isolated, one of them (PAL05) showed significantly greater stability in organic solvents. Crude lipase maintained its activity with 25% and 50% levels of different organic solvents. Benzene and ethanol truly enhanced the enzyme activity (Anbu and Hur 2014). Tripathi and Singh (2014) reported that lipase activity was not affected by Na^{+} and Ba^{+} salts, while Li^{+}, K^{+}, $Mg^{2+,}$ and Zn^{2+} ions decreased the activity. Ca^{2+} was found the best activator by increasing lipase activity by 50%. Das et al. (2016) studied the effect of organic solvents on fungal lipase, which revealed an extreme reduction in its activity than that of the control due

to dehydrating action of organic solvents that resulted in precipitating the enzyme, which severely affected its activity.

4.5.4 *Kinetic Parameters*

Lipases have been reported to obey Michaelis–Menten kinetics in many cases, which is characterized by K_m and V_{max}. K_m represents the enzyme affinity towards its substrate, while V_{max} denoted the maximum reaction rate (Qamar et al. 2020; Bilal et al. 2019). Michaelis-Menten kinetic constants for lipases might be different according to the reaction being catalyzed. Santos et al. (2014) calculated the K_m and V_{max} of free and carrier-supported lipase. The free lipase had K_m of 117 mM which showed its higher affinity to the substrate than the immobilized enzyme having K_m of 170 mM. On the contrary, V_{max} for free and immobilized enzymes had similar values, i.e., 0.0276- and 0.0216-mM min.$^{-1}$, respectively. Tripathi and Singh (2014) determined the K_m and V_{max} values at optimal values of variables, like temperature and pH using diverse pNPP substrate several concentrations. The K_m and V_{max} were estimated to be 3.2 mM and 50 μM/min/mg, respectively. Vasiee et al. (2016) identified *Bacillus cereus* by Rhodamine B agar plate method. K_m and V_{max} for the enzyme were 5.3 mM and 0.367 μM/min.mL, respectively.

4.6 Lipase-Assisted Degradation of Polyester

Polyesters are versatile synthetic polymers having ester linkages. Many factors can have an influence on the biodegradation and depolymerization of aliphatic polyesters. The chemical bonds and repeating units in polyesters are in such order that exhibits different physical properties like crystallinity, melting temperature (*Tm*) and glass transition temperature (*Tg*), which have decisive out-turns on the degradation of polyesters (Amin et al. 2020a). In addition, pH, surface condition, temperature, and chemical structure of the polyester have also a great influence on the degradation rate (Tokiwa and Calabia 2007). However, these factors are still not very clear that controls the ability of enzymes to break the ester bond in one polyester but not in the other. Exhaustive work was carried out to explain the mechanism of the enzymatic degradation of polyesters. These efforts were managed not only to design new biodegradable plastics as well as to use new enzymes as catalysts to recycle the bulk polyesters (Mueller 2006; Danso et al. 2018; Gan and Zhang 2019).

Extracellular hydrolytic enzymes like lipases have the ability to decompose films of aliphatic polyesters. Enzymatic degradation comprises simple and multiple enzymatic systems secreted by various microbial strains (Calil et al. 2007). These biocatalysts are capable of weakening the chemical association in the polymers to reduce the degradation activation energy (Amin et al. 2020b). The reaction mechanism is a very common characteristic of hydrolases that uses three amino acid

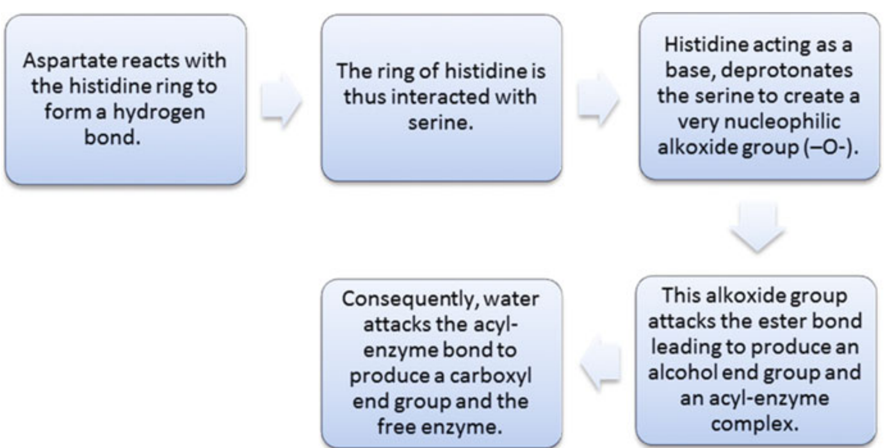

Fig. 4.3 Reaction mechanism of hydrolases for biodegradation of polyesters

residues i.e., aspartate, serine, and histidine (Abou-Zeid et al. 2001; Belal and Belal 2003). The specific arrangement of these amino acid residues is termed a catalytic triad, which is described in Fig. 4.3.

Biodegradable polymers can lessen the solid waste problems as they can be converted into monomers like CO_2, H_2O, or biomass, and their main chain can easily be broken by microorganisms. These properties have brought forth the attention towards biodegradable polymers, which prove themselves environmentally-friendly (Vidaurre et al. 2008). Most of the degradable polymers have various characteristics, such as mechanical strength, morphology, degradation rate, etc., and oxidizable and hydrolyzable or linkages along the main chain (Sivalingam et al. 2003). After degradation, biodegradable polymers can easily be included in carbon and nitrogen cycles under some specific environmental conditions. This biodegradation is carried out largely by microbial enzymes, which can be categorized into hydrolytic degradation and enzymatic degradation according to the characteristics of the covalent bond where cleavage occurs (Amin et al. 2020a, b). Collagen/chitosan are natural polymers that undergo enzymatic degradation whilst, among other types of polymers, polyesters could be hydrolyzed by lipases or esterases enzymes. Other classes of polymers can be disintegrated majorly by nonbiological methods, such as ultraviolet radiation and oxidation (Albertsson and Karlsson 1992).

Lipases are the enzymes that can catalyze the ester bonds hydrolysis in polyesters in the presence of an aqueous media. Nagata et al. (2008) prepared biodegradable poly(ester carbonate)s through the melt polycondensation process to prepare pre- and post-polymers for 40–80 min at 270 °C. Transparent and flexible films were achieved by this process. These films were biodegraded by *Rhizopus delemar* lipase at 37 °C. The weight loss in the films of polymers was observed to increase with time. Hydrolysis of the ester linkages in the polymers by lipase was confirmed by GPC curves. The biodegradation of poly(octamethylene suberate) was reported by

Casas and Puiggalí (2009). Different attack mechanisms by *Rhizopusoryzae* lipases in degrading crystalline spheres were observed. Depending on crystallization conditions like temperature fluctuations, the enzyme attacked the lamellar fold surfaces or lateral crystal growth faces.

Mallepally et al. (2009) reported the enzymatic degradation of hyperbranched polyesters. *Pseudomonas cepacia*, Cal-B, Novozym 388, and Lipomod 34P lipases were employed for degradation. The degree of polymer degradation was carried out by gas chromatography. It was revealed that the rate of degradation was diminished by increasing the alkane chain length of the end groups. Reflected electron microscopy was employed to assess the surface morphological alterations. After degradation, the crystallinity changes in the polyesters were determined by DSC. Karunanidhi et al. (2010) synthesized a series of aliphatic copolyesters. Characterization of the polyesters was conducted by XRD, DSC, viscosity measurements, and gel permeation chromatography. The biodegradation of polyesters was studied by using the buffer solution with lipases from *Mucor miehei* and *Candida cylindracea*. The structural morphological changes were determined by using SEM. The biodegradation was influenced by the crystallinity of polyester, and the polyester with little crystallinity index showed the highest rate of degradation by both enzymes.

Peng et al. (2010) explored the synthesis of copolymers based on PCL. Porcine pancreatic lipase was reported for the first time to degrade the PCD electrospun mats up to 92% weight loss in 7 days of incubation but no visible impact on PCL Ems was observed. The analysis of degradation products revealed that a fast rate of degradation was achieved due to the presence of a higher percentage of the amorphous region in PCD. Introducing the PEG segment improved the hydrophilicity of PCD, but it caused to decrease in the degradation rate. Tsai et al. (2010) investigated the synthesis of a series of co-polyesters (PBSCs). Techniques like wide-angle XRD, gel permeation chromatography, TGA, DSC, and ^{1}HNMR were employed to characterize these co-polyesters. *Pseudomonas cepacian* lipase was employed to examine the biodegradation of PBSCs. Crystallinity had no significant effect on biodegradation while surface hydrophilicity was proved to be a key factor to influence enzymatic hydrolysis. Jecu et al. (2013) degraded PHB by fungi, bacteria, and actinomycetes. SEM confirmed the attachment of fungal growth on the film's surfaces, the existence of filaments network, and conidiophores. FTIR spectra exposed a small increase in absorption of the C=O band in the 1750–1700 cm^{-1} region, which was more prominent for composites incubated for 60 days, representing the degradation of PHB-PVA composites.

Microbial biodegradation of PHA was carried out by different communities. PHA degradation was affected by specimen shape, polymer chemical composition, and microbial properties. PHA degradation was also followed by a decline in the molecular mass of polymer and, frequently, a rise in crystallinity degree, indicating favored degradation of the amorphous region (Boyandin et al. 2013). Shah et al. (2013) used enrichment techniques to isolate a bacterial strain MZA-75 having the ability to degrade polyurethane. The PU film degradation process in the medium containing mineral salts was evaluated by SEM, FT-IR, and gel permeation chromatography. SEM exposed the presence of extensive flaws on the surface. FTIR

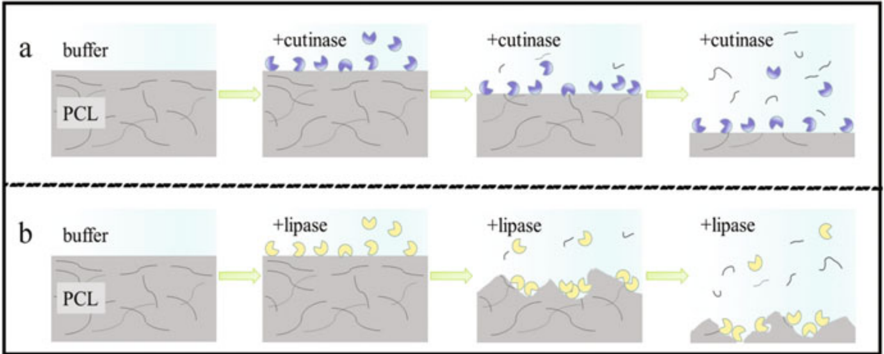

Fig. 4.4 Schematic illustration of PCL degradation by a) cutinase and b) lipase. (Reprinted with permission from Shi et al. (2020). Copyright © 2019 Elsevier B.V)

spectrum determined the reduction in the peak intensity of the ester functional group. In the presence of lipase, the effect of enzymatic degradation on fiber morphology and mechanical properties of PCL and PGC was studied. Changes in tensile strength were found significant after 1 month of degradation. The cracks in fiber were visibly marked by SEM after 4 weeks.

Of most recent, poly(ε-caprolactone) were exposed to biocatalytic depolymerization by cutinase from *F. solani* and lipase from *C. antarctica* (Shi et al. 2020). The experimental result revealed that the catalytic action of lipase showed a superior degradation of PCL films in terms of weight loss compared to cutinase catalyzed reaction under identical levels. No noticeable decrease was observed in PCL crystallinity after cutinase action, whereas lipase-mediated degradation markedly reduced the crystallinity of PCL. Notably, the degradation rate and mode were found to be distinct by both enzymes. Cutinase-mediated hydrolysis of PCL occurred layer by layer, whereas some large pores were observed on the surface of lipase-assisted degradation of PCL films (Fig. 4.4).

4.7 Conclusion

Due to restricted foreign exchange and a small number of substrates to produce lipases, the synthesis and import of these enzymes are very expensive. By 2017, the industrial demand for lipolytic enzymes was reported to rise to 6.2%. For that reason, it is needed to continue to develop the enzyme-related technology. During industrial processes, most of the lipolytic enzymes have poor stability, and some of these have lost their properties at high temperatures or extreme pH. The industrial requirement for lipolytic enzymes that are active in extreme conditions has mainly encouraged the search for suitable microorganisms as a source of reliable enzymes. Various efforts have been made for this purpose to discover and identify new

lipase-producing microorganisms, which can synthesize enzymes with enhanced properties for their applications in the catalysis of different processes. In this chapter, we review the biosynthesis, purification, and characterization of lipases and their applications for the degradation of polyesters to overcome the solid waste problem. Literature studies and passement reveal that the application of microbial lipases for the degradation of polyesters is considered suitable candidate for solid waste management. Additional research efforts on sophisticated immobilization and molecular approaches to improving the stability, reuse, and recycling traits could outspread the application scope of lipases for an array of biotechnological applications.

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Conflict of Interest The authors declare that they have no conflict of interest.

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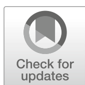
Chapter 5
Application of Microbial Biofilms in Biocatalysis and Biodegradation



Mohd Faheem Khan and Cormac D. Murphy

Abstract Biofilms are colonies of microorganisms that adhere to a surface, often in a flowing system. They convey advantages to the community of cells within, such as access to nutrients, protection from physicochemical stress, intercellular communication and horizontal gene transfer. In many instances, biofilms are viewed as problematic, for example, causing difficult-to-treat infections or corroding metal surfaces. However, the properties that make biofilms a challenge in some circumstances are beneficial in others. As biofilms have mechanical stability, are resistant to the toxic effects of xenobiotics and are stable for extended time periods, they have potential applications in the continuous production of chemicals and the biodegradation of pollutants. In this chapter, we will present examples of the research that has been conducted on examining single-species biofilms of bacteria and fungi in the biosynthesis of commodity and fine chemicals and the biodegradation of xenobiotics. The merits and disadvantages of biofilms in different applications are discussed.

Keywords Biofilms · Biotransformation · Bioremediation · Fermentation

5.1 Introduction

Biofilms exist as a natural form of immobilisation, in which the microbial cells produce an extracellular polymeric substance (EPS) that adheres them to a suitable surface, provides mechanical stability and a barrier against toxic compounds. Extensive work has been conducted with various microorganisms in relation to their growth as biofilms and common stages of biofilm development are recognised (Fig. 5.1). Biofilms are most frequently associated with surfaces submerged in an aqueous solution to which free-floating, or planktonic, cells might adhere. Following

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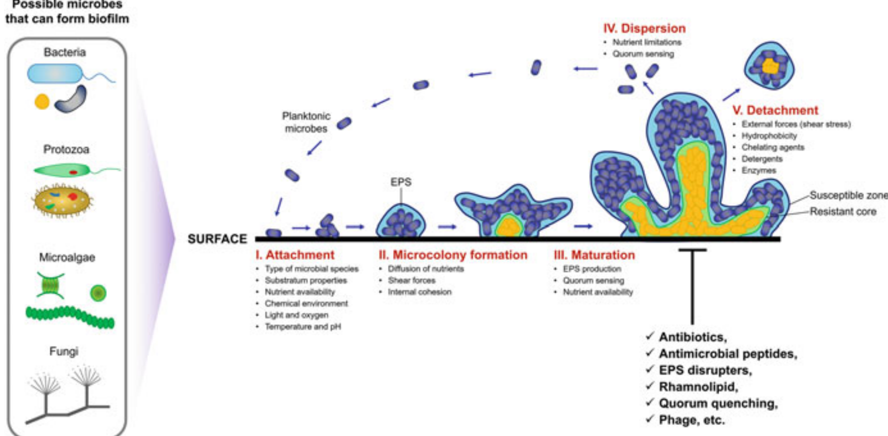

Fig. 5.1 Stages of biofilm development

initial attachment, the cells start to produce EPS, which adheres the colony to the surface and allows further development to form a mature biofilm that contains channels that allow the distribution of nutrients and signalling molecules. Eventually, parts of the biofilm may detach (dispersal). The nature of the surface is a key factor in biofilm development and in particular the degree of hydrophobicity. This is reflected in the protocols used to cultivate biofilms in the laboratory which often employ polystyrene microtitre plates, glass slides and silicone as surfaces to promote attachment (Azeredo et al. 2017). The EPS is typically comprised of polysaccharide, protein and/or extracellular DNA (eDNA). In nature, multispecies biofilms are normal, and there is genetic exchange between the cells as well as syntrophy, facilitating a more complex suite of metabolic reactions. This is in contrast to most studies on biofilms conducted in the laboratory, which often focus on a single species. The importance of biofilms in secondary wastewater treatment, for example, in trickling filters, to decrease the biochemical oxygen demand has been well established; however, in recent years, the attention of researchers has been on how to treat biofilms that cause infection or foul industrial pipes and metal surfaces; thus, there is a widespread perception that biofilms are 'bad'. In this chapter, we will illustrate the advantage of biofilms (mainly single culture) in the areas of fermentation, bioremediation and biocatalysis and highlight the importance of understanding and optimising/controlling their growth so that novel, sustainable methods of pollution removal and biochemical production can be developed.

5.1.1 *Useful Biofilms*

Suspended culture is the main form of microbial growth that is used in the application of microorganisms in the production of valuable compounds, either by

fermentation or biotransformation. However, these cultures can suffer from relatively short periods of activity and are sensitive to the toxic effects of accumulating metabolites or the starting substrate; thus, they may not be sufficiently productive to replace an effective chemical method of production (van Beilen et al. 2003; Garzón-Posse et al. 2018). At the other end of the pipeline, microorganisms are important for the biodegradation of xenobiotic pollutants, but although there are many successful examples, the introduction of a new strain directly into a polluted environment to promote bioremediation (bioaugmentation) often results in the strain being out-grown by indigenous organisms (Mrozik and Piotrowska-Seget 2010). Furthermore, as the number of specialised chemicals that we use increases, traditional wastewater treatment facilities are unable to completely eliminate them from contaminated influents, thus they become persistent in aquatic environments.

It has been known for several decades that artificially immobilising microbial cells and enzymes, e.g. by encapsulating in a hydrogel, has proven to alleviate the problems associated with using suspended or planktonic cultures (Es et al. 2015). By extension, naturally immobilised cells would be expected to have similar features, with the advantage that no additional manipulation is required.

5.1.2 Bioreactor Configuration

Small-scale cultivation of biofilms is mainly done in multiwell-plates, but to operate biofilms for longer time periods and study their characteristics in relation to productivity, other bioreactors are employed, which enable operation for longer periods of time with controlled parameters. In addition to high reaction rates, biofilm reactors also have some other advantages like improved mixing, improved mass transfer, high shear stress, controlled biofilm growth, high product yield, providing resistance to toxic compounds, high product recovery, reduced fermentation time, high operational stability, compact technology, etc. Biofilm reactors have various configurations the most common of which are shown in Fig. 5.2. Different biofilm reactors have different applications depending upon their configuration and size. Biofilms grow attached to a range of materials that are incorporated into the reactor, including glass, metal, plastic and ceramics; the surfaces can be continuous, such as in a membrane or tubular reactor, or particles of the biofilm carrier that might be found in a moving or fluidised bed reactor. The nature of both the surface and reactor type depends on the microorganism and the application.

5.2 Biofilms Applied to Fermentations

Microorganisms that are used industrially in the production of valuable compounds and enzymes are most commonly cultivated in batch or fed-batch fermentors as suspended cultures. While this approach is effective, one major drawback is the

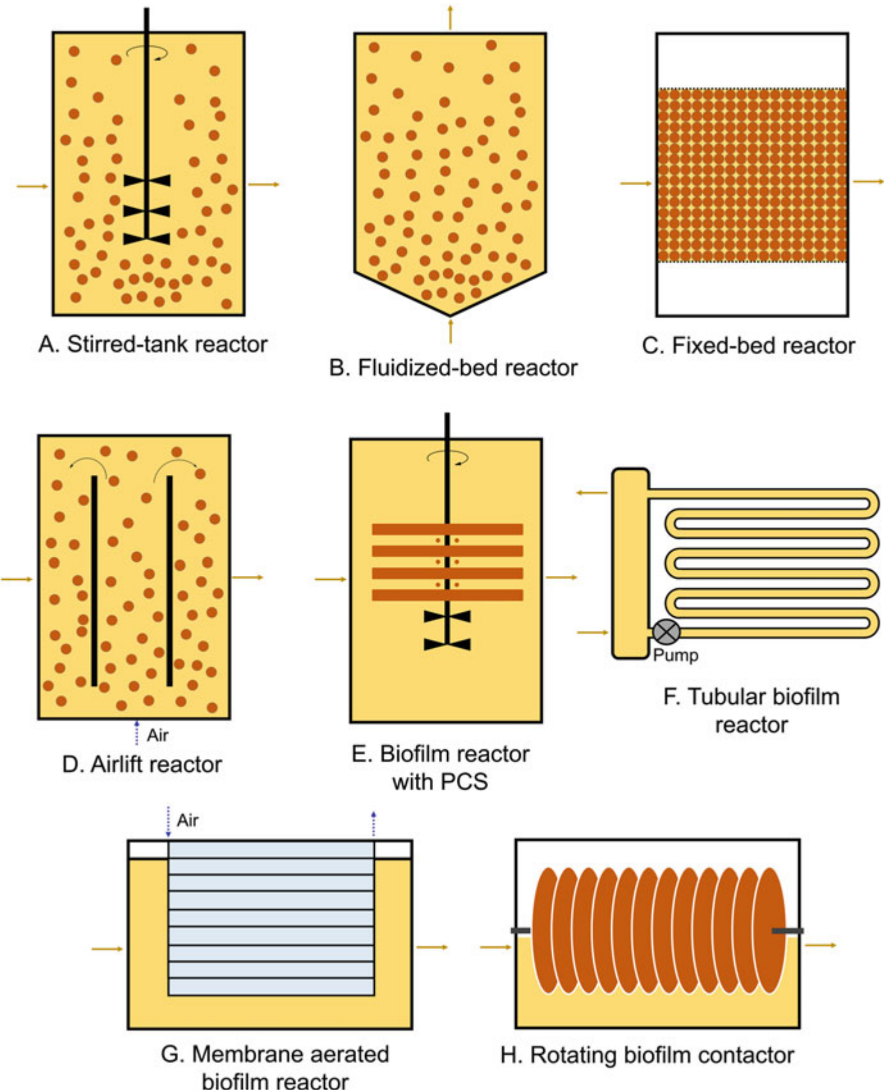

Fig. 5.2 Configuration of different biofilm reactors

repeated stopping of the reactor followed by a re-establishment of the culture, resulting in time delays and additional consumption of resources. One potential application of biofilms is in the continuous production of small molecules and enzymes, and several examples of research in this area are given below.

5.2.1 Saccharomyces cerevisiae

This unicellular yeast is widely studied as a eukaryotic model organism for cell and molecular biology and has been instrumental for the production of beverages and biofuels as it can convert sugars to ethanol at a high rate (Dombek and Ingram 1987).

Saccharomyces cerevisiae has been extensively studied for biofilm formation on numerous surfaces like plastic, mat and ore (Reynolds and Fink 2001). To unveil the mechanism of biofilm regulation requires transcriptomic and proteomic analyses of *S. cerevisiae*, since biofilm enhances fermentation rates and ethanol resistance which has industrial relevance for full-scale production. The switch from planktonic to biofilm growth of *S. cerevisiae* is regulated by Cyc8p, Tup1p and Flo11. The latter is a surface glycoprotein involved in cell adhesion and Tup1p regulates Cyc8p-mediated *FLO11* repression and prevents Flo11p degradation (Van Nguyen et al. 2020). Additionally, a *FLO11* deletion mutant abated ethanol tolerance of yeast cells in the biofilm fermentation (Gu et al. 2018). Along with *FLO11* the other *FLO* genes like *FLO1*, *FLO5*, *FLO9* and *FLO10* encode glycoproteins, which are responsible for cell-cell adherence, and their deletion mutants decrease biofilm formation during fermentation (Halme et al. 2004). Another study showed a transcription factor, *MIG1* expresses more in *S. cerevisiae* biofilms than in their planktonic cells and it induces yeast morphology as filamentous growth, which is confirmed primarily under the control of *FLO* genes (Yang et al. 2018). A low concentration of nitric oxide can also induce *S. cerevisiae* biofilm growth by activation of a transcriptional factor Mac1p that regulates a transmembrane protein Ctr1 which is independent of *FLO11* genetic system. Furthermore, *MAC1* and *CTR1* contribute to the intracellular tolerance to ethanol (Yang et al. 2019a).

In earlier work, Demirci et al. (1997) used *S. cerevisiae* ATCC 24859 as biofilms in a continuous and batch-culture biofilm reactor to compare the ethanol production on a polypropylene support (PPS) and plastic composite-supports (PCS; containing soybean hull, flour, yeast extract-salt and polypropylene). Their study showed immobilised yeast on the PCS produces 2–10 times more ethanol (30 g L^{-1} with low-cost, low-nutrient ammonium sulphate medium) than PPS (5 g L^{-1}), as PCS supplies nutrients to the immobilised cells. In contrast Liu et al. (2015) showed a maximum of 2.90 g L^{-1} ethanol using suspended cells of *S. cerevisiae*. More recently, Izmirlioglu and Demirci (2016) optimised growth parameters of *S. cerevisiae* like pH, temperature and agitation theoretically using Box-Behnken design of response surface methods (RSM) for a similar kind of PCS-biofilm reactor, then reported further improvement in the bioethanol production from potato waste hydrolysate to 37.05 g L^{-1} at pH 4.2 and incubation temperature of34 °C and agitation of 100 rpm. Kana et al. (1989) used very cheap and abundant mineral ores of kissiris for immobilisation of *S. cerevisiae* cells and found improved ethanol production (53 g L^{-1}; 115 g L^{-1} d^{-1}) when immobilised cells inoculated in the glucose-containing media for up to 29 repeated cycles of batch fermentations. Roukas and Kotzekidou (2020) used a rotary biofilm reactor (RBR) to enhance bioethanol production using agro-industrial waste (non-sterilised beet molasses).

They achieved an ethanol concentration of 52.3 g L^{-1} and productivity of 1.45 g L^{-1} h^{-1} in a repeated-batch reactor for 60 days with 84.8% energy recovery.

5.2.2 Zymomonas mobilis

This bacterium ferments glucose to ethanol and has potential application in the production of biofuel. Early work by Kunduru and Pometto (1996a, b) demonstrated that biofilms of this bacterium grown on polypropylene support in a packed bed arrangement, continuously operated for 60 days, dramatically outperformed planktonic cultures with respect to ethanol production (536 g L^{-1} h^{-1} compared with 5 g L^{-1} h^{-1}). Li et al. (2006) subsequently demonstrated that *Z. mobilis* biofilms were more resistant to the toxic effects of benzaldehyde than planktonic cells, retaining 45% metabolic activity after exposure to 50 mM of the solvent, whereas the planktonic cells were inactivated. Interest in *Z. mobilis* biofilms for the production of ethanol has continued, and researchers have investigated lignocellulose hydrolysates as fermentable substrates on different support surfaces such as DEAE cellulose and corn silk (Todhanakasem et al. 2015; Todhanakasem et al. 2016). Furthermore, it has been shown that the biofilm is more resistant than planktonic cells to toxic compounds that are typically found in the hydrolysates, such as furfural (Todhanakasem et al. 2018; Todhanakasem et al. 2014).

5.2.3 Bacillus subtilis

Bacillus spp. are industrially important bacteria; *B. subtilis* is particularly relevant owing to its GRAS status and is applied in the production of enzymes and pharmaceutically important compounds (Gu et al. 2018). For example, menaquinone-7 (MK-7), or vitamin K2, is produced industrially by *Bacillus subtilis* natto in static fermentation, where it forms pellicles (Mahdinia et al. 2019). To investigate if biofilm growth might improve productivity and alleviate problems associated with heat and mass transfer, Mahdinia et al. (2017) developed a biofilm rector using plastic composite supports arranged in a grid formation (Fig. 5.3), but the MK-7 concentration was low compared to the static fermentation in a glycerol-based culture medium, with concentrations of approx. 1 mg L^{-1} in biofilm compared with over 30 mg L^{-1} in static fermentations. However, further medium optimisations using a glucose-based medium resulted in improvement of productivity to 20 mg L^{-1} (Mahdinia et al. 2018).

Bacillus spp. also produce the lipopeptides surfactin, iturin A and fengycin (amongst others) that have significant biological activity. They are also biosurfactants, and their production in suspended cultures can result in foaming, which is detrimental to the fermentation, and one potential solution to this problem is to employ biofilms. Several bioreactor configurations have been investigated to

Fig. 5.3 Grid arrangement of PCS used to support *B. subtilis* natto biofilms. (Reprinted by permission from Springer: Applied Microbiology and Biotechnology (Biofilm reactors as a promising method for vitamin K (menaquinone-7) production, Mahdinia et al.), © 2019)

promote biofilm growth and production of lipopeptides, for example, Chtioui et al. (2012) used a rotating disc bioreactor to cultivate *B. subtilis* ATCC 21332, which produces surfactin and fengycin. In this bioreactor, the discs were partially submerged in the culture medium enabling surface aeration, thus avoiding foam formation and favouring the production of fengycin over surfactin. Using 14 discs and an airflow of 100 L h^{-1}, fengycin productivity reached 838 mg L^{-1} after 72 h, which was greater than that of planktonic cultures and an improvement on other bubbleless reactors studied up to that point. A further alteration in the design to include agitators to improve oxygen transfer further improved the selectivity of the reactor for fengycin production (Chtioui et al. 2014).

Bruck et al. (2019) investigated the genetic changes required for *B. subtilis* 168 to grow effectively as a biofilm and produce surfactin. This is a domesticated strain that is widely studied but has key mutations that both prevent surfactin production and impede biofilm growth. These researchers repaired the *sfp* and *epsC* genes, which enabled the cells to produce surfactin and EPS; they also deleted *sepF*, which codes for a protein involved in septation, resulting in cell filamentation. The resulting mutants had much improved biofilm-forming capabilities, which was mainly down to the production of EPS, and the biofilms demonstrated ten-fold enhanced surfactin production compared with strains that could only grow planktonically but had a functional *sfp*.

The same strain could be manipulated to produce another lipopeptide, iturin A, by horizontal transfer of the *itu* operon and introduction of the *sfp* gene plus the pleiotropic regulator *degQ* (Tsuge et al. 2005). Rahman et al. (2007) investigated

this strain for its biofilm-forming capability and found that it formed thick biofilm in a multiwell plate; furthermore, it produced approx. Double the concentration of iturin A of planktonic cultures (~800 mg L^{-1}). Productivity of iturin A in planktonic and biofilm cultures is also impacted by the medium used: Zohora et al. (2013) found that by using a medium containing 12% maltose and 5% fish protein, *B. subtilis* RB14 biofilms grown in Erlenmeyer flasks could produce over 5 g L^{-1} of the lipopeptide.

5.2.4 Propionibacteria

Propionibacterium spp. are anaerobic, Gram-positive bacilli that are found in milk, dairy products, skin, soil, plants or digestive tracts of ruminants (Zárate 2012). Probiotics and cheese industries employ *Propionibacterium* to produce vitamin B12, tetrapyrrole containing compounds and propionic acid (Kiatpapan and Murooka 2002). These bacteria are capable of catabolizing various polyols (like glycerol), carbohydrates (like glucose, trehalose, etc.), lactic acid and pyruvic acid into succinic acid, propionic acid, acetic acid and CO_2; however, propionic acid is a major product in the fermentation (Roy 2019). Various species of *Propionibacteria* (*P. freudenreichii*, *P. acnes*, *P. acidipropionici*, *P. acidifaciens*, *P. namnetense*, *P. propionicus*, etc.) can form a biofilm, so have potential application in large-scale production.

Propionibacteria acnes can cause blood infections in immunocompromised patients (like pneumonia and chronic granulomatous disease) and endocarditis patients and like infections in ventriculoperitoneal shunts, pacemaker devices, prosthetic joints, catheters, etc. (Bayston et al. 2007; Holmberg et al. 2009; Tyner and Patel 2016). Coenye et al. (2008) cultured in vitro biofilms from planktonic *P. acnes* obtained from skin infections. The biofilms hydrolyzed triglycerides yielding free fatty acids that were subsequently catabolised to short-chain fatty acids (like propionic acid and butyric acid) depending upon the bacterial subtype and free fatty acid length (Linfante et al. 2018).

Propionibacteria freudenreichii, *P. acidipropionici* and *P. arabinosum* can immobilise as biofilms and have been exploited for propionic acid and acetic acid production (Xu et al. 2011). Dishisha et al. (2012) used polyethylenimine-treated Poraver® (glass beads) matrix for *P. acidipropionici* DSM 4900 immobilisation and obtained propionic acid productivity of 0.35 g L^{-1} h^{-1} in batch or fed-batch fermentation and 1.40 g L^{-1} h^{-1} in continuous mode. In another experiment, a maximum propionic acid productivity of 1.63 g L^{-1} h^{-1} was obtained from 90 g L^{-1} glycerol in sequential batch fermentation (Dishisha et al. 2015). Chen et al. (2013) assembled a plant fibrous-bed bioreactor for immobilisation of *P. freudenreichii* CCTCC M207015 for effective production of propionic acid. Up to 41.20 ± 2.03 g L^{-1} propionic acid was obtained from 80 g L^{-1} glucose in 108 h, which was 21.07% more than that produced in suspended cell fermentation. Furthermore, they optimised the fermentation by a constant fed-batch process which led

to 136.23 ± 6.77 g L^{-1} propionic acid in 108 h. Recently, Cavero-Olguin et al. (2019) used biofilms of *P. acidipropionici* in recycle batch reactors (Poraver®) for improved fermentation of carbohydrates and glycerol for propionic acid production. Citric acid and NaCl were used to induce bacteria to form biofilm as these increased the biofilm-forming capacity (BFC) index, EPS production and trehalose production with increased expression *treY* gene. They obtained the highest productivity of 0.7 g L^{-1} $h^{-1}$with citric acid and 0.78 g L^{-1} h^{-1} with NaCl.

5.2.5 Actinobacillus succinogenes

This rod-shaped, facultatively anaerobic bacterium, isolated from the bovine rumen, produces succinic acid from pentose and hexose sugars and is promising for its wide utilisation of carbon (Jiang et al. 2019; Pateraki et al. 2016). It can also yield other organic acids (e.g. pyruvic and lactic acids) under microaerobic conditions (Li et al. 2010; Wang et al. 2016). Recently, Yang et al. (2019b) reviewed metabolic engineering strategies to improve succinic acid synthesis with reduced accumulation of the by-product, via elimination of the metabolic pathways involved in by-product formation. However, there are many key factors (such as media components, trace substances, neutralizing agents, CO_2, redox potential, temperature, pH, stirring) that can affect bacterial growth and significantly improve the succinic acid accumulation without the formation of any by-product. For instance, Zhu et al. (2012) employed Box-Behnken design method of RSM to optimise the fermentation media compositions for the production of maximal succinic acid, and they achieved 52.7 ± 0.8 g L^{-1}.

Actinobacillus succinogenes is well studied for biofilm formation via self-immobilisation to the support surfaces under prolonged operation (Bradfield and Nicol 2014). *Actinobacillus succinogenes* biofilm has immense potential for improved yield of succinic acid over free-cell fermentations since both cell growth and maintenance rates in free-cell fermentation are drastically decreased with increased succinic acid titre. In contrast to suspended cells in a chemostat, biofilm was capable of achieving higher cell densities and improved succinic acid yield (Brink and Nicol 2014). Mokwatlo and Nicol (2017) characterised the cellular structure and viability of *A. succinogenes* biofilms as it exhibits a heterogeneous structure: The top layer cells have increased cell viability, and biofilm sessile cells have different morphology compared to planktonic cells and extensive connection fibres. In earlier experiments, Urbance et al. (2003, 2004) evaluated succinic acid production by *A. succinogenes* in both continuous and repeat-batch in a plastic composite support bioreactor for biofilm formation. As in continuous fermentation, repeat-batch can operate in the biofilm mode as it retained biomass after every batch cycle. More recently, Longanesi et al. (2018) used Glaxstone®, which is a sintered glass, as the support material in a 1 L packed-bed reactor for succinic acid production with grown *A. succinogenes* biofilms. The attached cells were fed with cheese whey (lactose-rich by-product of cheese processing), and maximum productivity of

0.72 g L^{-1} h^{-1} was achieved. Similarly, Ferone et al. (2018) also used a packed-bed biofilm reactor for continuous succinic acid production for over 5 months and evaluated different dilution rates and carbon sources. The bioreactor showed maximum productivity of 43 g L^{-1} h^{-1} at a dilution rate of 0.5 h^{-1} with glucose as the substrate.

5.2.6 Lactobacillus

Lactobacilli are anaerobic (aerotolerant), non-spore-forming, Gram-positive bacteria, present in the human mouth, associated with dental caries, gastrointestinal tract and genital tract of females (Makarova et al. 2006). Currently, the use of *Lactobacillus* spp. is expanding towards other dairy products such as energy bars, chocolate, juices, shakes, etc. This extensive use of *Lactobacillus* spp. requires reduction of foodborne pathogenic contaminations and their biofilm formation in the food industries. These bacteria are mostly employed for the production of lactic acid in the food, cosmetics and pharmaceutical industries (Taskila and Ojamo 2013). One other major use of lactic acid is in the production of biodegradable and renewable raw material such as poly lactic acid (PLA) for household applications (Krishna et al. 2018) and in the synthesis of environmentally friendly solvents, such as butyl lactate (Wee et al. 2006).

The biofilms of *Lactobacillus* are more resistant to acetic acid/vinegar and ethanol (used as a food preservative) than planktonic cultures. Kubota et al. (2008) investigated biofilm formation by 40 different species of lactic acid bacteria on the glass coverslips and found longer cells than in their planktonic cultures. Among these species, *L. plantarum* M606 have maximum survival percentage in 10% acetic acid and 30% ethanol. In earlier work, Demirci et al. (1993) compared pure- and mixed-cultures of *Streptomyces viridosporus* T7A and *Lactobacillus casei* subsp. *rhamnosus* for biofilm formation and continuous production of lactic acid through fermentation in a biofilm reactor with plastic composite support (PCS) chips. Their work showed two to five times faster lactic acid production in the pure- and mixed-culture bioreactors than those of their suspension culture (control). Similarly, Cotton et al. (2001) designed a biofilm reactor with six PCS tubes composed of agricultural products (50% w/w) and polypropylene (50% w/w) for continuous lactic acid fermentation using *Lactobacillus casei* subsp. *rhamnosus*. The study found that biofilm growth on the PCS tubes can be controlled by the speed of agitation and reported the PCS biofilm reactor improved the yield of lactic acid by 70%. The PCS biofilm reactor achieved a 9 g L^{-1} h^{-1} optimal average production rate, whereas the control reactor without PCS tubes achieved only 5.8 g L^{-1} h^{-1} at 125 rpm agitation and 0.4 per hour dilution rate. Cuny et al. (2019) investigated a strain of *L. delbrueckii* that was a high producer of lactic acid, for its ability to form biofilms in a tubular reactor and reported higher cell densities and productivity of 9 g L^{-1} h^{-1}. The biofilm could be maintained for more than 2 weeks.

5.2.7 Aspergillus niger

Lignocellulolytic enzymes are of intense interest for the digestion of lignocellulose biomass, which is comprised of lignin, cellulose and hemicellulose, to simple sugars that can be used in the production of biofuel. *Aspergillus niger* elaborates a number of these enzymes and their activities have been investigated in biofilm cultures of the fungus. Villena and Gutierrez-Correa (2006) cultivated *A. niger* biofilms on polyester cloth with lactose as a growth substrate, either in flasks or in microbioreactors, showed that the biofilms produced up to three times more cellulase than the free mycelium cultures. Similarly, when biofilm was grown on perlite, the production of cellulase, endoglucanase and xylanase was greater than that in either submerged culture or solid-state fermentation (Gamarra et al. 2010). Comparison of cellulase gene expression in *A. niger* grown as biofilm and submerged fermentation demonstrated a higher level of expression in biofilm, which is reflective of the enzyme activity measurements (Mahmoud et al. 2016). Izmirlioglu and Demirci (2017) established a dual biofilm with *A. niger* and *S. cerevisiae* to couple saccharification and fermentation, generating bioethanol directly from food waste. Biofilms were established on plastic composite support and under optimised conditions almost 38 g L^{-1} ethanol was produced after 72 h.

A. niger is also known for the industrial production of citric acid for the food and pharmaceutical industries. The current method involves submerged fermentation, but efforts have been made to investigate the possibility of using biofilms to enable continuous production of this important organic acid. Earlier work examined the *A. niger* biofilms growing in rotating biological contactors, for example, Wang (2000) used polyurethane foam-covered discs to grow the fungus and observed citric acid productivity of 0.896 g L^{-1} h^{-1}, compared with 0.33 g L^{-1} h^{-1} in submerged culture. More recently, Yu et al. (2018) employed a porous foam carrier, comprised of polyurethane and carbon black, in repeated fed-batch fermentation. Stable citric acid production was achieved over 600 h, with 8 fed-batch cycles, yielding 2.26 g L^{-1} h^{-1}.

5.3 Biofilms in Biotransformation

Biocatalysis describes the use of an enzyme or whole cell to afford the biotransformation of a substrate to a valuable intermediate or product and is increasingly important in the synthesis of fine chemicals, since the reactions are more environmentally-friendly than those of classical organic chemistry. Industrially important chemicals such as acrylamide, synthons such as *R*-2-chloropropionic acid and drugs such as L-DOPA rely on biocatalysis for their production (Murphy 2012); increasing applications are inevitable as manufacturing processes require sustainability. Bacterial biofilms have been studied for their potential use in biocatalytic processes since they typically have a longer effective half-life compared with

suspended cells, have a higher tolerance to the often unnatural substrates that are required, can be easily reused and require no separation step from the product.

5.3.1 Pseudomonas taiwanensis *VLB120ΔC*

The metabolic diversity in *Pseudomonas* spp. has resulted in these bacteria being used in numerous biocatalytic applications. *Pseudomonas taiwanensis* VLB120ΔC biofilms have been extensively investigated for the continuous production of valuable compounds in a variety of bioreactors. The bacterium was originally identified as a potentially useful biofilm biocatalyst following a screen of strains, including those isolated from biofilters and contaminated soils, in a well-plate assay (Gross et al. 2007). As it demonstrated comparatively good biofilm growth and had been already investigated for its ability to stereospecifically transform styrene to (*S*)-styrene oxide, it was selected for further investigation in a tubular bioreactor. The biofilm was established on the inner wall of the silicone tubing that was partially submerged in liquid styrene; the substrate diffused into the biofilm and was biotransformed continuously over a 55-day period. As with *Z. mobilis*, exposure to the solvent (styrene) impacted planktonic cells more than biofilm cells, which rapidly adapted to solvent exposure by producing more extracellular polymeric substances (Halan et al. 2011). Further experiments demonstrated that oxygen was a major limiting factor of styrene oxide productivity in the biofilm, which was affected by membrane area and tube wall thickness (Gross et al. 2010); biofilm growth on microporous ceramic material was shown to enable efficient oxygen transfer (Halan et al. 2010), demonstrating the impact of the support material when cultivating biofilms. Additional iteration of the bioreactor design employed an aqueous-air segmented flow arrangement (Fig. 5.4) (Karande et al. 2014), whereby the biofilm was allowed to develop in a tube in single-phase flow for 72 h; air segments were introduced, which caused biofilm detachment, then regrowth of a stable second generation biofilm. When the biofilm was exposed to styrene, a volumetric productivity of (*S*)-styrene oxide of 46 g ${L_{tube}}^{-1}$ day^{-1} was achieved, which was 21-fold greater than the initial single-phase flow used in the original experiments. Further improvement of the biofilm biocatalyst was achieved by using a mutant strain of the bacterium, designated VLB120ΔCeGFP 04710, in which a gene responsible for the degradation of the second messenger c-di-GMP was deleted (Schmutzler et al. 2017). This strain adhered more strongly owing to higher EPS production and increased hydrophobicity, resulting in higher cell numbers in the initial phase.

In addition to the biotransformation of styrene, VLB120 has been metabolically engineered to produce other important chemicals, either via biotransformation or from the growth substrate/carbon source. For example, Lang et al. (2015) engineered the bacterium to produce (*S*)-3-hydroxybutyric acid fermentatively from glucose by eliminating 3-hydroxyisobutyric acid dehydrogenase activity through random mutagenesis, heterologously expressing *kivD* from *Lactococcus lactis*, which codes for

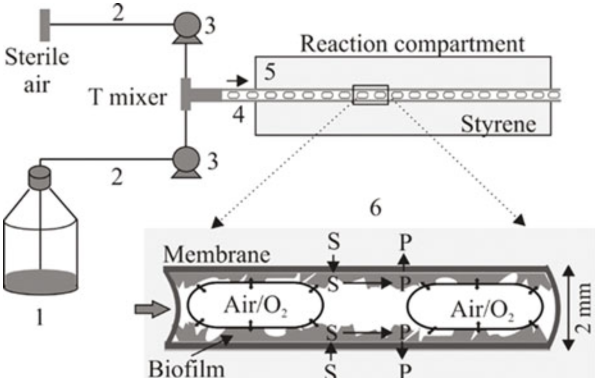

Fig. 5.4 Schematic set-up of the aqueous-air segmented flow biofilm reactor. (1) Medium reservoir; (2) 1.5 mm silicone tubing; (3) peristaltic pump; (4) silicone tubing as growth surface (2 mm inner diameter and 2000 mm long); (5) styrene phase; (6) magnified sketch of aqueous-air segments describing the mass transfer scheme of substrate and products in the reactor. S—styrene; P—(S)-styrene oxide. (Copyright © 2014 WILEY_VCH Verlag GmbH & Co. KgaA, Weinheim. Used with permission from Rohan Karande et al., "Segmented flow is controlling growth of catalytic biofilms in continuous multiphase microreactors", Biotechnology and Bioengineering, John Wiley and Sons)

2-ketoacid dehydrogenase, and homologously expressing genes in the pathway for 2-ketoisovalerate synthesis. The bacterium was engineered to express other enzymes to expand the capabilities of the biofilm in the continuous production of other important chemicals such as n-octanol (Gross et al. 2013).

Finally, the strain was employed in a dual-species biofilm alongside the cyanobacterium *Synechocystis* sp. PCC6803 to alleviate oxidative stress and allow the high cell density growth of the photoautotroph (Hoschek et al. 2019). Both strains expressed a cyclohexane monooxygenase, and the biofilm was applied in the production of cyclohexanol and was active for at least 1 month.

5.3.2 Escherichia coli *PHL644*

This strain of *E. coli* is a mutant strain of a K-12 strain (MC4100) that overproduces curli adhesin, which enables it to readily form biofilm. Tsoligkas et al. (2011) engineered the strain to express a tryptophan synthetase from *Salmonella enterica* sv Typhimurium so that it could biocatalytically produce 5-halotryptophans from 5-haloindoles. After growing the bacterium as a biofilm on poly-L-lysine-coated glass slides, using a spin-coating method to improve biofilm growth, the biotransformation of halotryptophans was compared with that in free cells and immobilised enzyme, and found to be superior. Furthermore, the biofilm lost little activity through three 12 h biocatalytic cycles. The longevity of the enzyme activity was explored by Tong et al. (2016) who used stable isotopic labelled amino acids in cell cultures

(SILAC) to follow the fate of the recombinant tryptophan synthetase. Surprisingly, the reason for the extended enzyme activity was not due to the stabilising influence of the extracellular matrix, as might have been expected, but because the enzyme was continually replenished.

5.3.3 Acinetobacter *ST-550*

The biocatalytic production of the dye indigo from indole has been the subject of numerous investigations but is complicated by the toxicity of the substrate. As has been noted already, one advantageous characteristic of biofilms is their improved resistance to the toxic effects of certain compounds. *Acinetobacter* ST-550 is a bacterium originally isolated from soil and demonstrated efficient indigo production in organic solvent (Doukyu et al. 2002). However, the bacterium does not readily form biofilms under conventional conditions, so they would not be a candidate for such investigations. However, Ishikawa et al. (2014) employed a novel approach to improve the biofilm-forming characteristics of ST-550 by expressing the gene coding for the adhesin AtaA from another *Acinetobacter* strain (tol 5). This protein makes the tol 5 strain extremely sticky and enables it to adhere to numerous abiotic surfaces (Furuichi et al. 2018). The transformed ST-550 was subsequently able to adhere to polyurethane and the resulting biofilm displayed an improved tolerance for the starting material and catalysed the production of indigo at a rate that was five-fold faster than planktonic cells. The strategy of expressing the *ataA* gene in bacteria already known to catalyse industrially important reactions could speed up the development of biofilm reactors for continuous processes. Indeed, the same approach was taken with the hydrogen-producing bacterium *Enterobacter aerogenes* (Nakatani et al. 2018), which was immobilised on polyurethane foam and could continually produce hydrogen from glucose. Most recently, a truncated AtaA was employed to adhere an artificial cell (liposome) to polystyrene and glass (Noba et al. 2019); the liposomes also had β-glucuronidase encapsulated, the activity of which could be measured after adherence. Such artificial biofilms might be useful in circumstances where genetically engineered organisms are prohibited.

5.3.4 Pseudomonas diminuta

Glycolic acid can be used as skincare agent in pharmaceutical products, as a preservative and flavouring agent in food processing, as a dyeing/tanning agent in textile processing and as an additive in adhesives, polymers, paints, plastics, inks, etc. (Hua et al. 2018). *Pseudomonas diminuta* (or *Brevundimonas diminuta*) can biotransform ethylene glycol to glycolic acid without the formation of by-products (Li et al. 2007); thus, it is of industrial interest. Furthermore, it can grow efficiently as a biofilm and the ability of the bacterium to continually produce glycolic acid has

been examined by Li et al. (2013). These researchers evaluated glycolic acid production in a trickle-bed biofilm reactor fitted with Montz-pak A3–500 stainless steel structured packing for improved catalyst stability (up to 60 days) and efficient gas-liquid exchange. A steady state productivity was achieved up to 1.6 g L^{-1} h^{-1} which compares favourably to that of planktonic cultures, for example, Kataoka et al. (2001) examined glycolic acid production in planktonic cultures of different organisms and observed up to 0.92 g L^{-1} h^{-1}.

5.3.5 Cunninghamella elegans

The recognition that filamentous fungi can grow as biofilms is relatively recent. Harding et al. (2009) proposed criteria for defining these biofilms, which are based on differences in structural features (complex aggregated growth of hyphae, surface-associated growth, production of extracellular matrix) and physiology (enhanced tolerance to biocides, changes in enzyme and metabolite production). One fungus capable of biofilm growth is the zygomycete *Cunninghamella elegans*. This fungus is a model of mammalian drug metabolism, and many examples exist in the literature reporting the production of mammalian-equivalent phase I (oxidative) and phase II (conjugative) metabolites upon incubation of the fungus with different drugs (Asha and Vidyavathi 2009). Recently, the complement of cytochromes P450, which are responsible for the oxidation of drugs, were identified in the fungus (Palmer-Brown et al. 2019a). Most studies focus on using planktonically-grown cells to biotransform xenobiotics, but Amadio et al. (2013) and Mitra et al. (2013) described how the fungus can be grown as a biofilm, either on a spring included in an Erlenmeyer flask

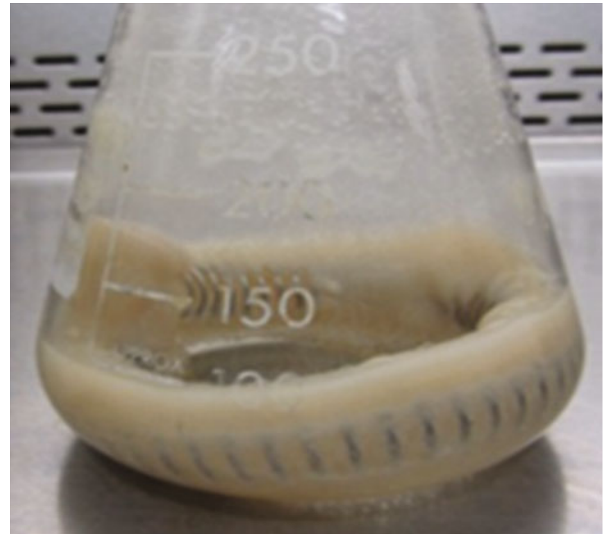

Fig. 5.5 *C. elegans* grown as biofilm in an Erlenmeyer flask with a spring included. The spring must be touching the glass for the biofilm to properly form. (Reprinted by permission from Springer: Applied Microbiology and Biotechnology (Filamentous fungal biofilm for production of human drug metabolites, Amadio et al.), © 2013)

(Fig. 5.5) or on polymethylmethacrylate. Under these conditions, biotransformation reactions are enhanced; for example, Mitra et al. (2013) observed that fluoranthrene biotransformation was enhanced by 22-fold in biofilm compared with planktonic cultures. Amadio et al. (2013) demonstrated the convenient reusability of the biofilm by using it to catalyse multiple rounds of biotransformation of the drug flurbiprofen. Furthermore, the biotransformations could be performed in water rather than culture medium, and by shortening the incubation time with the drug and introducing rejuvenation steps with a fresh culture medium, Quinn et al. (2015) improved the productivity of phase I metabolites in biofilms from 1 mg L^{-1} h^{-1} to over 4 mg l^{-1} h^{-1}.

5.4 Biofilms in Biodegradation

For several decades, bacteria and fungi have been studied for their ability to biotransform a wide range of xenobiotics. They are able to do this since many xenobiotics are similar in structure to naturally occurring compounds and microbial enzymes have a relaxed substrate specificity. For example, some *Pseudomonas* bacteria have evolved pathways to catabolise biphenyl, which occurs naturally from the pyrolysis of lignin; the same bacteria are able to biotransform anthropogenic polychlorinated biphenyl (Field and Sierra-Alvarez 2008). Since microbes are environmentally benign, this gives them an advantage over chemical and physical approaches to remediation of contaminated sites (bioremediation) and waste streams. The advantages that immobilisation conveys are particularly relevant to biodegradation, and in this section, examples of how biofilms can remove pollutants from toxic waste streams are presented.

5.4.1 Pseudomonas knackmussii *B13*

Many drugs and xenobiotics contain fluorine, which makes them particularly resistant to biodegradation owing to the strength of the carbon-fluorine bond. *P. knackmussii* was studied several decades ago for its ability to biodegrade fluorobenzoate. Schreiber et al. (1980) demonstrated that the bacterium could use 4-fluorobenzoate as a sole carbon and energy source, yielding fluoride ion. This characteristic was later exploited by Misiak et al. (2011) who grew B13 in a membrane-aerated biofilm reactor (MABR, Fig. 5.6) for up to 600 h. Two growth phases were observed: an initial period of fast growth up to 200 h, followed by a period of much slower growth. By measuring the concentration of fluoride ion and 4-fluorobenzoate concentration in the effluent it was clear that approx. 15% of the available fluorine remained in the biomass. Subsequent experiments using a tubular bioreactor showed that fluoride ion accumulated in the biofilm and inhibited growth. These experiments revealed that while biofilms can be advantageous for the

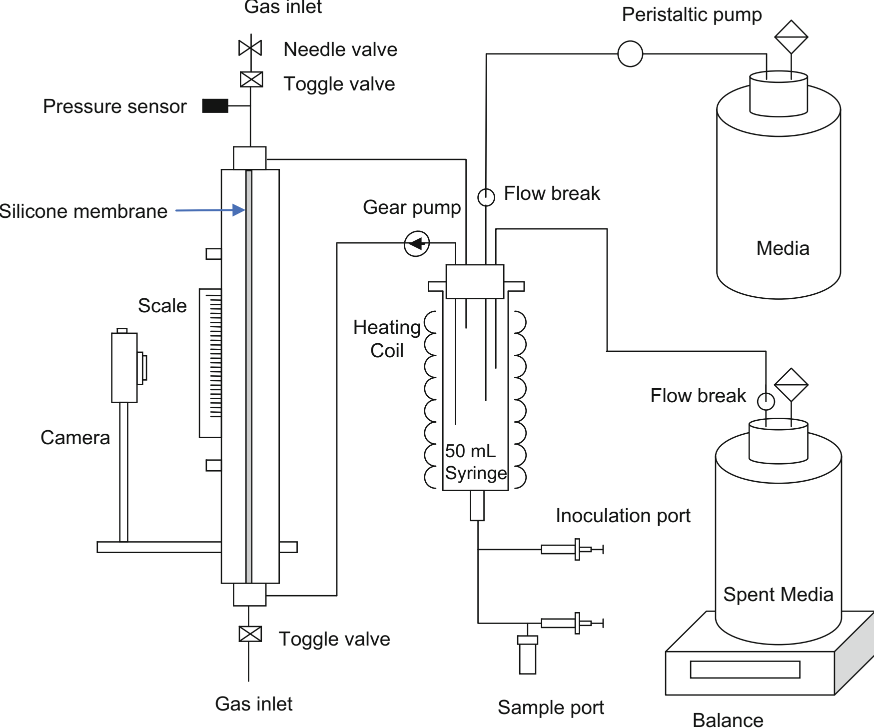

Fig. 5.6 Schematic diagram of the MABR used to grow *P. knackmussii* B13 biofilm on 4-fluorobenzoate. The biofilm grew on the outer surface of the silicone tubing. (Reprinted from Water Research, Volume 45, Misiak et al., Factors influencing 4-fluorobenzoate degradation in biofilm cultures of *Pseudomonas knackmussii* B13, Pages 3512–3520, Copyright (2011), with permission from Elsevier)

continuous degradation of persistent chemicals, the toxic intermediate may accumulate and impact on performance.

5.4.2 Pseudomonas stutzeri *T102*

This bacterium was isolated from the sludge in an oil tank in Okinawa, Japan and degrades PAHs including naphthalene (Hirano et al. 2004) and was selected for further investigation by Shimada et al. (2012) as it readily formed biofilms on a number of surfaces. These researchers compared naphthalene degradation in biofilm and planktonic cultures of the bacterium and found that although initially degradation was faster in planktonic cultures, after 4 h the biofilm culture degraded naphthalene quicker. Sloughing of cells was noted in the biofilm cultures, which is common, and Shimada and colleagues found that the expression of *nahAc*, which

codes for the large subunit of naphthalene dioxygenase, was much higher in these cells than in planktonic cells. Thus, it was concluded that the rapid degradation of naphthalene in biofilms was due to the detached cells rather than the cells remaining in the biofilm.

5.4.3 Cunninghamella elegans

In addition to the application of the fungal biofilm to biocatalytic production of drug metabolites, its usefulness in the biodegradation of pollutants has been investigated. Hussain et al. (2017) investigated the bioremediation of water containing the dye malachite green and the metal Cr (VI). An earlier study had shown that *C. elegans* could degrade malachite green via leucomalachite green and *N*-demethylated and *N*-oxidised products (Cha et al. 2001). The biofilm rapidly (15 min) absorbed the dye from the water, which was followed by a slower decolourisation of the biomass; the biofilm could be used repeatedly for dye decolourisation. Furthermore, when Cr (VI) was also included in the water, this was simultaneously removed, even in the presence of high salt concentrations, demonstrating that the biofilm was robust and could be applied to the removal of several pollutants under harsh environmental conditions.

Planktonically-grown *C. elegans* is able to biotransform agrochemicals such as the insecticides fenitrothion and diazinon (Zhao et al. 2020; Zhu et al. 2017), and the fungicide mepaniprim (Zhu et al. 2010). Palmer-Brown et al. (2019b) compared planktonic and biofilm cultures of the fungus in the biodegradation of the pyrethroid insecticide cyhalothrin and found that similar to malachite green, the compound was initially biosorbed in the biofilm before being more slowly degraded. Interestingly, planktonic cells were more effective at degrading the pesticide as only 6% of the initial material remaining after 120 h, whereas, in the biofilm, 22% of the starting substrate remained after the same incubation period. Repeated additions of pesticides were not investigated in this study.

5.4.4 Bacillus subtilis *N4*

Acetonitrile is a toxic chemical that is biotransformed to hydrogen cyanide and acetaldehyde. It can be degraded by some microorganisms, such as *Pseudomonas aeroginousa*, *Mesorhizobium* sp. and *Rhodococcus rhodochrous* (Feng and Lee 2009; Chapatwala et al. 1990; Li et al. 2012), via the actions of nitrile hydratase and amidase. The genes coding for these enzymes in *R. rhodochrous* BX2 were cloned into *B. subtilis* N4, which grows well as biofilm. The strain was grown on amine-modified polypropylene carriers, which were added to a movable bed biofilm reactor (MBBR) that had been seeded with activated sludge (Li et al. 2018). The biofilm was able to withstand repeated additions of synthetic wastewater containing

acetonitrile and community analysis of the biofilm reactor revealed that the recombinant strain was the dominant organism after 50 days' operation, demonstrating the applicability of the organism to a wastewater treatment system.

5.5 Future Prospects

The research on productive biofilms is limited compared with the efforts that have been made to investigate other methods of immobilisation. Nevertheless, the studies consistently demonstrate that compared with suspended cultures, biofilms are more robust, are resistant to toxic effects, are more productive and are stable over much longer timeframes. However, there are obvious challenges to applying biofilms at larger scales, notably sloughing, which is difficult to predict and control and might lead to blockages in tubing or contamination of the product. Additionally, the biofilm bioreactors are quite specialised; thus, considerable investment would be required from industry if they were to be adapted. Nevertheless, there is sufficient evidence that research on biofilms that might be applied in industry should be accelerated.

In relation to biodegradation, although single-species biofilms might play an important role in the decolourisation of dyes (Cerron et al. 2015; Malachova et al. 2013; Mawad et al. 2016; Munck et al. 2018; Novotny et al. 2012), the structural complexity of many xenobiotics means that a single species is unlikely to have the catabolic capacity to fully degrade a particular pollutant. Thus, mixed-species biofilms are more useful in this regard. For instance, the degradation of isomeric dinitrotoluene (DNT), i.e. 2,4-dinitrotoluene (2,4-DNT) and 2,6-dinitrotoluene (2,6-DNT), was studied by Lendenmann et al. (1998) using mixed culture bacteria that are capable of growing on these isomers as the sole carbon and nitrogen source. In an aerobic fluidised-bed biofilm reactor, the mixed culture had a degradation efficiency of 98% for 2,4-DNT and 94% for 2,6-DNT. Similarly, Oh and Tuovinen (1994) studied biodegradation of the phenoxy herbicides methylchlorophenoxypropionic acid (MCPP) and 2,4-dichlorophenoxyacetic acid (2,4-D) using a mixed culture of herbicide-degrading bacterial biofilm in fixed-film column reactors and found overall efficiency of degradation of MCPP was partial and 2,4-D was complete. A major problem with current waste-water treatment facilities is that pharmaceuticals and personal care products are not effectively removed and consequently are released back into waterways where they are a potential hazard. Bioaugmentation of mixed-species biofilms that are found in wastewater treatment plants with strains developed for the biodegradation of specific xenobiotics, such as that described in Sect. 5.4.4, is therefore an important strategy.

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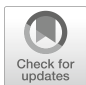
Chapter 6
Pyrethroid-Degrading Microorganisms and Their Potential Application for the Bioremediation of Contaminated Environments



Yaohua Huang and Shaohua Chen

Abstract Long-term and extensive application of synthetic pyrethroid (SP) insecticides indoor has resulted in a large increase in the number of people reported to have detected residues of pyrethroids and their major intermediate metabolite 3-phenoxybenzoic acid (3-PBA) in body fluids. The neurotoxicity and reproductive toxicity of pyrethroids to nontargets have attracted extensive attention. The microbial degradation of pyrethroids has been reported frequently in the past 30 years. In recent years, based on the development of biomolecular tools and materials science, the mode of action of microorganisms and their functional enzymes has been expanded. This chapter summarizes the pyrethroid degradation microorganisms that have been published in the past and proposes the metabolic pathways of pyrethroids. In addition, we also discussed the degradation mechanisms of pyrethroids based on the catalytic triad of the pyrethroid hydrolase.

Keywords Pyrethroids · Toxic effect · 3-Phenoxybenzoic acid · Bioremediation

6.1 Introduction

Pyrethroids are a synthetic organic compound similar in structure to natural pyrethrums extracted from *Chrysanthemum cinerariaefolium*. Their acid and alcohol parts are bound by ester bonds and usually contain 1–3 chiral centers. As a chiral compound, pyrethroids usually have 4–8 stereoisomers (Bhatt et al. 2020a). Different isomers exhibit different insecticidal activities and have enantioselective degradation characteristics during microbial metabolism (Garcia et al. 2017). Pyrethroids

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can be divided into two categories based on the presence or absence of cyano-group on the α-chiral carbon: type II pyrethroids with cyano-group and type I pyrethroids without cyano-group.

Allethrin is the first type I pyrethroid synthesized in the USA in 1949, which is to control domestic sanitary pests (Bhatt et al. 2020d). Compared with type II pyrethroids, pyrethroids of type I had structural diversity and were generally less toxic than type II due to the absence of cyano-group (Zhang et al. 2016). In addition to the cyano-group, pyrethroids have introduced halogens such as fluorine, chlorine, and bromine to increase insecticidal activity (Tang et al. 2018b; Zhan et al. 2018; Cycoń et al. 2014).

Pyrethroids have been used worldwide for more than 40 years since they were synthesized in the middle of the last century. With the increasing restrictions on organophosphorus and organochlorine pesticides by governments, the use of pyrethroids has increased year by year, from 25% of the world pesticide market in 2010 to more than 30% in 2018 (Zhai et al. 2012; Cycoń and Piotrowska-Seget 2016). A pyrethroid is a broad-spectrum insecticide with selective toxicity to Diptera, Hymenoptera, and mammals (Thatheyus and Gnana Selvam 2013). To improve the insecticidal activity and environmental stability of active ingredients, piperonyl butoxide and piperonyl sulfoxide were added to commercial pyrethroids (Thatheyus and Gnana Selvam 2013).

Pyrethroids are highly hydrophobic and will be adsorbed in sediment rapidly after entering the water body (Delgado-Moreno et al. 2011). Subsequently, some of them are utilized by indigenous microorganisms, and the other part enters the aquatic food web, which may eventually accumulate in the human body through biological amplification (Liu et al. 2012; Cycoń et al. 2017). A large number of results have confirmed that pyrethroids have high toxicity to aquatic organisms (Mendis et al. 2018; Mulla et al. 2017; He et al. 2008). Long-term exposure to pyrethroids can affect the quality of human semen (Ham et al. 2020). A series of environmental problems caused by the irrational use of pyrethroids has aroused public concern in recent years. At present, diverse methods have been developed to remove pyrethroids from the environment.

In this chapter, we are summarizing the previously isolated and characterized degrading microorganisms and introduce the metabolic pathways of common pyrethroids. In the last two decades, many researchers have reported pyrethroid hydrolases encoded by genes with open reading frames (ORF). We will discuss these functional enzymes and describe the metabolic mechanisms that catalyze the triad (Serine, Histidine, Aspartate) at the active site of the degrading enzyme.

6.2 Potential of Microbes in Pyrethroid Degradation

Pesticide-degrading microorganisms have become a scientific research hotspot and effective strategy to treat pesticide residues over 30 years of development due to their unique advantages. Microbes can catabolize the large-molecular heterologous

pollutants remaining in the environment into nontoxic or low-toxic inorganic small-molecule compounds in various ways (Huang et al. 2021; Lin et al. 2020; Mishra et al. 2020; Chen et al. 2012a). Generally, it is an effective way to use enrichment culture technology to screen functional microorganisms from soil, water, and activated sludge contaminated by pesticides (Zhang et al. 2021; Zhan et al. 2020; Lin et al. 2011). There are also reports that it is feasible to obtain degrading functional bacteria from resistant insects and fermented food (Cho et al. 2009). Type II pyrethroids are lethal to aquatic organisms which are often used as ship detergents to prevent the adhesion of marine organisms (Feng et al. 2009). Therefore, seawater has also been reported commonly as an important source of pyrethroid degrading microorganisms.

To date, a great number of studies have confirmed that bacteria and fungi can effectively degrade pyrethroid residues in water and soil (Table 6.1). There are also a few reports on yeast and algae (Pang et al. 2020). Microorganisms can use pyrethroids as the sole energy source or with the help of other nutrients to remove pyrethroids through co-metabolism (Zhao et al. 2019b). *Bacillus subtilis* 1D has been reported to hydrolyze 95% of cypermethrin within 15 days. And 700 bp esterase and 1200 bp laccase were extracted from strain 1D, which indicates that esterase and laccase are involved in the cypermethrin metabolism (Gangola et al. 2018). Wang et al. (2019) isolated a strain of *Photobacterium ganghwense* 6046 from seawater. This strain can grow with cyfluthrin as the sole carbon source and degrade 60% of cyfluthrin (100 mg·L^{-1}) for 72 h (Wang et al. 2019). The intermediate products of cyfluthrin were identified, and the cyano-group was not found. It is speculated that cyano-groups may be metabolized by strain 6046 first. *Paracoccus acridae* SCU-M53 is an epiphytic bacterium isolated from locusts, which can metabolize 79.84% of cyhalothrin in 48 h (Tian et al. 2018).

Response surface methodology (RSM) is a common method to optimize microbial growth and degradation conditions. Based on the Box-Behnken design, Zhan et al. (2018) used RSM to optimize the degradation conditions of *Acinetobacter baumannii* ZH-14. Under the conditions of 30 °C and pH 7, permethrin with a concentration of 50 mg·L^{-1} was completely removed within 72 h. *Bacillus thuringiensis* ZS-19 obtained from activated sludge can completely eliminate 100 mg·L^{-1} cyhalothrin in 72 h and can continue to work effectively when the concentration is as high as 800 mg·L^{-1}. In addition to cyhalothrin, strain ZS-19 can also efficiently metabolize fenpropathrin, deltamethrin, beta-cypermethrin, and cyfluthrin (Chen et al. 2015). Allethrin is the earliest synthesized pyrethroid, but reports on allethrin degradation bacteria are not common. *Acidomonas* sp. degraded 70% of allethrin at a concentration of 16 mM in MSM medium during 72 h and proposed a metabolic pathway (Paingankar et al. 2005). Recently, Bhatt et al. (2020b) manifested that *Sphingomonas trueperi* strain CW3 utilized allethrin as the sole source of energy and remove 93% of 100 mg·L^{-1} allethrin after 7 days. The optimal culture conditions are pH 7, temperature 30 °C, and inoculation amount of 0.1 g L^{-1}.

While earlier studies focused on the degradation kinetics of single strains, more studies now suggested that microbial consortia may exhibit higher degradation

Table 6.1 Pyrethroid-degrading microbes obtained from various sources

Pyrethroids	Microbial strains	Species	Results	References
β-Cypermethrin	*Pseudomonas aeruginosa* CH7	Bacteria	1. 25–900 mg/L β-cypermethrin is metabolized over 90% in MSM within 12 days 2. The optimal culture condition is 25–35 °C and pH 6–9, inoculation amount is 0.15 g/L	Zhang et al. (2011)
	Bacillus licheniformis B-1	Bacteria	1. 50% degradation observed in a liquid medium within 72 h 2. Degradation rate can be improved by adding surfactants Tween-80 and BRIj-35	Zhao et al. (2015)
	Aspergillus Niger YAT	Fungus	1. 54.83% degradation obtained with 50 mg·L^{-1} β-cypermethrin using strain YAT after 7 days	Deng et al. (2015)
	Brevibacillus parabrevis BCP-09	Bacteria	1. 75.87% of β-cypermethrin was removed by strain BCP-09 in 3 days 2. The optimal inoculation condition was pH 7.4, 38.9 °C	Tang et al. (2018a)
	Pseudomonas aeruginosa GF31	Bacteria	1. More than 80% of 50 mg/L β-cypermethrin was removed after 7 days 2. Supplementation of peptone significantly increased the degradation	Tang et al. (2015)
	Bacillus subtilis BSF01	Bacteria	1. 89.4% of 50 mg/L β-cypermethrin was eliminated after 7 days	Xiao et al. (2015)
	Ochrobactrum lupini DG-S-01	Bacteria	1. 90% of β-cypermethrin was degraded in MSM within 5 days and concentration was 50 mg/L	Chen et al. (2011a)
	Bacillus thuringiensis SG4	Bacteria	1. Approximately 80% β-cypermethrin (50 mg/L) was removed by strain SG4 after 15 days	Bhatt et al. (2020c)
	Bacillus sp. ISTDS2	Bacteria	1. β-cypermethrin with a concentration of 50 mg/L was complete catabolism in MSM within 8 days 2. Completely removed 100 mg/L beta-cypermethrin in soils after 30 days	Sundaram et al. (2013)
	Bacillus sp. SG2	Bacteria	1. 81.6% of β-cypermethrin (50 mg/L) were eliminated in MSM after 15 days	Pankaj et al. (2016)
	Bacillus subtilis 1D	Bacteria	1. 160 mg/L of β-cypermethrin was metabolized by 95% within 15 days	Gangola et al. (2018)

(continued)

Table 6.1 (continued)

Pyrethroids	Microbial strains	Species	Results	References
Fenvalerate	*Bacillus licheniformis* CY-012	Bacteria	1. When the culture was in the optimal condition at pH 7.48 and the initial concentration was 44 mg/L, about 80% fenvalerate was eliminated during 60 h	Tang et al. (2018b)
	Bacillus flexus XJU-4	Bacteria	1. Fenvalerate with a concentration of 2 mM was completely degraded within 6 days 2. Fenvalerate can be utilized as the sole source of carbon	Mulla et al. (2017)
	Stenotrophomonas sp. ZS-S-01	Bacteria	1. 100% removal of 50 mg/L fenvalerate was accomplished in 6 days 2. About 80% of fenvalerate was metabolized when concentration was 500 mg/L after 5 days	Chen et al. (2011c)
	Cladosporium sp. HU	Bacteria	1. More than 90% of fenvalerate with 100 mg/L was eliminated during 5 days	Chen et al. (2011d)
	Pseudomonas aeruginosa JQ-41	Bacteria	1. About 92.3% of fenpropathrin with an initial dose of 50 mg/L was degraded after 7 days	Song et al. (2015)
	Bacillus sp. DG-02	Bacteria	1. 93.3% degradation of fenpropathrin was achieved in a liquid medium within 72 h 2. Strain DG-02 can degrade a variety of pyrethroids	Chen et al. (2014)
Deltamethrin	*Bacillus cereus* Y1	Bacteria	1. About 99.4% deltamethrin was metabolized in MSM within 96 h 2. When deltamethrin in soils, the degradation rate is 74.9% after 24 days	Zhang et al. (2016)
	Streptomyces aureus HP-S-01	Bacteria	1. 100% initial dose of deltamethrin with a concentration of 300 mg/L was removed by strain HP-S-01 within 7 days 2. Deltamethrin can be used as a sole source of carbon	Chen et al. (2011b)
Cyhalothrin	*Bacillus thuringiensis* ZS-19	Bacteria	1. 100 mg/L of cyhalothrin was degraded completely in a minimal medium within 72 h	Chen et al. (2015)
	Paracoccus acridae SCU-M53	Bacteria	1. 79.84% of 75 mg/L cyhalothrin was degraded within 2 days, and the carried-out condition is 28 °C and 180 rpm	Tian et al. (2018)
	Cunninghamella elegans DSM1908	Fungus	1. Most of the 100 mg/L cyhalothrin is hydrolyzed within 120 h	Palmer-Brown et al. (2019)

(continued)

Table 6.1 (continued)

Pyrethroids	Microbial strains	Species	Results	References
	Aspergillus sp. CBMAI 1829	Fungus	1. 44.8% cyhalothrin was degraded when concentration was 100 mg/L in 14 days	Birolli et al. (2018)
Allethrin	*Sphingomonas trueperi* CW3	Bacteria	1. Approximately 93% allethrin (100 mg/L) was metabolized in the liquid medium after 7 days of incubation	Bhatt et al. (2020b)
	Fusarium proliferatum CF2	Fungus	1. Completely removal of allethrin (50 mg/L) was achieved after 5 days and used as a sole corban source	Bhatt et al. (2020d)
Permethrin	*Acinetobacter baumannii* ZH-14	Bacteria	1. Completely metabolism of chlorpyrifos with a concentration of 50 mg/L was achieved after 72 h 2. Permethrin can be utilized by strain ZH-14 for growth as a sole source of energy	Zhan et al. (2018)
Flucythrin	*Brevibacterium aureum* DG-12	Bacteria	1. About 88.6% of degradation was observed in 5 days	Chen et al. (2013a)
Cyphenothrin	*Staphylococcus succinus* HLJ-10	Bacteria	1. 92.8% cyphenthrin was metabolized in MSM within 7 days 2. Strain HLJ-10 can use cyphenthrin as the sole carbon source	Huang et al. (2020)
Phenothrin	*Pseudomonas fulva* P31	Bacteria	1. Strain P31 was able to completely degrade 100 mg/L phenothrin within 72 h 2. Phenothrin can be utilized as the sole carbon source by strain P31	Yang et al. (2018)

capabilities than single strains (Feng et al. 2020a, b). The previous results of Tang et al. (2020) showed that when the ratio of *Klebsiella pneumoniae* BPBA052 and *Acinetobacter Junii* LH-1-1 was 2.5:7.5, the dissipation of 75 mg·L^{-1} deltamethrin in 96 h was 94.25%. *Streptomyces aureus* HP-S-01 and *B. Cereus* ZH-3 can 100% metabolize cypermethrin (50 mg·L^{-1}) within 72 h (Chen et al. 2012b). A consortium composed of four beta-cypermethrin-degrading bacteria, which was *Streptomyces* sp. GXZQ4, *Enterobacter* sp. GXZQ6, *Streptomyces* sp. GXZQ7, and *Pseudomonas* sp. GXZQ13, was obtained by enrichment culture and high-throughput sequencing. The consortium's degradation rate of beta-cypermethrin (100 mg·L^{-1}) was up to 89.84% after 96 h (Qi and Wei 2017).

Among the published pyrethroid degrading bacteria, only a few strains can degrade pyrethroid 100% in a short time. Pyrethroids are similar in structure,

which means that the same strain may degrade many different pyrethroid pesticides (Bhatt et al. 2020f). The essence of microbial metabolism is an enzymatic reaction, in which complex macromolecular compounds are gradually broken down under the action of various enzymes (Feng et al. 2020a; Zhang et al. 2020; Bhatt et al. 2021a). 3-Phenoxybenzoic acid is the main intermediate metabolite of most pyrethroids. 3-PBA has high water solubility and antibacterial properties, which is one of the reasons limiting the further metabolism of pyrethroids (Zhao et al. 2019a). It is a new attempt to solve the toxicity of 3-phenoxybenzoic acid (3-PBA) through co-culture. Two strains of *Aspergillus oryzae* M-4 and *B. licheniformis* B-1 were combined by Zhao et al. (2016). After 72 h of cocultivation, 100 mg·L^{-1} β-cypermethrin was removed by 78.85%. The toxic intermediate product 3-PBA formed by the hydrolysis of β-cypermethrin with *B. licheniformis* B-1 was effectively utilized by *A. oryzae* M-4, the gallic acid produced by the metabolism of beta-cypermethrin with *A. oryzae* M-4 was effectively degraded by *B. licheniformis* B-1.

6.3 Genes and Enzymes Involved in Pyrethroid Metabolism

Carboxylesterase is a very important class of pyrethroid hydrolases. It is a subtype of esterase and had classified in subtype 3.1.1 by the International Union of Biochemistry (Bhatt et al. 2021b). Carboxylesterase/lipase is divided into eight families (I–VIII). Group I esterases are true lipases, while group II–VIII esterases are carboxylesterase (Zhan et al. 2020). Carboxylesterase is the most studied enzyme among pyrethroid hydrolases. It is widely found in resistant insects, mammals, and microbial cells. It can hydrolyze a variety of organic compounds containing esters, such as carbamate and pyrethroid, and produce nontoxic acids and alcohols (Liu et al. 2017). The active site of esterase contains serine residues, which are located in the conserved pentapeptide motif (Gly-X-Ser-X-Gly) (Diegelmann et al. 2015).

Many pyrethroid degradation genes, such as *pye3*, *pytY*, *estA*, *pytZ*, *pytH*, *est3385*, *mes1*, and *sys410*, have been cloned and identified (Wang et al. 2009; Li et al. 2008; Ruan et al. 2013; Luo et al. 2018). Phylogenetic analysis indicated that Est3385 derived from *Rhodopseudomonas palustris* PSB-S belong to the esterase group I, Sys410 belongs to esterase V family, and the pyrethroid carboxylesterase PytY encoded by *pytY* gene is a member of esterase VI families (Luo et al. 2018; Ruan et al. 2013; Fan et al. 2012). Most pyrethroid hydrolase activities have been reported to require no cofactor, but the presence of some metal ions can severely inhibit enzyme activity. The pyrethroid hydrolase extracted from *A. niger* ZD11 has a pI value of 5.4 and a molecular weight (MW) of 56 kDa; a pyrethroid esterase, Pye3, with an open reading frame (ORF) of 819 bp and an MW of about 31 kDa, was obtained from the soil by using metagenomics tools; and the esterase EstP with an ORF of 1914 bp was extracted from *Klebsiella* sp. strain ZD112 cells. These three enzymes can be strongly inhibited by Hg^{2+} and Ag^{+} (Wu et al. 2006; Li et al. 2008; Liang et al. 2005).

Due to the similarity in the structure of pyrethroid pesticides, most pyrethroid functional enzymes show broad-spectrum substrate specificity to pyrethroids. Pyrethroid hydrolytic esterase (EstP) was isolated from *Klebsiella* sp. ZD112 and encoded by gene *estP*, has an ORF of 1914 bp. The molecular weight of EstP is about 73 kDa, which contains 637 amino acid residues (Wu et al. 2006). No similarity was found with the reported nucleotide sequences of esterase/lipase family members by multiple sequence alignment. The purified EstP has a broad spectrum of substrate utilization. The K_m and k_{cat} values of EstP hydrolyzing *trans*- and *cis*-permethrin indicated that EstP hydrolyzes pyrethroids more efficiently than carboxylesterases obtained from insect-resistant insects and mammals.

The ORF of the pyrethroid hydrolytic gene *est 3385* contains 963 nucleotides, and the optimum pH and temperature are 6.0 and 35 °C, respectively (Luo et al. 2018). The enzyme can metabolize a variety of pyrethroid pesticides, and fenpropathrin is the best substrate. The enzyme degradation kinetics indicated that the V_{max} and K_m values of hydrolyzed fenpropathrin were 0.918 ± 0.025 U/microg and 0.734 ± 0.013 mmol/L, respectively. The *pytH* cloned from *Sphingobium* sp. strain JZ-1 encodes the carboxylesterase PytH. In addition to hydrolyzing a variety of pyrethroids, PytH can also convert short-chain fatty acids (Wang et al. 2009). Studies have shown that transferring the carboxylesterase encoding gene *pytH* into *Pseudomonas putida* KT2440 can completely hydrolyze 0.2 mM permethrin, fenpropathrin, and cypermethrin within 48 h (Zuo et al. 2015).

Previous results showed that there is enantioselective degradation of pyrethroid hydrolase. The pyrethroid hydrolase extracted from the *A. Niger* ZD11 was able to detoxification various pyrethroids, but compared with *cis*-permethrin, the substrate utilization of *trans*-permethrin was higher (Liang et al. 2005). A pyrethroid hydrolase esterase gene *pytY* containing 897 bp ORF was isolated from *Ochrobactrum Anthropi* YZ-1 by Ruan et al. (2013). PytY can hydrolyze different pyrethroids, but it showed the highest hydrolysis activity with *lambda*-cyhalothrin as the substrate. The kinetic constants of *V*max and *K*m were 56.33 nmol/min and 2.34 mmol/L, respectively.

The *beta*-cypermethrin-degrading monooxygenase CMO was first identified by Chen et al. (2013b) from environmental microorganisms. The natural enzyme showed that the CMO has a pI of 5.4 and an MW of 41 kDa. The enzyme exerted the greatest activity against *beta*-cypermethrin at 30 °C and pH 7.5. Fe^{2+} can significantly enhance the activity of CMO, while Cu^{2+}, Al^{3+}, and Ag^{+} have a strong inhibitory effect on CMO activity. Most of the pyrethroid hydrolases are extracted from the cells of organisms. It has been proved that it is feasible to extract pyrethroid metabolizing enzymes from extracellular regions. A functional enzyme was isolated from *Pseudomonas aeruginosa* strain GF31 cells. The molecular weight of the enzyme was 53.7 kDa, and the pI value was 7.67. The ORF contained a 1611 bp DNA fragment, encoding 536 amino acids. Through phylogenetic analysis, it was highly similar to aminopeptidases (Tang et al. 2017).

6.4 Catalytic Mechanisms of Pyrethroids

Pyrethroid hydrolases are members of the *α*/*β* superfamily. Most of the active site amino acids of carboxylesterase have conservative sites, with the active site amino acids contain a catalytic triad composed of nucleophiles, basic groups, and acidic groups (Bhatt et al. 2020a). Hydrolase folds into a complex three-dimensional structure in space, and the different residues of the catalytic triad (Ser-His-Asp) come together during the folding process. The triad is hidden in the enzyme protein molecule, and the serine residue at the active site is masked by the alpha helix. Through the folding of enzyme protein, the catalytic triad of carboxylesterase activity site is combined with a pyrethroid.

Aspartate and histidine combine with two hydrogen bonds to increase the *pKa* value (acidity coefficient) of the triad and activate the nucleophile serine. After the hydroxyl molecule of the serine is activated by catalytic histidine/aspartate, the latter obtains electrons from the hydroxyl molecule of the nucleophile. The active site of pyrethroid hydrolases contains serine residues, which is situated in the common pentapeptide sequence Gly-X-Ser-X-Gly of esterases. It acts as a nucleophile to attack the carbonyl group of pyrethroids through hydroxyl (OH), then releases alcohols, and produces a covalent intermediate of acylation (Bhatt et al. 2019). The basic group (His) and the acidic group (Asp) obtain hydrogen ions from the OH of serine, and the carbonyl group's nucleophilic attack of pyrethroid is performed by hydroxyl anion generated from serine. These two processes are carried out simultaneously.

The carbon atom of the ester bond of pyrethroid is attacked by serine (nucleophile) and forces the oxygen atom of the ester bond to accept electrons, forming a tetrahedral intermediate. Restoration of the intermediate carbonyl leads to the transfer of histidine protons to the carbon atoms of pyrethroids adjacent to the *α*-chiral carbons. Subsequently, water molecules replace serine as a nucleophile to supply a proton to histidine, and the remaining OH attacks the carbonyl carbon atom to form a complex intermediate (Bhatt et al. 2020a). Furthermore, the serine in the enzyme regains protons from the basic group histidine, which further hydrolyzes the complex intermediate into nontoxic acids and alcohols. The specific metabolic process is shown in Fig. 6.1.

6.5 Metabolic Pathways of Pyrethroid Biodegradation

The main intermediate metabolites detected in pyrethroids during microbial metabolism are 3-phenoxybenzyl alcohol, 3-phenoxyebenzoic acid (3-PBA), and 3-phenoxybenzaldehyde. The metabolite α-hydroxy-3-phenoxybenzeneacetonitrile is a unique intermediate product of pyrethroid II due to the absence of cyanogen in I pyrethroid (Guo et al. 2021; Bhatt et al. 2021c). Under alkaline conditions, pyrethroids are easily hydrolyzed into cyclopropane-containing acid and α-hyroxy-3-

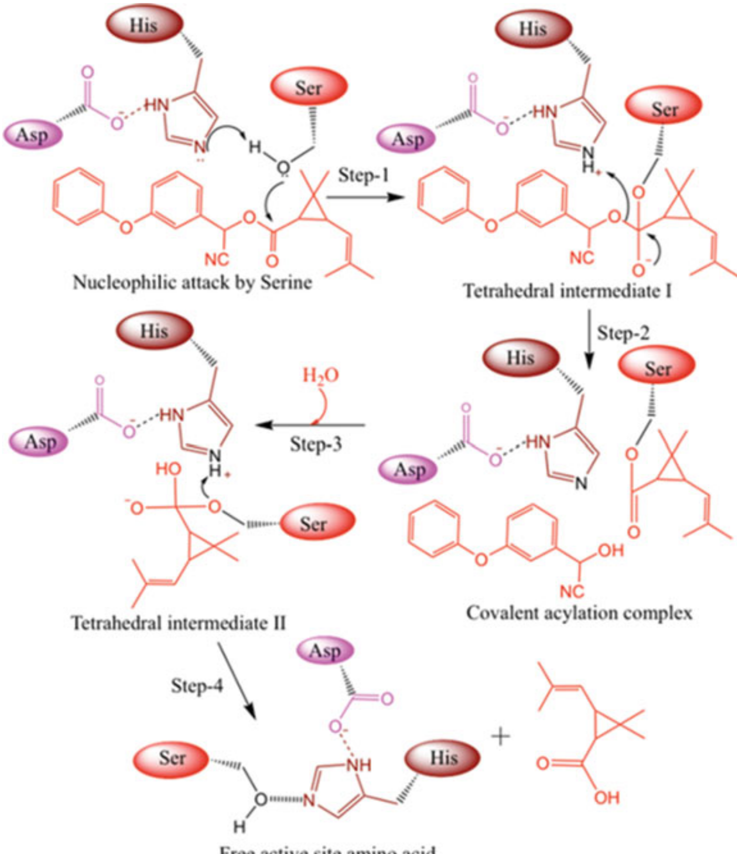

Fig. 6.1 Catalytic mechanisms of pyrethroids

phenoxybenzeneacetonitrile, and then quickly converted into 3-phenoxybenzaldehyde (Chen et al. 2011a). In the presence of dehydrogenase, 3-phenoxybenzaldehyde is further oxidized to 3-PBA (Deng et al. 2015). 3-PBA is an endocrine disruptor which stables in the environment, has higher water solubility than the parent compound, and is frequently detected in human urine. When monitoring the residues of pyrethroids, 3-PBA is often used as a detection indicator (Hongsibsong et al. 2019).

Most of the time, microorganisms follow the same metabolic pattern and metabolize the parent pesticide into 3-PBA (Chen et al. 2012d). *Staphylococcus succinus* HLJ-10 converts *D*-cyphenothrin through the cleavage of ester bonds and diaryl bonds. 3-PBA, 3-phenoxybenzaldehyde, and α-hyroxy-3-phenoxybenzeneacetonitrile were detected in this process (Huang et al. 2020); *B. licheniformis* B-1, *A. niger* YAT, *B. subtilis* BSF01, and *Brevibacillus parabrevis* BCP-09 have been reported to have similar metabolic steps on beta-cypermethrin (Tang et al. 2018a; Deng et al. 2015; Xiao et al. 2015). However, after the formation

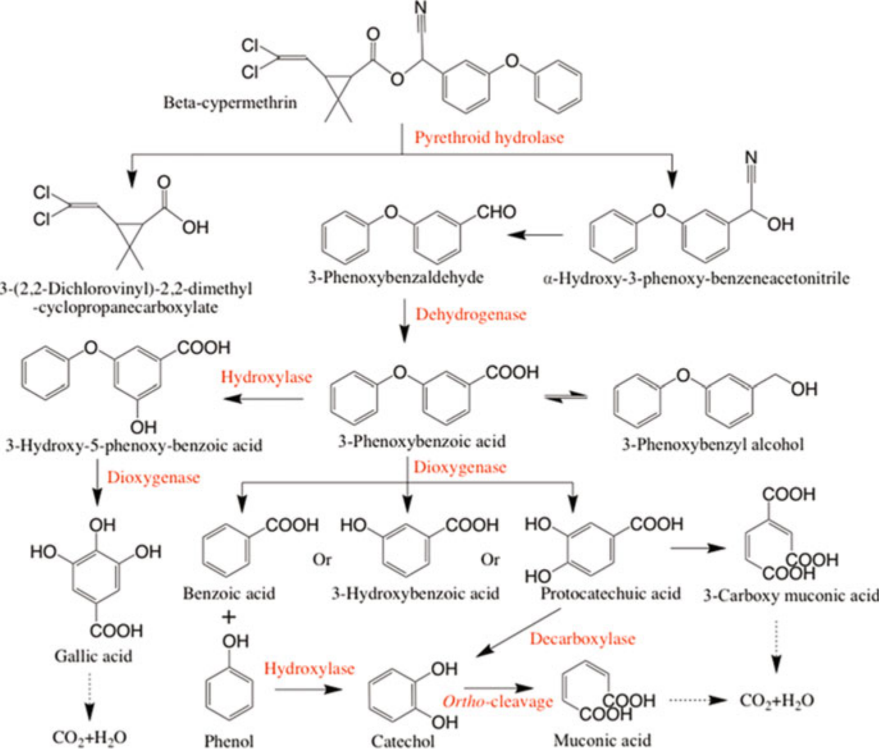

Fig. 6.2 Microbial degradation pathways of pyrethroids

of 3-PBA, different strains hydrolyze 3-PBA in completely different ways (Fig. 6.2). Under the catalysis of dioxygenase, 3-PBA may have a variety of downstream metabolic mechanisms. *Bacillus licheniformis* CY-012 can hydrolyze 3-PBA into benzoic acid and phenol (Tang et al. 2018b). Subsequently, phenol is hydrolyzed to catechol by hydrolase, and benzoic acid is further generated into 3-hydroxybenzoate. Another approach is performed by *A. niger YAT*; 3-PBA was hydrolyzed into protocatechuic acid and phenol (Deng et al. 2015). Protocatechuic acid undergoes aromatic ring cleavage with the assistance of dioxygenase to form 3-carboxy-muconic acid.

3-Phenoxybenzoic acid is one of the most studied pyrethroid intermediates. An interesting phenomenon was observed by Zhu et al. (2016) that 3-phenoxybenzyl alcohol and 3-PBA can be converted to each other, but will soon be hydrolyzed further. In addition, *Candida pelliculosa* ZS-02 and *A. oryzae* M-4 revealed another possibility of transforming 3-PBA into 3,5-dimethoxyphenyl or gallic acid (Chen et al. 2012a). After the formation of phenol into catechol, dioxygenase played an important catalytic role in the further decomposition of the aromatic ring to muconic acid (Zhao et al. 2019a). Ultimately, it is mineralized into nontoxic water molecules and carbon dioxide.

Until now, there are few reports on the biodegradation pathway of type I pyrethroids. In recent years, microbial degrading strains which can efficiently degrade permethrin, bifenthrin, d-cypermethrin, and permethrin have been screened from different sources, and their metabolic pathways have been demonstrated. Bhatt et al. (2020b) first hydrolyzed the ester bond of permethrin by *S. trueperi* CW3 to produce chrysanthemic acid and alcohol 2-(1,4,4-trimethyl-cy-clohex-2-enyl) ethanol. Then, the alcohol was oxidized to 1,4,4-trimethylcyclohex-2-ethylene carboxylic acid, which was further transformed into chrysanthemyl alcohol. Under the metabolism of *Acinetobacter baumannii* ZH-14, permethrin first formed 2,2-dimethyl-3-(2,2-dichlorovinyl) cyclopropanecarboxylic acid and 3-phenoxybenzenemethane, and then transformed into 3-phenyl benzaldehyde by redox. Finally, 3-Phenoxybenzaldehyde converted to 1,2-benzenedicarboxylic acid with the diaryl cleavage (Zhan et al. 2018).

6.6 Bioremediation of Pyrethroid-Contaminated Environments

The large-scale application of pyrethroids has caused ecological pollution of soil, sediments, and surface water, as well as serious indoor residues (Yoshida 2009; Chen et al. 2012c). After applying pyrethroids in the environment, as long as a small part of the active ingredients reach the target organism, most of them remain on the surface of plants and soil (Bhatt et al. 2020e; Huang et al. 2019). Most of the pyrethroid residues that contact the surface of plants and soil will be degraded by solar radiation. A small proportion of pyrethroids are utilized by indigenous microorganisms as a carbon source for growth. However, there are reports that the presence of deltamethrin can interfere with indigenous microbial communities (Braganca et al. 2019).

The long-term residues of pyrethroids in the soil force indigenous microorganisms to induce the expression of pyrethroid-related metabolic genes, thereby accelerating the metabolism of pyrethroid residues in contaminated environments. In addition to their own enzyme activities, soil temperature, pH, initial pyrethroid concentration, soil water content, and organic matter content also affect the soil bioremediation process by pyrethroid-degrading microbes (Bhatt et al. 2021d; Mishra et al. 2021). Zhang et al. (2016) collected a *B. cereus* strain Y1 from soil contaminated with deltamethrin, which can metabolize 74.9% of deltamethrin within 24 days. *Bacillus* sp. ISTDS2 was isolated from the marble mining area and was observed to use beta-cypermethrin as the sole source of nitrogen and carbon for growth (Sundaram et al. 2013). The β-cypermethrin with a concentration of 100 mg/L is thoroughly hydrolyzed by strain ISTDS2 in the field after 30 days. Recently, Bhatt et al. (2020c) obtained a *B. thuringiensis* SG4 from farmland. The experimental results suggested that 83.3% of 100 mg/L cypermethrin was removed from soil after 15 days of incubation.

6.7 Conclusions and Future Perspectives

Microbial degradation of pesticides in the environment faces many different problems. The metabolic activity of purified strain in soil has been the focus of previous research. Studies have shown that some microorganisms will give priority to the use of nutrients in soil media, leading to a decrease in the utilization of pyrethroids. The biodegradative efficiency of hydrophobic pyrethroids by microorganisms is related to the bioavailability of cells to pesticide molecules. The degradation rate of pyrethroids can be improved by adding appropriate surfactants Tween-80 and BRIj-35. The transformed pyrethroids pose another challenge to the environment. Pyrethroids metabolites are more water-soluble and have biological toxicity than the original pyrethroids. In the past 30 years, although a variety of microorganisms have been screened out and have the ability to hydrolyze pyrethroids. However, only a few strains can metabolize 3-PBA and pyrethroids simultaneously.

In order to solve these problems, many governance schemes have been proposed in recent years. Microorganisms that can utilize different substrates were combined to form consortia, which often exhibits a higher biodegradation effect than a single strain. Most microorganisms in nature cannot be obtained directly. Metagenomics provides a powerful tool for obtaining novel microbial enzyme resources. At present, the main pyrethroid degrading enzymes reported are carboxylesterase, monooxygenase, CYP, and laccase. However, not all enzymes have all the characteristics of high stability, high productivity, and high enzyme activity, which is essential for practical field application. Random mutagenesis, as another powerful tool, can enhance the catalytic activity and stability of enzymes through molecular modification, providing the possibility to obtain more potential enzymes.

Based on multidisciplinary results, many studies on immobilized degradation strains have been reported. Using calcium alginate in the form of microcapsules can immobilize a single purified strain or a multi-strain consortium. Compared with free cells, the immobilized strain has a higher substrate utilization rate. By regulating the flow rate, the number of repetitions of fixed strains can also be prolonged. With the development of materials science, many kinds of substrates for fixing cells have been developed, showing different advantages. In addition to immobilizing living cells, the immobilization of degrading enzymes has also been reported. Most of these experiments are currently in the laboratory research stage, and there are still many studies that need to be further carried out before the real large-scale application.

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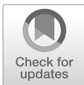
Chapter 7
Bacterial Biodegradation of Phenolic Hydrocarbons



Youssof Sefidi-Heris and Nader Hajizadeh

Abstract Phenolic hydrocarbons are among the most important pollutants with mutagenic, toxic, and carcinogenic potentials. Because of being thermodynamic stable, these pollutants are recalcitrant to degradation, making them persistent in the environment. There are some physicochemical methods to remove these compounds from the environment, each of which is suffering from limitations such as high costs, complexity, and difficult handling. Biodegradation is an alternative bioremediation technology, which efficiently removes aromatic pollutants using the potential of indigenous organisms. As a group of microorganisms, bacteria possess considerable potentials for application in the biodegradation of these substances. Application of bacterial enzymes is also an interesting option for bioremediation processes. The present chapter mainly focuses on bacterial biodegradation of phenolic hydrocarbons under different conditions, the metabolic pathways of their biodegradation, genetic backgrounds and regulation of the bacterial aromatic hydrocarbon biodegradation, the potential applications of genetically-engineered bacterial degraders of phenolic hydrocarbons, and the possible obstacles of this application.

Keywords Bacteria · Biodegradation · Phenolic hydrocarbons · Aromatic compounds

7.1 Introduction

Recently, environmental pollutants have been a focus of attention. As a group of major environmental pollutants, phenolic hydrocarbons are organic compounds with one or more aromatic moieties, especially benzene rings. They have genotoxic,

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carcinogenic, mutagenic, and toxic effects. These compounds include aromatic rings with different arrangements, and their properties differ with the number of these rings. Because of their stability, this group of pollutants can accumulate in food chains, making them worthy of concern as potentially dangerous substances for humans. United States Environmental Protection Agency (US EPA) has enlisted some of these compounds as priority environmental pollutants (Seo et al. 2009; McGuinness and Dowling 2009; Basha et al. 2010; Boll et al. 2014; Sefidi-Heris et al. 2014; Hajizadeh et al. 2015; Ghosal et al. 2016; Bello-Akinosho et al. 2016; Monzón et al. 2018; Mulla et al. 2019a, b). These compounds belong to a group of persistent environmental pollutants, which, at least in part, is due to the thermodynamically stable benzene moiety (Monzón et al. 2018).

Phenolic hydrocarbons fall into three groups: polycyclic aromatic hydrocarbons (PAHs), substituted aromatics, and heterocycles. PAHs contain two or more aromatic rings with different arrangements. They introduce into the environment from biogenic, pyrogenic, and petrogenic origins. Substituted aromatics bear some substitutions such as alkyl groups. Heterocycles can be found simultaneously with PAHs and other phenolic compounds and include substitutions like sulfur and chlorine (Seo et al. 2009; Ghosal et al. 2016).

As mentioned, phenolic compounds are stable and resistant to degradation in the environment. Consequently, it seems crucial to remove them from the environment (Seo et al. 2009; Díaz et al. 2013; Ghosal et al. 2016). There are some physical and chemical ways to solve this problem, including UV oxidation, solvent extraction, base-catalyzed dechlorination, fixation, incineration, and so on. However, these solutions are complex and costly. Besides, sometimes they do not remove the contaminants thoroughly and only transfer them between environments. Here, bioremediation appears as an appealing eco-friendly, and competent alternative. Bioremediation techniques take advantage of living organisms to convert hazardous materials into harmless agents (usually carbon dioxide and water) (Seo et al. 2009; Ghosal et al. 2016; Monzón et al. 2018). These techniques eliminate problems of physicochemical processes and have become fascinating remedial alternatives to remove environmental pollutions including aromatic compounds (Ghosal et al. 2016).

Biodegradation is a bioremediation technique for the removal of environmental pollutants. It is known that microorganisms can degrade organic pollutants in different settings (Hoskeri et al. 2014; Megadi et al. 2010; Mulla et al. 2016; Mulla et al. 2017; Mulla et al. 2019a, b; Mulla et al. 2020; Tallur et al. 2015; Talwar et al. 2014). They can co-metabolize pollutants through different catabolic pathways to efficiently clean up the environment. Biodegradation is a vast field of research with a high variety of microorganisms breaking chemical bonds. As a large group of organisms, bacteria possess many different metabolic tactics to degrade aromatic compounds and convert them into less toxic materials (Seo et al. 2009). The present chapter mainly discusses bacterial biodegradation of phenolic hydrocarbons under both aerobic and anaerobic conditions, the metabolic pathways of their biodegradation, genetic backgrounds and regulatory mechanisms of the bacterial aromatic hydrocarbon biodegradation, the potential applications of

genetically-engineered bacteria for biodegradation of phenolic hydrocarbons, and the possible limitations of this application.

7.2 Phenolic Hydrocarbons Are Present in the Environment

With their diverse properties, phenolic hydrocarbons are abundantly distributed throughout different environments. They are present in living beings as aromatic amino acids tyrosine, tryptophan, and phenylalanine. Besides, phenolic compounds like toluene, benzene, ethylbenzene, and xylene are some petroleum-derived aromatic pollutants. Plants produce natural aromatics, but do not have the metabolic pathways necessary for recycling them. Excepting aromatic amino acids and some other compounds, animals also have limited potential to metabolize these substances. Therefore, some bacteria and fungi are dominant aromatic-hydrocarbon-degrading organisms. (Fuchs et al. 2011).

Phenolic hydrocarbons fall into two groups: biological and nonbiological. The production of the biological group occurs in plants and microorganisms via shikimic acid and malonic acid pathways, respectively. Some famous compounds of such an origin include aromatic amino acids, caffeine, gallic acid, capsaicin, salicylic acid, and monolignols. Monolignols are among the most important natural aromatics, because of their huge amounts in the environment in the form of lignin and humic acids. Nonbiological aromatic compounds originate from human activities such as chemical, fuel, explosive, plastic, ink, metal, pharmaceutical, and electric industries. Because of their nonbiological origin, this group is called xenobiotics (Basha et al. 2010; Das and Chandran 2011; Kumar et al. 2013; Arora et al. 2014; Mulla et al. 2014; Ladino-Orjuela et al. 2016; Ito et al. 2016; Kamimura et al. 2017).

Chemically looking, there are three groups of phenolic hydrocarbons present in the environment: polycyclic aromatic hydrocarbons (PAHs), substituted aromatics, and heterocycles. PAHs include two or more aromatic rings with linear, cluster, or angular arrangements. Their physicochemical properties differ with the varying number of rings and molecular weight, consequently affecting their distribution, environmental fate, transport, and effects on biological systems. PAHs are stable and persistent in soils, making their degradation and removal hard. PAHs originate from biogenic, pyrogenic, and petrogenic sources. Biogenic PAHs are lignin, aromatic amino acids, and their derivatives. Pyrogenic PAHs are products of the combustion process and are mainly not substituted. Petrogenic PAHs are components of petroleum and its derivatives and often alkyl-substituted. Substituted aromatics bear some substitutions like alkyl groups. Because some substitutions decrease solubility, this group of phenolic hydrocarbons tends to accumulate in biological systems. These phenolics are abundant in petroleum-derived products, fossil fuels, and crude oil. Heterocycles are compounds found in the oil and its derivatives and sometimes coexist with PAHs and other phenolic compounds. They include compounds such as carbazole and dibenzothiophene and contain sulfur, nitrogen, etc. atoms in their

Table 7.1 Three groups of environmental phenolic hydrocarbons. (Inferred from Seo et al. 2009)

Type of phenolic hydrocarbons	Source	Properties	Examples
Polycyclic aromatic hydrocarbons (PAHs)	Biogenic Pyrogenic Petrogenic	Two or more aromatic rings, persistent in soils	Aromatic amino acids and lignin
Substituted aromatics	Petroleum-derived products, crude oil, and fossil fuels	Some substitutions like alkyl groups, decreased solubility, bioaccumulative	Methylnaphthalene
Heterocycles	Oil and its derivatives	Include sulfur, nitrogen, etc. atoms in their aromatic rings	Carbazole and dibenzothiophene

aromatic rings. (Seo et al. 2009). Table 7.1 summarizes three groups of environmental phenolic hydrocarbons.

7.3 Removing Phenolic Hydrocarbons from the Environment

7.3.1 Physicochemical Removal

Some physicochemical methods are common for clearing phenolic compounds from the environment. These include fixation, removal, UV oxidation, transfer to landfills, adsorption, excavation, flocculation, incineration, alteration, membrane separation (Kumar et al. 2013; Sefidi-Heris et al. 2014), stabilization, solvent extraction, vitrification, and base-catalyzed dechlorination. These methods are costly, complex, and difficult to handle. Also, these methods may remove the pollutants incompletely and even transfer them between different environments (McGuinness and Dowling 2009; Sefidi-Heris et al. 2014; Hajizadeh et al. 2015; Ghosal et al. 2016; Haritash and Kaushik 2016; Monzón et al. 2018; Shahsavari et al. 2019).

7.3.2 Biodegradation

Bioremediation is an efficient, eco-friendly technique to clean up environmental pollutants. This technique is increasingly used to fight against environmental pollution and detoxifies polluted sites by the aid of living organisms converting hazardous substances into harmless products (often water and carbon dioxide). Bioremediation destroys several organic pollutants at a low cost and under natural ambient conditions, making it an appealing alternative for pollution removal (Ghosal et al. 2016). The efficacy of bioremediation techniques depends on the type of pollutants, its level of bioavailability, and the capability of microorganisms in the natural contaminated environment (Shahsavari et al. 2019). As a bioremediation technology,

biodegradation is a biological method for removing organic pollutants. Microorganisms can omit pollutants via degradation. Bioremediation uses the metabolic potential of natural microbiota to eliminate hazardous polluting agents. This transforms organic contaminants into safe metabolites or metabolizes them to form carbon dioxide and water. Microorganisms used in an effective biodegradation technology can quickly adapt to the pollutants and use them in a reasonable period. As a broad field of study, biodegradation exploits a variety of microorganisms to break chemical bonds. This is a very active field of study with new data increasingly being added to the literature (Seo et al. 2009). Biodegradation is the most satisfactory method to remove environmental pollutants in many cases (Monzón et al. 2018).

Several groups of microorganisms, including bacteria, fungi, and yeasts can use environmental pollutants as the sole carbon and energy sources. This mainly affects the fate of pollutants such as PAHs in ecosystems (Brzeszcz and Kaszycki 2018). Some plants can also carry out PAH biodegradation. In eukaryotes, PAH is only transformed through reactions including cytochrome P450 and only some basidiomycetes can mineralize PAHs (Baboshin and Golovleva 2012). Only microorganisms have the biological potential necessary for the complete mineralization of aromatic compounds (Boll et al. 2014). It is worth noting that some plants and bacteria can cooperatively remove organic contaminants. This includes symbiotic systems such as rhizospheres and endophytic bacteria (McGuinness and Dowling 2009; Zahid et al. 2015). As a group of major environmental pollutants, microbial biodegradation of phenolic compounds has been under the focus, because it is important in the carbon cycle (Díaz et al. 2013; Kamimura et al. 2017). Growing ecological importance is given to microbial pollutant biodegradation and technologies relying on native microorganisms are broadly used in waste managing (Monzón et al. 2018).

With their versatile characteristics, bacteria are among the first groups of microorganisms responding to environmental pollutions and take part in the process of hazardous material degradation and several reports are available about bacterial degradation of pollutants. Bacteria have several metabolic pathways to metabolize environmental pollutants, including aromatic compounds (Nojiri et al. 2001; Janssen et al. 2005; Seo et al. 2009; Monzón et al. 2018; Brzeszcz and Kaszycki 2018; Shahsavari et al. 2019). In contrast to eukaryotes, bacteria can use some aromatics as the exclusive carbon and energy sources (Baboshin and Golovleva 2012). Bacterial elimination of aromatics from soil has been reported to enhance soil fertility and shows the restorative role of this biodegradation process (Bello-Akinosho et al. 2016). Depending on oxygen availability, these microorganisms use two major strategies in degrading phenolic compounds: reductive reactions occur in the absence of oxygen, while when the conditions are aerobic, oxygen plays the role of final electron acceptor and simultaneously, as a co-substrate for some critical catabolic pathways (Díaz et al. 2013). Under aerobic conditions, bacterial biodegradation is very rapid and complete, and reactions are principally different in aerobic and anaerobic conditions (Baboshin and Golovleva 2012). Bacteria able to degrade aromatic compounds are scattered extensively in different environments and can

degrade both natural compounds and xenobiotics. These bacteria are critically important for the carbon cycle (Suenaga et al. 2007).

During the next sections, to simplify the concept of phenolic hydrocarbon biodegradation by bacteria, we focus on bacterial biodegradation of phenolic hydrocarbons such as benzene. However, the reader must be cautious that because phenolic hydrocarbons include an extremely diverse group of substances, the real realm of bacterial phenolic hydrocarbon biodegradation goes so much farther than the perspective presented here.

7.4 Bacterial Biodegradation of Benzene

7.4.1 Anaerobic Biodegradation

Several studies have examined the anaerobic biodegradation of benzene. Bacteria able to degrade benzene grow very slowly. This may be due to the small energy amounts gained from oxidizing such a hydrocarbon under anaerobic conditions. Anaerobic biodegradation of benzene occurs under methanogenic conditions, or with sulfate, ferric iron, Mn(IV), CO_2, or nitrate as electron acceptors (Ladino-Orjuela et al. 2016; Meckenstock et al. 2016).

Firstly, Wilson et al. reported anaerobic biodegradation of benzene under methanogenic conditions in 1986. They observed slow benzene disappearance taking up to several weeks (Wilson et al. 1986). Radioactive isotope labeling helped to clarify metabolic pathways of benzene biodegradation under these conditions and it was suggested that benzene was mineralized through phenol to carbon dioxide and methane (Vogel and Grbić-Galić 1986; Grbić-Galić and Vogel 1987). Later, the role of phenol as an intermediate in methanogenic benzene biodegradation was established (Weiner and Lovley 1998; Caldwell et al. 1999). However, abiotic phenol production can also take place via reactions including benzene and reduced compounds (Kunapuli et al. 2007).

The first report showing the simultaneous sulfate reduction and anaerobic benzene biodegradation was published in 1992. Using ^{14}C-labeled benzene, it was established that benzene was converted into CO_2, but decreased sulfate level was not observable (Edwards and Grbić-Galić 1992). Later, another study demonstrated the role of sulfate as an electron acceptor for anaerobic benzene degradation. As a strong sulfate reduction inhibitor, molybdate inhibited ^{14}C-labeled CO_2 production from ^{14}C-benzene. Furthermore, when sulfate was exhausted, benzene was not metabolized and sulfate addition restored this metabolism (Lovley et al. 1995). Benzene is converted into benzoate by some sulfate-reducing bacteria (Caldwell and Suflita 2000; Phelps et al. 2001; Abu Laban et al. 2009).

It was also demonstrated that ferric iron can act as a terminal electron acceptor for anaerobic benzene biodegradation, converting benzene into carbon dioxide (Baedecker et al. 1993; Cozzarelli et al. 1994). Ligands complexing with Fe(III), such as EDTA, antraquinonedisulfonate (AQDS), humic acids,

N-methyliminodiacetic acid, nitrilotriacetic acid, ethanol diglycine, and phosphates can facilitate benzene biodegradation via ferric iron (Lovley et al. 1994; Lovley et al. 1996; Lovley and Woodward 1996; Caldwell et al. 1999; Lovley 2000; Jahn et al. 2005). Radioactive carbon labeling has identified phenol and benzoate as an intermediate for benzene degradation (Botton and Parsons 2007; Kunapuli et al. 2007). Mn (IV) and graphite can also act as electron acceptors for benzene biodegradation, showing diverse bacterial strategies in benzene biodegradation (Villatoro-Monzón et al. 2008; Zhang et al. 2010).

A direct relationship has been demonstrated to exist between nitrate reduction and benzene biodegradation (Burland and Edwards 1999). Under nitrate reduction, benzene is converted into benzoate and toluene, which occurs through an initial methylation step yielding toluene and the subsequent transformation to benzoate (Ulrich et al. 2005).

7.4.2 Aerobic Biodegradation

Some bacteria like the model organism *E. coli* (Mukherjee et al. 2019), *Methyloversatilis* and *Zavarzinia* (Rochman et al. 2017) can degrade benzene under aerobic conditions. Bacteria possessing this potential can belong to both *Gammaproteobacteria* and *Betaproteobacteria* (Posman et al. 2017). Under aerobic conditions, benzene could be catabolized via multi-component bacterial enzymatic complexes (Millacura et al. 2017). Since oxygen is the most prevalent and frequent oxidizer in nature, bacteria should firstly use oxygen as a terminal electron acceptor. This includes the application of enzymes such as monooxygenases and dioxygenases (Ladino-Orjuela et al. 2016). It should be considered that bacteria can degrade benzene and other phenolic hydrocarbons even when mixed (Hocinat et al. 2020).

Benzene biodegradation if feasible in the presence of appropriate oxygen levels. However, supplying such an oxygen level is energy-consuming and can affect benzene biodegradation. It is while anaerobic bacteria degrade benzene at the expense of using other terminal electron acceptors and thus, lower the cost of energy and the exposure risks (but as mentioned previously, the growth rate is slower instead) (Liu et al. 2018). When aerobically degraded, benzene may go through the catechol pathway to be mineralized (Atashgahi et al. 2018).

7.5 Metabolic Pathways of Phenolic Hydrocarbon Biodegradation in Bacteria

The biggest challenge for bacterial degraders of phenolic hydrocarbons is the high resonance energy of the aromatic ring. Therefore, aromatics are recalcitrant to reduction or oxidation, and bacteria require intricate tactics to degrade such compounds. The solution depends mainly on the accessibility of oxygen. Aromatic compounds are extremely diverse, and there should be as many metabolic pathways as these different substances to enable an organism to degrade all of them. Nevertheless, organisms like bacteria transform them into some key intermediates entering reactions. These unifying pathways are called upper, channeling, or peripheral pathways. In contrast, lower or central pathways subsequently convert the central intermediates into intermediate metabolites like pyruvate, acetyl-CoA, or succinyl-CoA. The main challenging problems all through anaerobic and aerobic degradation of these compounds are different, requiring different sets of tools to overcome (Fuchs et al. 2011). Therefore, oxidation is the starting point for the upper pathways and the formation of central intermediates (catecholic or non-catecholic compounds) is the end (Ladino-Orjuela et al. 2016).

Oxygen is the most ubiquitous and potent natural oxidizing agent (DeLaune and Reddy 2005), and bacteria use O_2 as a terminal electron acceptor for aromatic hydrocarbon degradation (Ladino-Orjuela et al. 2016). Monooxygenases or dioxygenases catalyze an oxidation reaction as the first stage in upper biodegradation pathways in aerobic bacteria (Parales and Resnick 2004; Huijbers et al. 2014). Monooxygenases cleave oxygen-oxygen bonds. One of the oxygen atoms is incorporated into the aromatic ring and the other experiences reduction to yield H_2O. According to their structures, there are eight groups of monooxygenases. Group A-B monooxygenases are enzymes with flavin adenine dinucleotide (FAD) as a cofactor, and their electron donor is nicotinamide adenine dinucleotide phosphate NAD(P)H. They can catalyze hydroxylation, oxidative decarboxylation, heteroatom oxygenation, sulfoxidation, and N-hydroxylation. Group C-D monooxygenases use FAD or flavin mononucleotide (FMN) as a cofactor and $FMNH_2$ or $FADH_2$ as an electron donor to catalyze oxidation, hydroxylation, epoxidation, desulfurization, and sulfoxidation. In Group E–G, FAD is the cofactor, and $FADH_2$ or a substrate plays the role of electron donors. Internal flavoprotein monooxygenases are members of this group and reduce the flavin cofactor at the expense of substrate oxidation. Enzymes of this group can catalyze oxidative decarboxylation, sulfoxidation, and halogenation (Huijbers et al. 2014). Monooxygenases can catalyze the oxidation of both single-ring aromatics and polyaromatic hydrocarbons. Mobooxygenases consecutively add hydroxyls to the aromatic ring to form phenols and then, catechols. While catalyzing reductive dihydroxylation of the aromatic ring to form *cis*-dihydrodiols, which are then directed to catechols by specific *cis*-dihydrodiol dehydrogenases (Ladino-Orjuela et al. 2016).

In aerobic bacteria, oxygen attacks the aromatic ring with the aid of oxygenases to form central intermediate compounds such as catechol and protocatechuate. Following this, the central ring is cleaved through the catalytic activity of dioxygenases. Oxygen is a potent oxidizer to cleave aromatic rings. But this event needs O_2-activating oxygenases, activating and arranging it to produce hydroxylated species such as catechol, protocatechuate, homogentisate, or gentisate. Since electron-rich substituents are present with *ortho* or *para* positions, activation of these intermediates leads to oxidative ring cleavage. Therefore, benzene and similar aromatics such as phenol, toluene, and benzoate are converted into catechol or protocatechuate. Next, the central intermediates produced aerobically in peripheral pathways encounter ring-cleaving dioxygenases. Cleavage can take place between hydroxyl groups, in the *ortho* position, or the *meta* position, near the –OH groups. As the central intermediate is derived from different phenolic compounds, protocatechuate is converted into β-ketoadipate, and catechol gives rise to the same substance as well. During the *ortho*-cleavage pathway, both catechol 1,2-dioxygenase and protocatechuate 3,4-dioxygenase join both oxygen atoms of O_2 to the ring cleavage products, namely *cis*,*cis*-muconate, and the β-carboxy analog, respectively. Cyclo-isomerization transforms these to five-membered muconolactone and its 4-carboxy analog, respectively. Subsequently, a double-bond shift unstable enol-actone (decarboxylated for the 4-carboxy derivative). Lactone is readily converted into β-ketoadipate, and afterward, is transformed into tricyclic acid intermediates after two additional steps (Fuchs et al. 2011).

A second O_2-dependent pathway of aromatic ring cleavage ends in the formation of a nonaromatic epoxide. This epoxide experiences a rearrangement to be cleaved through hydrolysis. Fascinatingly, the substrate metabolism in these pathways goes ahead through CoA thioesters. Bacterial degradation of phenylacetate, benzoate, and their precursors often occurs in such a manner. This pathway seemingly helps bacteria adapt to low or unstable oxygen conditions. Conditional to the O_2 concentration, benzoyl-CoA and phenylacetyl-CoA can go through aerobic or anaerobic pathways. Class I di-iron protein family monooxygenases like the multicomponent bacterial monooxygenases are the key enzymes necessary for this metabolic route. Members of this family share very slight similarity in their sequences but have conserved secondary and tertiary structures and conserved di-iron-binding site. They catalyze a variety of reactions dependent on molecular oxygen (Fuchs et al. 2011).

CoA thioesters act as intermediates in the aerobic biodegradation of benzene and its relative compounds. Molecular oxygen is used for de-aromatization and cleavage of the aromatic ring and this may seem pointless. But alternative pathways are as energy-consuming as classical ones (Fuchs et al. 2011). As well, CoA thioesters can facilitate several reactions, including BoxC-catalysed hydrolytic reactions (Bains et al. 2009; Rather et al. 2010), PaaG-mediated oxepin-CoA formation (Teufel et al. 2010; Teufel et al. 2011), and 2-aminobenzoyl-CoA monooxygenation and reduction (Hartmann 1999; Torres and Bruice 1999). Besides, the membrane is impermeable to CoA-bound intermediates, and therefore, they are trapped inside the cell, while free intermediates can leave the cell. Moreover, processing enzymes have CoA-binding motifs for the rapid recognition of CoA-bound intermediates (Rather

et al. 2011). During epoxidation reactions, highly reactive epoxide-CoA products are synthesized and thus, this can control these epoxides and inhibit their possible harms to the cell. Aerobic pathways including CoA thioesters are designed for facultative anaerobes able to use benzoyl-CoA, 2-amino-benzoyl-CoA, or phenylacetyl-CoA during anaerobic periods. Electron withdrawal by CoA-thioesters can also favor anaerobic catabolism to facilitate the reduction of the aromatic system (Fuchs et al. 2011).

Two classes of benzoyl-CoA reductases exist: class I benzoyl-CoA reductases and class II benzoyl-CoA reductases. Class I benzoyl-CoA reductases are ATP-consuming enzymes present in facultative aerobic bacteria, and class II benzoyl-CoA reductases are ATP-independent enzymes found in strictly anaerobic microorganisms. The main product of both classes is 1,5-dienoyl-CoA. But when class I benzoyl-CoA reductases are catalyzers, there must be a reduced ferredoxin as an electron donor, two ATP molecules, and two water molecules (Ladino-Orjuela et al. 2016). The electron donor for class II benzoyl-CoA reductases is unknown (Holmes et al. 2012).

Under anaerobic conditions of, for example, sediments, soil, and groundwater, different strategies are necessary to cleave the aromatic ring. There are two anaerobic strategies, both of which reduce aromatic rings and require waterless arrangements and sodium as a reducing agent (Birch reduction) (Birch 1944). These two approaches necessitate the role of de-aromatizing reductases acting on benzoyl-CoA as the central intermediate, which accepts two electrons to produce cyclohexa-1,5-diene-1-carboxyl-CoA (1,5-dienoyl-CoA). This product may have the lowest known redox potential among substrate-product complexes of the enzymatic world (Kung et al. 2010). Benzoyl-CoA reduction is extremely endergonic with ubiquitous electron donors such as ferredoxin. Consequently, it must be supported by an exergonic reaction. There are four possible solutions to this problem. To do a biological Birch reaction, first, the active sites of enzymes provide a water-free environment. Second, the functionality of thioesters makes it possible to produce supplementary resonance structures in extremely reactive radical intermediates (Buckel and Keese 1995; Möbitz and Boll 2002). Accordingly, the one-electron redox potential of a benzoyl-thioester is less negative than benzene (Boll et al. 2000; Boll 2005). However, still, this is not at a physiological range. Third, the direct coordination of the aromatics to tungsten cofactors or Fe-S stabilizes radical intermediate species extensively. Fourth, having electron-withdrawing properties—for example, in Zn^{2+}Lewis acid—or partial protonation in carboxyl groups can enhance electron transport to the aromatic ring (Fuchs et al. 2011).

In the third aromatic ring cleavage strategy, ATP hydrolysis drives benzoyl-CoA reduction, catalyzed by class I benzoyl-CoA reductases (Boll and Fuchs 1995; Boll et al. 1997; Boll et al. 2000; Unciuleac and Boll 2001; Boll 2005). The resultant nonaromatic dienoyl-CoA subsequently goes through benzoyl-CoA degradation routes that differ from aerobic pathways of benzoyl-CoA oxidation. This includes a set of adapted β-oxidation reactions, cleavage of the ring via hydrolysis, and decarboxylation (Egland et al. 1997, Breese et al. 1998, Laempe et al. 1998, Laempe et al. 1999). This reaction hydrolyzes two ATP molecules to produce ADP and P_i.

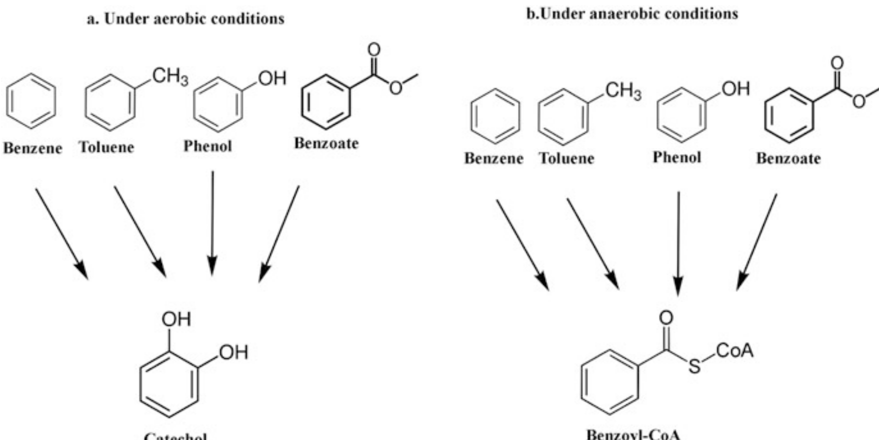

Fig. 7.1 Bacterial upper pathways of phenolic hydrocarbon biodegradation for some model compounds. (**a**) Under aerobic conditions, catechols are produced as central intermediates by bacterial degraders of aromatic hydrocarbons. (**b**) when the conditions are anaerobic, bacterial biodegradation of phenolic hydrocarbons continues via benzoyl-CoA as the central intermediates

Coupling the ring reduction to ATP hydrolysis makes it irreversible (Fuchs et al. 2011).

Three acetyl-CoA molecules are produced during anaerobic aromatic compound catabolism through benzoyl-CoA degradation. Strict anaerobic bacteria lacking a respiratory chain use these acetyl-CoA molecules to produce three ATP molecules. The formation of benzoyl-CoA consumes more ATP molecules, two ATP equivalents during the AMP-forming CoA ligase function, and another two ATP molecules for ATP-driven ring reduction. As a result, fermentative bacteria and other obligate anaerobic organisms exploit ATP-independent mechanisms to reduce the aromatic ring (Peters et al. 2004). Here, class II benzoyl-CoA reductases act as key enzymes, which do not have homology with class I benzoyl-CoA reductases and may have eight protein subunits, seemingly with flavin, selenocysteine, zinc, Fe-S clusters, and tungsten, as cofactors (Fuchs et al. 2011). Although their compositions are not the same, both class I and class II benzoyl-CoA reductases produce 1,5-dienoyl-CoA. Consequently, the following steps of 1,5-dienoyl-CoA catabolism are often catalyzed by comparable enzymes in all anaerobic aromatic compound-degrading bacteria (Wischgoll et al. 2005; Löffler et al. 2011). Figure 7.1 elucidates bacterial upper pathways of phenolic hydrocarbon biodegradation for some model compounds such as benzene, toluene, phenol, and benzoate.

7.6 Genetic Background and Regulation of Phenolic Hydrocarbon Biodegradation

Enzymes participating in the degradation of phenolic hydrocarbons are encoded by chromosomal genes in many bacteria. These genes are also found in some plasmids, enabling them to spread into new hosts during the process of horizontal transfer. Transposons can also set the stage for the evolution of new aromatic compound degradation pathways. There are different arrangements for the genes coding for key enzymes acting in the peripheral pathway of phenolic catabolism, and these differences are sometimes even at the strain level (Rucká et al. 2017). For peripheral catabolism of phenol its byproducts, these genes are often clustered with sequences encoding the enzymes of central catabolic pathways. These gene clusters usually include regulatory elements. This type of arrangement is indicative of the common regulatory interactions within these clusters. But in some cases, regulatory proteins for peripheral pathways are encoded by genes very distant from genes of the central phenol degradation pathways. In some aromatics-degrading bacteria, there is a redundancy of paralogous genes encoding enzymes of phenolic catabolism, able to recognize very similar or even identical substrates. Genes that encode analogous enzymes acting in different genetic backgrounds facilitate the expression control of individual paralogs to set the stage for optimal bacterial survival under unfavorable environments. (Nešvera et al. 2015; Aisami and Allamin 2017). To develop genetically.

Engineered, more competent bacterial degraders of phenolic compounds, regulation mechanisms during the expression of phenolic degradative genes must be elucidated. Catabolic genes usually form operons that are regulated by transcription factors encoded by a neighboring gene. Sometimes, several clusters are parts of a regulon under the control of a regulatory factor encoded by a distant gene (Rucká et al. 2017).

Different aerobic and aerobic bacteria use diverse mechanisms to regulate the biodegradation of phenolic hydrocarbons. There are different sequential regulation systems for peripheral and central pathways, which are achieved through a variety of regulatory mechanisms responding to respective substrates or intermediates. These systems are classified based on the type of regulators, but an individual protein family can include both proteins regulating peripheral pathways and those controlling central pathways (Fuchs et al. 2011).

Regulators controlling the aerobic degradative pathways progressing through catechol or protocatechuate are members of LysR or acetate operon repressor (IclR) protein families, while anaerobic and aerobic benzoyl-CoA pathways in denitrifying bacteria are controlled by the regulator of anaerobic benzoate metabolism (BzdR) family, by members belonging to multiple antibiotic resistance protein R (MarR) or the fumarate and nitrate reduction regulatory protein (Fnr) families in phototropic degraders of benzoate, and by a regulatory mechanism including a two-component benzoate sensor system (BamVW), inducing an RNA polymerase

σ^{54}-dependent regulator called BamY138 in strict anaerobes like *Geobacter* species (Fuchs et al. 2011; Rucká et al. 2017).

Different regulatory systems specifically responding to their corresponding substrates control the operons transcribing the enzymes involved in peripheral pathways. Regulators functioning in these systems are members of different protein families of transcription regulating factors. Binding of acyl-CoA derivatives (not free acids) controls the functionality of regulators in different pathways including CoA thioesters. By contrast, the proteins regulating CoA-independent pathways can directly respond to several kinds of free substrates (Fuchs et al. 2011).

Catabolite repression is an additional regulatory mechanism in most aromatic compound-degrading bacteria. Contrary to well-known catabolite repression mechanisms, this phenomenon in aerobic and anaerobic degraders of aromatic compounds includes the inclination to non-sugar metabolites like nonaromatic amino acids or nonaromatic organic acids over aromatic compounds or preference of degradable aromatic compounds like benzoate over more invert substrates like toluene. However, the molecular mechanisms of these regulatory systems are not clear. It should be remembered that facultative aerobes, which can degrade aromatic compounds under both aerobic and anaerobic conditions need sensor systems detecting the presence of O_2 or other electron acceptors (Fuchs et al. 2011).

7.7 Why Are There Different Strategies for Phenolic Hydrocarbons Degradation?

Diverse strategies used for the degradation of aromatic compounds seem reasonable. Given only the well-studied oxygen-dependent pathways, about ¼ of Earth's biomass might be kept under anaerobic anoxic conditions, inaccessible for metabolism. Anaerobic pathways are inherently sensitive to O_2, which is due to the sensitivity of their component proteins to oxygen-rich conditions and their required, extremely low redox potentials. As a result, anaerobic pathways cannot proceed under oxic conditions. Probably, anaerobic pathways of metabolism evolved first and biological oxygen generation was a late phenomenon in evolution. Additionally, strict anaerobes cannot provide two ATP equivalents to reduce the ring, and consequently, the functions of ATP-dependent class I benzoyl-CoA reductases are impossible under these low-energy conditions. The evolution of benzoyl-CoA reductase might have been a later event in phototrophic bacteria and facultative anaerobes using anaerobic respiration pathways producing high amounts of ATP (for example, denitrification). Epoxidation of the ring under aerobic conditions activates the ring using only one O_2 molecule and includes CoA thioesters, which can also act as intermediates of anaerobic pathways. As a result, this approach seems acceptable for fluctuating or very low oxygen levels. After the formation of the CoA thioester substrate, its metabolism can continue through aerobic or anaerobic pathways. Generally, anaerobic pathways yield lower growth rates than aerobic ones. But, despite the low

energy rates produced via anaerobic respiration and fermentation, these pathways quickly turn aromatic compounds over. Facultative anaerobes can grow at almost equal rates when aerobically or anaerobically grown on aromatics such as benzoate (Fuchs et al. 2011).

7.8 Genetically Engineered Bacteria, Degrading Phenolic Hydrocarbons

Besides the description of bacterial genes and enzymes of phenolic hydrocarbon biodegradation, genetic engineering helps us construct new bacterial degraders with the ability to degrade these major ecological pollutants. Cloning the corresponding genes into high-copy number plasmids simplifies the construction of strains with higher biodegradation potentials (Rucká et al. 2017). The engineered strains constructed this way degrade phenolic compounds as the only carbon and energy source much more efficiently during short periods (Soda et al. 1998; Zídková et al. 2013). However, nonselective conditions sometimes make recombinant plasmids vanish from bacterial hosts, causing a lack of favorite capabilities in engineered strains. Here, a potential solution could be the targeted insertion of biodegradative genes into the bacterial chromosomes. This way, genetically stable strains are constructed with high phenolic hydrocarbon biodegradation efficiency (Hu et al. 2014).

Protein engineering approaches can assist in extending the biodegradation potentials of bacterial degraders of aromatic compounds by broadening the substrate range for the key enzymes involved in these biodegradation pathways. In some cases, it has been reported that the resultant recombinant enzymes have higher activity for the original substrate and simultaneously, can convert more new substrates (Leungsakul et al. 2006). Some recombinant strains constructed by these methods show higher growth rates, higher resistance to the target phenolic hydrocarbon and its metabolites, increased biodegradation rates, and constitutive expression of the target biodegradative genes (Dai and Copley 2004).

Nanotechnology can also help to improve bacterial phenolic hydrocarbon biodegradation and efficient removal of these pollutants from the environment (Rucká et al. 2017). Nanoparticles synthesized by adding a thermally-responsive polymer to hollow mesoporous silica (TRP@HMS) are loaded to the cell surface of bacterial phenol degraders. The equipped (namely, armed) cells can remove phenol completely after some hours. It is while the native cells need more time to accomplish the task (Yang et al. 2016).

Regulatory mechanisms controlling the expression of phenolic hydrocarbon pathway genes can be a foundation for constructing systems able to detect and monitor the environmental presence of phenolic hydrocarbons and their derivatives. For example, some *Pseudomonas* strains can be used as biosensors for phenolic hydrocarbon detection in the environment (Rucká et al. 2017). Mutations used to

construct such biosensors enable mutant strains to more sensitively detect the presence of aromatic compounds and identify a broader range of these hydrocarbons as well (Wise and Kuske 2000; Gupta et al. 2012). As a reality, ex situ application of genetically engineered bacteria degrading phenolic hydrocarbons would meet the terms of legal regulations, while in situ technologies taking these organisms into action may encounter some legal limitations (Rucká et al. 2017).

There are considerable amounts of scientific reports paying special attention to microorganisms with the potential biodegradation capabilities, their genetic information and biodegradation pathways, regulatory mechanisms, and potential applications in fields such as biotechnology and nanotechnology. This encourages us to optimally use these organisms for bioremediation of polluted environmental sites. However, we should not forget that still some limitations are hindering the achievement of our hopes.

Many bacterial strains isolated from polluted sites carry the enzymes necessary for the degradation of natural or synthetic aromatics. But practically, these enzymes do not possess a satisfactory rate and efficiency. Exhaustion of organic material, inactiveness of responsible microorganisms, and evolutional backgrounds lower the rates of aromatic compound biodegradation by the native microorganism. Furthermore, the preference of some substrates such as carboxylic acids over phenolic hydrocarbons can inhibit the expression of genes involved in the biodegradation of aromatic rings (Nešvera et al. 2015). Other limitations could be the high toxicity of phenolic or other contaminants (e.g., heavy metals) and unforgiving environmental conditions (extreme pH, temperature, or salinity), applying stress on bacterial cells able to degrade aromatic pollutants. From the other side, low concentrations of the target phenolic compounds may be insufficient for the induction of respective catabolic genes (Rucká et al. 2017).

Genetic engineering solves these problems by cloning the target metabolically important genes in high-copy number plasmids (Soda et al. 1998, Zídková et al. 2013), bringing new degradative pathways together by the aid of heterologous genes (Hu et al. 2014), broadened substrate ranges for catabolic enzymes (Leungsakul et al. 2006), and overexpression of target genes by designing potent promoters and modifications of regulatory regions. These modified bacterial strains and technical solutions ensure the efficient degradation of pollutants under experimental conditions. However, ex situ processes are costly, and therefore, in situ bioremediation could be an alternative for environmental cleanup. Again, there are some problems limiting the application of engineered bacterial strains for in situ bioremediation: (1) availability of the pollutant in the soil is a major problem for a bacterial degrader; (2) anoxic conditions limit the function of aerobes; (3) under uncontrolled environmental conditions, engineered bacterial degraders often are overwhelmed by indigenous microorganisms; (4) even if the engineered recombinant bacteria can adapt themselves to the environment, they cannot be better degraders than the native microorganism; and (5) natural environments are often polluted by a mixture of compounds, and a successful remediation strategy usually needs a consortium of bacterial degraders (Fu et al. 2017; Rucká et al. 2017).

Another limitation for the use of engineered bacteria to remove phenolic pollutants is that introduction of these organisms to the environment can spread unnatural genes in nature with unknown consequences. As a result, governments in many countries contradict the use of such microorganisms legally. Mutations of such synthetic microorganisms spread in nature can lead to unpredictable aftermaths, adding more concerns to their social reputation. Therefore, many social groups believe in stopping the application of these products of synthetic biology. However, the introduction of engineered bacteria to the mixed, sophisticated microbial communities residing in natural habitats has not been very fruitful, and these organisms often fail to express the target genes efficiently and cannot compete with endogenous communities. But there are not any reported cases of harm to humans, animals, plants, or biodiversity, and some scholars look at these social concerns as an exaggeration (Rucká et al. 2017).

We have to know more about the genetic, physiological, and enzymatic aspects of bacterial phenolic hydrocarbon degraders to develop bacterial strains more efficiently removing these pollutants from the environment. High-throughput genomic techniques are promising tools for helping the achievement of this hope. They provide us with a large amount of initial data, needing to be analyzed by bioinformatics. At the same time, the necessity of reductionist methods focusing on single genes, enzymes, metabolic pathways, and regulatory mechanisms for a comprehensive characterization of biodegradation potentials in microorganisms cannot be underestimated. Here, in-silico modeling could be a helpful tool for developing predictive platforms. Application of these complementary methods can end in advances in the development of strong and effective bacterial phenolic hydrocarbon degraders with minimum risks to the natural environment (Rucká et al. 2017).

Developing an in situ bioremediation approach requires the analysis of the polluted site's physical and chemical properties, including the detection of contamination and nutrition sources and measurements of their concentrations, osmotic pressure, temperature, and oxygen resource (Rucká et al. 2017). These analyses are fundamental for designing biological stimulators surrounding bacterial degraders. It is also important to microbiologically analyze and select microorganisms able to function as primary sources of bioaugmentation and engineering. Bacterial strains isolated from different sources can be the basis of designing possible bacterial degraders at the organism level by the aid of gene shuffling (Dai and Copley 2004) or adaptive evolution (Yoneda et al. 2016). Hence, intricate analyses performed by omics approaches can provide multidimensional perspectives towards the efficiency of bacterial aromatic compound degraders and guide us to well-characterized biodegraders capable to be used in the bioremediation process. However, to change bioremediation into real feasible systems, complicated technologies should complement microbiological and bioinformatic approaches (Rucká et al. 2017).

7.9 Bacterial Enzymes Used for Degradation of Aromatic Compounds

Some bacterial enzymes have been used for the removal of aromatic compounds from the environment. For example, bacteria (under both aerobic and anaerobic conditions) show great potential for the degradation of azo dyes as a group of hazardous environmental aromatic pollutants. Through anaerobic degradation, azo dyes are transformed into intrinsically carcinogenic colorless amines, which are further processed via aerobic pathways. Two bacterial enzymes can be efficiently used for the degradation of industrial azo dyes: azoreductase and laccase. Also, when the environmental conditions are not favorable, besides their regular metabolic functions, bacterial oxidases and peroxidases can partially degrade azo dyes. These enzymes can act both intracellularly and extracellularly (Chauhan et al. 2017; Sarkar et al. 2017).

Bioremediation approaches using partially purified and purified enzymes do not rely on the growth of bacterial cells in the polluted sites and instead, are dependent on the secretion of catalytically active enzymes. Therefore, some toxic byproducts present in the bacterial bioremediation approaches are not produced. Also, enzymes are substrate-specific molecules with a higher mobility rate in the natural context because of their smaller particle size (Sharma et al. 2018).

Oxidoreductases and hydrolases are some examples of bacterial enzymes with possible applications in bioremediation technology. From the oxidoreductase category, oxygenases (catalyzing aromatic ring oxidation through adding one or two oxygen molecules to make the ring unstable), laccases (catalyzing aromatic ring cleavage and reducing one oxygen molecule in the water to produce free radicals), and peroxidases (which catalyze reductive reactions including peroxides such as H_2O_2 to generate reactive free radicals after the oxidation of organic molecules) have the potential of bioremediation applications. Bacterial hydrolases potentially used in this field include lipases, cellulases, carboxylesterases, phosphotriesterases, haloalkane dehalogenases, atrazine dechlorinase, triazine hydrolase, and so on (Sharma et al. 2018).

Bacterial laccases are enzymes containing multiple copper atoms in their structure and catalyze the oxidation of a broad range of aromatic compounds with or without a mediator. Fungi and plants also produce laccases (benzenediol: oxygen oxidoreductase), which are more well-studied than similar bacterial laccases. However bacterial laccases have some advantages over fungal counterparts; for example, bacterial laccases are stable in high-temperature and high-pH conditions. Produced by bacteria in both intracellular and extracellular forms, laccases can be functional in a wide range of pH and temperature. These characteristics make it possible to use laccases in bioremediative processes, including pollution removal, textile waste treatment, biobleaching, and even in biosensor technology (Chauhan et al. 2017).

Enzymes have the appealing potential for waste treatment and removal of phenolic hydrocarbons from the environment; since they have a broad spectrum of substrate specificity, easily immobilized, and highly efficient. Given that the

conditions are controlled, the application of enzymes in bioremediation processes like decolorization of azo dyes in the textile industry can enhance the speed of many reactions, and the candidate enzymes can work for long periods after being immobilized in a proper matrix. Furthermore, because of the substrate-specific nature of enzymes, they can be easily used to remove only the desired aromatic compounds. It should also be considered that since bacterial enzymes are biodegradable, they pose minimal risks of environmental pollution. In addition, because of lower temperatures and pressures necessary for enzymatic activity, enzymes can help us save energy during remediation processes (Sarkar et al. 2017).

However, there are some drawbacks, limiting the use of bacterial enzymes for removing phenolic hydrocarbons from the environment. First, the production of bacterial enzymes for bioremediation purposes is a costly process. Second, enzymes are really temperature-sensitive, which again, increases the costs of their application in biodegradative processes. Of course, the costs are reducible through strategies such as using waste biological products as supplements for supporting microbial growth (Sarkar et al. 2017).

7.10 Conclusions

Pollution of the environment with different types of phenolic hydrocarbons is a major concern for humanity today since these compounds can exert toxic, mutagenic, and carcinogenic effects. On the other hand, these hydrocarbons are difficult to handle with current physicochemical methods. It has been well-established that bacteria can degrade phenolic hydrocarbons under anaerobic and aerobic conditions. Different aspects of metabolic pathways necessary for bacterial biodegradation of phenolic hydrocarbons and the genetic or biochemical mechanisms regulating these pathways are also clear to some extent. Today, complemented with other fields like nanotechnology, high-tech realms of biology such as genetic engineering are trying to exploit genetically modified bacterial strains for more efficient removal of aromatic compounds from our surroundings. Unfortunately, several studies regarding the effective use of these modified organisms in realistic or nearly realistic environmental conditions have not been as successful as expected. Of course, it should be cautioned that such applications have emerged some global concerns related to this field of bioremediation, including unknown fate and interactions of these engineered (synthetic) microorganisms, interventions with natural ecosystems, etc. Bacterial enzymes have been used for bioremediation processes such as azo dye decolorization. This solution has some advantages such as a wide substrate range of these enzymes, high efficiency, higher rate of degradation, long-term usage, being eco-friendly, and saving energy. But high production fees and being temperature-sensitive limit the application of bacterial enzymes in bioremediation approaches. More comprehensive and multidisciplinary investigations are necessary to shed more light on the negative and positive aspects of this technology, alleviate its

limitations, and use it more effectively to clean up aromatic contaminants from the environment.

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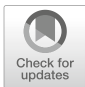
Chapter 8
Biosorption of Industrial Wastewater by Microalgae



Halleshappa Gurumurthy, Gurumurthy Dummi Mahadevan, and Sikandar I. Mulla

Abstract The heavy metals in wastewater are hazardous to aquatic animals, and it will start accumulate in the food web, and its removal from the wastewater is necessary. There are a number of techniques that emerged to remove heavy metals. The utilization of biomass is highly advantageous over conventional methods. Biofuel production is achieved by subjecting biomass into transesterification process. Algal biomass is a highly potential feedstock for the biofuel industry.

Keywords Biosorption · Microalgae · Carcinogens · Oxidoreductases

8.1 Introduction

Industrial wastewater is one of the most common by-products of industries; nearly all phases of industry use water to make commercial products. Once the water is used for processing, it passes through a number of processes, and different types of chemicals and industrial waste will be added to it. This polluted water is treated before it is discharged to nearby water bodies. Industrial wastewater carries pesticides, chemicals, oil, traces of metals, and other waste processing particles (Bilal et al. 2021; Edalli et al. 2018; Edalli et al. 2016; Hoskeri et al. 2014; Mulla et al. 2019a; Mulla et al. 2020a; Mulla et al. 2019b; Tallur et al. 2015).

The organic and inorganic pollutants of water released by industrial production must be properly treated. Organic matter and metals found in the wastewater must be removed before being discharged to back bodies of water or to land or reused in lawn. In order to reduce the overall effect of wastewater released from industries to

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the environment, it is redirected to water treatment plant where the water is treated to remove hazardous chemicals and finally released to water sources (Mulla et al. 2021; Mulla et al. 2019c; Mulla et al. 2018, b). At present the treated water is fulfilling a number of demands within the industry. This overcomes the utilization of water from natural sources (Kuyucak and Volesky 1988).

The degree to which wastewater is treated depends on the environmental conditions and standards in that region. If the wastewater is treated to avert the deterioration of the waterbody, it is released to the lake, river, stream, pond, or sea; stream standard is applied to treat the industrial wastewater. When the treatment is based on stream standards, the wastewater is treated for oxygen, acidity, turbidity, toxic chemicals, and microorganisms. On the other hand, when the wastewater is discharged into the sewer, it is treated only for acidity, microbes, suspended solids, and biochemical oxygen demand (BOD) (Becker 1994).

8.2 Industrial Wastewater and Its Characteristics

Wastewater is a common by-product of industrial or commercial activities (Mulla et al. 2018, b; Mulla et al. 2020b; Tallur et al. 2015). All industrial production processes use water to make commercial products; the water crosses nearly all phases of production. Once this water enters the production process, it is treated as wastewater, and it needs to be treated before it is discharged. Industrial wastewater is a type of water generated by various chemicals, oil, gas, or mining chemical manufacturing companies and food and beverage processing industries (Saxena et al. 2020).

Before releasing the wastewater back into the land or into bodies of water, many inorganic and organic pollutants of water used in the industry process for production must be managed. Organic matter and metals found in wastewater must be removed before the water can be safely discharged or reused in plant operations.

8.2.1 Sources of Industrial Wastewater

8.2.1.1 Metal Industries

The metal industrial process is involved in many operations and produces a slurry containing metals dissolved in liquid; particularly, manufacturing operations like printed circuit board and metal finishing release a number of metal hydroxides like ferric hydroxides, magnesium hydroxide, nickel hydroxide, zinc hydroxide, and copper hydroxide. Using current treatment techniques, wastewater must be treated before it is released into the environment and nearby water bodies.

8.2.1.2 Chemical Industries

Treating the wastewater effluents is of utmost importance for chemical industries in order not to face formidable environmental regulatory challenges. Petrochemical plants and petroleum refineries carry hazardous pollutants like oil, grease, suspended solids, phenols, sulphides, ammonia, and chromium; these pollutants when released to the environment cause adverse effect to the biofilm and reduce the normal growth of the flora and fauna by affecting soil fertility.

8.2.1.3 Mining

Mining processes, such as construction, operation exploration, expansion, abandonment, decommissioning, and repurposing, can have negative impact on the environment and to humans and societies; for example, mining affects the traditional practices of indigenous peoples living very near to mining points, and the community systems and native ecosystem will be affected by pollution. The remediation process on wastewater from mining will be the best method to reduce the pollution in environmental systems. The technical approaches on the basis of research evidence is required to overcome the adverse effect given by mining.

8.2.1.4 Steel Industries

Water is used as a basic lubricant and coolant for the production of iron, steel, wire, or rods, along with tallow particulate solids and hydraulic oils. Hydrochloric acid and sulfuric acid are required for galvanizing steel. Water is also used for cooling and by-product separation. The steel industrial process wastewater is comprised of a number of contaminates like phenols, benzene, anthracene, naphthalene, etc. and also includes acidic waters as well as waste acid.

8.2.1.5 Oil and Gas Fracking

The chemical-mixed water is injected into the well to achieve drilling, and it contains high concentrations of chloride, manganese, sodium, magnesium, strontium, barium, iron, methanol, sulphate, and other substances. The water used in fracking also includes hydrocarbons like ethylbenzene, benzene, xylene, and toluene; this contaminated water is subjectable to remediation to convert toxic effects of chemicals into nontoxic.

8.2.1.6 Power Plants

The coal-fired plant is a major source of industrial wastewater. These plants discharge wastewater with higher number of metals such as arsenic, selenium, lead, mercury, cadmium, chromium and nitrogen compounds (nitrates and nitrites). The wet scrubbers typically transfer the captured pollutants to the wastewater stream.

8.2.1.7 Wastewater Treatment Plants

The common practices of wastewater treatment in plants are producing wastes with many potential contaminants (Saxena et al. 2020). Chlorine is used to disinfect the water after the treatment; its by-products like trihalomethanes and halo acetic acids enter into water. The biosolid, the residue of wastewater treatment, contains common fertilizers, household products, heavy metals, and synthetic organic compounds.

8.2.1.8 Food Processing and Agricultural Process

Water is the main source for food and agricultural processing system, which uses water to add a number of insecticides, pesticides, and fertilizers; these hazardous chemicals enter into soil and water bodies. It is major concern to address the problem given by polluted water in both soil and is a water to reduce risk attained to soil fertility and aquatic animals. The water used in the processing of raw materials in food industry is adding high loads of particulate matter and soluble organic chemicals. The bodily fluid, intestinal matter, organic waste from animal slaughter and processing, and blood contaminants need to be treated (Table 8.1).

Table 8.1 Sources of heavy metals resulting from industrial operations

Heavy metals	Industry	Reference
Chromium	Steel industry, electroplating, leather tanning, mining, industrial coolants, chromium salt manufacturing industry	Gabarron et al. (2017) Acosta et al. (2010)
Lead (pb)	Lead is used in pipes and gasoline, cosmetics, and paint industry, and it also used in thermal power plants, bangle industry, lead acid batteries, and smelting operations	
Mercury (Hg)	Batteries and semi-conductors. Medical appliance, production of fluorescent lamps and electrical appliances	
Arsenic (As)	Geogenic/natural process, smelting operations, and thermal power plants	
Fuel burning copper (Cu)	Mining, electroplating, and smelting operations	
Cadmium (Cd)	Zinc smelting, waste batteries, e-waste, paint sludge, incinerations, and fuel combustion	
Molybdenum (Mo)	Spent catalyst, zinc smelting, and electroplating	

8.3 Treatment of Industrial Wastewater

To reduce the negative environmental impact and for conserving precious water resources, it is necessary to apply properly designed and engineered filter press system – the customized and optimized process is presently required to address the effects caused by polluted water. Based on industrial treatment requirements, manufacturing companies need to develop customized method, and to achieve the particular treatment, the proper selection of the parts of optimizing operation devices is required. Filter presses are an important option for on-site solution for treating industrial wastewater and effluent wastewater for many industries; there are many more industrial applications of filter presses for wastewater treatment which can efficiently and effectively dewater their sludge in compliance with EPA, state, and local regulations. (Acién Fernández et al. 2001).

8.4 Biosorption

Biosorption is an approach to remove toxic metals from wastewaters, and the mechanism of biosorption process is involved in metal binding capacities of various biological materials. The wastewater containing heavy is accumulate through metabolically mediated and by physicochemical pathways of uptake (Fig. 8.1) (Volesky and Holan 1995). Algae, bacteria, and fungi and yeasts proved to be potential metal biosorbents (Liu et al. 2009; Mulla et al. 2019c). The biosorption process is

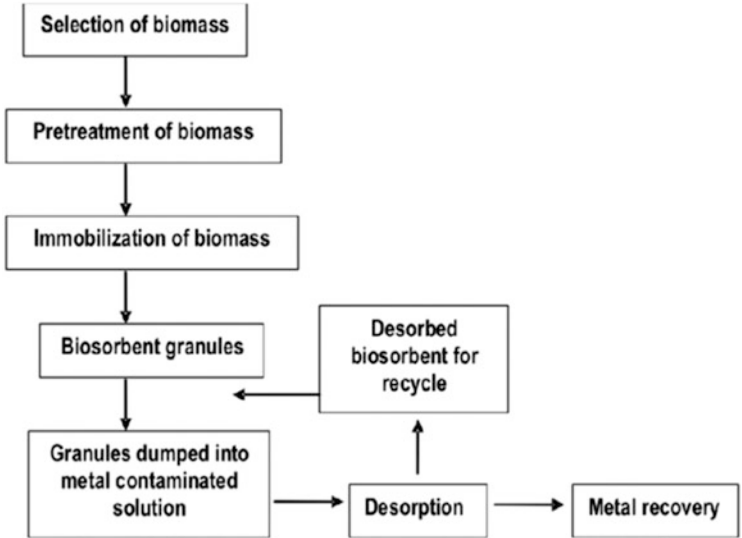

Fig. 8.1 Schematic representation of the biosorption process

advantageous over conventional treatment; it minimizes the utilization of chemicals and is one of the low-cost processes. This process is known to the best method with high efficiency to regenerate the biosorbent and higher possibility of metal recovery (Holan et al. 1993).

The biosorbent process is involved in both solid phase and liquid phase. Water is used in the liquid phase of the biosorption process. The higher affinity of the sorbent for the sorbate species is attracted and is achieved by different mechanisms. The process of biosorption continues until equilibrium is established between the amount of solid-bound sorbate species and its portion remaining in the solution. The degree of sorbent affinity for the sorbate determines its mode of distribution between the solid and liquid phases (Basibuyuk and Forster 2004).

8.4.1 Biosorbent Material

The efficacy of biosorbent depends on the uptake of chemicals of microorganisms and the binding capacity of various heavy metals by microbial cells. This type of sorbent process consists of dead and metabolically inactive cells. Some types of biosorbents are involved in the binding of the majority of heavy metals without specificity toward metals, and it is achieved through specificity toward certain metals by some strains of microorganisms. Compared to available biomass and isolated strains of microorganisms, the processed biomass is considered as the best, and it improves their biosorption properties (Kratochvil and Volesky 1998).

Recent biosorption experiments were conducted to understand the utilization of various by-products through large-scale industrial operations; waste mycelia from fermentation processes, biosolids, activated sludge treatment from sewage treatment plants, olive mill solid residues, and aquatic macrophytes are another alternate options to achieve good biosorption. The marine macro-algae are represented as an inexpensive source of biomass compared to other types of biomass. Live marine algae have a higher amount of uptake of toxic metals, and lesser amount of uptake was observed in freshwater algae. The live, metabolically active algae showing biomass metal accumulation is a pollution indicator (Fig. 8.1).

The biosorption mechanism, like ion exchange, chelation, and adsorption by physical forces, are the basic mechanism involved in the biosorption and is achieved through inter- and intrafibrillar capillaries and spaces of the structural polysaccharide network, and adsorption process will be complete as a result of the concentration gradient and diffusion through cell walls. The functional groups in molecules like phosphate groups in nucleic acids, sulfhydryl and carboxyl groups in proteins, hydroxyls in polysaccharide, and carboxyls and sulphates in polysaccharides of marine algae, acetamido groups of chitins, and structural polysaccharides of fungi can participate in the chemical reactions to complete the adsorption process (Acién Fernández et al. 2001).

8.4.2 Choices of Metals for Biosorption Process

On the basis of environmental threatening and for mode of recovery point of view, heavy metals would be divided into four major categories: (I) toxic heavy metals, (II) strategic metals, (III) precious metals, and (IV) radio nuclides.

8.5 Algal Description

The live and dead algal cells are increasingly used as biosorbents to achieve adsorption of various chemicals including heavy metals (Table 8.2) from aqueous solutions; the sorption efficacy is high and has unlimited quantities in the seas and ocean (Liu et al. 2009; Mulla et al. 2019c). Algae are autotrophic organisms composed of a diverse and simple groups from unicellular to multicellular. Seaweeds are the largest and most complex marine forms and are photosynthetic, and they lack many distinct organs found in land plants; the various groups of algae play significant roles in aquatic ecology. Microscopic forms are suspended in the water column and provide the food base for most marine food chains. Some are used as human food or harvested for useful substances such as agar (Sharma and Sharma 2017)

8.5.1 Toxic Heavy Metals

Heavy metals are toxic and carcinogenic agents making serious damage to the human health and in the water, interm affects flora and fauna (Saxena et al. 2019). Heavy metals have a great tendency to bioaccumulate, having nondegradable, persistent nature, and enter to the environment through the food web. If heavy metals are discharged to receiving bodies without treatment, it can have long-lasting effects to both human and aquatic life. There are several techniques available to treat industrial wastewater. The techniques like electrocoagulation, filtration, and various aerobic and anaerobic biological treatments can be used for heavy metal removal (Gupta et al. 2016; Keskinkan et al. 2003; Mustapha and Normala 2015) (Fig. 8.2).

Table 8.2 Biosorption capacity of algal species

Metal removed	Algal species	Biosorption capacity (mg/g)	Reference
Lead	*Spirogyra* sp.	94.34	Olal (2016) Cheng et al. (2017) Ibrahim et al. (2016)
Cadmium	*Spirogyra* sp.	22.52	
Copper	*Ulva lactuca* sp.	84.7	
Chromium	*Chlorella miniata*	34.60	
	Spirulina platensis	67.93	

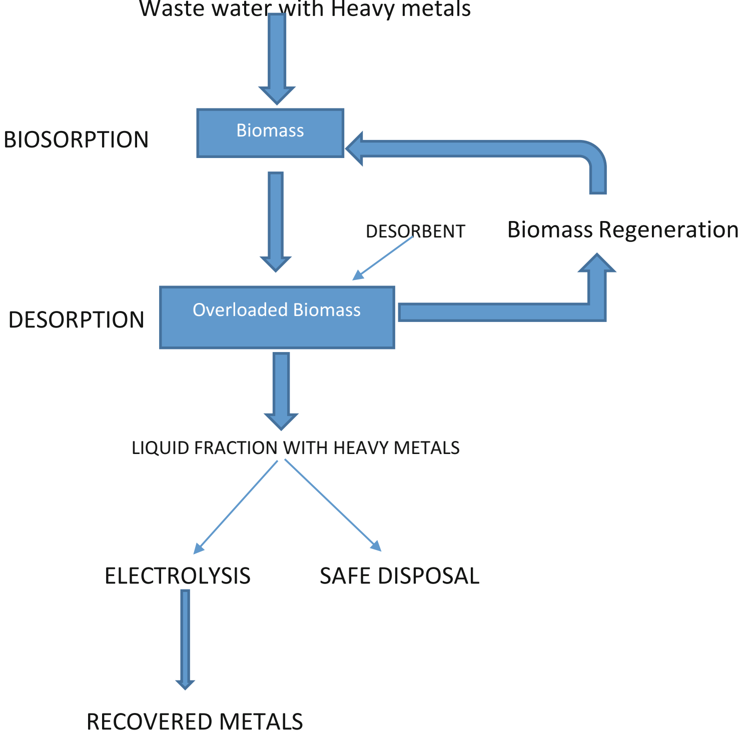

Fig. 8.2 The process of microbial biosorption and recovery of heavy metals

8.6 Microalgae for Wastewater Treatment

The removal of heavy metal by microalgae is one of the very efficient and convenient method; microalgae is an adaptable and economically feasible option for the treatment of effluent. The methods of heavy metal removal such as ion exchange or lime precipitation process are often ineffective and very expensive when used for the reduction of heavy metals; the effective utilization of suitable mechanism for achieving maximum level of removal of heavy metals is a challenging work.

The algal biosorption process is a method to enhance the environmental and economic performances of the process. Cultivation of algae is a one of best method for improving water qualities and for producing biomasses. Microalgae are absorbing nutrients and converts biomass into biofuel (Larsdotter 2006). Microalgae are absorbing the trace elements, and these are needed for their growth and their photosynthetic activities, and it freely releases oxygen which is utilized by bacteria in the wastewater. The elements like nitrogen, phosphorus, and other nutrients present in the wastewater are effectively absorbed by microalgae.

The microalgal-based wastewater treatment technology with membrane technology is one of the options for enhancing the efficacy of treatment. The microalgae-

based treatment depends on light and temperature and shows proper growth in optimum light and temperature. This technology is not suitable in temperate region where there is low sunlight, but this limitation can be overcome by providing adequate sunlight.

8.6.1 Cost-Effectiveness

Microalgae growth in wastewater is a cost-effective process than conventional wastewater treatments. Organic loads in microalgae are suitable for their growth, and this process is one of the conventional methods for achieving sustainable and low-cost wastewater treatment. Several species of microalgae are capable of capturing nutrients from wastewater.

8.6.2 Energy Requirement

Microalgae releases oxygen as their by-product, utilized by aerobic bacteria for degrading the remaining organic load. This process is natural and reduces the needed energy required for aeration during conventional wastewater treatment, and this mode of treatment does not require any extra energy input to reduce BOD from wastewater, and it produces electric power by converting algal biomass into methane.

8.6.3 Reductions in Sludge Formation

The treatment of sludge is the main objective in waste treatment plant. The chemicals used in a number of water-related processing are rescindable in producing sludge. This produces hazardous solid waste, and the entry of this solid sludge into environment should be avoided. The cultivation of algae in wastewater is the best method, and the cultivated algae are absorbing all the components of waste for their growth. Sludge is accumulated as a biomass (Hammaini et al. 2003), and biomass intern is subjected for the production of biofuel by using the conventional chemical process method.

8.6.4 Greenhouse Gas Emissions

Global warming is one of the major threats and involved in rising the temperature around the world. The carbon diooxide mitigation is achieved by methods like

chemical and biological. Chemical process involves separation, transporting, and sequestration. This approach consumes energy and is costly. To avoid this limitation, alternative cost-effective method is needed to make sustainable means to curb the threat.

Microalgal-based treatment emerged as one of the best and promising methods. A number of biotechnology techniques are currently used to develop a better solution, where are helpful in isolation and characterization of species strains to achieve the best result. The strain improvement program of biotechnology is one of the promising methods to develop the best strain to absorb all hazardous chemicals released by industrial process; the developed new strain of microalgae is very useful to produce good biomass, and it is able to capture more sunlight, showing faster growth rate, responsible in making higher rate of CO_2 fixation.

The supply of the required amount of CO_2 is crucial to achieve optimal algal growth. Supplying pure CO_2 may be very expensive; the CO_2 produced from industrial production addresses these challenges. The supply of flue gas from industries is the best way to fulfil the demand. Microalgal-based wastewater is utilizing sunlight to reduce tons of CO_2 compared to conventional treatment.

8.7 Wastewater Detoxification Using Enzymes

The limitation of the biosorption method can be overcome by utilizing oxidative enzymes; the oxidoreductases enzyme catalyzes the oxidation-reduction reaction and is effective in the treatment of heterogeneous effluents. The oxidative enzymes form the radicals instead of mineralizing the substrate, and radicals will be disintegrated into fractions of transformation products. The transformation products of enzymes catalyzed the treatment method, exhibit minimal toxicity, and are more biodegradable than the parent compounds.

8.7.1 Types of Microbial Enzymes

The enzymes oxidoreductases, oxygenases, monooxygenases, dioxygenases, peroxidases, microbial cellulase, and microbial protease are the different types of enzymes involved in the detoxification of toxic organic compounds by oxidation or in the metabolism of organic compounds. The oxygenase enzyme mediates the dehalogenation of halogenated methanes and ethanes (Pandey et al. 2017). There is a potential advantage of enzymatic treatment like the enzymatic treatment, very useful in making operation at high and low containment concentrations, avoiding delay of acclimatization of biomass, reducing the sludge volume, and making the simple controlling in process.

8.8 Conclusion

This chapter concluded that in industrial production process, water is a major component of many operational works and all the operated process like chemicals, raw materials, and production of products generate high strength of wastewater. This wastewater includes heavy metals, chemicals, and waste with Biochemical oxygen demand (BOD) and Chemical oxygen demand (COD). This type of wastewater is hazardous to the flora and fauna of the ecosystem. If industrial wastewater is released to water bodies without proper treatment, it causes oxygen depletion, and in the soil the wastewater with heavy metals reduces the soil alkalinity and affects vegetation by disturbing seed germination. Industrial wastewater is involved in the discoloration of water, and the melanoidin pigments are toxic to microorganisms present in the soil and water.

The entry of toxic chemicals into food chain will cause severe disasters to human beings and aquatic and nonaquatic species. This chapter revealed the effect of biosorption and the potential of microalgae in wastewater treatment of available heavy metals released by industrial wastewater using algal culture.

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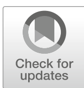
Chapter 9
Plastic Degradation and Utilization by Microbes: Challenges and Scope



Amit Kumar Verma, Ashok Kumar Nadda, Arun Gupta, and Swati Sharma

Abstract After the discovery of plastics in 1950, it has been used expeditiously throughout the world, which leads to its overaccumulation in the ecosystem. Microplastics are widely affecting the life of terrestrials and marine ecosystems. Natural depolymerization of plastics is very slow and progression takes a long time. Physical and chemical methods are quite worthwhile, but the biological plastics degradation has gained an interest in recent decades. In the biological degradation of plastics, the microorganisms attach to the surface of the plastics and the enzymes convert long chain of polymers into non- or less toxic forms. This review summarizes progressive data on microorganisms degrading plastics, physical and chemical methods, mechanisms for biofilm formation onto plastic materials, enhancement of microbial depolymerization by enzymatic catalysis, the engineering of enzymes, pathways modification, and microbiome's role in plastic depolymerization.

Keywords Microbial depolymerization · Biofilms · Biodegradation · Depolymerases · Free radicals · Crystallinity and hydrophobicity · Pretreatments

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9.1 Introduction

Plastic is a derivative of a long chain of the petrochemical synthetic polymer of higher molecular weight (Ahmed et al. 2018). Plastics are one of the troublesome pollutants to the environment (Guinand et al. 2004). Some crucial characteristics of plastics like hydrophobicity, crystallinity, long chain, and high molecular weight made it lightweight, resistant, and widely applicable for various usage (Wilkes and Aristilde 2017). After the production of plastics started in 1950, there were extensive applications in the different fields that statically increased the production and utilization graph. Numerous applications of plastics increased the synthesis of polythene 20-fold more from 1964 to the next 5 years. In 2018, the production of plastics was calculated as 360 million metric tons (MMT) and estimated to be double in 2050 (Ellen MacArthur Foundation 2017). The substantial production of some of the economically important plastics includes polyurethane (PUR), polyethylene (PE), Polyamides (PA), Polystyrene (PS), Polyethylene terephthalate (PET), Polyvinyl chloride (PVC) and Polypropylene (PP), and their production data during 2015 is given in Fig. 9.1. Moreover, the consumption and environmental leakage were brought around 5–13 MMT of plastics into the oceans (Geyer et al. 2017; Plastics Europe 2018; Ellen MacArthur Foundation 2017). 110,000–730,000 tones of plastics from the household and industrial waste were accumulated to agricultural landscape or green land (Nizzetto et al. 2016). These plastics on land or in the oceans show adverse effects on plants, animals, and microorganisms (Ritchie and Roser 2018; Thompson 2019). Terrestrial plastic pollution contains chlorinated plastics which contaminates the groundwater and can cause serious harm to the animal's health (Horton 2017). The toxic chemicals which are released from plastics are also included biphenyls, phthalate, and bisphenol (Bryant et al. 2016). It is

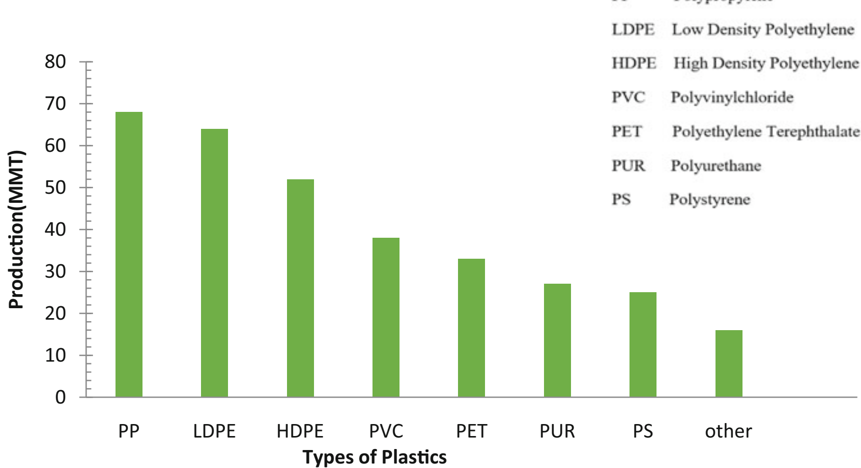

Fig. 9.1 Different types of plastics polymers produced in 2015

estimated that 8 MMT of plastics enter into the oceans annually (Jambeck et al. 2015). A Great Pacific Garbage Patch (GPGP) covers an estimated area of 1.6 M KM^2 which is around thrice the area of France (De Wolff 2014; Lebreton et al. 2018). Over 250,000 tons of fragmented pieces or microplastics floated at sea level. Microplastics formation due to abiotic stress caused by UV radiation, oxygen, temperature, and physical stress (Geweret et al. 2015).

The development sees toward plastics as a measure concern, and for that, they were moving toward alternative ways: firstly, by introducing biodegradable plastics (hydrodegradable, compostable, biocomposite, pre-oxidant additive-containing and oxo-degradable plastics) and secondly, depolymerization of plastics which are leaked into the environment. Depolymerization through physical and chemical processes which are performed at higher temperature releases toxic substances (Hauenstein et al. 2016). Biological degradations (BD) are an environmentally friendly process end up with byproducts which are not harmful (Florez et al. 2015). But BD was limited to the physical and chemical properties of polymers used (Das and Kumar 2013). Both natural and synthetic polymers were degraded by attaching to surface, and colonization was given the ability to break ester bonds in plastics via enzymatic hydrolysis (Arutchelvi et al. 2008). Peroxidase enzyme breaks down the polymer, and the host microbes utilize it as a carbon source (Raziyafathima et al. 2016). Some characteristics of plastics that affect biodegradability are molecular weight, chain length, functional group, solubility, crystallinity, hydrophilicity, and hydrophobicity (Okada 2002; Jaiswal et al. 2020). The increase in the crystallinity of plastics provides fewer sites for the action so that degradability decreases (Slor et al. 2018). Microbial degradation by bacteria, fungi, and actinomycetes includes the formation of biofilm, plastics pretreatment before microbial action, engineered processes, and designing consortia. The biofilm formation crucially helps in the colonization of microbes on the hydrophobic surface, and the biofilm was found to be more effective than the individual performance (Jenkins et al. 2019). Further recapitulation of the techniques and advancements served a better understanding of plastics degradation.

9.2 Pollutant Degradation Using Enzymes

Increasing population and industrialization give us more pollutant deposition throughout the environment. The impact of these pollutants were seen clearly in soil, air, water, animals, plants, and humans. These hazardous compounds present in organic and inorganic forms which are the most common polluting agents are listed in Table 9.1 (Rao et al. 2010). Therefore, the implication of efficient strategies is mandatory for an immaculate environment. The major implications of pollutants are seen at the soil and the water level, so an effective remediation methodologies are inferring to utilized waste water and clean groundwater and enhancing soil fertility. Previously, research and studies were performed from many decades to find out a

Table 9.1 Common environment pollutants (Rao et al. 2010)

Organic	Inorganic	Air pollutants
Pesticides	Cyanide	Smog-forming compounds
N and P derivatives	Lead	Greenhouse gases
Plastics and biopolymers	Cadmium	Particulates
Phenols, chlorophenols	Chromium	
Chloroanilines, dyes	Copper	
PAHs, BTEX, PCPs, NAPL	Arsenic	
Nitro compounds		

Table 9.2 Most commonly used thermosets and their structures

S. no.	Thermoset	Structure
1.	Polyurethane	
2.	Epoxy resin	
3.	Silicone resin	
4.	Acrylate	
5.	Cyanoacrylate	

suitable and the efficient remediation techniques, and in these studies, it was seen that the enzymes play the key role (Gianfreda et al. 2016).

9.3 Types of Plastics

In the "plastic foam age," most of the natural polymers are replaced by both thermoplastics and thermosetting plastics (Landrock 1995; Crawford and Martin 2020). This classification is based on the melting properties of plastics (Shah et al. 2008). Some economically important thermosets used frequently are polyurethanes, epoxy resins, silicone resins, acrylate, and cyanoacrylates (Sastri 2014) (Table 9.2). Thermosets are synthesized in two stages: firstly, formation of a long chain of

monomer molecules and secondly, the crosslinking in chains formed in molding at high temperature and UV exposure (Crawford and Martin 2020). The centralized chain of the thermoset formed of ester or amide makes them more biodegradable. These thermosets are not having properties of plasticity, but they are thermal resistance, hard, and more cross-linked.

Thermoplastics are long-chain-based polymers which developed fluidity at high temperature and harden gradually with decrease temperature. The property of reheated, flexibility, rapid cooling, and modifying their shapes without a change in the chemical and mechanical properties made it a recyclable material. Some economically important plastics which are commonly used are polystyrene (PS), polyethylene (PE), polyvinylchloride (PVC), polystyrene, polyethylene terephthalate (PET), and polyurethane (PU) shown in Table 9.3. A greater part of these polymers are used in industrial processes due to their flexibility, strength, lightweight, chemical resistance, and recyclability. Their significant applicability is found in the packaging, construction, and automobile industries (Elias 2009). Around 40% of plastics were used by the packaging industries, and over 500 billion single-use plastics (short life of 15 min) were released annually (Plastic Oceans International 2008). These accumulations in landfills and marine environments impact on animals, macroenvironments, and human health-related problems. Decades of improving in the microbial degradation show the potentials to use in plastic degradation.

9.4 Plastics' Biodegradability

Based on their degradation properties, plastics are classified as biodegradable and nonbiodegradable. Biodegradable plastics are the polymers that are broken down due to the action of living organisms, and their end up result must have a less harmful effect than the traditional plastic. Biodegradability mainly involves some factors like the morphological arrangement, molecular weight, crystallinity, and hydrophobicity. These involve the naturally occurring polymers like polysaccharides and lipids; starch; cellulose; alginates; protein—animal proteins; collagen/gelatin; plant protein, gluten. A new type of plastics created as bioplastics is much easier to degrade using microbes in natural conditions, e.g., starch-based bioplastics, polylactic acid (PLA), poly-3-hydroxybutyrate (PHB), and polyhydroxyalkanoates (PHA). For the degradation of PHA, microbes produce such enzymes like polyhydroxyalkanoates depolymerases utilized in the process of degradation (Trivedi et al. 2016; Mukherjee and Chatterjee 2014).

Nonbiodegradable plastics do not break down under natural conditions and act as pollutants to the environment. Nonbiodegradable plastics have higher molecular weight, and high crystallinity index results in higher hydrophobicity. The surface with increased water-repellent properties does not allow the attachment of microbes and further degradation. Mostly conventional plastics like polyethylene, polypropylene, polystyrene, polyvinyl chloride, and polyethylene terephthalate are nonbiodegradable. Extensive use of these plastics leads to the wide deposition in

Table 9.3 Widely used commercial thermoplastics and their structures. Discussed with microbes showing the deterioration of different plastics

Plastics	Structures	Microbes	Sources	References
PE (polyethylene)		*Phormidium* sp., *Rivularia*	Microplastic from the North Atlantic	Zettler et al. (2013)
		Pseudophormidium sp., *Phormidium* sp.	Plastics particles from costal area of the UK, Germany, and Denmark	Oberbeckmann et al. (2014, 2016)
		Zalerion maritimum	Marine environment	Paço et al. (2017)
		Pseudomonas aeruginosa and *Brevibacillus* species	Soil sample from a dump site	Gumbi et al. (2019)
PET (polyethylene terephthalate)		*Phormidium*, *Lewinella*	Bottles submerged in the north sea off the UK coast	Oberbeckmann et al. (2016)
		Stanieria, *Pseudophormidium*	Drinking bottles submerged in the north sea off the UK coast	Oberbeckmann et al. (2014)
		Ideonella sakaiensis 201-F6	A PET bottle-recycling factory in Japan	Liu et al. (2018)
LDPE (low-density polyethylene)		Arcobacter *Colwellia* spp.	Coastal marine sediments within the Humber estuary, UK	Harrison et al. (2011)
		Stenotrophomonas sp.	Plastic debris in soil	Peixoto et al. (2017)
		Staphylococcus massiliensis and *Clostridium novyi* type A	From petroleum and garbage site soils	George (2019)
		Paenibacillus sp.	Landfill and solid waste incinerator from Brazil	Bardají et al. (2019)
PVC (polyvinyl chloride)		*Pseudomonas citronellolis* and *Bacillus flexus*	Leibniz institute DSMZ-German collection of micro-organisms and cell cultures (Germany)	Giacomucci et al. (2019)
		Anaerobic marine consortia	Marine samples were collected from Eleusis bay (Greece)	Giacomucci et al. (2020)
PU (polyurethane)		*Pseudomonas* sp.	S site rich in brittle plastic waste	Espinosa et al. (2020)

land and marine environments. The natural polymer-degrading enzymes have the potential for plastic removal. For example, cutin-hydrolyzed enzyme is involved in the hydrolysis of the ester bond of PUR and PET. Also, lignin-degrading enzymes were used for the degradation of LDPE (Sen and Raut 2015).

9.5 Processes and Pathways of Biodegradation

The process of change in chemical and physical properties of the material by the microbes such as bacteria, fungi, actinomycetes is shown in Table 9.2. Microorganisms can catalyze the breakdown of bonds in plastic polymer by oxidation-reduction mechanism (Tokiwa et al. 2009). The action of enzymes on the plastic material requires the attachments of microorganisms to the surface. Many biotic and abiotic factors (topography, molecular weight, crystallinity, and hydrophobicity) are affecting the binding probability to the surface of plastics (Leja and Lewandowicz 2010). The higher the crystallinity, the lesser the amorphous region where the vulnerability of the microorganisms to bind is decreased. To understand the degradation mechanism, understanding the biochemical changes is important.

Biodegradation by microbes mainly involves four stages: biodeterioration, fragmentation, assimilation, and mineralization. Biodeterioration involves the microorganism's access to the surface of plastics, which is crucially supported by chemical and physical oxidation (e.g., UV-induced oxidation) (Vivi et al. 2019). Next, fragmentation by microorganisms catalyzes the reaction using enzymes (monooxygenases and dioxygenases) to add oxygen to form alcoholic and peroxyl groups which are less toxic to the environment and help in the mixing of materials into environments. Further, mineralization and assimilation include the formations of the monomer units and integration to the microbial cell biomass. Degradation of metabolites inside the cell leads to the generation of secondary products like CO_2, N_2, CH_4, and H_2O (Devi et al. 2016). Many enzymes are reported being diversely acted upon plastics. There are enzymes include lipase, catalase, laccases, peroxidase, PETase, MHETase, and α- or β-hydrolases from different microorganisms which help in catalyzing the degradation of many plastics. In a study two enzymes of hydrolase (PETase and METHase) of *Ideonella sakaiensis* identify as complete degradation of PET within 6 weeks at 30°C. The mechanism involves PETase, firstly, breaking PET into mono-2-hydroxyethyl terephthalate (MHET) and then METHase catalyzed to convert MHET into terephthalic acid and ethylene glycol (Palm et al. 2019). The formation of the consortium of fungus in which pioneer colonization species of *Chaetomium globosum* (ATCC 16021) were seen which results in 75% for PCL (polycaprolactone) films and 9% for PVC films loss weight in 28 days (Vivi et al. 2019).

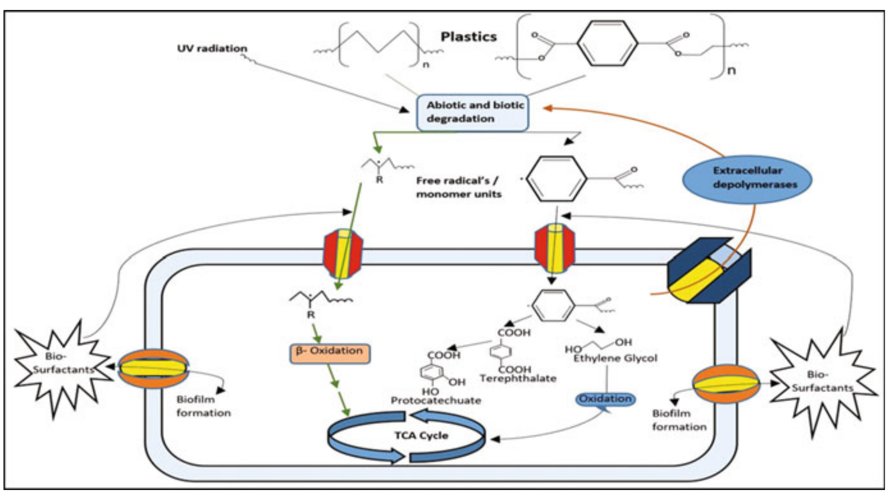

Fig. 9.2 The microbial biofilm natural degradation of plastics

9.5.1 Microbial Natural Pathways of Degradation

Natural pathways include the microbial biofilm formation onto the surface of plastics. Microbes containing enzyme depolymerases are subjected to biodegradation. The bio-surfactants secreted help to maintain the biofilm formation on plastics. In a microenvironment, the polymers are broken down into oligomers units by both abiotic factors and enzymes secreted by microorganisms, and these short oligomers are transported to the cytosol through channels. Further, through the sequential oxidation process, they were entering in the TCA cycle and utilized as a carbon source and energy source for microorganisms (Fig. 9.2) (Koutny et al. 2006), for example, the PET degradation in *Ideonella sakaiensis* by enzyme PETase into monohydroxyethyl terephthalate (MHET) transported to the cytosol of a microorganism, in which the MHET broke down into terephthalate and glycol in further stepwise oxidations through the TCA cycle (Joo et al. 2018; Ghosh et al. 2019). *Pseudomonas* sp. AKS2 degrades 300 mg of LDPE film to 5% without pretreatment in 45 days (Tribedi and Sil 2013).

9.5.1.1 Biofilm Biodegradation

Naturally, most of the microorganisms degrade plastic via biofilm formation – it is an organism's community in which they produce a matrix of extracellular substances. Microorganisms in biofilms show different characteristic properties than that from the free-living cells. Crystallinity and hydrophobicity affect the most the microbial growth on the surface of plastics. The amorphous region between crystalline structures helps in the formation of biofilm. The rate of colonization on the floating

plastics is found that a very slow modification in the procedure brings a higher rate of microbial growth. Most of the biofilm formation is seen in bacteria, and biofilms of fungal and yeast were also found. *Pseudomonas* and *Rhodococcus* spp. involve in PCL film degradation had a 53% weight loss in 30 days. *Bacillus* sp. degrades 4% of PP (polypropylene) in 40 days of incubation (Auta et al. 2018). Some other bacterial species like *Enterobacter asburiae*, *Cryomorphaceae*, *Bacteroidetes*, *Ideonella*, *Escherichia*, *Azotobacter*, *Actinomycetes*, and *Mycobacterium* were reported to degrade plastic (Joo et al. 2018; Ghosh et al. 2019). In the formation of biofilm, the bacterial species are pioneers, but some of the entangled fungal species share functionality in the process of degradation. In a study, the *Aspergillus niger* was used for plastic bag degradation and observed to lose 36% of weight in the incubation period of 60 days (Mohan and Suresh 2015). *Penicillium* species, *P. chrysogenum* and *P. oxalicum*, degrade HDPE with 58.60 and 55.34% weight loss and LDPE with 34.35 and 36.60% in the 90-day incubation (Ojha et al. 2017). Biofilm degradation involves the interaction of microbes to plastic surface to bring phytochemical changes, increased bioavailability, cell viability, and degradation efficiency.

9.5.1.2 The Effect of Physical and Chemical Factors in Plastic Biodegradation

Without a pretreatment a plastic degradation takes decades; it is a complex process that includes plastics and microbes for depolymerization. Mainly physiological stress, polymer hydrophobicity, chain length, crystallinity, and breakable bonds of esters and amides affect degradation (Díaz et al. 2014). Other factors such as surface attachment, surfactant secretion, and types of enzymes also play an important role. The primary degradation processes of polymer oxidation and hydrolysis substantially affect the rate of degradation due to physiochemical or biological origin (Smith 2005). For improving the degradation of plastics, different techniques were used including pretreatments, mixing with highly degradable polymers, and the use of surfactants produced by microorganisms. Most of the pretreatment is in vitro studies for the decrease of the time course between the polymers to be degraded. The pretreatments substantially include photothermal oxidation, the use of chemical oxidants, UV oxidation, and surfactants by microorganisms (Devi et al. 2016). The photo- and thermal oxidations are mainly involved with the formation of free radicals which initiate the chain scission of polymer into smaller units. The photo- and thermal oxidation of PE, PP, and PET are delineate by Fotopoulou and Karapanagioti (2017). The introduction of chemical oxidants like sulfuric acid, nitric acid, hydrochloric acid, and hydrogen peroxide generates free hydroxyl radical which helps in the reduction in chain length in polymers (Sen and Raut 2015). UV oxidation comes under photooxidation which cleaves the bond of the polymer with decreased molecular weight and increases the number of carboxylic acid groups that subsequently enhances the biocatalytic depolymerization of plastics. In a study UV-pretreated LDPE has been placed for biodegradation for 28 days, and *A. pittii* was shown having the highest weight loss of 26% (Montazer et al. 2018). In contrast

to the formation of free radical and adding of carbonyl group, the surfactant produced by microorganisms helps decrease the hydrophobicity of polymer. Having an amphoteric nature, biosurfactants increase the availability of the hydrophilic group and the mobility of polymer. Substantially plastic-degrading microorganisms produce the biosurfactants which also help in biofilm formation. The yeasts *Pseudozyma antarctica* are known to produce a biosurfactant, mannosylerythritol lipid (MEL) (Fukuoka et al. 2016). Actinomycetes and *Pseudomonas* sp. are also able to produce biosurfactants (Duddu et al. 2015; Kádár and Fonseca 2019). The addition of alternative sources and additives for surfactant production in the procedure helps to increase depolymerization. Biodegradable substrate like hydroxypropylated starch mixed with PE enhances the degradation ability of *P. aeruginosa* ATCC 13388 (Kim 2003). For *Pseudomonas* sp. AKS2 external substitute ammonium sulphate in media provides slightly increases the hydrophobicity of polymer.

9.5.1.3 Microbial Consortium for Plastic Degradation

Microbes show function-based symbiotic interactions are considered as the microbial consortium. The interaction of microorganisms and secretion of compounds help in resource exploitation (Madigan et al. 2017). The consortium in biofilm microorganisms is sharing the products of metabolism, enzymes, and genetic information (Mitri and Richard Foster 2013). The characteristic feature of efficient exploitation of resources, economical preparation of consortium, and complex procedures followed simultaneously make applicable the depolymerization of plastics. The consortium preparation is an alternative to genetically modified microorganisms use in degradation (Varrone et al. 2018). The synthesis of consortium focuses on some key factors: inter- and intraspecies interaction, biocontainment measures, modeling, developing population control, and spatiotemporal dynamics (Johns et al. 2016; Jenkins et al. 2019). *P. putida* VM15A and *Pseudomonas* sp. VM5C are not able to degrade PVA individually, but co-culturing helps in the growth onto the PVA surface (Shimao 2001). *Flavobacterium* sp. produces toxic byproducts in PEG degradation but when cultured with *Pseudomonas* sp., was able to remove toxin and show feasible degradation of PEG (Gu 2003). Satlewal et al. (2008) reported that the microbial consortium was prepared with the potential to degrade and reduce the weight of HDPE and LDPE by 22.40% and 21.70%, respectively. The consortium of *Bacillus subtilis* MZA-75 and *P. aeruginosa* MZA-85 showed increased weight loss than the individual species used for the depolymerization of PU (Shah et al. 2016). *Bacillus* sp. and *Paenibacillus* sp. consortium showed a 14.7% weight loss of dry microplastics in 60 days of incubation (Park and Kim 2019).

9.5.1.4 Genetic Engineering for Plastic Biodegradation

Developments in genetic engineering, rDNA technology, gene editing, gene cloning, and cell biology lead to the generation of modified strains of microorganisms. Plastic biodegradation is based on enzymatic activity. Most of the microbial plastic degradation involves enzymatic depolymerization, and the efficiency for enzymatic action was inadequate. Natural production of these enzymes in microbes is very less; the overwhelming expression of enzyme approaches increase the degree of efficacy. Protein-specific pathway manipulation concerns with the enzyme's binding specificity and affinity to the groups on a plastic surface (Dourado et al. 2017). More often, in *P. putida* and *Nitrosomonas europaea*, pathway manipulation performed with suitable host *E. coli* extended in the bioremediation (Rajakaruna and Robinson 2016). The plastic (LDPE and HDPE)-degrading microorganisms *Pseudomonas mediterranea* and *Bacillus megaterium* undergo a xenogeneic expression of enzymes in *E. coli* (Liu et al. 2015). For *Pseudomonas* sp. directly put for the manipulation of gene and some of the gene of interest expressed in *E. coli*. The crystallinity structure of PE and PET plastics is more rigid, and the enzymes PETase and MHETase form *I. sakaiensis* recently identified for better degradation ability. Protein engineering is approached in two ways: directed evolution and rational design. Directed evolution used for strain and population improvement includes random mutagenesis by providing microenvironment and selective pressure to microbes, screening to find the desired protein-modified strain. The rational designing modification of protein performs with good knowledge of their structure and functions. It is a new discipline that includes the amino acid changes and impact on the structural and functional behaviors to improve the microorganism's pathway. The two fields of molecular modeling and computational protein designing assist more confined rational changes. Recently the PETase found to have more potential in enzyme-based degradation gives a futuristic perspective to protein engineering and manages redundant PET debris.

9.5.1.5 Microbiome-Assisted Biodegradation of Plastics

The bioremediation of plastics widely opens the field of research on microorganisms. Studies claim the depolymerization of different plastics through bacteria and fungi, even decades of study on different microbes still the efficiency degradation being slow or lower than expected (Restrepo-Flórez et al. 2014; Krueger et al. 2015). Recently, an invertebrate's worm larvae known as plastic-eater have been reported. The invertebrates were capable of breaking large pieces of plastics into smaller ones which increase surface area to enhance the binding area for microorganisms. The mechanism involves, in this occurrence, plastic chipping and grinding by the insect larvae into smaller pieces, and then gut microbes were attached to perform degradation. In one primary report in 2017 by a Spanish researcher, wax worm (*Galleria mellonella*) shows a high level of PE depolymerization (Bombelli et al. 2017). A

labeling study of polystyrol through α- ^{13}C and β-^{13}C evidenced that the carbon of PE was used to form lipid residues (Yang et al. 2015b). An Indian mealworm containing *Bacillus* sp. YP1 in their microbiota can degrade PE (Yang et al. 2014; Yang et al. 2015a). The *Exiguobacterium* sp. YT2 in mealworm brokens down approximately half the eaten PS within 12–15 h which was higher than most of the other species (Yang et al. 2015a. Yang et al. 2015b). *Tenebrio molitor* is able to degrade both PE and PS assisted by gut microbes *Citrobacter* and *Kosakonia* (Brandon et al. 2018).

9.6 Conclusion

Plastics accumulation in the environment causes a diverse problem to the ecosystem and the living organisms. The resistant property of plastics to environmental conditions makes it very hard to degrade naturally. Most of plastic products used in packaging industries are hardly recycled and buried in a landfill or incinerated. Recycling of plastics has limitations for its execution areas. The use of physical and chemical processes for the depolymerization of plastics is always considered less important than the biodegradation of plastics, because it can perform the degradation more naturally and products are less toxic to environments. Microorganisms (bacteria, fungi, and invertebrates) having depolymerase are used directly for the degradation or after some genetic manipulation.

The major approaches of biodegradation of plastics include biofilms formation, physical and chemical enhancement, consortia formation, protein and pathway modification, and microbiomes of invertebrates for degradation. Biofilm formation is the phenomenon of attachment of microbial colonies to the surface of the plastics and breaking down polymers into smaller unit, so they can use it as their carbon sources. Physical and chemical pretreatments have been used to enhance the degradability of plastics. The symbiotic interaction of microbes in a microenvironment forms the consortium. Microbiomes of vertebrates degrade more efficiently than when they degrade in an isolated system.

The research and development in the approaches toward biodegradation of plastics is still in a progressive state. But the diversity of microorganisms degrading plastics were limited. The efficiency of microbial degradation is still low. Microplastics are being more concerned for the environment and organisms level deposition a field open for the research. The rate of biodegradation should be increased to combat the overburden of plastic on the Earth's surface.

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Chapter 10
Marine Microorganisms: From Pollutant Degradation to Added Value Products

Fuad Ameen, Mona S. Al Tami, Khawla Alsamhary, and Peijun Zuo

Abstract Marine microorganisms are microscopic organisms that live in oceans. In the oceans, more than 98% of the biomass is contributed by marine microorganisms. They play a role in earth for the mass and energy balance by mass and energy flow worldwide. Almost all of the marine products are made of marine microorganisms. In marine ecosystems, biomass keeps the dynamic balance between the producers and the consumers. The deepest part of the ocean is beneath 10,984 m. The pressure is 1100-fold of the standard air pressure. Because of the different metabolic pathways, marine microorganisms are sources of novel biomolecules, such as bio-surfactants. In the battle to degrade the pollutants, they are key players. Currently, more than 30,000 chemicals have been discovered in the marine. Many of them are useful substances in industries. For example, they have the abilities to degrade pollutants like antibacterial, antifungal, antivirus, and antitumor properties, and have functions in foods and enzymes. Marine microorganisms are used as pollutant degraders in other added value products.

Keywords Bio-surfactant · Cyanobacteria · Polysaccharides · Pollutants · Microalgae

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10.1 Introduction

Many researchers are looking for novel microbial strains, bioactive substances, and enzymes with special property from special microorganisms (Birolli et al. 2019). The effort was focused on forests (Pajares and Bohannan 2016), savannas (Noriler et al. 2018), the Arctic (Malard and Pearce 2018) and the Antarctic Poles (Duarte et al. 2018), deserts (Cui et al. 2018), and finally the sea.

Marine microorganisms are microscopic organisms that live in oceans. In the oceans, more than 98% of the biomass is contributed by marine microorganisms. They play a role in earth for the mass and energy balance by mass and energy flow worldwide. Almost all of the marine products are made of marine microorganisms.

However, marine microorganisms have not been studied as much as the microorganisms on land. According to statistics, valuable compounds discovered from marine weigh only 1% more than terrestrial environments. Discovery of new compounds from marine is a field still with high possibilities (Ameen et al. 2021; Chen et al. 2019).

In marine ecosystems, biomass and energy keep a dynamic balance between the producers and the consumers. The deepest part of the ocean is beneath 10,984 m. At that point, the pressure is 1100-fold of the standard air pressure. This means that the marine microorganisms from the surface would not be the same as those on the seafloor.

Marine microorganisms are abundant in seafloor sediments as well as in seawater. The microbial communities are comparable to each other (Bacosa et al. 2018).

Besides the microorganisms themselves, man-made wastes are also spread into the ocean. There is an estimated 800,000 tons of oil cast to the Gulf of Mexico. Of this, 4.9% is deposited in the Deepwater Horizon (DWH). In the northern Gulf of Mexico (nGOM), the cold seeps are formed by the cast oil. In the environment with high concentration of hydrocarbon, the in situ bacteria are altered to adapt for surviving. They likely already have the abilities to destroy the surrounding hydrocarbons (Mills et al. 2004). The hydrocarbon-contaminated DWH was studied for the possible finding in the sediments. After only half a year, the DWH was found containing rich bacteria such as Gammaproteobacteria and *Colwellia* species. The bacteria were uncultured. The sequenced nucleotides expected had the ability of degrading hydrocarbons with aliphatic and aromatic groups (Handley et al. 2017; Mason et al. 2014).

Surfactants are molecules having both hydrophilic and hydrophobic parts (Araujo et al. 2019). They are classified as chemical surfactants (petroleum origin) or bio-surfactants (microbial origin).

The uniquely distributed marine microorganisms have their own unique characters in metabolic routes and physiological functions. They form in key sources for the discovery of novel substances with biological activities. Kinds of these biomolecules are bio-surfactants. They are important for degradation of the pollutants in the ocean.

After 1980, drug development shifted the study to marine (Khalifa et al. 2019).

Currently, more than 30,000 chemicals have been discovered from the marine source. Many of them are useful substances in industries. For example, they have the abilities to degrade pollutants like antibacterial, antifungal, antivirus, and antitumor properties, and have functions in foods and enzymes. Marine microorganisms are used as pollutant degraders in other added value products.

10.2 Marine Microorganisms: Their Role in Pollutant Degradation

Marine biosphere provides bacterial community, which is a raw material to produce industrial grade products. Bio-surfactants (BS)/bio-emulsifiers (BE) are attractive products. The special properties of surface-active compounds lead them to have applications in various industries (Satpute et al. 2010).

In our planet, oceans account for 70% of the whole area on the surface. At the huge interfaces between the oceans and the air, chemicals in gas form are exchanged between the water bodies and the air body (Nizzetto et al. 2012). The most global air pollutant is CO_2. To control the source of CO_2 is important as well as to emit the existing CO_2.

Marine phytoplankton consists of only about 1% of the global plant biomass. But it fixes half of CO_2 and produces half of the oxygen. The net global primary CO_2 production is about 50×10^{15} grams of carbon (Pg C) per year. In comparison with land plants such as trees, marine phytoplankton covers a bigger surface area on the earth, has less seasonal difference, and has significant faster turnover rates (days versus decades) (Cavicchioli et al. 2019).

The whole global air pollutants are collected in oceanic water bodies, which further gradually fall down to the lower waters and finally get fixed and bonded with the solid ground (Dachs et al. 2002).

The term biodegradation is misleading as to convert polymers such as plastic materials to monomers. However, according to Lucas et al. (2008) and Harrison et al. (2018), biodegradation of synthetic polymers should include biodeterioration, bio-fragmentation, and assimilation.

Synthetic plastic wastes are generated by general industries and plastic producers (Cole et al. 2016).

Marine biofouling is the procedure of plastics, when they merge in sea. They are attached with microorganisms and other creatures. Bacteria are one of the microorganisms. They play a main role in biofouling. Their work is to perform the four procedures, adsorption, immobilization, consolidation, and micro-fouling (Urbanek et al. 2018). Besides these four procedures, other creatures perform the fifth procedure, macro-fouling.

Biofilm is formed by bacteria binding to solid surfaces such as teeth or other materials. Bacteria in the biofilm have better ability to be alive than the bacteria not in the biofilm. These are observed in dentistry as well as in marine science (Junge

et al. 2004). The protecting mechanism of the biofilm should include organizing ecosystems, sharing information, enriching nutrients, and protecting toxic or other harmful substances.

Hours after they meet together, microorganisms are assembled to cover the plastic. This procedure is called attachment. In such a procedure, assembled bacteria help to enhance the turnover of enzyme activity that finally degrades targeted microplastic-associated complex. Or they even break down the complex itself.

Besides the solid waste, the liquid waste also frequently occurs in the oceans. One accident happed in Kuwait coastal line, and the cast oil contaminated the sea. About 2 years later, one strain of *Nocardioides* sp. (strain KP7) was found in the site. It had the ability to degrade phenanthrene (Iwabuchi and Harayama 1998).

Bio-surfactants help in oil solubilizing and make oil into tiny drops in the hydrophase. These small oil drops facilitate in providing hydrocarbons (HCs) as carbon source and energy to the bacteria which cannot express bio-surfactants on their own. Bacteria and fungi which can express bio-surfactants generally make anionic bio-surfactants as well as neutral bio-surfactants. Normally, on the surfaces of oil-rich water, main problems in the global oceans, like lots of Acinetobacter, Bacillus, Pseudomonas, and Alcanivorax, are found, because they can generate glycolipid bio-surfactants (Yakimov et al. 2007).

Aureobasidium pullulans YTP6–14, a marine yeast, can generate massoia lactones. Massoia lactones are a kind of fatty acids with lactonized ring in the structure. They are bio-surfactants (Luepongpattana et al. 2017) usually used for fragrances. Besides the lactonized ring structure, linear 3-hydroxy fatty acids are secreted by a kind of a *Gammaproteobacteria*, *Cobetia* sp. (Ibacache-Quiroga et al. 2013). Rubiwettin R1 is a commercial name for the dimers of the hydroxy fatty acid. The rubiwettin R1 can be secreted by *Serratia rubidaea* (Matsuyama et al. 1990). It was reported in 1944 that another similar *Serratia*, the *Serratia marinorubra*, did the same work.

The ocean microbes have a key function in pollutant degradation.

10.3 Marine Microorganisms: Products in Food

The oceans are resources of carbon and energy. These resources contain unexploited and/or unknown microorganisms (Nichols et al. 2005; Poli et al. 2010). Furthermore, the products generated by the microorganisms, new biomolecules which have used for pharmaceutical, medical products (Zanchetta et al. 2003). They have also been used in cosmetic, food, feed, and other enzyme products (Satpute et al. 2010).

Cyanobacteria and microalgae produce metabolic products. They have been analyzed for decades. These biotechnological products have high commercial value (AlNadhari et al. 2021). They have been served as food from the very beginning by the Chinese. Nowadays, ingredients from them can be used as cosmetic raw material and supplements for food and feed. Some metabolic products are

raw materials for anti-inflammatory products or even used to treat disease in plants (Oliveira et al. 2020).

Conventionally, these compounds or elements were extracted from samples using organic solvents. The disadvantages were time consumption, lower yield, and more impurities. The procedure seemed not good for the environment, because it used large amounts of organic chemicals. These organics would finally go to the environment and add the weight to pollution. To minimize the disadvantages, five novel extract methods were discovered: enzyme-assisted extraction (EAE), microwave-assisted extraction (MAE), pressurized liquid extraction (PLE), supercritical fluid extraction (SFE), and ultrasound-assisted extraction (UAE) (Getachew et al. 2020).

Researchers are encouraged to develop new techniques to minimize waste generation. The achievements include centrifugal partition extraction (CPE), deep eutectic solvents (DES), ionic liquids (IL), natural deep eutectic solvents (NDES), ohmic heating, pulsed electric field extraction (PEF), and surfactant-mediated extraction (SME) (Anaelle et al. 2013).

Microalgae from marine are a source to produce marketable compounds. They are detailed as polysaccharides, like β-glucan; polyunsaturated fatty acids (PUFA), like docosahexaenoic acid (DHA); β-carotene; astaxanthin; sterols; chlorophylls; and phycobiliproteins. They can be used in food and drug production (Torregrosa-Crespo et al. 2018).

10.3.1 Polysaccharides

Microalgae and other ocean microbes can produce special oligo- or polysaccharides. They normally have special groups on their molecules. The groups can be formed by the reaction of sulfation and carboxylation. These ocean sugars are expected to be a source to obtain prebiotic products (Sardari and Nordberg 2018), including exocellular polysaccharides (EPS) and sulfated polysaccharides (sPS). Polysaccharides (PS) are good anti-inflammatory agents such as antiviral agents, antioxidants, immunomodulatory compounds, and health foods. They are also lubricants (Raposo et al. 2013). Bio-surfactants (BS)/bio-emulsifiers (BE) and exopolysaccharides (EPS) can be produced by *Acinetobacter*, *Alteromonas* sp., *Arthrobacter*, *Bacillus*, *Corynebacteria*, *Halomonas*, *Myroides*, and *Pseudomonas* (Satpute et al. 2010). Exopolysaccharides (EPS) have broad applications. These EPS are xanthan, dextran, gellan, and curdlan and can be synthesized from the microbes of *Alcaligenes* genera, *Leuconostoc, Pseudomonas*, and *Xanthomonas*.

The most common EPSs are pentose such as xylose from wood; hexoses such as D-allose, D-galactose, D-glucose, D-mannose, L-fucose, and L-rhamnose; sugars with amino group; and uronic acids.

Recently, a *Pantoea* sp., strain BM39, was reported to make unexpected large amount of glucan, one of the exopolysaccharides (EPS) (Silvi et al. 2013).

Marine polysaccharides (MPs), not only from microorganisms in marine, but also including marine plant and marine animal, can help in metabolic syndrome (MetS) by pathway regulation (Wang et al. 2018).

Glycogen works as a natural carbon source. It is a storage form of glucose in liver. When the concentration of glucose is lower than needed, the glycogen will release glucose to the blood. The glucose is the final pool of carbon and energy and is the most important factor in Calvin-Benson-Bassham (CBB) cycle. Like other bacteria, cyanobacteria can be modified to be more efficient to produce glycogen (Luan et al. 2019).

Gracilaria lemaneiformis was reported to produce sulfated *Gracilaria lemaneiformis* polysaccharides (GLP). It also produced agaro-grouped *Gracilaria lemaneiformis* oligosaccharides (GLO). When compared with control, either GLP or GLO induced more production of short-chain fatty acids (SCFAs). Both of them regulated the intestinal microorganisms' population. Overall, GLP and GLO were useful for gastrointestinal tract, through either increasing SCFAs or regulating intestinal microorganisms. Thus, GLP as well as GLO, and their producer, *Gracilaria lemaneiformis*, might be the candidate of prebiotics in health products (Zhang et al. 2020a, b).

Enterobacter sp. is able to biosynthesize a nanostructured iron-polysaccharide complex (nano-IPC). The biological and physicochemical characteristics were evaluated. Nano-IPCs can be used to treat iron-deficiency anemia (IDA) (Kianpour et al. 2020).

10.3.2 Docosahexaenoic Acid (DHA)

Ocean organisms could also produce various novel enzymes and metabolites. They are potential foods and drugs (Rahman et al. 2010; Lee et al. 2011; Martins et al. 2014).

Without omega-3, omega-6 fatty acids, or sufficient amounts, human health will be a question. Humans are unable to synthesize essential fatty acids with sufficient amounts (Kaur et al. 2011).

One of omega-3 fatty acids is docosahexaenoic acid. It is good in the development of human brain. For this reason, some advertisements even call docosahexaenoic acid as PHD.

In the cell level, DHA synthesizes cell membranes and other tissues (Lazzarin et al. 2009; Kiecolt-Glaser et al. 2012).

Thraustochytrids can synthesize high amounts of DHA. Thraustochytriida is an order name, and Thraustochytriidae is a family name. It can be subdivided into 11 genera. The *Aurantiochytrium*/*Schizochytrium* genera are of particular interest (Raghukumar 2008).

In 2012, demand for DHA accounted for $350 million. In 2017, this demand was increased 12 times in only 5 years. The amount reached to $4.212 billion. That is to

say, it is clear that the market demands high-quality DHA, especially from microorganisms (Bannenberg et al. 2017).

DHA from oil-producing microbes is promising, because such heterotrophic microbes can increase the oil consumption in the ocean. But to culture them is a major challenge because of the elevated cost. Glucose, some special elements such as organic acids, yeast extract, or cheaper materials such as corn steep liquor are needed for culturing (Patel et al. 2020a, b). If using glucose, the cost of DHA is more than that of fish oil (Li et al. 2007). This might be possible to be solved by replacing the glucose with corn steep. Because the price of sugar is rising, efforts have to be made to find more cheap carbon sources for DHA (Park et al. 2014; Leong et al. 2019).

Aurantiochytrium sp. T66 is an ocean product. It could be fermented in food waste and meanwhile produce DHA. The waste from food was hydrolyzed first and then used to release carbon and energy. This bacterium was analyzed after 5-day fermentation. The bacterium's dry weight reached 14.7 g/L, compared with the normal *E. coli*'s only around 2 g/L. The Aurantiochytrium sp. T66 was really a high producer. In the 43.13% (w/w) of the whole-cell dry weight, 6.34 g/L consisted of fat lipids. In the total fat lipids, DHA weighted 34.05%. It reached 2.15 g/L in the whole fermentation. Or the DHA production rate was 0.15 g/g cell dry weight (g/gCDW). The other product was squalene. The product level reached 1.05 g/L or 69.31 mg/gCDW. Thus, thraustochytrids, a group of bacteria rich in oil-contaminated ocean, are considered to use the waste of food and it is an economic method for fermenting. The by-product of DHA might be more interesting for the operator (Patel et al. 2020a, b).

Docosahexaenoic acid (DHA; C22:6) is one of the polyunsaturated fatty acids (PUFAs). Another PUFA is eicosapentaenoic acid (EPA; C20:5). Both of them are good for human body. Some PUFAs have been reported to be good for dissolving brain problem (Maki et al. 2017; Canhada et al. 2018).

10.3.3 Essential Amino Acids

Essential amino acids are not available in humans for synthesis. Acyl adenylates and amines can be combined to make new products. These final products are *N*-acyl amides, a kind of essential amino acids. The *N*-acyl histidine is made by an enzyme involved in the procedure (Marchetti et al. 2019).

Another essential amino acid is diketopiperazine L-leucine. It has the function to inhibit the enzyme to hydrolyze α-glucoside (Abd et al. 2017).

The amino acid lysine is the most important biotechnological product. This essential amino acid can be made from mannitol by fermentation of *Corynebacterium glutamicum*. Ocean macroalgae are now found and may be planted everywhere in the worldwide water. They are considered a reproducible energy pool for the next generation. These algae, rich in mannitol with up to 30% and higher, produce mannitol in some oceans with higher temperature (Hoffmann et al. 2018).

In the genomes of all organisms, simple sequence repeats (SSRs) often exist in the genes. In A/T-rich species, Oligohymenophorea and Spirotrichea, homopolymers are the ones that mostly show SSRs. With AAA, the codon is for lysine, a charged amino acid. It is also an essential amino acid. This codon's frequency is highest among other coding SSR regions. In *Tetrahymena*, lysine is found to be expressed in high level in alveolin (Li et al. 2020a, b).

10.4 Enzymes

10.4.1 Oxidases

Lactic acid bacteria can produce oxidases, such as NADH oxidase, pyruvate oxidase, lactate oxidase (Udaka et al. 1959), and α-glycerol phosphate oxidase (Parsonage et al. 1998).

Melanin is made from phenols. One of the oxidases that involve phenol conversion was normally named polyphenol oxidase (PPO). There are so many oxidases produced by marine microorganisms; we have just picked out three of them to discuss.

10.4.1.1 Sulfite Oxidase (EC 1.8.3.1)

Sulfite oxidase (EC 1.8.3.1), one of the oxidases, is located in the mitochondria, a cellular organ of most cells including eukaryotes. ATP is generated by oxidative phosphorylation. In this procedure, sulfite is oxidized to sulfate by sulfite oxidase. Cytochrome c helps to conduct electrons by the electron transport chain (Cohen et al. 1972; Tan et al. 2005; D'Errico et al. 2006). Sulfate is the final product to break down from sulfur chemicals. It is released from the body by excreting.

Reduced ammonia, CH4, H2, iron, manganese, and sulfur can be oxidated by alpha-, gamma-, and epsilon-proteobacteria (Huber et al. 2003; Huber et al. 2006; Huber et al. 2007; Opatkiewicz et al. 2009; Dick and Tebo 2010; Sylvan et al. 2012). Gamma-proteobacterial sulfur oxidizer is shortened by GSOs. SUP05/Arctic96BD-19 is one of the GSOs (Sunamura et al. 2004; Anantharaman et al. 2013; Anderson et al. 2013).

10.4.1.2 Cytochrome c Oxidase (EC 1.9.3.1)

Cytochrome c oxidase, EC 1.9.3.1, is a kind of oxidase. It is found in bacteria, archaea as large transmembrane protein complex, and eukaryotes (Castresana et al. 1994) in their mitochondria. Such enzyme diversity depends on species. In metazoan, cytochrome c oxidase I (COI) is used as a biological marker to determine the species (Suter et al. 2020).

This enzyme is expressed in the cell surface. In electron transport chain, cell respiration has several enzymes. Cytochrome c oxidase acts as the last enzyme. Four cytochrome c molecules provide four electrons to this oxidase molecule. The four electrons are then transferred to one dioxygen molecule. Thus, it turns the molecular oxygen over to two molecules of water. By translocating another four protons across the cell membrane, the difference of proton increases electrochemical potential. The energy is used by ATP synthase to synthesize ATP.

Cytochrome *c* oxidases are especially expressed well in alpha-, beta-, and gamma-proteobacteria. These bacteria are grown well at oxygen minimum zones (Wright et al. 2012) followed by the Chlamydiae-Verrucomicrobia group.

Ribosomal small subunit (SSU) rRNA and gene of cytochrome c oxidase were used in phylogenetic analyses for amoeba and *Vannella ebro*. It was found that the sequences were similar to each other. But they belonged to two totally different creatures (Kudryavtsev et al. 2019). Comparing of the cytochrome c gene in the mitochondria provided a reliable method to recognize different species. The phylogenetic map could also be reconstructed (Singer et al. 2018).

Plastocyanin is associated with copper. When copper is deficient, *Ostreococcus tauri* adjusts its copper usage by producing less plastocyanin. Cytochrome *c* oxidase will work keeping the regular physiologic function of *Ostreococcus tauri* (Scheiber et al. 2019).

Shewanella sp. Arc9-LZ is a bacterium. This kind of bacterium is able to synthesize nanoparticles from silver in dark environment. After analysis with the online software of CAZy, COG, KEGG, NR, TCDB, and Swiss-Prot, 64 genes belonging to 9 kinds have been found to belong to 9 clusters. All of them are involved in silver nanoparticles (AgNPs). Besides the nanoparticles, such genes have the capability to synthesize many vitamins and enzymes. These vitamins and enzymes include b-type cytochrome, c-type cytochrome, coenzyme Q, cytochrome c oxidase, cytochrome c reductase, NADPH dehydrogenase, nitrate reductase, nitroreductase, and riboflavin (Li et al. 2020a, b).

10.4.1.3 Polyphenol Oxidases

Mangroves are normally grown on the seaside. The rotten wooden materials are accumulated beneath the water. For degrading the wooden materials, several enzymes are expressed by the in situ microbes. Some of the enzymes are β-*N*-acetylhexosaminidase (EC 3.2.1.52), hexosyltransferase (EC 2.4.1.-), and polyphenol oxidase (PPO, EC 1.10.3.-) (Zhao et al. 2019).

Alternaria tenuissima is a kind of fungus. It can grow on potatoes in storage and leads tuber to be rot. It is a big problem for economic plants. To solve this problem, it was reported that a dose of 1.25 g/L chitosan could induce the translation of several protective enzymes. These enzymes were catalase, chitinase, β-1,3-glucanase, peroxidase, and polyphenol oxidase. Besides the enzymes, the production of two additional components, flavonoids and lignin, is also elevated (Liu et al. 2019).

High-pressure processing (HPP) can inhibit PPO, and has the ability to enable a more wholesome, fresh product with extended shelf-life of fruits and vegetables (Tsikrika et al. 2019).

Polyphenol oxidase activity of fruits and vegetables is significantly inhibited by nitrogen treatment.

Enzymes which have lysozyme-like activity (LYS), like exochitinase (EXOC), peroxidase (POX), and polyphenol oxidase (PPO), all of them do have protective activities against plant pathogens. PPOs are involved in the innate immune system of plants (Fuerst et al. 2014) and take part in enhancing the oxidation procedures of monophenols and/or *o*-diphenols. The oxidation products are *o*-diquinones.

In recent years, many wind electric power generators have been built very close to the seas or even in the seas. The generated extremely low-frequency electromagnetic fields (ELF-EMF) are released to the aquatic lives. Exposure of ELF-EMF is a factor to enhance the expression levels of immune-associated enzymes checked in coelomic fluid of *Onchidium struma*. The enzymes are acidic phosphatase, alkaline phosphatase, catalase, polyphenol oxidase, and superoxide dismutase. They are statistically significant ($P < 0.05$) (Zhang et al. 2020a, b).

PPO immobilized on gold nanoparticles showed more stability and reusability than the non-gold control (Wang et al. 2020).

Tyrosinases are also called as catechol oxidases (EC 1.10.3.1). These enzymes are richly found in most plants, including fruits. Catechol oxidases are found in most plants, such as in fruits. When the plants become injured or mature, such PPOs in them are responsible for the browning reaction. Such PPOs are catechol oxidases (Sanchez-Amat et al. 2010).

10.4.2 L-Asparaginase (EC 3.5.1.1)

There are many traditional enzymes used in food processing. They are important to health also (Rasmussen and Morrissey 2007).

L-Asparagine can convert to L-aspartic acid by the enzyme L-asparaginase (EC 3.5.1.1). This enzyme is useful in medicine and the food industry. L-Asparaginase offers the possibility to develop a method to treat cancer patients. Acrylamide is formed from baked and fried foods. It is a confirmed carcinogenic substance. L-Asparaginase can avoid the forming of acrylamide and further avoid cancer development (Ameen et al. 2020; Prihanto and Wakayama 2016).

It is interesting that this L-asparaginase holds cross activities with some L-glutaminase (El-Bessoumy et al. 2004; Labrou and Muharram 2016). This enzyme is mainly expressed by microorganisms. About 33% of antileukemia/anti-lymphoma agents are made from L-asparaginase.

Among the leukemia patients, children are most likely to have acute lymphoblastic leukemia (ALL). L-Asparaginases are the first-line drugs to treat acute lymphoblastic leukemia (ALL). This is extremely better established than other therapeutic enzymes. It is also a good antitumor agent for acute myelocytic leukemia, acute

myelomonocytic leukemia, chronic lymphocytic leukemia, lymphosarcoma, pancreatic carcinoma, and reticulum sarcoma. It is even good for bovine lymphoma sarcoma (Broome 1963; Abd and El 2016).

Food and drug are associated with each other, and they are even administered by the Food and Drug Administration (FDA). L-Asparaginase can inhibit acrylamide production. Although this is a concern for food hygiene, the effect of inhibiting carcinogen generation is exactly the work of an anticancer drug (Cachumba et al. 2016; Xu et al. 2016).

10.4.3 Alpha-Amylase

Alpha-amylase is widely applied in clinical, medical, and analytical chemistries and food, detergent, textile, brewing, and distilling industries (Suriya et al. 2016; Wang et al. 2016).

The deep-sea environments have extreme temperatures, salinities, pHs, and pressures. The deep-sea microorganisms must contain extremozymes which are stable in the extreme condition. These microorganisms can be used to screen extremozymes for special industrial applications. Because they retain their activities and stabilities in extraordinary environments, such extremozymes are suitable for industrial applications. In the pharmaceutical, bio-mining, food, beverage, feed, agricultural, detergent, leather, textile, and pulp industries, normal enzymes are used in such ordinary biotechnological processes. Definitely, the extremozymes will do a better job if they replace the ordinary enzymes in the industrial biotechnological processes. These will bring considerable economic benefits to the industries (Raddadi et al. 2015).

Halophilic bacteria can express enzymes when working in high-salt solutions. Such bacteria are useful in extraordinary environments or special aims. *Ulkenia* sp. AH-2 is an ocean straminipilan thraustochytrid. It can grow in middle levels of a salt solution. It is originally identified from mangrove environments. The halophilic alpha-amylases are its characterized enzymes (Shirodkar et al. 2020).

Some α-amylases are also found in *Aeromonas salmonicida, Bacillus aquimaris* MKSC 6.2 (Puspasari et al. 2013), *B. subtilis* S8–18 (Kalpana and Pandian 2014), *Geobacillus* sp. 4j (Jiang et al. 2015), and *Halothermothrix orenii* (Tan et al. 2008). It has also been recognized from a marine bacterial metagenome (Lei et al. 2012).

The natural extract from *Spirulina* sp. has higher inhibition to amylase. The dose of the inhibition is 0.08%/(min·μg). Compared with the control, it is statistically significant ($p < 0.05$). Microalgal phenolic compounds have inhibitory activity against amylase. These activities can be used to antifungal agent for grain before harvest. The advantages might differ case by case (Scaglioni et al. 2018).

Streptomyces sp. S2A is an ocean actinobacteria and found in India. The active fraction can inhibit α-glucosidase, with the IC_{50} of 21.17 μg/mL. Such active fraction can also inhibit α-amylase, with the IC_{50} of 20.46 μg/mL (Siddharth and Vittal 2018).

Table 10.1 Enzymes that have activities at low temperature from marine microorganisms

Bacterium	*Martelella mediterranea*	*Exiguobacterium oxidotolerans*
Isolation	Deep-sea water	Deep-sea sediment
Enzymes	β-Glucosidase	β-Glycosidase
Activities below 10 °C	50% (4 °C)	61% (10 °C)
References	Mao et al. (2010)	Jin et al. (2019)

On earth, Antarctica is the coldest, windiest, and driest continent. So the microorganisms that live in Antarctica are already adapted to the extraordinary environments. For example, the enzymes which are isolated from such microbes are still active in the extraordinary environments. These enzymes are hydrolases and oxidoreductases. The hydrolases are α-amylase, cellulase, chitinase, glucosidase, invertase, lipase, pectinase, phytase, protease, subtilase, tannase, and xylanase. The oxidoreductases are laccase, as well as superoxide dismutase (Duarte et al. 2018).

In practice, enzymes that have activities at low temperature can be used in energy-saving factories. Table 10.1 contains two examples.

Hydrolases, lyases, oxidoreductases, transferases, isomerases, and ligases can be used in pharmaceutical and food applications (Lima and Porto 2016).

10.5 Marine Microorganisms: Products in Anti-Inflammatory Drugs

10.5.1 Drugs for Antibacteria

The global amount of antibiotics used is huge and has reached the level to cause drug-resistant problems. According to the World Health Organization (WHO)'s report in 2016, Brazil was the country that used most quantity of antibiotics. Turkey, Iran, Russia, and France were the countries from second to fifth, following the trend to use antibiotics in descending order. Over 2000 tons of antibiotics are used in Brazil per year. About 1000 tons are used in Turkey and Iran (Alves et al. 2020).

Around 67% of antibiotics from clinical units are made from microorganisms or modified products by semi-synthesizing. The ratio of microorganism-generated antiproliferative drugs has reached 33% (Newman and Cragg 2016). In these microorganisms, actinomycetes serve as the main strains for searching substances which may be potential drugs (Jensen et al. 2005).

The use of next-generation genome sequencing together with the help of advanced bioinformatic tools, such as antiSMASH (Blin et al. 2019) and MIBiG (Medema et al. 2015), makes it easier to discover novel gene products (Naughton et al. 2017; Malmierca et al. 2018). For example, this genome mining has identified new antibiotic scaffolds from 21 marine actinomycetes (Schorn et al. 2016).

Table 10.2 The marine bacteria which have activities against fungi

Kingdom	Bacteria	Bacteria
Order	Actinomycetales	Bacillales
Genus	*Micromonospora*	*Bacillus*
Strain	FCO_2	B9987

10.5.2 Drugs for Antifungi

The marine microbes were mixed in the same culture. The activities against fungi were analyzed. Orthogonal design was used for selection of the best condition using a single medium. The aim of this approach was to optimize the composition in the medium for mixed fermentation. Compared with monoculture, the mixed culture was showing significantly obvious antifungal activity. The minimal inhibitory concentration (MIC) reduced by mixture of monoculture fermentation. The inhibition increased from 200% to 300% with mixed culture (Tian et al. 2005). Table 10.2 shows the bacteria that were used for analyzing the antifungal activity in this study.

10.5.3 Drugs for Antivirus

Glycosaminoglycans (GAGs) are endogenous polysaccharides. GAGs are polymerized saccharides with a linear chain. They can be synthesized inside cells, on the surface of cells, and outside of cells. By interacting between other factors, they take part in many routes of signal transduction. Polysaccharides have the capability to act against virus and to enhance immune response, with remarkable track records. They are expected to be applied against life-threatening viral infections (Chen et al. 2020).

Polymerized saccharides with sulfate group do have other functions besides against virus. These additional functions are anticoagulant, anti-inflammatory, and antitumor (Cumashi et al. 2007; Fitton 2011; Kwak 2014).

Marine fungi account for 27% of all the marine-related natural products (Gao et al. 2013; Ma et al. 2016; Jia et al. 2019). Azaphilones can be synthesized by fungi. They express the functions of anti-inflammatory, antimicrobial, antioxidant, antiviral, cytotoxic, and nematocidal capability (Gao et al. 2013).

P. sclerotiorum OUCMDZ-3839 is a fungus which is related to sponge. A total of 16 chemicals were analyzed from the cultural medium of this fungus. Sclerotiorins A–D (**1**–**4**) have been recognized as azaphilones and reported for the first time. Analogues (**5**–**16**) have been reported before. Compared with the normally used antivirus drug, ribavirin, compounds **5**, **7**, **10**, **12**–**14**, and **16**, about 44% (7/16) showed stronger inhibition to the virus of H1N1, when the virus infected MDCK cells (Jia et al. 2019).

Violapyrones S exhibited the best activities among other violapyrones (Hou et al. 2018). IC50 concentration of violapyrones S for the inhibition of H1N1 was only 30.6 μM, compared with other IC50 of 58.8–68.4 μM.

Table 10.3 The aromatic steroids which have antitumor activity

Name	Number	Total
Ring A monoaromatic steroids	24	24
Monoaromatic steroids	25	49
Di- and triaromatic steroids	31	80

10.6 Marine Microorganisms: Products in Antitumor

Streptomyces sp. strain SP 85 is a strain of actinobacterium. It was found to grow in ocean sponge *Dysidea avara*. After analyzing with ^{13}C NMR, ESI-MS, and UV spectroscopic instruments, SP 85's product has been found to be very similar to "olivomycin A," which is considered as an antitumor drug (Gozari et al. 2019).

Eighty aromatic steroids have an antitumor activity (Dembitsky et al. 2018). Table 10.3 shows the details of the 80 steroids.

10.7 Conclusion

Marine microorganisms work as a reservoir of marine microbial natural products. Antifouling substance is one of such products. It is helpful to create a friendly environment. Many such compounds are chemicals that can act against bacteria, fungi, HIV, tumor, algae, helminth, protozoan, and allergic substances.

Ocean microbes are useful for pollutant degradation, food production, enzyme production, and drug production.

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Chapter 11
Biodegradation of Pesticides Used in Agriculture by Soil Microorganisms

Namadev K. Pujar, H. G. Premakshi, Madhu P. Ganeshkar, and Chandrappa M. Kamanavalli

Abstract The phenyl carbamates such as chlorpropham (CIPC) and phenmedipham (PMP) are selective systemic herbicides. CIPC used as a preemergence and PMP used as postemergence herbicides have been widely used for weed control in crops. CIPC is used as a sprout suppressant on crops during the lengthy period storehouse of potatoes. The widespread use of CIPC in agriculture continuously increasing the contamination of natural resources. The metabolites of CIPC are 3-chloroaniline and catechol, which are highly toxic. Chlorpropham has toxic properties both for the environment and consumer's health and is still widely used. Phenmedipham and its metabolic products disrupt electron transport at the photosystem II (PS II) receptor site. Therefore, its usage can impact on terrestrial and semiaquatic plants and invertebrates, including mollusks, fishes, and birds.

Keywords Biodegradation · Chlorpropham · Phenmedipham · Sprout suppressant · Preemergence

11.1 Introduction

Current agricultural practice is mainly contingent on the use of various pesticides. Pesticides are substances purely to possess an effect on nature. Unfortunately, natural effect is a relatively usual side effect of societal use. Impact on natural living beings does occur when pesticides are liberated. Nature itself takes care of lives and appearing toxics, and additionally, their conversion results are of great attentiveness to the present eco-toxicologists. Various types of manufacturing chemical substances such as plasticizers, pesticides, stains, detergents, medicine, and petroleum by-products and manufacturing toxics like oils, cooling agents, nonconductors,

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hydraulic fluids, and solvents are constantly liberated into the natural bodies causing the contamination of nature (Hoskeri et al. 2014; Megadi et al. 2010; Mulla et al. 2017; Mulla et al. 2019a, b, 2020a, b; Mulla et al. 2021; Hutzinger and Veerkamp 1981; Tallur et al. 2015). One of the recognized nature complications was "chemical stress," which is one after the other showed to a not worthy site for human and environmental safe life growth (Rockström et al. 2009). Pesticides and preservatives are two categories of chemicals whereby the design's use is various compared to other chemical substances. These two chemical categories are knowingly liberated into the atmosphere with a plan to stop (Brock et al. 2004) or knockdown any contagion examined to be damaged (WHO 2006). Discharged from cultivated lands and sprinkled with pesticide on the environment. Even-now, results on environment give safe life additionally, prospective responsibility due to damage and evaporation. Nature and environmentally safe life may be run-offs by such damaged live and waste design (Brock et al. 2004). Insecticides are chemicals utilized to stop, knock-down, revolt against any contagion leveling from pests, rodents and weeds to microbes. These pesticides are of essential significance in the combat against infection, for the producing and repository of food nourishment living extensively applied for pest prevention in crops, planting, houses, and farmland (Crespo-Corral et al. 2008; Janssen 1997). All of the large scales utilize, approximately one-third percentage of generating vanishes universal (Janssen 1997). Several other compounds belonging to different aromatic classes such as haloaromatics, nitroaromatics, sulfoaromatics, polychlorinated biphenyls, polycyclic aromatic hydrocarbons (PAH), phthalates, and its derivatives are also considered to be environmental toxics. A large of these pesticides and their biotransformation metabolites are studied to be poisonous, carcinogenic to humans and animals. Complete mineralization of these chemicals in the natural ecosystem is primarily due to microorganisms.

Microbes lead a vital character in the utilization of toxic substances by the process of biodegradation (Mulla et al. 2012). The indigenous microbial populations present in soil possess a versatile mechanism to degrade various pesticides and organic chemicals of natural and synthetic origin and help environmental cleanup. Bacteria and fungi are the chief agents for the biodegradation of organic toxins (Talwar et al. 2014; Mulla et al. 2016; Mulla et al. 2020a, b). Hence, phenyl carbamate pesticides' microbial degradation has been studied to develop microbial technology for environmental protection. The common point of view of depolluting is the increased natural breakdown by indigenous microbes by practicing healthy food and oxygenation or microbes.

11.2 Environmental Pollution by Pesticides

Agriculture chemicals have facilitated more than double the food production during the nineteenth century and the present need to enhance the crop rising to supply a rapidly increasing population. To maintain the force on the agriculture, modern irrigation practices, synthetic fertilizers, pesticides, herbicides, and genetically modified crops are used in the green revolution. The pesticides are mainly classified into four categories. The most important group of toxic organochlorines are dichlorodiphenyltrichloroethane (DDT), heptachlor, hexachlorobenzene, etc. Organochlorines (OC) are used in various types of crop protection from the pest in the 1940s. During 1950–1970s, these insecticides interfered with food and non-food plants such as corn, wheat, and tobacco. The chlorinated benzenes and cyclohexanes are used as fungicides and antimicrobials. The toxicity of organochlorine pesticides depends on their mechanisms and chemical structures. These fat-soluble compounds are stored in the higher tropic levels, which promote bio-amplifications in the agriculture products (Poon et al. 2006). These compounds' toxicity affects the human and animal systems because of their ability to react to the endocrine gland. These compounds are transformed to offspring during lactation period via breastfeeding (Munozde-Toro et al. 2006). Higher DDT levels and their products may be available in the natural bodies (Boul 1995; Miersma et al. 2003; Yanez et al. 2002; Shen et al. 2005).

Among pesticides, organophosphates are the type-II main groups. The basic approach of these pesticides is to control the acetylcholinesterase activity on the central nervous system. Phenyl carbamates are the third essential category of pesticides since their produce into the agricultural chemical business in the mid-nineteenth century (Tomlin 1997) and are consumed yearly in a vast quantity globally (Paige et al. 2009). These are made up of various chemicals used as insecticides and pesticides, including fungicides, nematocides, acaricides, molluscicides, sprout inhibitors, or herbicides. Comparatively, less accumulation and low toxicity, treated as hazardous to the living system, is incorporated in the United States Environmental Protection Agency (1992) list. Based on their persistence, the importance of carbaryl, propoxur, and carbofuran, these may be toxic chemical agents were recently surveyed on initial studies in vitro using trypsinized squamous cell carcinoma. They inhibit cellular metabolism, thereby bringing about cell arrest and dying (Amanullah and Hari 2011). In addition to natural group pyrethrins, and few organic pyrethroids are present in different market brands. These have quick trample results and are commonly used against flying insects.

11.3 Phenyl Carbamate Pesticides

Phenyl carbamates are classified into three categories: i) Ester derivatives ii) Common R1NHC (O) OR2 (R1 and R2 are aromatic and/or aliphatic) groups, and iii) Aromatic imidazole category (IPCS 1986). The presence of toxic chemicals and their by-products is highly persistent compared to the parent compounds in the biosphere (Crespo-Corral et al. 2008). These pesticide residues of carbamates are potentially toxic to beneficial microbes compared to invaders and infections (Gilden et al. 2010). The properties of carbamates are formed carbamoylation in the catalytic site of acetylcholinesterase priming to the non-function it has a vital character in the human nervous system including animals (Ecobichon 2001). Carbamates are also named as anticholinesterases. The acetylcholinesterase is progressively blocked and cannot hydrolyze from acetylcholine to choline and acetic acid (Jokanovic 2009). Therefore, acetylcholine deposits on cholinergic receptor sites and causes parallel to the entire central and peripheral nervous system's enormous incentive.

The severe poisoning of the various carbamates is from higher to lower or safe (IPCS 1986). Regarding the major carbamates in use, their respective poisonous vigor determined human rates, (Erdman 2003) different from higher poisonous ($LD_{50} < 0.050$ g/kg), moderate toxicity ($LD_{50} = 0.050–0.2$ g/kg); and less toxicity ($LD_{50} > 0.2$ g/kg). Many carbamates have been used as pesticides or pharmaceutical compounds. Some major carbamates are listed in Table 11.1. Kidd and James (1991) and Dupont de Nemours (1991) reported that carbamate pesticides are most poisonous to birds, fishes, insects, and earthworm's population.

11.4 Biodegradation of Pesticides

The metabolic effect of pesticides in nature is a vital role in the vanish of such chemicals' tenacity, which estimates its hazardous results practically. Pesticides are a breakdown in nature primarily by microbes' action, through the activity of microbial enzymes. The biodegradation rate is varied in the various systems widely. A few insecticides like carbofuran and diazonium are readily biodegradable and degraded in the soil. This may not be valid for pest measures (Felsot 1989).

The biodegradation of a pesticide has severe results on nature due to the effects and ability of chemicals. When insecticides break down at an appreciable rate, there will be no toxicity to control the pest and do not raise any pollution. In case pesticides break down in a too short period, they may not effectively control the pest. If fast biodegradation of pesticides has been shown in soil property, then we have to face the problem of incorporating some pesticides frequently. Many pesticides are unable to prevent the pest and therefore applied continuously for several years. Because of this, there will be loss of income from failed crops. When frequently using these toxic substances, the pest may develop resistance to the

Table 11.1 Phenyl carbamates

Common name (Trade name)	Structure	LD_{50} (mg/kg of body weight)
Chlorpropham (CIPC)		5000
Phenmedipham (Betanal)		8000
Desmedipham (Betanil AM)		9600
Propham (IPC)		5000
Propoxur (Baygon)		5000
SWEP		522
Carbofuran		8
Aldicarb (Temik)		1
Carbaryl (Sevin)		307
Barban (Carbyne)		600
Aminocarb (Matacil)		30
Phenisopham		400

breakdown of those chemicals. Those microbes are capable of sustaining the toxic chemicals in the soil.

To know the effect of toxic chemicals in the nature, it is crucial to understand the degradation roots used to break down toxic substances by the microbes (Somasundaram and Coats 1990). This knowledge is required to indicate the effect of the toxic chemicals and the formation of metabolite. The microbial degradation of pesticide byproducts is those higher poisonous than the origin. A few metabolites may deposit in nature, for example, carbofuran phenol (Cain and head 1991).

Many of the aerobic and anaerobic microorganisms can break down toxic chemicals that have been isolated and identified from the soil, water, and other natural habitats. A vast array of microbes, including *Alcaligenes, Flavobacterium, Pseudomonas, Rhodococcus, Bacillus*, and *Ochrobactrum*, are utilized for pesticides. The microbes can utilize the pesticides as the sole carbon source or alternatively co-metabolizes. Biodegradation not only use bacteria with specific enzymes but wide variety of external conditions. Many have reported on the biodegradation of pesticides (Roberts and Standen 1977; McRae 1989; Somasundaram and Coats 1991; Cork and Krueger 1991; Aislabie and Lloyd-Jones 1995; Parekh et al. 1995; Gao et al. 1996; Feng et al. 1997; Ogram et al. 2000; Chaudhary et al. 2002; Bano and Musarrat 2004; Zhang et al. 2006; Qiu-Xiang et al. 2007; Fritsche 2007).

11.5 Microbial Degradation of Phenyl Carbamate Pesticides

Phenyl carbamates like carbaryl, carbofuran, and propoxur have been extensively used to prevent different kinds of insect pests. These compounds are environmentally hazardous because they are effectively controlled by acetylcholinesterases (Fahmy et al. 1970) and N-nitrosocarbamates that are formed as potent mutagens (Elespuru et al. 1974). However, carbamate pesticides are not highly toxic in nature for a longer duration. Microbial degradation plays a crucial job in the pesticide decontamination. However, degradation may be so rapid as to reduce the efficiency against the target pests. Therefore, lot of attention has been focused on the microbial degradation of carbamate pesticides.

Microbes are the ability to break down carbamates are the concept is the key role in the degrading and removal of toxicity in nature. Such microbes have received great awareness because of their crucial use in removing pesticide toxicity and their efficacy on pesticides' metabolic effect in nature. A single step involving hydrolysis of *N*-methylcarbamate linkage is a key point to nullify the pesticides' toxicity (Cain and Head 1991).

11.5.1 Biodegradation of Carbaryl

Carbaryl is the toxic chemical of the pesticide, which has been utilized widely to save crops. Bollag and Liu (1971, 1972) studied that *Fusarium solani* and two unknown soil microbes hydrolyzed carbaryl to 1-naphthol and break down the latter. Diethyl ether extraction of three days incubated a culture of *Achromobacter* grown on carbaryl it gives 1-naphthol, quinol, catechol, pyruvate, and all the metabolites were confirmed by chromatographic technique (Sud et al. 1972). The accumulation of 1-naphthol and 1, 4-naphthoquinone has been shown to occur during catabolism of ^{14}C-labeled carbaryl by enrichment cultures and *Bacillus* species (Rajagopal et al. 1984). Larkin and Day (1986) reported that biodegradation of carbaryl by three bacterial strains, *Pseudomonas* sp. (NCIB 12042 and 12,043) and *Rhodococcus* sp. (NCIB 12038), was shown to metabolize through 1-naphthol and salicylic acid via gentisate pathway. But, the *Pseudomonas* sp. (NCIB 12042) was shown to degrade carbaryl through 1-naphthol and salicylic acid via catechol pathway rather than gentisate. The various degradation routes for the bacterial breakdown of carbaryl are showed in Fig. 11.1.

11.5.2 Biodegradation of Carbofuran

Carbofuran is a pesticide that is extensively utilized in crop protection. There are several studies on bacteria's capability to breakdown carbofuran (Williams et al. 1976; Venkateswarlu and Sethunathan 1984; Felsot et al. 1981; Rajagopal et al. 1984a; Karns et al. 1986; Chaudhry and Ali 1988). Bacteria of *Achromobacter*, *Pseudomonas*, and *Flavobacterium* have been the utilization of carbofuran as a substrate. A number of microbial strains reported utilizing methylamine, which is produced during the hydrolysis of the methylcarbamate ester linkage of carbofuran by hydrolase, used as carbon or nitrogen unique source. They are made into three categories, based on their mode of cleavage. Category I used the pesticide as a sole source of nitrogen and categories II and III utilized carbofuran as the sole source of carbon. The strain of the categories I and II degrade carbofuran to carbofuran phenol. But category III degraded carbofuran rapidly by the oxidative pathway, but this pathway's detail has not been reported. On the other hand, during carbofuran breakdown by enrichment cultures of *Bacillus* sp. three metabolites were reported. 3-Hydroxycarbofuran and 3-ketocarbofuran were again degraded quickly, but carbofuran phenol was not degraded further (Rajagopal et al. 1984b). The proposed route for the bacterial degradation of carbofuran is shown in Fig. 11.2.

In contrast with bacteria, fungal cultures, viz. *Aspergillus niger*, *Trichoderma viride,* and *Helminthosporium* sp., were capable of forming an intermediary compound, hydroxycarbofuran and rest of the degraded products could not be identified as they formed part of unknown spots (Kandasamy et al. 1977).

Carbaryl

Hydrolase — NH2CH3

1-Naphthol

Salicylic acid

Catechol — O2 — Catechol 2, 3 dioxygenase — Ring Cleaving

Gentisic acid — O2 — Catechol 2, 3 dioxygenase — Ring Cleaving

Fig. 11.1 Bacterial degradation of carbaryl (Chapalamadugu and Chaudhry 1991)

Fig. 11.2 Microbial breakdown of carbofuran by *Sphingomonas* sp. strain SB5 (Kim et al. 2004)

11.5.3 Biodegradation of Propoxur

Propoxur (2-isopropoxyphenyl-*N*-methylcarbamate) is an insecticide and acaricide toxic against a wide range of insects particularly those that source harm/ destruction via draw and crunch. It is also used for flea prevention on household insects. It is a powerful controller of cholinesterase and is a severe poison to humans and animals, having an LD_{50} of 100 mg/kg in rats (Kuhr and Dorough 1976). Soil microbes that are frequently applied to toxic chemicals may adopt new abilities to utilize such toxics. *Pseudomonas* species degraded carbamate pesticide propoxur by a hydrolytic pathway to yield 2-isopropoxy phenol and methylamine. However, 2-isopropoxy phenol was not further metabolized and acquired as end product of propoxur metabolism. The methylamine released by hydrolysis of *N*-methylcarbamate linkage

Propoxur

Hydrolase

2-Isopropoxyphenol + CH_3NH_2 + CO_2

Methylamine

Crbon and Nitrogen source

Catechol

Catechol 2,3 dioxygenase

Ring cleavage

Fig. 11.3 Microbial degradation of propoxur by *Pseudomonas* species (Kamanavalli and Ninnekar 2000)

Fig. 11.4 The general structure of organophosphate pesticide

O (S)

OR_1 P OR_2

X (OX or SX)

of propoxur was used as a carbon and nitrogen source for microbe development (Fig. 11.3).

11.6 Organophosphate Pesticides

Organophosphate pesticides are the major category utilized globally to manage insects. OPs are the derivatives of ester or sulfur, phosphoric, phosphonic, and phosphoramidic acids (Fig. 11.4).

Structurally, both P=O/oxons (OX) and P=S/thions (SX) exhibit different single/double-bonded, R_1 and R_2 are alkoxys, aryloxy, and thioalkoxy groups, X linked to the phosphorus atom is the complex group specific to the pesticide and it is referred to as the leaving group.

Currently, more than 140 organophosphate pesticides are used for crop protection. Many of these compounds are commonly obtainable like paraoxon, parathion, profenofos, chlorpyrifos, and quinalphos. OPs are highly poisonous and act on acetylcholine esterase, a key enzyme in the central nervous system. On contact with OPs, the enzyme has altered the function to bring about acetylcholine deposition, which involved transferring the nerve signals at the nerve endings (Donarski et al. 1989; Sultatos 1994; Grimsley et al. 1998; Bakry et al. 2006). In animal systems, OP toxicity indicates some common symptoms noticed. In extreme cases, respiratory failure may lead to death. These are used to protect the various types of crops from pests, including gardening plants. These are commercially selling from famous global agro-industries.

The wide utilization of insecticides has shown to pollute nature. These compounds are highly toxic to insects and mammalians (Singh and Walker 2006). Organophosphate pesticides are environmental pollutant is a key matter that captivates universal general concern because of the big threat of humans showing to these toxics. The threat of these OPs is a continuous warning and they are accountable for many poisoning cases in the mammalian toxicity (Laham et al. 1986; Fautz and Miltenburger 1994). Gupta (2004) reported that more than 70% of death might occur worldwide due to pesticide toxicity.

11.7 Biodegradation of Organophosphate Pesticides

Biodegradation breaks pollutants in the soil and water by indigenous microorganisms (Liu et al. 2007). Pesticide pollutants are capable of breakdown by living and nonliving organisms. Therefore, microbial detoxification is the basic process of degradation and removal of toxicity in the farmlands and helpful in the development of the plan of action for removal of pesticides polluted soil and aqueous systems (Qiu et al. 2006). Thus, the microorganisms may affect the determination of maximum toxic chemicals in the soil used to kill insects (Surekha et al. 2008). Several reports are available on the breakdown of various toxic chemicals (Horváth et al. 1972; Inayat-Hussain et al. 2007; Lakshmi et al. 2009). The degradation of insecticides by the inclusion of bacteria (bioaugmentation) had been studied previously for various chemicals, such as OPs (Singh et al. 2004). The separation of native bacteria's ability to degrade organophosphate pesticides has undergone significant notice since these are environmentally safe techniques of in situ remediation (Richins et al. 1997; Mulchandani et al. 1999). A few organophosphate pesticides polluted sites and indigenous microorganisms have been developed over a long period to adjust to these pollutants (Pahm and Alexander 1993). These places are more suitable for biodiversity spots to exist and separate strains' ability to breaking organophosphate

pesticides (Ramos and Rojo 1990; Oshiro et al. 1996; Ortiz-Hernández et al. 2001; Horne et al. 2002). The essential key enzyme phosphotriesterase established in *Pseudomonas diminuta* MG and *Flavobacterium* Sp. ATCC 27551 is accomplished to hydrolyze many OPs (Mulbry et al. 1986; Serdar et al. 1989; Mulbry 2000). Organophosphorus hydrolase (OPH) (Mansee et al. 2005) shows very activator hydrolyzing a wide mixture of organophosphates via breaking of P-O and P-S bonds (Ang et al. 2005).

Hydrolysis is the crucial stage in organophosphate pesticides removal of toxicity, which causes chemicals more unsafe to again breakdown (Kumar et al. 1996). The hydrolysis process and kinetic studies are documented (Brown 1980; Lewis et al. 1988; Mulbry and Karns 1989; Dumas et al. 1989; Dumas and Rauschel 1990; Ortiz-Hernández et al. 2003). Phosphotriesterase has the energy to utilize in the removal of OP-polluted nature.

11.8 Biodegradation of Herbicides

Herbicides are the group of compounds called pesticides, which kill or inactivate any pest. Herbicides are the chemicals that are used to intentionally destroy weeds in different crops. Phenyl carbamates herbicides are a limited number of compounds. The number of reports indicates that herbicides are specifically dispersed via the apoplastic in plants (IPCS 1986) and are known for endocrine disruptor (Schulte-Oehlmann et al. 2011).

Herbicides such as pendimethalin, trifluralin, chlorpropham, phenmedipham, desmedipham, and phenisopham and dinitroanilines are extensively used to prevent different types of grasses and broadleaf weeds. They are breakdown to non-photo toxic ranges within a developing duration in little hot, moist soil and undergo series of processes like *N*-dealkylation, nitro reduction, and cyclization, once included in the farmland (Marquis et al. 1979). Biotransformation is one of the vital processes through which herbicides are degraded from the ecosystem. These transformation mechanisms lead to the removal of herbicidal molecules (Kole et al. 1994).

11.8.1 Chlorpropham

Chlorpropham (CIPC) pesticide was launched in mid-nineteenth century, primarily *N*-phenyl carbamate, which is approximately 11% of the total pesticide trading globally (Farawela 2009). It is a scrupulous essential pesticide, used as a pre-emergence control of weeds in the cultivation of crops, and sprout suppressant on crops in the warehouse of potatoes and lives for tobacco inhibition (Gamez-Castillo et al. 2013). Due to this, quick enhance in a body mass loss of stockpile tubers is most harmful to the dietary level and standard (Mani et al. 2014). The result of the extensive use of CIPC can source developing stages of pollution in nature with the

degradation of products, particularly with regard to 3-chloroaniline (Angioi et al. 2005) and 4-chlorocatechol. Among the herbicides, it is the most poisonous to worms and comparatively high detrimental to birds, aquatic life in the environment (Paul et al. 2014). As per the U. S. Food and Drug Administration, this is a highly widespread herbicide in adults' foods (Daniels-Lake et al. 2011; Aml et al. 2014).

CIPC acts as a mitotic obstacle by intrusive action of spindle development through the cell breakup in the living organization. It is familiar to control β-amylase activity, protein, and RNA formation. Anatomical/physiological activities are suppressed by this herbicide (US EPA 1987; Vaughn and Lehnen Jr 1991). Continuation of this affects man mortal wellbeing as well as future harm to the liver, kidney, spleen, and erythrocytes (Nakagawa et al. 2004). Marty et al. (1986) studied in *Pseudomonas alcaligenes* was revealed in related aniline and alcohol by cometabolism (Fig. 11.5).

Rouillon et al. (1989) studied the breakdown of the weed killer CIPC by few *Ectomycorrhizal* fungi. 3-Chloroaniline is an intermediary product, regarded as dangerous natural toxics and very poisonous to all lives processes and animal lives, including chemicals themselves (Sihtmae et al. 2010; Smith and Bucher 2012; Mohammed et al. 2015). According to European Community Pollutant Circular No 90–95 (1990), 3-chloroaniline is identified as poisonous, water-dissolved toxics and dangerous to living beings in water (David et al. 1998). 3-Chloroaniline is one of the main intermediary compounds mainly formed by microbial degradation of phenylurea, acylanilide, and phenyl carbamate weed killer in cultivated land (Zeyer and Kearney 1982; Häggblom 1990). Therefore, little knowledge is obtained on the total degradation by microbes.

11.8.2 Phenmedipham

Phenmedipham (PMP) is generally called broad-spectrum herbicide, which is manufactured progressively at a higher quantity, and is needed to protect the crops for profits. It is a judicious essential post-development PMP which has been extensively utilized for weed management in cultivated farms (Lewis et al. 2006; Tomlin 2006). Beginning, it is absorbed through leaves with translocation essentially in the apoplast in order to control photosynthesis. Phenmedipham breaks electron transport at the photosystem II receptor site, delicate to natural pressure components, a few non-biodegradable compounds and it may show depletion in its process (Ikeda et al. 2003). The electron transfer is negatively persuaded and along with the formation of intermediate power compounds like ATP and $NADPH_2$ and interrupt carbon dioxide absorption. The activity trend is electron shift obstruct joining the QA and QB by attaching to the D1 protein of photosystem II in the chloroplast (Jursík et al. 2011). PMP is less dissolved in water, somewhat moving in soil. It has a greater capacity for biomagnification (Kegley et al. 2009). The U.S.EPA has grouped phenmedipham and its by-products are lasting biomagnification poisonous chemicals disperse in nature (Edwards 2005). It has an oral LD_{50} of 0.8 g/kg in rats that reveal that RBCs

Chlorpropham
Chlorpropham hydrolase
3-Chloroaniline
Isopropyl alcohol
CO_2
O_2
Acetic acid
4-Chlorocatechol 1,2-dioxegenase
NH_3
CO_2
Dechlorination
Cl-
Catechol
4-Chlorocatechol
Catechol 2,3 dioxygenase
$Cl_2 + CO_2 + H_2O$

Fig. 11.5 Biodegradation of chlorpropham by *B. licheniformis* NKC-1 (Pujar et al. 2018)

are the basic earmark of such development in hemolytic anemia. Persistent dosage development in a consistent and concentration-contingent and depletion in body mass (Jursík et al. 2011). So, its utility may negatively act on endangered species of geographical, amphibiotic herbs and notochord (Kamrin 1997). PMP is most poisonous to *Vibrio fischeri* bacteria, *Pseudokirchneriella subcapitata, Chlorella vulgaris*, *Chlamydomonas pseudostate* microalgae, *Lamna minor* macrophyte, and Cladocerans family (Vidal et al. 2012). Sonawane and Knowles (1971); Pohlenz et al. (1992); and Perret et al. (2001) reported that the PMP breaks down by *Arthrobacter P52* and *Pseudomonas putida* strain by hydrolysis to form methyl-*N*-(3-hydroxyphenyl) carbamate, *m*-aminophenol, and *m*-toluidine. However, total degradation steps have not been reported (Fig. 11.6).

Further, the extensive utilize and liberation of like poisonous products showed atmosphere contamination and soil richness deprivation. This is a developing route of a universal affair. Therefore, continuous crop cultivation possesses a set of preferences in the twenty-first century (FAO 2008). The corrective of polluted soils, floating mass is crucial for preserving the ecosystem and protecting natural inherent assets. Therefore, the microbes rollick a key character in removing the poisonous agents and their metabolites in the nature (Ishag et al. 2017).

11.9 Conclusion

This chapter concluded that agricultural chemicals are used in huge quantity, and various types of toxic chemicals and raw materials are used in the production of pesticides. The excessive use of these pesticides and their products pollute the soil and water leading to environmental pollution. This creates various problems in the environment, when used in the agriculture, and they create oxygen depletion. These pesticides are highly toxic to living beings in the ecosystem. Microbes play an active part in the decontamination of like chemicals by the action of biodegradation. Indigenous microorganisms potential to remove toxics have been characterized from agricultural soil. The microorganisms were ability break down to degrade a large array of environmental pollutants. Thus, there was complete mineralization of these toxic compounds by the microbes. It is therefore necessary to explore the catabolic effect of such compounds in the environment.

Phenmedipham
Phenmedipham hydrolase
Methyl- N- (hydrooxyphenyl) carbamate
m-Toluidine
+ CO_2
NH_3
Toluidine dioxygenase
m-Aminophenol
4-Methylcatechol
Deamination
4-Methyl catechol 1,2-dioxygenase
Catechol
2-Hydroxy-5-mrthyl-6-oxohexa-2, 4-dienoate
Catechol 2,3 dioxigenase
Ring cleavage
TCA cycle

Fig. 11.6 Bacterial detoxification of phenmedipham by *O. anthropi* sp. strain NC-1 (Pujar et al. 2019)

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Conflict of Interest The authors report no conflicts of interest.

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Chapter 12
Probiotic Enzymes in Biodegradation and Value-Added Products

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Abstract Probiotics are a group of active microorganisms, providing health benefits to the host. It is of great significance to promote the development of human gastrointestinal nutrition and health by regulating the host mucosal and systemic immune function or regulating the balance of intestinal flora. Many enzymes produced by probiotics help for the biological activity especially in value addition in the food industry, for example, beverages and mushroom industry. Microbial agents can supplement, adapt, and maintain the balance of intestinal microorganisms, which can be used for disease prevention and treatment, promoting health and enhancing productivity. The rational utilization of edible fungus bran became an urgent problem in the edible fungus industry, and the production of probiotics from mushroom bran provided a solution. In the present report, we have tried to tabulate the enzymes from probiotic bacteria and their significance in the food industry.

Keywords Esterase · Laccase · Lipase

12.1 Introduction

Probiotic foods are a category of functional foods with increasing market share and significant commercial interest (Arvanitoyannis and Van Houwelingen-Koukaliaroglou 2005). It has been recommended for a long time to increase the value and usage of probiotics, and there is concern that they have often been implemented without critical evaluation. The concept of probiotics comes from Latin, meaning "life." Probiotics are live microorganisms that give the health

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benefits to the host when administered in adequate quantities. Sometimes probiotic organism produces secondary metabolites (organic substances), which might get involved in the cascade of biological events, further promoting health beneficial activity to host (Leroy et al. 2008).

Fermented milk was found to be the first food which contains active microorganisms since the beginning of human civilization. Probiotics have been used in fermented dairy products for centuries. There has recently been a growing interest in food applications of probiotics, the selection of new probiotic strains, and the development of new applications (Prado et al. 2008).

The World Health Organization (WHO) describes probiotics as "live microorganisms that confer a health benefit on the host when administered in adequate amounts." In order to be considered a probiotic, clinical evidence need to be reported for the health benefit. These are live microbes that can be formulated into several products of pharmaceutical and/or nutraceutical grade (FAO/WHO 2001). Probiotic is a relatively new term used to refer to bacteria that are associated with beneficial effects on humans and animals. The most common microorganisms used as probiotics are from the genera *Lactobacillus* and *Bifidobacterium* which are widely *used* in food and feed (Panduru et al. 2015). As probiotics, other microorganisms such as yeast *Saccharomyces cerevisiae* and certain species of *Bacillus* are often used (Jacobs 2017). Lactic acid bacteria (LAB) that have been used since ancient times for food fermentation may serve a dual purpose by acting as a food-fermenting agent and probiotic. *Lactobacillus* have been identified as "generally recognized as safe (GRAS)" with no pathogenic or virulence properties. Some of the desirable characteristics for the use of *Lactobacillus* as probiotics include acid tolerance, bile tolerance, adhering capacity, and low cost, preserving its viability during processing and storage and facilitating the application in the products (Balasingham 2017). These probiotics also produce enzyme which can employ many more value additions. Some of the enzymes are listed below.

12.2 Different Enzymes

12.2.1 Malolactic Enzyme

Yeast and some beneficial species such as *Lactobacillus*, *Leuconostoc oenos*, and *S. cerevisiae* can synthesize malolactic enzyme. As a single enzyme, the malolactic enzyme is involved in the conversion of L-malic acid to L-lactic acid and is used in fermentation systems, also in studies of expression. The bioreactor containing NAD, Mn ions, and *Leuconostoc oenos*, a malolactic enzyme achieved a conversion rate of 62–75% for L-malic acid to L-lactic acid in wine (Formisyn et al. 1997). Incomplete conversion was due to the inactivation of the enzyme and instability of the NAD cofactor at the pH of the wine (Colagrande et al. 1994; Gestrellius 1982).

Expression of the malolactic enzyme encoded by the mleS gene from *Lactococcus lactis* in *S. cerevisiae* wine yeast has enabled it to simultaneously effect malolactic fermentation and alcoholic fermentation (Colagrande et al. 1994, Gestrellius 1982).

The major function of *Lactobacillus* is the conversion of L-malic acid to L- lactic acid during the malolactic fermentation (MLF). This conversion may be achieved by one of the three pathways (Henick-Kling et al. 1993). Decarboxylation of L-malic acid to L-lactic acid and CO_2 is catalyzed by the malolactic enzyme without the release of intermediates by most of the wine-borne *Lactobacillus*. However *L. casei* and *L. faecalis* are exceptions (Van Vuuren and Dicks 1993). These *Lactobacilli* use malic enzyme (malate dehydrogenase) to metabolize L-malic acid to pyruvate, and then L-lactate dehydrogenase acts on pyruvate to produce L-lactate. Second exception can be seen in *L. fermentum*, in which the metabolism of L-malate yields D-lactate, L-lactate, acetate, succinate, and CO_2 (Volschenk et al. 1997; Crapisi et al. 1987a, b; Divies 1989).

12.2.2 Proteolytic and Peptidolytic Enzymes

Proteolytic and peptidolytic enzymes were produced from probiotics like *Lactobacillus* and *Pediococcus*. Proteases have been assessed as a substitute to eliminate uninvited proteins and also redeeming assimilable N_2 for exploitation by yeast. Commercially prepared proteolytic enzymes like trypsin and pepsin are not best at low temperatures and pH during wine preparation (Henschke 1993). Yeasts are used as alternate sources of such enzymes in the preparation of wine and beer as these organisms are suitable to the respective conditions during the corresponding fermentation process. For the successful fermentation and quality of the product, nitrogen compounds are essential. The bulk of the nitrogenous fractions are comprised of the α-amino acids (AA) and ammonium (Henschke 1993) along with peptides having five AA residues (Becker et al. 1973; Mougin et al. 2003) represent the assimilable N_2 that is essential for the growth of yeast and fermentation activity and suppression of H_2S (Julien et al. 2000; Monteiro and Bisson 1992). *Lactobacillus* is fastidious in their AA requirements, and there is clear indication that some *Lactobacillus* produces the events needed to obtain peptides and AA to meet these requirements. Reviews on proteolytic and peptidolytic activities among the genera of *Lactobacillus* are available. These enzymes are directly involved in increasing flavor and texture and are indirectly involved in the maximization of microbial cell growth by providing essential AA (Sanz and Toldra 2002).

Organisms whose growth is most specious toward the conclusion of the primary fermentation are improbable to have influence on yeast growth. At this time yeast needs negligible as similable N_2; therefore, any peptide lytic activity of *Lactobacillus* is beneficial mainly for smog reduction. Conversely, the sensitivity of *Lactobacillus* and *Pediococcus* in ethanol fermentation (Sanz and Toldra 2001) relegates their growth in mixed cultures with yeast in primary fermentation. Decomposition of

Table 12.1 Description of proteases

Enzyme	Optimal activity	
	pH	Temperature
Protease I	4.0	30 °C
Protease II	5.5	40 °C

Table 12.2 Components of glycosidal substrates

Yeast	Enzyme	Substrate	Terminal sugar
S.cerevisiae	Glycosidases	Mono or disaccharides	-D-glucopyranoside -L-arabinofuranoside -D-apiofuranoside -D-xylopyranoside

proteins and peptides at initial stage could affect protein haze formation in the completed wine and release solubilized N_2 for yeast benefit. A series of studies conducted by Rollan et al. (1993) described proteases I and II, which are produced by several strains of *Oenococcus oeni* during the early and final phases of growth (Table 12.1).

Both proteases were apparently repressed by ammonium, tryptone, and casein hydrolysates; further induced by nutrient starvation; and were able to liberate detectable concentrations of AA from protein and polypeptide extracts from red and white wines. When applied to sterile grape juice, a concentrated, purified exoprotease is found to degrade proteins at a high rate (Colagrande et al. 1994).

12.2.3 Glycosidases

S. cerevisiae is known to produce intracellular glycosidases. Amusingly, *Lactobacillus* has received little awareness as a potential source of glycosidic enzymes. The glycosidase that detaches the sugar moiety from glycosides has a significant effect on a wine's sensory profile. The occurrence of many types of such enzymes is a reflection of the complexity of their glycoside substrates, as shown in Table 12.2 (Gunata et al. 1988).

In the case of a disaccharide, a -D-glucopyranoside enzyme is required (Gunata et al. 1988). The second enzyme is essential for the liberation of aglycones from all diglycosides and _-D-glucopyranosides; therefore, research efforts are concentrated more on this enzyme compared to other enzymes. In many microbes, including both wine-related and non-wine-related, glycosidases have been studied extensively. These enzymes directly influences the color of wines (Sanchez-Torres et al. 1998).

The liberation of aroma components from natural grapes and the usage of aroma glycosides are still in the early stages of development. *Lactobacillus* appears to have the full array of glycosidases needed to hydrolyze many of the glycosides, thus determining the specific sensory significance of glycosidic activities.

12.2.4 Polysaccharide-Degrading Enzymes

The polysaccharides such as cellulose (primarily glucans), hemicelluloses (xylans), and pectic substances present in higher plant cell walls and middle lamellae affect the wine production (Whitaker 1990). These compounds are released by the action of degradative enzymes from the grape juice resulting in the berry disruption.

The hydrolyzation of the abovementioned macromolecules are carried out by various numbers of enzymes (Table 12.3) (Van Rensburg and Pretorius 2000).

Enzymes capable of degrading polysaccharides have the potential to improve juice yields and wine processability. The presence of polysaccharides in the wine preparation reduces juice extraction and are responsible for fouling of filters during clarification process and also affects wine quality and may influence on taste and aroma. These degrading enzymes increase wine quality by breaking down the cell wall of grapes to yield better extraction color and aroma (Langourieux and Crouzet 1994).

An extracellular glucanase obtained from *Oenococcus oeni* was capable of hydrolyzing yeast cell wall macromolecules. This enzyme is produced early in the stationary phase of cell growth and plays an important role in yeast cell autolysis followed by alcoholic fermentation (Hansson et al. 2001).

12.2.5 Esterases

Esters are a large group of volatile compounds which are present in wine and produced by yeast as secondary products of sugar during fermentation. Esters can also be derived from grapes (Hansson and Hills 1997) and also during chemical esterification of alcohols and acids during wine aging (Etievant 1991). These esters play an important role in determining the quality of wine by giving desirable fruity aroma (Bakker et al. 1999). The presence of excessive ester concentration affects the aroma of wine (Ganga et al. 1997). The important wine esters (Etievant 1991) include:

Table 12.3 List of enzymes involved macromolecule degradation

Macromolecules	Enzymes	Examples
Pectic substances	Pectinases	Protopectinase Pectin methylesterase Polygalacturonase Pectin Pectate lyase
Cellulose	Cellulases	Endoglucanase Exoglu canase Cellobiase
Hemicellulose	Hemicellulases	-D-galactanase -D-mannase -D-xylanase

Table 12.4 List of eaters and corresponding aroma

Wine esters	Aromas
Isoamyl acetate	Banana
Ethyl hexanoate	Fruity violets
Ethyl octanoate	Pineapple. Pear
Ethyl decanoate	Floral

1. Ethyl esters of organic acids.
2. Ethyl esters of fatty acids.
3. Acetate esters.

Ethyl acetate is an important ester in wine with a low sensory threshold which contributes to the aroma of wine. The low concentration of ethyl acetate gives a desirable fruity aroma. Higher concentration gives an undesirable nail-polish-remover aroma to the wine (Bartowsky and Henschke 1995). The activity of esterases during wine production results in an increase or decrease in wine quality depending on the ester involved (Davis et al. 1988). Some of the esters and aroma are listed below (Table 12.4).

The compounds liberated by the esterases (fatty acids and higher alcohols) could contribute to the aroma of the wine (Lambrechts and Pretorius 2000). *Lactobacillus* esterases are used in dairy industries which contribute to the characteristics of flavors and defects in cheeses. These enzymes can synthesize and hydrolyze esters (Liu 2002). Ethyl butanoate and ethyl hexanoate are synthesized by dairy *Lactobacillus*. Different strains ($n = 23$) were found which were able to hydrolyze an ester substrate, but the characterization of those enzymes was not carried out which determined their synthesizing ability (Davis et al. 1988). Many researches have been carried out in order to study the flavor of wine. Changes in concentration levels of individual esters during MLF were studied such as increase in ethyl acetate (Maicas et al. 1999), isoamyl acetate, and ethyl lactate (Maicas et al. 1999). Some studies also reported decrease in concentration levels of some esters (Zeeman et al. 1982). Esterases of wine LAB are involved in synthesis and hydrolysis of esters like esterases of dairy isolates.

12.2.6 Ureases

The hydrolysis of urea is catalyzed by ureases (Schlegel and Kaltwasser 1985). Ethyl carbamate is a known carcinogen and is formed in beverages by spontaneous acid ethanolysis of some carbamyl precursors along with urea and citrullin. To avoid dangerous and illegal concentrations of ethyl carbamate, urea-degrading enzymes act as a potential tool. Commercial urease products are derived from different sources like beans which has low pH optima (Kodama et al. 1991). To remove excess urea present in beverages, the use of *Lactobacillus* as alternate source has also been investigated.

12.2.7 Phenoloxidases

Phenoloxidases are a group of enzymes which occurs in *Lactobacillus* strains, commonly associated with brewing. These enzymes are present in fermentative *Lactobacillus* strains (Benz et al. 1998).

Laccases (*p*-benzenediol:oxygen oxidoreductase) and tyrosinases (monophenol monooxygenase) are two groups of phenoloxidases ubiquitous and also found in probiotic bacteria. These enzymes catalyzes the transformation of a large number of dyes, polyphenolic and nonphenolic aromatic compounds used in bioremediation of paper, pulp, tanning process, and also used in food industries mainly olive mill and brewery wastewater (Bagewadi et al. 2017; Bilal et al. 2021; Durán et al. 2002; Mougin et al. 2003). Laccases are also involved in the food industry for product stabilization in fruit juice, beer, and wine processing (Miele et al. 1993).

An innumerable phenolic compounds are found in musts and wine which ranges from hydroxybenzoate and cinnamic acid derivatives to more complex molecules, such as catechins, anthocyanins, flavonols, flavanones, and tannins (Durán et al. 2002). These compounds are responsible for enviable attributes such as color, astringence, flavor, and aroma of wine and also unwanted attributes like browning, flavor, and aroma alterations and also some forms of haze which lead to chemical oxidoreduction in white musts and wines.

Enzymes such as laccases, tyrosinases, tannases, and peroxidases have been considered an alternative to treatment with physical-chemical adsorbents in treating must (Maicas et al. 1999). Laccases are thought to be the most hopeful enzymes, because they have broader specificity for phenolic compounds and also product stability is achieved.

12.2.8 Lipases

Wine lipids are derived from the grape berry and are released from yeast during autolysis. Grape lipids originate from a number of sources within the berry, skin, seeds, and berry pulp. Red variety grapes have greater lipid concentrations compared to white varieties as lipid profile depends on grape maturation climate and variety and also concentration and composition of neutral lipids, glycolipids, and phospholipids (Gallander and Peng 1980). Autolysis of yeast following fermentation releases different types of lipids, such as tri-, di-, and monoacylglycerols and sterols, in amounts and proportions that varies with the yeast strain. Such lipids not only influence the sensory profile of sparkling wine but also form characteristics (Pueyo et al. 2000).

The action of lipases on lipids yields a range of volatile compounds, including fatty acids. The low aroma thresholds of fatty acids allow them to contribute to wine aroma, but since their odors are described as vinegar, cheesy, and sweaty, their impact might not be desirable (Etievant 1991). When the volatile compounds like

esters, ketones, and aldehydes are derived from these fatty acids, the aroma profile of the wine can be positively influenced.

The lipolytic activity of *Lactobacillus* strains has been extensively researched in food production areas. These enzymes contribute to flavor and processability in dairy foods (Urbach 1995). *Lactobacillus* strains are generally recognized as weakly lipolytic, and their lipases display substrate utilizing numerous substrates and various degrees of cell fractionation. *Lactobacillus* and *Pediococcus* are the microorganisms on which studies have been conducted to determine their capability in wine preparation. *Lactobacillus* has the potential to influence the wine lipid content when they are grown in grape juice or wine as lipases are located extracellularly and are associated with whole cells. The ability of these enzymes is determined as they attack yeast membranes and grape cells and also influence on wine aroma (Urbach 1995).

12.3 Conclusion

A significant contribution of probiotic enzymes has been studied to determine the enzymatic properties. Probiotic organisms possess an extensive collection of enzymes undoubtedly as many of these enzymes have the potential to influence the product processing. Probiotic organisms also serve as a source for the preparation of enzyme extracts that are able to function under the harsh and changing environmental conditions.

Enzyme preparation is a kind of safe, consistent, and pollution-free, green-feed preservative. Biocatalytic property improves the production efficacy of livestock and food products, reducing environmental adulteration and saving animal feed resources. As a kind of food additive, probiotics play an important role in the intestinal flora. It helps the host by controlling harmful bacteria and provides beneficial effects on intestinal flora. Probiotics also provide resistance to fight against various diseases.

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Chapter 13
Current State, Challenges, and Perspectives on Microbial Degradation of Dioxin and Furan

S. Prajwal and Satish Kumar Murari

Abstract Dioxin and furan are future catastrophes as their effects on human health and environmental risks have been proven beyond doubt. Both pollutants are highly toxic and stable with emission sources being local but have an impact on the world's living populations, so the upcoming requirement is accuracy in identification and monitoring. Specialized fungi and several gram-negative aerobic bacterial species degrade furan and dioxin to harmless components which is as of date both economical and environmentally sustainable when compared to physicochemical methods. Several microbial enzymes via redox mechanism with selected specific genes for degradation of furan and dioxin have been identified and characterized for remediation to attenuate contamination for many systems.

Keywords Polychlorinated biphenyls (PCB) · Polychlorinated dibenzodioxins (PCDDs) · Polychlorinated dibenzofurans (PCDFs) · Tetrachlorodibenzo-p-dioxin (TCDD) · Persistent organic pollutants (POPs)

13.1 Introduction

The world has been polluted since centuries by most chemicals (organic compounds), such as certain neglected organic compounds, namely, furans and also Dioxins (Hutzinger and Blumich 1985). To be more specific, the polychlorinated dibenzofurans (PCDFs) and also dibenzo-p-dioxins (PCDDs) are important threats to the environment. The sources of these hazards are chemical manufacturing company, combustion, and metal processing. These organic compounds are found everywhere (soil, water, and air), and it may get contracted via inhalation and dermal

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contact too (McKay 2002). Upon accumulation of PCDD and PCDF to the food items such as milk and red meat, an individual's well-being gets distorted gradually (Alcock and Jones 1996). The PCDD and PCDF contain two rings of six-membered aromatic benzene ring bonded by one or two atoms of oxygen. About eight chlorine atoms are attached adjacently in their respective positions. These halogenated chlorine atoms are stable and are enormous and hydrophobic in nature (Klimm and Schramm 1998; Krauss and Krauss 1994).

There are about 75 and 135 various analog of the PCDF along with PCDD, respectively. PCDF and PCDD are planar in shape. They are tricyclic in nature. It belongs to two families (Oberg and Rappe 1992). In the modern era, people started using these organic compounds as pesticides to their farms. But they ignored the fact that it was a byproduct from the manufacturers of the chlorinated phenols, which they were actually releasing to the atmosphere. The chemical inertness, toxicity, lipid solubility, and recalcitrance of these compounds make them more hazardous to the environment. Not only the environment, it affects the human health, causing gastrointestinal toxicity, neurotoxicity, developmental toxicity, and carcinogenicity, respectively (European Commission 1994; Gaus and Brunskill 2002).

By the action of chlorine atoms and incinerating certain organic compounds, the enormously toxic polychlorinated dibenzo dioxins are formed. The report states that agrochemicals had potentials of polluting the agricultural environment. In the presence of an enzyme called fungal peroxidases, the dioxins were formed by the polymerization of chlorophenol. It is very difficult to make them dioxin-free areas due to their long half-lives and the toxicity. Countless efforts were being put to remove and decompose the harmful compounds from the contaminated sites using efficient skills for rehabilitation (Buekens and Huang 1998). During composting and sewage treatment, the analogues of octachloro- and heptachloro-dioxins are formed naturally.

Dioxins are formed by the peroxidases during the coupling of chlorophenols, which helps in the formation of dioxins, and also forest fires can immensely produce chlorinated dioxins. The causative agents (PCDD and PCDF) continue to pollute the environment because they are very much stable and hydrophobic in nature. The tetrachlorodibenzo, the isomers of chlorine positioned at 2, 3, 7, and 8, caused adverse effects to mammals and also higher organisms. According to the toxicology point of view, the amount of analogues of dioxins are 17 polychlorinated dibenzo dioxins and 10 polychlorodibenzo furan (Davy 2004; Kerkvliet 2002) (Fig. 13.1).

The chief emission sources of polychlorinated dibenzo dioxane or polychlorinated dibenzo furan are, namely, coke plants, metal-producing plants (copper, aluminum, zinc, and lead), power-producing plants (coal, gas, crude, sewage sludge, and biomass), miniature incineration units, waste incineration units, automobile units, and mineral product production unit. These can be rehabilitated by using certain sophisticated methods such as bioremediation, thermal, and physicochemical, respectively (Ronald 2011).

Quite a few operative methodologies have been reinforced to protect the environment by eradicating hazardous dioxins and furans, but the effective application isn't as frugal as it should be. It was evident that the microbes were acting effectively

Fig. 13.1 Chemical structure of PCDD/Fs

against it naturally by converting the organic substances, as a result allowing them back to the environment. There are immense chances of degrading the dioxins by exposing it to particular microbe or a consortium of microbes.

According to subsequent evaluation done by the scientists Ward, Kearny, Matsumura, and Benezet, 2,3,7,8-TCDD could be degraded by the consortia of microbes. Certain aerobic microorganisms are having the capability of degrading the dioxins. It is due to the presence of the dioxygenase which has an hydroxylating aromatic ring in it (USEPA 1994). On the other hand, the anaerobic bacteria use a unique way of mechanisms such as reductive dechlorination to eradicate the dioxin from the environment. The lower organisms like fungi have also been able to degrade the dioxins through oxidation, utilizing certain enzymes, for instance, cytochrome P-450, diarylpropane oxygenase (LiP), Mn-dependent NADH-oxidizing peroxidase (MnP), and laccase, respectively. Although fungi are able to bioremediate the dioxins, their field-scale applications are still at the emergence. Apart from bioremediation, sophisticated methodologies like nanotechnology can give promising results in effectively removing dioxins from the environment.

At certain places like rivers, estuaries, and bays, it is believed that the degree of biodegradation of the dioxins and furans by the microcosms has surged. It is so because of the fact that there are plenty of anaerobic sediments which possessed congeners of dioxin which were spiked and get converted to lower concentration of dioxins which represents the biologically mediated reductive dechlorination (Van den Berg and Birnbaum 1998). Time period meant for bioconversion is 1 year. While in the field, it has been considered as many years. The difference in the time period can happen adequately to numerous concentrations of exorbitant dioxin utilized in the microcosms with a sufficient supply of electron-donating substrates.

According to the evaluation done on microcosm, the remains obtained from various places like rivers, bays, and estuaries have significant amount of evidence

based on biodegradation of PCDDs and PCDFs. Analogues of the polychlorinated dibenzo dioxins were found, where in a substrate with robust exchange of electrons was also found (Saibu and Adebusoye 2020). The microcosmic biotransformation may take place in a span of 1 year. In the fields, the time period may be several years. This variation can be due to the fact that the microcosm requires abundant concentrations of dioxins along with the proper stream of substrates which donate continuous flow of electrons.

At anaerobic conditions, dioxin was dechlorinated. A scientific report on microcosm states that in the soil, the chlorinated dioxins getting biodegraded are quite inconsistent. On the other hand, they are tenacious. It was so done by introducing the polychlorinated dibenzo dioxins into the soil by applying the sewage mud (Fagervold and May 2007; Mitrou and Dimitriadis 2001).

Compounds similar to dioxins containing the dirty dozen have around 2, 3, 7, and 8 substitutional arrangements whose toxicity is reduced accordingly. The time must be taken into consideration for the analogues of bromine, low-chlorinated and unchlorinated dibenzo-para-dioxin and PCDFs, respectively. It is due to the nuclei of the two aromatic ring attached in between the oxygen atoms by sharing common elements structurally.

Upon liberating the toxic compounds into the Earth's ecosphere through anthropogenic sources, there are chances of claiming legislative and executive activities. Since the toxicity levels are high, technologies have been improved to rehabilitate the polluted soil by the chlorinated dioxins released from industrial plants, old transformers, and so on. Most of the timber-preserving agents have also been affected with the polychlorinated dibenzo dioxins and polychlorinated dibenzo furans possessing pentachlorophenol. Though there are various methodologies including physicochemical methods to eradicate dioxins from the environment, incineration has alone been used extensively just because it was economically feasible (Reiner and Clement 2006). On the other hand, the substitutes of biological components were being used instead of thermal treatment due to the fact that it was quite expensive. Microscopic organisms like fungus and bacteria take an important part in carbon cycle worldwide by distorting the organic components. Sophisticated methodologies have been found to recover the environmental hazards like oil spills.

13.2 Health Risks

Exposure to 1,4-dioxane can occur during its manufacture or use as a stabilizer or solvent, via inhalation, eating the spoilt food, through polluted water, and dermal contact. Inhalation is the chief route of exposure, and it gets circulated all over the lungs, liver, kidney, spleen, colon, and skeletal muscle tissue very swiftly (Kulkarni and Afonso 2008). The highest risk of exposure is for workers at industrial sites that can inhale 1,4-dioxane repeatedly. Short-term effects include irritation of the eyes, throat, nose, and lungs as well as vertigo, anorexia, and headache. Long-term exposure effects include skin disorders like dermatitis, eczema, and drying and

cracking of the skin. 1,4-Dioxane has no known genotoxic or reproductive effects. The environmental protection agency states that the 1,4-dioxine is an effective human carcinogen. In the case of animals, 2,3,7,8-tetrachloro dibenzo dioxins are considered to be teratogens and toxic to fetus, and it leads to liver cancer in rats.

13.3 Noxiousness Assessment of the Fisk for Dioxins

Usually, dioxins get exposed by consortia of numerous analogues. The determination of the effects of a particular kind of analogues is quite intricate. The cell gets encountered at the very first step when there is any kind of interaction between the receptors of aryl hydrocarbon and the dioxin. The genetic data gets transcribed from the DNA to the RNA, when a protein (aryl hydrocarbon (Ah) receptor) binds on particular sequences of the DNA. The aryl hydrocarbon receptor ligands can be categorized into two types, namely, natural Ah receptors and synthetic Ah receptors. Some examples of synthetic Ah receptor ligands are dibenzo-1,4-dioxin, PCDFs, benzopyrene, phenylbenzene (halogenated aromatic hydrocarbons) and 3-methylcholanthrene, benzanthracenes, and 7,8-benzoflavones and 5,6-benzoflavones (polycyclic aromatic hydrocarbons), respectively (Reiner and Clement 2006). A few Ah receptor ligands were found naturally, namely, indigo and indirubin (Derivatives of tryptophan), bilirubin, polyunsaturated omega-6 fatty acid 20:4(ω-6) or 20:4(5,8,11,14), lipoxin A4, and unsaturated fatty acids derived from arachidonic acid (tetrapyroles). The cell gets affected only when there is any sudden alteration in the gene expression. As a result, the growth and functions of a cell gets distorted. This change happens usually when the aryl hydrocarbon receptor gets in contact with the halogenated aromatic hydrocarbons. Initially the aryl hydrocarbon receptor binds with the chaperone heat shock protein (Hsp70/Hsp90) and aryl hydrocarbon receptor (AhR) interactive protein within the cytoplasm of a cell.

The receptor gets translocated to the nucleus; as a result there is a discharge of Hsp90. It is tailed by the bombardment of the protein (AhR nuclear translocator) within it and the formation of the complex which in turn becomes active. The heterodimer complex binds to particular sequences in the double-stranded DNA which is near the gene CYP1A1 (Schecter and Birnbaum 2006). As a result, a series of events takes place like DNA bending, distortion of the chromatin, elevated promoter accessibility, initiation of the transcription process, and succeeding buildup of cytochrome P4501A1-specific mRNA. Hence, the polychlorinated dibenzo dioxin induces the gene expression of the cytochrome P450.

13.4 Dioxin Sources

Through anthropogenic resources, strains of dioxins can be found in the environment. These harmful substances get exposed in innumerable quantities based on the toxicity. Based on the chief emission sources, it has been categorized majorly into

three types, namely, very-high-temperature thermal combustion, industrial, and reservoir processes.

13.4.1 Very-High-Temperature Thermal Combustion

Metropolitan nonliquid waste incinerator is one of the chief sources of dioxins. Approximately 50 ng I-TEQ kg1 of dioxins and dioxin progenitor are found in municipal nonliquid and other than gas waste.

13.4.2 Hospital Waste Incinerators

In places like hospitals, organs of humans, used bandages, tubes contaminated with blood, test tubes, syringes including needles, tissue cell culture of tissues, and plastic equipment/s are found all over the waste bins. The chief source of dioxin emissions are waste incinerators at hospitals. It is due to the increment in number and continuous burning of high-chlorine-content waste.

13.4.3 Hazardous Waste Incinerator

In places like hospitals and industries, there are certain amounts of toxic emissions which are highly flammable, infectious, mutagenic, explosive, and carcinogenic. There is a unique way in order to incinerate this without causing any surplus effects.

13.4.4 Sewage Sludge Incinerator

Contaminated water processing plant produces thick nonliquid remains with elevated toxic metals and organic contents which is said to be “sewage sludge.” Land filling, recycling and there is a prohibition on sea disposal. As a result, sewage sludges are getting incinerated.

13.4.5 Wood Burning

As per the inventory of European emission, incinerating wood is the foremost chief emissions of dioxin. On an average, there is 945 g I-TEQ amounts of dioxin emission annually.

13.4.6 Diesel Vehicles

Norway and Sweden scientists have studied the after effects of diesel vehicles emitting dioxins. Since these assessments are done based on the use of fuel by a particular nation, detailed examinations are to be done in order to end up with a conclusive inference.

13.4.7 Crematoria

According to the reports from Europe and the United States, 0.3% and 0.24% of dioxin emissions were from crematoria. The procedures done during crematoria are the chief source of organic compounds.

13.4.8 Coal-Fired Utilities

Compared to burning of wood, emissions of dioxin-like compounds is relatively reduced. These are abundant and enormous in size. Its sky-scraping heaps specify that it will influence extremely and to enormous extents.

13.4.9 Uncontrolled Fires in Landfill Sites

Air, water, and soil can also get polluted through the emissions of dioxin. In particular, landfill is one of the chief sources of it. The amount of risk is based on the components which were masked inside the landfill, by geography, and also the behavior of the fire.

13.4.10 Pulp and Paper Mills as Factory Sources

According to reports, numerous amounts of waste are generated during the manufacturing process of pulp and paper. Pulp mill in China releases around 300 pg l1 I-TEQ concentrations of dioxin emission. It is later on released to the water and land. These components can also be formed via the chlorination of phenols.

13.4.11 Metal Industry

The classic sources of dioxins are elevated temperature at manufacturing sectors like steel factories, and also scrap metal smelting processes, including retrieval furnaces and sintering of iron ore, are also considered as chief sources.

13.4.12 Chemical Manufacturing

Chlorinated compound manufacturing produces byproducts similar to phenols with substituted chlorines, phenoxies, chlorinated organic solvents, chlorinated catalysts, and polybrominated diphenyl ethers (PBDEs). During the late 1970s, production of phenols with substituted chlorine intermediates and products was dismissed in the Unites States. Nevertheless, the manufacturing sustained till 1990. Restricted utilization and dumping of these complexes can lead to exposure of dioxins to the ozone at an extensive range (Schecter and Birnbaum 2006).

13.4.13 Combustion Sources in Cement Kilns

Burning up of hazardous waste materials such as fuels for cement kilns brought trouble to many individuals. Approximately 18% of the conveniences smolder hazardous waste as reserve fuel; Statistics suggest that kilns which burn hazardous waste like clinker dust and stack emission significantly have increased dioxin level.

13.5 Dioxin-Degrading Microbes

13.5.1 Bacterial Degradation of Dioxins

13.5.1.1 Biodegradation of Non-Chlorinated Dioxins

According to studies, it is believed that dibenzo dioxins and dibenzo furans are the best models for CDD and DF, respectively. At aerobic conditions, microbes degrade DD and DF and their congeners via hydroxylation and cleaving up of the aromatic rings via oxidative degradation. Hydroxylation occurs at lateral and angular positions in an aromatic ring (Young 2006). The degradation mechanism of naphthalene being the simplest aromatic hydrocarbon, polychlorinated biphenyls (PCBs), and other complicated polycyclic aromatic hydrocarbons (PAHs) compounds is very much related to the lateral deoxygenation pathway. Conversely, angular dioxygenation tends to happen at electronegative carbon atoms neighboring to an

ether bridge and is a fair breakdown mechanism. Eventually, it leads to the annihilation of the structure which is planar.

13.5.1.2 Dioxin Degradation by Bacterial Transformation

Microbes are useful to Earth's habitants which help organic components to get mineralized by utilizing it as carbon energy sources followed by degradation. Microbial enzymes help pollutants to get transformed into less toxic (Matsumura and Quensen III 1983). As a result, it forms products which gets mineralized and directed toward the biogeochemical cycle. It is so done because, in most cases, there is a formation of more toxic metabolites if the degradation process is incomplete. Since there is increased stability of chlorination to carbon skeleton, very minute number of microbes have the capability of degrading the non-substituted chlorinated dioxins aerobically.

13.5.1.3 Dioxin Degradation by Aerobic Bacteria

Microbes presented with aerobic conditions vitiate or breakdown dioxins via oxidative degradation by hydroxylation. Oxidation of dioxins is divided into two processes, namely, angular deoxygenation and lateral deoxygenation. In angular deoxygenation, it befalls at carbon atoms adjacent to ether bridge followed by the devastation of the planar structure. As a result, there is a severe decrease in the noxiousness of the compound. The enzyme dioxygenases possess an extensive array of substrate specificity which belongs to the aromatic hydrocarbon dioxygenases. On the other hand, lateral deoxygenation of the aromatic rings of the aromatic complexes like naphthalene being the simplest aromatic hydrocarbon, polychlorinated biphenyls (PCBs), and other complicated polycyclic aromatic hydrocarbons (PAHs) occurs at 1,2/2,3/3,4 positions on the carbon atoms.

13.5.1.4 Lateral Dioxygenation

Initially at the adjacent locus of the six-membered ring, two hydroxyl groups are added during the bacterial degradation of aromatic compounds aerobically. As a result, there is a production of cis-dihydrodiols (Yamazoe and Yagi 2004; Seo and Keum 2009). A peculiar trait of this pathway is to degrade, producing enol of 2-hydroxy-4-(3′oxo-3′H-benzofuran-2′-yliden)but-2-enoic acid (HOBB) which is as yellow-colored metabolite. Dioxygenases amalgamates molecular oxygen into aromatic nuclei. Upon degradation of cis-dihydrodiols, there is a formation of "catechol." Certain strains of bacteria have the capability of producing HOBB by degrading the compound using carbon as the sole source of aromatic compounds. Surprisingly, one of the strains of *Pseudomonas*, i.e., *P. putida* B6–2, degrades HOBB via salicylic acid (Miyauchi and Sukda 2008). Frin *Pseudomonas aeruginosa*

FA-HZ1enzye dioxygenase was isolated which later on augmented and exposed in *Escherichia coli*. It oxidizes 1,2-dihydroxy-1,2-dihydrodibenzofuran from dibenzofuran and also oxidizes 2,8-dibromodibenzofuran and 4-(4-bromophenyl) dibenzofuran.

13.5.1.5 Angular Dioxygenation

The Rieske aromatic hydrocarbon dioxygenases (angular dioxygenases) reacts showing angular positions adjacent to the dibenzofuran. It is then followed by the formation of an unstable molecule called "hemiacetal" by spontaneous cleavage (meta-cleaved). It also makes 2,2′,3-trihydroxybiphenyl (TrHB) or 2,2′3-trihydroxydiphenylether (TrHDE) by rearomatizing the unstable hemiacetal molecule. Catechol and salicylic acid are formed by the meta-cleavage of dibenzofuran and dibenzodioxin (Seeger and Camara 2001). At certain times, the polychlorinated dibenzofuran forms 2-methyl-4H-chroman-4-ones only after chlorine atoms are on both rings. This pathway is also said to be the "constructive pathway," because it is more efficient than the lateral dioxygenation. This step involves knocking down of planar structure of dioxin at a single step unlike the lateral dioxygenation.

In the dioxin pathway, dioxygenases catabolize at the angular position. They are three types explicitly, dibenzofuran 4,4a-dioxygenase (*dbfA* or *dfdA*), DD 1,10a-dioxygenase (*dxnA*), and carbazole 1,9a-dioxygenase (*carAa*) obtained using the *Terrabacter* sp. strain DBF63, *Sphingomonas* sp. strain RW1, and *Pseudomonas* sp. strain CA10, respectively. In a report, the dffA and dbfA genes were only expressed during the growth stage by the HA01 strain. Later on, it was evident not only during growth but also during the angular deoxygenation. Additional dioxygenases were found in *Nocardioides* sp. DF412 which coded for *dfdA1A2A3A4* gene. The biphenyl dioxygenase encoded by the *bphA* genes are very well known for the catalytic dihydroxylation of biphenyl. It also has the capability of hydroxylating ortho carbons which possess a wide variety of substituents. In a report, the bphA genes from *Rhodococcus* sp. RHA1 had all the capability of transforming the dibenzofuran via angular and lateral dioxygenation, while the dibenzodioxin was transformed exclusively via angular dioxygenation. Upon site-directed mutagenesis, the mutant *bphA* dioxygenases, a variant from LB400 *bphA* dioxygenase, enhanced the catalytic activity of the dibenzofurans (2-chlorodibenzofuran) wild type was way more than mutant (Wang and Yamazoe 2004; Iwasaki and Takeda 2007). Phenoxybenzoic acid 1′,2′-dioxygenase coded by *pbaA1A2B* gene helps the microbes to form 3-phenoxybenzoicacid metabolically. The utilization of the dibenzofuran and the dibenzodioxins was disabled. Hence the metabolism of polychlorinated dibenzofuran and the dibenzodioxin is not well known.

13.5.2 Dioxin Degradation Using Fungi or Fungus

13.5.2.1 The Use of Monooxygenase Cytochrome P-450 to Degrade Dioxins

Lately, groups of active researchers are working on a protein named cytochrome P-450 which can be seen in both kinds of organisms (prokaryotes and eukaryotes) which has the capacity to hydroxylate and degrade the dibenzofuran as well as the dibenzo dioxins. Upon conducting numerous experiments on it, they keenly observed the metabolism between the entities (CYP1A1 and CYP1A2). There was a series of events which were employed, for instance, hydroxylation at an unsubstituted location followed by relocation of a substituents made out of chloride and eradication followed by the exposing the dioxin rings. Surprisingly, it was found that CYP1A1 and CYP1A2 were binding effectively with 2,7-dichloro dibenzo dioxins and 2,3,7-trichlorodibenzo dioxins which acted as the substrates.

Recent discoveries provide researchers an ardent opportunity to utilize microbes which had a similar cytochrome P-450 as mammals to bioremediate soils which were polluted by the dibenzo dioxins. Microbes like fungi have also certain enzymes which are efficient in degrading the dibenzo dioxins in a broad spectrum like laccase and lignin peroxidase.

13.5.2.2 Degradation of Dioxins by Lignin Peroxidase (LiP)

A scientific report states that an enzyme named lignin peroxidase (Lip) was efficient in degrading dioxins and furans. Conversely, two scientists named Hammel, Joshi, and Gold (1994) explained the meticulous pathway of dibenzo dioxin degradation biologically by Lip extracted from the fungi named *Phanerochaete chrysosporium. This is the only report which suggests that the white-rot fungi can be a potential degrader of certain chlorinated dibenzo dioxins. To determine the potential fungus which distorts the chlorinated dioxins, detailed study was being done. In places like polluted soils and tropical forests, capable fungi were found in order to degrade dioxins. Potential fungi were screened based on* dye decolorizing ability and TCDD degradation. Phylogenetically, the fungi were extremely capable at degrading the complexes of chlorinated dioxins. *Phlebia brevispora* simultaneously degraded the herbicide (2,4,6-trichlorophenyl-p-nitrophenol). Laccases being a part of the lignolyte has an immense efficiency in oxidizing the recalcitrant contaminants of the nature. Information about laccase-mediated dioxin degradation are restricted. *Polyporus versicolor* laccase considerably never oxidizes 2,3,7,8-TCDD, whereas that of *Trametes versicolor* and *Pycnoporus cinnabarinus* transformed 2-hydroxy-DF. Additionally, laccase of *Pycnoporus cinnabarinus* altered 2-hydroxydiphenylether and 2-phenylphenol and dechlorinated chlorinated hydroxybiphenyls.

13.6 Methods for the Treatment of Dioxins

The environment is continuously being polluted with various emissions. Industries are the chief sources of these kinds of emissions not only to the ozone but also to the soil. The soil gets eroded by the disposal of dioxins. Few harmful gases like flue gases are formed by incineration which becomes the sole source of environmental pollution, in particular air pollution.

To reduce the dibenzo dioxin emissions, certain methodologies have been employed which are as follows.

13.6.1 Dust Collector

At higher temperatures of up to 100–150°C, the dibenzo dioxins which are bound to certain particles are filtered. This helps in overcoming with the synthesis by de novo method. In an iron-sintering plant, the dibenzo dioxins are degraded up to 80%.

13.6.2 Scrubbers or Spray Absorber

Incinerators are the chief choice for degrading methodologies. Few incinerators like scrubbers are used to minimize the harsh effects of dibenzo dioxins. The inert material (limestone) is heated up at very high temperatures in order to make them very minute particles by reducing it to atoms in the spray tower. Initially, gaseous matter was absorbed during the liquid phase followed by the solid phase. Upon mixing the inert material in a reactor with the gases which are combustible, the acidic content gets neutralized.

13.6.3 Flow Injection Procedures or Sorbent

The sole purpose in employing this methodology is to degrade the dioxins as much as possible. Similarly, at high temperatures of about 100 °C, the stemmed coke which was well grounded was subjected to the mixture of inert material and bituminous coal. By doing this, it settles by forming a layer. The merits of limestone are to avoid the ignition of the coke. Many alternatives can also be used to minimize or completely avoid the ignition. Materials like zeolites are also a good choice.

13.6.4 Adsorbent Reuse for Fluidized Bed Operations

During the above operation, the temperatures may raise very high, followed by exposing the flue gas. As a result, there is a formation of fluid bed of coke extracted from certain substances which is inert in nature and also some amounts of the bituminous coal. The dust collector was subjected to separate the adsorbent from the flue gas. It was then again sent back to the fluid bed in order to remove any chemical substances which come in contact with it. The fluidized bed has many more advantages when compared to others in cases like maintaining the mass and heat.

13.6.5 Fixed or Mobile Bed Operations

There is no much difference between this process and the fluidized bed reactor. Both of them utilize the adsorbents which are very much similar to each other. When the coke upsurges, the waste gas flows in the contradictory direction. The pollutants are collected by the coke which is in activated form. During this phase, a large number of matter is exchanged in between. This process may take up to 900–1000 hours. There are a lot of difference between the process of fixed bed and moving bed. When compared to a fixed bed, mobile bed reactors are much more efficient and convenient. And also, the efficiency in distorting the dioxins is up to 99% in moving bed reactors.

13.6.6 Dioxins Putrefaction Using Catalysts

Dioxin can be degraded by a method of selective catalytic reduction (SCR) where there is a usage of NO_2 gas. In a recent study, it was evident that about 80% of enzyme dioxinases are masked by catalysts. Catalysts can be effective in the elimination of the NO_2 as well as dioxins. At high temperatures, dioxins are degraded which is made up of Ti, V, Au, and W, respectively. One of the major advantages is dumping of the residues.

13.6.7 Dioxin Degradation Operations Using Electron Irradiation

The process of degradation of the dibenzo dioxin happens in a series of steps by associating with the flue gas. It possesses various features to rehabilitate the environment. By utilizing this methodology, there won't be any sort of pollution, the regulation of temperature is not needed, and also the process is quite feasible by

installing it to the already present incinerators. As a result, substances obtained after degradation are mainly organic compounds.

13.7 Formation of Furan

The industrial interest shown on furan is expressed in a large extent due to the existence of lignocellulosic hydrolysates. The amount of furan depends on the kind of lignocellulose utilized and pretreatment and hydrolysis process engaged. In nature, furfural is the plentiful form of the modest furan. Furanic compounds are allied with emaciated foods like honey, caffeinated items, and dried fruits. People are exposed to furfural through these foods. HMF is a furanic derivative found in human urine. Though it has carcinogenic effects, it has advantageous efficiency in therapeutizing diseases (Parawira and Tekere 2011).

It is vague to justify that the furanic aldehydes come into view solely from abiotic loss of hydration or enzymatic steps. Furfural and HMF are made abiotically via a series of process called "threefold dehydration of pentose." Sugars with six-membered rings are formed via catalyzing mineral acid. HMF and furfural are believed to be metabolites of fungus (Ding and Wang 2010; Almeida and Modig 2007). But it was vague that the formation was biogenic as its presence was recognized by heat sterilization of growth media. Furfural is chemically produced as solvent, building block for resins, flavor compounds, and also pharmaceuticals.

13.7.1 Furan-Degrading Microbes

Furan-degrading microbes were first isolated by Kakinuma and Yamatodani in 1964 which were proficient in developing furanic compounds as a carbon. Utmost efforts were put by the furan degraders to isolate the microorganisms which slowed down the phytotoxicity of torrefied grass, by means of an assay using germinated lettuce seeds. Interesting, only three fungal dibenzo furanic destroyers are known. Two of them were *Coniochaeta ligniaria* strains and *Amorphothecaresinae* ZN1. This might be triggered by the cultures which were enriched; as a result the bacteria grew rapidly. The fungi responsible for degrading furanic aldehyde were visually in greater count upon enriching the culture over a period of time. Long-term enrichment led to the documentation of a bigger number of furanic aldehyde-degrading fungus. Evidence of furfural regarding anaerobic degradation is correspondingly insufficient; merely two microbes, both the strains of *Desulfovibrio*, are identified to adapt furfural anaerobically, generating acetic acid.

13.7.2 Toxic Effects of Furan

In general, aldehydes are exceedingly sensitive molecules. It gets intensified and forms the reactive oxygen species. Cellular components like nucleic acids, cellular organelles, amino acids, proteins, and other components gets distorted when there is an association with the reactive oxygen species. Due to the expression of the genes in the aldehydic furans, it expresses stress response at a broader spectrum. And also, they applied numerous detailed effects (Koenig and Andreesen 1989). It leads to an amplified lag phase which could be overcome by significantly escalating the inoculum density. And also, the microorganisms help the aldehydic furans to get converted into alcohol through oxidation. But the COOH ameliorates its hazardous effects. As a result, there is an elimination of the aldehydes.

13.7.3 Biodegradation of Furan

Redox reaction of furanic aldehydes is observed to a certain extent. In the environment, there is resistance toward most aldehydes. But it does not mean that it gets degraded. And also, it is unsure about the magnitude of the metabolism of the complexes of furan. In certain scientific reports, it is seen that furans are slowly wiped out. As a result, it may lead to the misperception about the metabolic capability of furan "degrading" microorganisms. Hence, the different types of furans should be monitored in order to find out whether the aldehydic furans were converted or not into reduced noxiousness.

In 1969, P.W. Trudgill proposed a biochemical pathway for the aerobic degradation of the *Pseudomonas putida F2*. 2-Furoic acid is formed by the oxidation of furfural which is then tailed by the ligation by an enzyme named furoyl-CoA synthetase. In the fifth position of the carbon atom, furoyl-CoA dehydrogenase hydroxylatesfuroyl Co-A. The enol-CoA gets tautomerized to keto form. It has a lactone followed by opening up through spontaneous hydrolysis resulting in the formation of the oxoglutaroyl-CoA. 2-Oxoglutarate is released upon hydrolysis with the CoA thioester, which is metabolized through the TCA cycle.

In a scientific report published by Koenig and Andreesen in 1989, it is believed that upon adding the arsenite in the degradation pathway of *P. putida* Fu1 at enzymatic level, the end product (2-oxoglutarate) formed inhibited the 2-oxoglutarate dehydrogenase.

Based on the analysis done at the genetic level, an enzyme which is molybdenum dependent, i.e., furoyl dehydrogenase. The results attained from the strains (*P. putida* Fu1) reveal that in *C. basilensis* HMF14 growth wasn't hindered by the tungstate metal. And also, an amazing property of *Cupriavidus basilensis* HMF14 is avoiding going through the tungsten metal into the cell.

Apart from 2-furoic acid breakdown through the Trudgill pathway in *Cupriavidus basilensis* HMF14, HMF degradation pathway has also interpreted. In correspondence to furfural, HMF is initially oxidized to the analogous aliphatic monocarboxylic acid continued by the oxidation of 2,5-furandicarboxylic acid (FDCA) by highly selective oxidoreductase HmfH. 5-Formyl-2-furoic acid might form as transitional product upon oxidation. Currently, 5-formyl-2-furoic acid has not been seen in the supernatant of *Cupriavidus basilensis* HMF14 cultures on HMF (Koopman and Wierckx 2010; Wierckx and Koopman 2010) (Fig. 13.2).

Oxidation makes HMF and furfural from both alcohol and aldehydes by an enzyme named oxidoreductase. 2-Furoic acid and 5-hydroxymethyl-2-furoic acid were the breakdown paths of the furfural and HMF. But here the process of oxidation had been done by the enzyme, which was nonspecific and acted as a protective shield for the cellular organelles from the aldehydes (Figs. 13.3 and 13.4; Table 13.1).

Fig. 13.2 Schematic pathway for furan degradation via microbes

Fig. 13.3 Schematic pathway for dioxin degradation via microbes

Fig. 13.4 Mapping of the European *Environment Agency* (EEA) data on emission percentage

Table 13.1 Microbial degradation of dioxin and furan (2000–2020) (Armengaud and Timmis 1997; Thanh and Thi 2019)

Microorganism (bacteria, fungi, etc.) Species/genus name	Bacterial aerobic degradation-dioxygenase pathway	Substrate DF = dibenzo furans DD = dibenzodioxins	Reference
Sphingomonas wittichii RW1	Angular	Monochlorinated DDs/DFs	Armengaud et al. (2000)
Burkholderia sp. LB400	Angular	DD, DF	Seeger and Camara (2001)
Sphingomonas wittichii RW1	Angular	Trichlorinated DD/Fs	Hong et al. (2004)
Pseudomonas resinovorans CA10	Angular	DD, DF	Maeda and Nojiri (2003)
Cycloclasticus pugetii	Angular, lateral	DF	Fuse and Takimura (2003)
Janibacter sp. YY1	Angular, lateral	DF	Yamazoe and Yagi (2004)
Janibacter terrae XJ-1	Angular, lateral	DF	Jin and Zhu (2006)
Sphingomonas sp. XLDN2–5	Angular, lateral	DF	Gai and Yu (2007)
Rhodococcus sp. HA01	Angular, lateral	DF, 2-CDF, 3-CDF	Aly et al. (2008)
Pseudomonas sp. ISTDF1	Angular, lateral	DF	Jaiswal and Kohli (2011)
Rhodococcus sp. p52	Angular	DF	Peng and Haiyan (2013)

(continued)

Table 13.1 (continued)

Microorganism (bacteria, fungi, etc.) Species/genus name	Bacterial aerobic degradation-dioxygenase pathway	Substrate DF = dibenzo furans DD = dibenzodioxins	Reference
Agrobacterium sp. PH-08	Lateral, angular	DF	Le et al. (2014)
Pseudomonas aeruginosa FA-HZ1	Angular, lateral	DF	Ali and Hu (2019)
Paenibacillus naphthalenovorans 4B1	Angular	DF	Thanh and Thi (2019)

13.8 Conclusion

The US EPA has been consistently approving alternate test procedures for the detection of dioxins and furans. More stringent legislations must be implemented for the monitoring of dioxins and furans in global habitats to reduce both from the source. 90–95% of human exposure to dioxins and furans are via food chain which was acknowledged in the Stockholm Convention Article 5 which declares the decrease or removal of their releases in 2016. The need of novel technologies for reduction of the source using low-energy approaches has to be adapted for destroying/remediation dioxin and furan against the existing energy-intensive processes.

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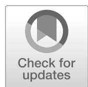
Chapter 14
The Management of Crude Oil Spill by Bioremediation Technique



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Abstract A process that uses decomposers and green plants, or their mixes, to improve the condition of polluted conditions is called bioremediation. Microorganisms can be used to clean up oil spills in the ocean through bioremediation. Unequivocal organisms can be used to bioremediate express contaminants, for instance, hydrocarbons, which are accessible in oil and gasoline. Oil spills in the ocean have a genuinely negative effect on marine life, especially seabirds and channel feeders. Oil in water gets concentrated inside and a while later accumulates in their predators in a higher concentration. With the sharp augmentation in people and modernization of society, regular sullying coming about in light of oil hydrocarbons has extended, achieving a basic prerequisite for remediation. Oil hydrocarbon-spoiling microorganisms are all inclusive in nature and can utilize these blends as wellsprings of carbon and essentialness. Infinitesimal creatures indicating such capacities are consistently manhandled for the bioremediation of oil-soiled circumstances. Starting late, microbial remediation advancement has developed rapidly and achieved noteworthy increments. Regardless, this development isn't extraordinary. It is affected by various common factors that irritated its helpful application, confining the tremendous degree of utilization of the advancement. The spillages of pipelines, oil wells, underground-accumulating tanks of the

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corner stores, ill-advised ejection of oil squander, and held oil slicks are the standard wellsprings of surface and groundwater contamination. Repairing measures for oil slicks are identified with disposing of oil to keep up a key decent ways from or turn ecological harm. Oil slicks can occur because of different causes, for example, gear disappointment, calamity, conscious immediate, or human blunder which portrays bioremediation as any cycle in which utilize microorganisms to tidy up and reestablish nature back to the past condition. The pattern of oil bioremediation by microorganisms where they use the trademark pollutions for their own absorption through biodegradation.

Keywords Bioremediation · Crude oil spill · Biodegradation · Bioaugmentation

14.1 Introduction

The activities in oil investigation, creation, and transportation may influence the world's condition to differing degrees. The spillages of pipelines, oil wells, underground stockpiling tanks of corner stores, ill-advised removal of oil squander, and held oil slicks are the principal wellsprings of surface and groundwater contamination. There are physical, synthetic, and natural strategies for the remediation of the oil slick. Natural strategies for the cleanup of these sorts of poisons are known as the bioremediation cycle. Bioremediation is a successful elective treatment apparatus that can be utilized in certain oil-defiled situations. During this cycle, microorganisms typically feed on the contaminations to get supplements and vitality for their development and propagation. Certain indigenous microbial networks may contain microbial populaces with various ordered connections that can debase raw petroleum spills. Certain hydrocarbons that are known to have unfriendly impacts are consequently specifically managed inside oil slick appraisal and tidy up work. The significant ones are BTEX (benzene, toluene, ethyl benzene, and xylene) and PAH (polycyclic fragrant hydrocarbons). Unpredictable natural mixes (VOCs) are the primary objective of the UNEP air quality review (UNEP 2011). Oil spills represent an immediate danger to the earth and require a fast and careful reaction. Healing measures for oil slicks are identified with disposing of oil to maintain a strategic distance from or turn around ecological harm. It is critical to begin eliminating oil from tainted regions promptly on the grounds that over the long haul and the oil climates, it will make more noteworthy harm the assets en route (Okereke et al. 2014). Bioremediation of unrefined petroleum-dirtied condition is a dull undertaking which is costly while applying the traditional technique for the purging oil, for example, utilizing dispersants, manual evacuation, utilizing synthetics, consuming, cutting vegetation, inactively gathering adsorbents, and eliminating garbage chips, digging, silt expulsion, mud and sandblasting, and so forth (Michel et al. 2010). As indicated by reference (Porto et al. 2011), the harm brought about by oil contamination to nature is hopeless. The above traditional techniques for limiting the effect

of unrefined petroleum spills in nature are of palliative arrangements which are consistently with no decisive decision about the issues caused. As the destructive impacts of oil slicks are getting more normal, more investigations were acted in the advancement of current innovations to manage the toxins brought about by the raw petroleum pills (United Nations Documentation Research Guide. United Nations 2011).

14.2 Role of Biofertilizers in Eliminating Hydrocarbon Pollutants

This is a successful, negligibly destructive, conservative, flexible, and ecologically benevolent elective treatment technique, called bioremediation (Finley et al. 2010). As a promising ecologically amicable treatment innovation for hydrocarbon remediation, bioremediation techniques are presently being very much advertised. Moreover, organic strategies can be more invaluable than physical and substance treatment techniques (Sathishkumar et al. 2008). The raw petroleum spills have become a significant irritating element for creating nations in the ongoing years. Oil slicks can happen because of numerous causes, for example, hardware disappointment, fiasco, conscious conduct, or human mistake (Anderson and LaBelle 2000), as the investigation exercises in the oil slicks ashore and ocean are expanding each spending years.

14.3 Bioremediation Technology

As per the reference (Lovley 2003) which characterizes bioremediation as any cycle in which the utilization of microorganisms to tidy up and reestablish nature back to the past condition. As indicated by the reports, the bioremediation innovation utilizing microorganisms was concocted in the 1960s by an associate area oil engineer in Santa Maria, California, named George Robinson (Meagher 2000), exploring different avenues regarding adaptable microorganisms with his tarnished container techniques. The oil bioredation cycle by microorganisms where they use the natural contaminations for their own digestion through biodegradation. The process of bioremediation in which biodegradation process was enhanced by bioaugmentation was described in the flowchart (Fig. 14.1).

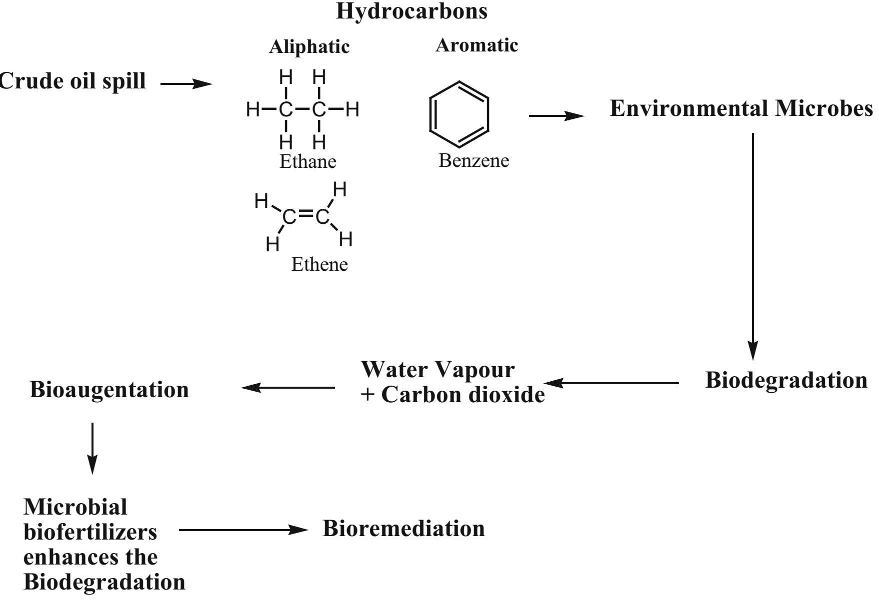

Fig. 14.1 The process of bioremediation of the crude oil spill flowchart

14.3.1 The Applications of Bioremediation Technique

The common biodegradation measure happened quickly in the Amoco Cadiz (Amoco Cadiz) unrefined petroleum spill which dirtied a huge territory of Brittany coastline in France in March 1978. Despite the fact that it might have been normal that the microbial populaces here will adjust to the debasement of hydrocarbons, since they are regularly presented to the arrival of counterweight water tanks, it isn't anticipated that the pace of corruption of low-atomic-weight hydrocarbons will be quicker or quicker than compound vanishing and disintegration. Prior to that spill, it was acknowledged that biodegradation just happened after a huge slack period and that the compound and physical enduring of raw petroleum consistently went before organic enduring (Atlas 1995). Notwithstanding mechanical reusing, the seashore likewise utilizes four distinctive bioremediation items. They will just prompt restricted and questionable outcomes. Anyway it isn't sure whether it was taken out by physical or natural media (Swannell et al. 1999). In June 1990, the Mega Borg oil slick took out bioremediation in uncovered water away from the shore of Texas, including the use of seed societies created by Alpha Corporation. The spilled oil has additionally been treated with a dispersant, and the oil is to some degree consumed. The Texas Land Department reports that the utilization of Alpha medium in the Mega Borg oil slick can adequately eliminate a lot of oil. In any case, there is no precise or autonomous adequacy observing. Despite what might be expected, the investigation demonstrated likely issues in marine bioremediation applications (Swannell et al. 1999). The Exxon Valdez oil spill in March 1989 caused the greatest

oil spill ever, with more than 2000 km of refueling coastline. Cleanup tasks include departure of mass oil, manual oil and adsorbent pads, mechanical developing, ejection or oiling of leftovers, and the process of bioremediation technique (Sugai et al. 1997). With respect to the last strategy, both microbial development and vaccination and natural improvement are viewed as bioremediation methods. The mishap in Galveston Bay, Texas, in July 1990, the spilled oil from the Apex flatboat was additionally exposed to an organic treatment of Alpha strains. The Texas Land Department indeed revealed that bioremediation was compelling. Nonetheless, autonomous perceptions demonstrate that the physical appearance of the treated oil has changed and may have been emulsified because of the expansion of Alpha items. The synthetic examination of tests from influenced and reference locales neglected to demonstrate that preparing with Alpha items has improved petroleum biodegradation. No noteworthy contrast in the C18/phytane proportion was found between the Al18-treated and untreated destinations, which demonstrates upgraded biodegradation. Thusly, experimentally substantial ends can't be attracted to affirm the viability of planting vast waters or beachfront spills. To tackle the issue of the adequacy of oil slick cultivating in untamed waters, clear plan and broad examinations are required, and proper control measures are embraced (Atlas 1995; Swannell et al. 1999). A trial dissected the viability of a specific bioremediation specialist in debasing oil spilled from the Arabian gulf. Financially accessible bacterial items comprise of a blend of common microorganisms. Oil corruption has been seen under various convergences of oil and included supplements and microorganisms (Fayad et al. 1992). The outcomes from the investigation show that adding supplements and microbes to the oil can upgrade the biodegradability of the n-paraffin portion in the oil. At the point when supplements are included alone, the level of upgrade is little, and without supplements or microscopic organisms, the microbial debasement of oil isn't self-evident. It is accepted that just including supplements can improve the biodegradability of oil, which is ascribed to the upgrade of the biodegradability of oil. The examination moreover found that the process of bioremediation perfectly works even more suitably at lower oil centers. At upper oil centers, qualifications were unreasonably little which uncommonly propose using the minuscule living beings developing over enhancement extension in a manner of speaking. Another examination concentrated on the connection among indigenous and cultivated microbial societies. The outcomes demonstrated that cultivating with nearby or unfamiliar oil-debasing microscopic organisms didn't prompt upgrade of hydrocarbon debasement and brought about emotional reductions in the quantities of the overwhelming, indigenous, oil-corrupting microorganisms, and hornet microscopic organisms in seawater (Fayad et al. 1992). Nearby microorganisms can undoubtedly build up their place in the sand on the Gulf Coast, while unfamiliar microscopic organisms are diminished or not endured. In any case, they despite everything lead to the debasement of hydrocarbons (Radwan et al. 1997). As a rule, the test ends up being effective, on the grounds that 1 year later, creepy crawlies and worms occupy the seashore. The way that the whole sullied zone of Kuwait (50 square kilometers of desert) was found to neglect to recoup agreeably is because of the absence of H_2O, significant for the indigenous microbial flora. Investigation presumed that

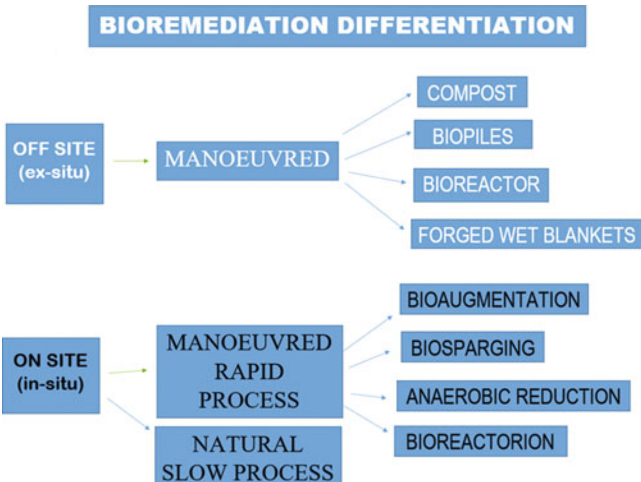

Fig. 14.2 The differentiation of bioremediation technique

indigenous microorganisms are appropriately overseen; bioremediation is ideal, which implies that dry territories must be watered if essential (Radwan et al. 1997). The differentiation of bioremediation process was explained in Fig. 14.2 in which both on-site (in situ) and off-site (ex situ) principles were mentioned.

14.4 Biodegradation Process

As per reference (Diaz 2004), the measure in which microorganism's changes over the unpredictable natural poisons into more straightforward substances by the enzymatic and metabolic movement is named as biodegradation, and these creatures are regularly named as "the best physicist on Earth" because of its geo ability profile (Madigan et al. 2012). The essential rule of the biodegradation of oil-polluted condition by microorganisms is to have the option to use the metabolic action of these microorganisms in the indigenous habitat that may influence their sustenance. Microbial biodegradation is a fundamental movement for the course of substance, and biodegradation is an ominous action that makes microorganisms decimate articles or materials with use and reason (Singleton and Sainsbury 2006). As indicated by the reference (Joanne et al. 2008), biodegradation can be portrayed in three fundamental changes of particles. It can depict minor changes in utilitarian gatherings joined to natural mixes, for example, supplanting chlorine with hydroxyl gatherings. It can likewise allude to the genuine cleavage of natural mixes into natural parts. Remaking the first atoms can at last portray the total debasement of natural mixes into minerals, otherwise called biomineralization.

14.4.1 Microbes in the Hydrocarbon Degradation Process

The commitment of microorganisms in the biodegradation cycle of unrefined petroleum spill is a fluid blend of different hydrocarbons from old green growth and plant remains and exists in stores underneath the surface (American Academy of Microbiology 2011) from the many individual segments with a few classes depending on related structures. Because these hydrocarbons from raw petroleum are normally happening in natural mixes, it isn't unexpected or astounding that microorganisms have built up the capacity to use these mixes. In amphibian and earthly territories, different microorganisms are identified with the biodegradation of oil hydrocarbons (Coronelli 1996; Ijah and Antai 2003). When the normal biological system is dirtied by the hydrocarbons of the unrefined petroleum, the indigenous microbial network may contain microbial populaces with various ordered connections, which can debase the sullied hydrocarbons (Atlas 1981). As per the reference (American Academy of Microbiology 2011), microorganisms that use the hydrocarbons as a vitality source have without a doubt existed for countless years, as long as this vitality rich substance that as of now exists. In the Gulf of Mexico where oil normally exists on the ground, aggregate microorganisms that feed on microorganisms have been built up, and these microorganisms on the whole tidied up all the various mixes contained in the oil by using the hydrocarbons as their storage facility of energy (American Academy of Microbiology 2011). Indeed, even where the foundation oil level is low, there consistently is by all accounts a few microorganisms with the capacity to debase oil. A wide range of sorts of microorganisms display the capacity to use oil, some of which are more adaptable than others. As per the reference (Ekpo and Udofia 2008), in which an examination on the biodegradation pace of raw petroleum by microorganisms disengaged from the slime condition indicated that the individual microbial types of *Pseudomonas aeruginosa*, *Micrococcus wacker*, and *Bacillus subtilis*. It was recorded that Pseudomonas aeruginosa has the most noteworthy debasement rate among them. The intricate blend of oil hydrocarbons, for example, raw petroleum and processing plant ooze, is required to change the action and structure of common microbial networks (Van Hemme et al. 2000). As indicated by the reference (Song and Bartha 1990), it was found that the extent of hydrocarbon-debasing microorganisms will enormously increment after presentation to hydrocarbons, which mirrors the selectivity of microorganisms to the carbon source. The capacity to corrupt oil hydrocarbons isn't restricted to a couple of microorganisms. An assortment of microorganisms and parasitic species have indicated promising results (Atlas 1981). The reference (ZoBell 1946) portrays that an excess of 100 species speaking to 30 microbial genera has demonstrated the capacity to use hydrocarbons. As per the reference (Bartha and Atlas 1977), there are 22 genera of microorganisms equipped for using hydrocarbons. Every one of these microorganisms are isolated from the oceanic condition. In the amphibian condition, the most significant genera for hydrocarbon clients (in view of the recurrence of confinement) are *Pseudomonas*, *Nocardia*, *Vibrio*, *Achromobacter*, *Corynebacterium*, *Flavobacterium*, *Arthrobacter*, *Micrococcus*, *Acinetobacter*, *Brevibacterium*,

Candida, *Rhododendron*, and spore fungus (Bartha and Atlas 1977). Some cyanobacteria and green growth have been tried for their capacities to use the hydrocarbons in the unrefined petroleum group. As per the reference (Walker et al. 1975), the utilization of algal protoplasts of chlorophyll strains for hydrocarbons was tried for hydrocarbon debasement. As per the reference (Cerniglia et al. 1980), the naphthalene-oxidizing capacity of nine cyanobacteria, five green growth, one red green growth, one earthy colored green growth, and two diatoms was illustrated. The study showed that the *Oscillatoria* microbes, *Microcoleus* microorganisms, *Anabaena* microscopic organisms, *Agmenellum* microscopic organisms, *Coccochloris* microscopic organisms, *Nostoc* microbes, *Aphanocapsa* microscopic organisms, *Chlorella* microorganisms, *Dunaliella* microscopic organisms, *Chlamydomonas* microbes, *Ulva* microbes, *Cylindrotheca* microscopic organisms, *Amphora* sp., and *Porphyridium* sp. Furthermore, *Petalonia* have the capacities to oxidize naphthalene. Their outcomes indicated that the capacity to oxidize sweet-smelling hydrocarbons is generally circulated in cyanobacteria and green growth (Atlas 1981). As indicated by the reference (Wolfgang 2007), *Pseudomonas putida* and *Pseudomonas fluorescens* have more noteworthy capacities to debase the hydrocarbons. The extraordinary capability of *Pseudomonas* depends on catabolic catalysts, yet additionally on their metabolic guideline capacity (Wackett 2003). There are numerous sorts of dioxygenases in the *Pseudomonas* species. Also, related strains have extraordinarily extended the scope of substrates that can be catabolized (Palleroni et al. 2005). The substrate explicitness of dioxygenase that follows up on sweet-smelling hydrocarbons is extremely wide (Wackett and Hershberger 2001).

14.4.2 Process of Aerobic and Anaerobic Degradation of Hydrocarbons

The nearness or nonattendance of oxygen for the most part decides the sort of biodegradation pathway and the sort and number of microbes associated with the biodegradation of explicit mixes (Wackett and Hershberger 2001). In genuine biodegradation, it is regularly essential to advance the decision of high-impact or anaerobic conditions. As per the reference (Wackett and Hershberger 2001), information has been aggregated about oxygen-consuming and facultative high-impact microorganisms. Pseudomonas and different forms. E. coli can be become for the time being with basic hardware to create high cell thickness. Interestingly, anaerobic advanced societies may at first show a long slack time before critical biodegradation happens. In the rehashed transmissions, the postpone period is generally abbreviated ceaselessly. It might take numerous years to arrive at a high biodegradation rate, and they may never accomplish an equivalent high-impact biodegradation rate (Wackett and Hershberger 2001). High-impact measures ordinarily produce more vitality, produce a comparing measure of ATP identical, and produce more biomass per unit of compound (Dagley 1975; Thauer and Rosazza 1977). Hence, exacerbates that

have a critical vitality yield when oxygen (O_2) is utilized to CO_2 and H_2O are commonly quickly biodegraded under high-impact conditions. The polycyclic fragrant hydrocarbon (PAH) mixes are normally corrupted under vigorous and anaerobic conditions. In the two cases, the key advance is the actuation of the inactive fragrant ring (Walter 2001). Within the sight of oxygen, this is finished by oxygen-subordinate proteins. Nonetheless, under anaerobic conditions, the rate and degree of hydrocarbon biodegradation are diminished, and the kinds of substrates that are debased are generally smaller (Coates et al. 1997; Bertrand et al. 1989). As per the reference (Wolfgang 2007) which characterizes high-impact biodegradation as the disintegration of natural poisons by microorganisms when oxygen is available explicitly alludes to a microbial reactant measure that happens or exists just within the sight of oxygen. Along these lines, the concoction qualities of the framework, condition, or living being are oxidizing conditions. Under oxygen-consuming conditions, numerous natural toxins will quickly debase. High-impact microorganisms have oxygen-based digestion, in which vigorous microscopic organisms are created through cell breath, and oxygen is utilized to oxidize substrates to get vitality. Before cell breath starts, glucose atoms are separated into littler particles in the cytoplasm. Cell oxygen is utilized in a synthetic response that separates little particles into H_2O and CO_2 in a response that discharges vitality. As per the reference (Wolfgang 2007), the most significant classes of natural toxins on Earth are mineral oil segments and halogenated results of petrochemical items. The capacity of vigorous microorganisms is especially applicable to the biodegradation of such mixes, as portrayed in the depiction of corruption. The study on aliphatic and sweet-smelling hydrocarbons and their chlorinated subordinates (Wolfgang 2007). The microorganisms equipped for the vigorous biodegradation incorporate *Rhodococcus* sp., Burkholderia xenovorans, and *Pseudomonas* sp. (McLeod and Eltis 2008). The defiled conditions are normally hypoxic, for example, springs, oceanic residue, and overwhelmed soil. In this condition, severe anaerobes or facultative microorganisms utilize elective electron acceptors for biodegradation (e.g., nitrate (de-nitrification creatures), sulfate-diminishing operator, and Fe(III) (iron particle-reducing specialist), and CO_2 (methane)) or different acceptors (e.g., chlorate, manganese, chromate, etc.) (Gibson and Harwood 2002; Lovley 2003; Widdel and Rabus 2001). Anaerobic biodegradation includes a progression of cycles where microorganisms deteriorate biodegradable materials without oxygen (Murphy 2004). Notwithstanding oxygen, the utilization of electron acceptors depends on the accessibility of electron acceptors and the opposition of electron benefactors by microorganisms of various respiratory sorts. For instance, the decrease of Fe(III) is the most widely recognized instrument for the oxidation of natural issue in the underground condition. Because of the high centralization of sulfate in seawater, it is the principal electron acceptor for the anaerobic corruption of contaminations in the marine condition (Lovley 2003). In terms of vitality, utilizing nitrate and Fe(III) as terminal electron acceptors to debase aromatics is nearly as powerful as utilizing oxygen, while sulfate-lessening specialists and methanogen conditions create substantially less vitality (Field et al. 1995). Thusly, the molar cell yield under states of methane creation and sulfide creation is very low. The microbial application of

sweet-smelling mixes. The distinctive terminal electron acceptors in the breath appeared in strong and lined up with the redox likely bar. The correct side shows the vitality (free vitality change) for high-impact and anaerobic debasement of the model fragrant compound benzoate. Methane creation should be combined with the aging response. Accordingly, the job of oxygen-consuming and anaerobic corruption measure fills in as a significant shelter in the evacuation of hydrocarbon debasement to limit the raw petroleum spill contaminations.

14.5 Major Technical Study of the Biodegradation of Crude Oil Spill

In a basic effort to choose the lifestyle that may be relevant to the cleanup of Prince William Sound, the EPA picked ten associated things for the research office stage testing. A couple of things have delayed biodegradation. Right when customary defilement occurs, most basic corruption will start after a concede season of 3–5 days and show up at a basic level after 20–30 days. Among the attempted things, two things were picked for extra field testing on the coastline impacted by the break in Prince William Sound. In field primers, four little plots were used to survey the reasonability of developing. These field tests fail to show that these things improved the oil's biodegradability of the grungy oil, which was significantly degraded (Atlas 1995). In development, the EPA has moved a thorough and huge extension that dares to use different manures on the dirtied shoreline of Prince William Sound. The explanation behind this is to display the update of biodegradation by including extraordinary kinds of excrements as nitrogen and phosphorus: lipophilic manure condition InipolTM EAP22 and granular moderate conveyance fertilizer Customblen. Lipophilic techniques are genuinely venerating oil. Inipol™ contains surfactants and enhancements expected to hold quick to the oil on the stone substrate and give supplements at the oil/air interface where microbial degradation occurs. A couple of watching ventures have assessed the reasonability of these manures in diminishing oil tainting and assessed anticipated regular impacts, for instance, supplement progression in adjacent waters and hurtfulness to marine life. The most faulty pieces of Prince William Sound's bioremediation application revolve around the 2-butoxyethanol fragment of Inipol™ and its normal hurtfulness to untamed life and cleaners. Workers' security rules should be followed while using Inipol™, and normal life obstructions should be used to deal with this issue inside the underlying 24 h when hurtfulness is of most concern (Hoff 1993). In the field tests, Inipol™ conveyed striking results, vivifying biodegradation, causing the smooth dim stone surface on the coastline to turn white inside in 10 days after the treatment, and there is apparently no surface oil (Pitchard et al. 1992). The eye-getting visual results solidly maintain the view that oil degradation in Prince William Sound is confined by enhancements, and fertilizer application is an important bioremediation philosophy (Atlas 1995). On account of its thriving, Inipol™

was asserted for shoreline treatment and used as a critical bit of cleanup work. Besides, Customblen has been applied. In around 2–3 weeks, the oil outwardly off the cobblestone coastline treated with Inipol™ and Customblen is debased, so these coastlines are essentially cleaner than non-bioremediated coastlines. Assessments have exhibited that the utilization of blend composts on the refueling coastline can keep up a higher number of oil-debasing microorganisms and augmentation the biodegradation rate, which is a result of the substance changes distinguished in the oil recovered from the treated and untreated reference centers attested (US Environmental Protection Agency 1990). As an outcome of the EPA-Exxon and joint checking adventures, bioremediation of oil-contaminated beaches is a secured cleanup strategy. The choice of fertilizers doesn't cause eutrophication, has no extraordinary hurtfulness to fragile marine test species, and doesn't cause the appearance of undegraded oil development on the beach (US Environmental Protection Agency 1990). Another field study focused on the effects of including composts. Studies have found that the pace of biodegradation prevalently depends upon the centralization of nitrogen, oil content, and the degree of ordinary biodegradation in the coastline. The more the oil has been tainted, the less incredible the bioremediation was found. Nevertheless, in light of the imbalance of the coastline and the oil level, the ideal fertilizer estimation will vacillate from site to site, and the ideal portion can't be foreseen early.

14.6 Issues Involved in the Application of Microbes in Crude Oil Biodegradation

There are snags in the application microorganisms for raw petroleum biodegradation. The biodegradation of unrefined petroleum contamination or ecological spillage is a mind-boggling cycle, and its quantitative and subjective viewpoints rely upon the nature and amount of the poisons present, the general condition and occasional ecological conditions, and the creation of the indigenous microbial network (Leahy and Colwell 1990; Hinchee and Olfenbuttel 1991). As per the reference (Sathishkumar et al. 2008), an examination was directed to investigate the chance of utilizing chosen bacterial societies and blended bacterial consortiums to corrupt unrefined petroleum at different pH, temperature, and oil, as outrageous pH and temperature relied upon to deliver a negative effect on the capacity of microbial populaces to debase hydrocarbons (Rahman et al. 2008). As indicated by the reference (Sathishkumar et al. 2008), the ideal temperature and pH of microorganisms show the best biodegradation potential. Hence natural conditions are not ideal for the microbial debasement species present on the oil contamination point, since the destiny of biodegradation to a great extent relies upon the nearby condition where the cycle of biodegradation eases back down as the ecological conditions that influence microbial development and protein movement, the ingestion and mineralization pace of numerous natural mixes by the microbial populace

relies upon the grouping of the compound (Olivera et al. 1997). The high grouping of undispersed unstable hydrocarbons may repress biodegradation. Because of high groupings of harmful toxins, poisonous solvents, outrageous pH, temperature, ionic quality, and so on, microorganisms used to remediate contaminations may endure ecological weight (Timmis and Pieper 1999). So as to decide the impact of fixation on microbial debasement, the reference (Sathishkumar et al. 2008) directed tests on choosing strains and blended greenery for various convergences of unrefined petroleum (1, 3, 6, 9, and 12%). For all fixations, tests were performed at 35 °C and pH 7. The carafes were vaccinated and hatched for 25 days, and bacterial development and raw petroleum debasement were assessed. The raw petroleum fixation was tried on the development of single-bacterial culture and blended bacterial consortium and their effect on unrefined petroleum debasement. The outcomes indicated that in 1% BH unrefined petroleum, blended bacterial consortium had 76% debasement, trailed by 72% (3%), 63% (6%), 52% (9%), and 41% (12%). A solitary culture likewise indicated great debasement potential in 1% BH unrefined petroleum, while corruption was decreased in higher centralizations of raw petroleum. Another significant test to the industriousness of certain fragrant hydrocarbon mixes in the earth is their restricted bioavailability (Diaz 2004). Generally, the current oil hydrocarbons frequently stick to soil particles, making them incapable to be utilized by corrupting microorganisms. Microorganisms that debase hydrocarbons will deliver biosurfactants with various compound properties and atomic sizes (Jagadevan and Mukherji 2004). These surface-dynamic substances increment the surface region of hydrophobic insoluble substrates and increment their bioavailability, in this manner expanding the development and bioremediation speed of microbes. The special marvel of surfactants is the self association of particles into dynamic bunches, called micelles (Jagadevan and Mukherji 2004). Volkering et al. (1993) tried to expand the biodegradation rate because of the creation of surface dynamic mixes delivered by microorganisms. Shockingly, since the solubilization of surfactants is essentially credited to the development of micelles, their investigation didn't improve the biodegradation rate.

14.7 Scope of Bioremediation of Crude Oil Spill

Despite the fact that the utilization of microorganisms in oil bioremediation has preferences, it is because of the way that microorganisms can utilize polyaromatic hydrocarbons as carbon and vitality sources. Their productivity in eliminating such contaminations may not be the most ideal approach to eliminate current contamination (Diaz 2004). Indeed, microorganisms have created toward biological versatility as opposed to biotechnology proficiency. Hence, it will take a long effort for microbes that can wipe out human contamination to develop through normal choice (Diaz 2004): phenomenal-modernized perspectives, pieces of information about the development of debasement pathways, and atomic transformation procedures that utilization microorganisms to change natural contamination conditions (Diaz 2004).

As a whole, these new advances can adequately lessen costs, decrease the utilization of synthetic compounds, and improve the money-saving advantage (Peixoto et al. 2011; Michael et al. 2020). Accordingly, examining the physiology, organic chemistry, and hereditary qualities of catabolic pathways has gotten pivotal for modifying and quickening regular cycles in test tubes and finishing their objective tasks to structure more viable biocatalysts for various biotechnological applications.

These include (i) Bioremediation of defiled destinations, (ii) natural change of harmful mixes into fine synthetic compounds and other high-worth included items (green science), and (iii) improvement of on the location of organic-observing hardware and biosensors to screen contaminations' bioavailability (De Lorenzo 2001; Schmid et al. 2001;Timmis and Pieper 1999). The advantages gave by atomic apparatuses can open up unlimited lucky chances, as it is conceivable to utilize undescribed catalysts to identify qualities from cultivable or non-culturable life forms (utilizing metagenomics) and express these qualities in cultivable creatures. While transferring recombinant microorganisms into any open condition, biosafety is a significant issue. To take care of this issue, a few hereditary circuits have been created to exist just in the defiled site and permit recombinant microorganisms to get by for the time required to eliminate the contaminant (biocontainment). So as to evade the spread of recombinant qualities from recombinant microorganisms to regular microbial populaces, distinctive quality incorporation circuits dependent on poisons and their homologous remedies have likewise been created. Such a functioning regulation framework fundamentally diminishes the potential dangers that the arrival of recombinant microbes may cause in the biological system (Ramos et al. 1995; Torres et al. 2003).

14.8 Conclusion

Bioremediation is a successful elective treatment method that can be applied in the specific raw petroleum-contaminated conditions where the microorganisms for the most part feed on the natural poisons, changing it into their vitality hotspot for their development and propagation. This is a generally moderate cycle, and it takes a long time for tidying up. Whenever done accurately, albeit no inside and out monetary investigation has been directed up until now, it might be very expensive and effective. The cycle can be vigorous or anaerobic, contingent upon the microorganism and accessible electron acceptors. This cycle might be regular (interior bioremediation), or it might be misleadingly upgraded (built bioremediation). The exceptional consequences of the genuine bioremediation application on location checking affirmed the hypothetical data establishment set up by past logical examination. Analysts regularly demonstrate that indigenous microorganisms ordinarily have an upper hand over unfamiliar or presented strains. Realities have demonstrated that adding supplements to nearby microorganisms as composts can viably advance biodegradation for a more secure condition. It has additionally been seen that there are microorganisms fit for debasing oil in practically all waterfront situations.

Despite supplements, normal limits similarly impact the certified corruption pace of oil fields. As such, the field usage of enhancements is so far impacted to some degree because of temperature, H_2O flood, the substrates, and common limits, which were not totally seen or easily estimated. In any case, as experience has indicated that no single innovation is appropriate for all occasions that require a reaction after an oil slick, bioremediation is as yet required in the cleanup of marine oil spills. Finally, numerous advantages can be picked up from the fast cleanup of oil slicks, some of which have nothing to do with marine biological systems however with others. These incorporate the financial effect brought about by the recreation utilization of the coastline, the settlement of lawful obligations and necessities, and tasteful contemplations. Bioremediation has been demonstrated to be effective for oil and hydrocarbon contamination. Its points of interest ordinarily exceed its detriments. The extent of bioremediation of raw petroleum spill is constantly considered as an aid to tidy up the dirtied conditions; however, more exploration should have been accomplished for the headway of this method to adapt up to the ecological requests.

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Chapter 15
Bacterial Pigments: An Untapped Colorful Microbial World

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Abstract Microbes are serving as the precious sources of diverse molecules used in the discovery of various drugs. Among them are the microbial pigments – colored metabolite produced by microbes like bacteria, fungi, algae, and actinomycetes. Microbial pigments are preferred over all the other sources for its simple culturing methods using cost-effective substrates, easy pigment extraction procedures, and mass production. Bacterial pigments are nontoxic, biodegradable, noncarcinogenic, safe for human use, and have a diverse range of applications in food industries, textile, cosmetics, and pharmaceuticals. Identification of new microbial sources, utilization of low-cost substrates, and optimization of process parameters are the areas under focus toward economical pigment production.

Keywords Microbial pigments · Textile · Cosmetics · Food industry · Therapeutic applications

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15.1 Introduction

Human being are always attracted toward colors in many products as foodstuffs, clothes, paintings, beauty products, and many more. The use of natural colors over synthetic is seen since prehistoric times; the nails of Egyptian mummies were found to be dyed with the henna leaves. Until the mid-nineteenth century, all dyes were obtained from animal or plant extracts. The textile industry used natural pigments, such as turmeric, cochineal, wood madder, or henna. From centuries the extraction of colors was seen from various natural sources as from plant roots (*Rubia tinctorum*), insects (Kermes, *Laccifer lacca*), and rhizomes (*Rheum emodi*, *Curcuma longa*) (Venil et al. 2013a, b; Yusuf et al. 2017) for its safe application. The first industry for the production of synthetic dyes was established by H. Perkin in the year 1856. Following the artificial synthesis, the discovery of diazotization and a coupling reaction by Peter Griess was a major advancement in the color industry (Socaciu 2007). Extensive research and developments in the production of synthetic organic dyes by nineteenth century enhanced the quality, cost-effectiveness, and applicative range of colorants. The economic significance of the color industry is clearly reflected in the large number of synthesized compounds; as many as 700 colorants are currently available (Sikorski et al. 2006).

Due to severe criticism suffered for food components predominantly food pigments, today all food colorants are caustically and meticulously regulated by federal authorities to ensure the safety of food production (Malik et al. 2012a, b; Socaciu 2007). To overcome this issues, the use of pigments from natural sources for various applications is of worldwide interest (Sikorski et al. 2006). It is, therefore, essential to explore various natural sources of food-grade pigments and their potential role (Socaciu 2007). The utilization of natural pigments in foodstuff, dyestuff, and cosmetic and pharmaceutical manufacturing processes has been mounting in recent years (Mortensen 2006; Venil et al. 2013a, b). Natural colorants or dyes derived from flora, fauna, and microbes are believed to be safer, being nontoxic, noncarcinogenic, and biodegradable in nature.

Due to a few constraints as solubility and stability of pigments from plants and insects, microbes (Fig. 15.1) have got attention as a pigment source and thus have

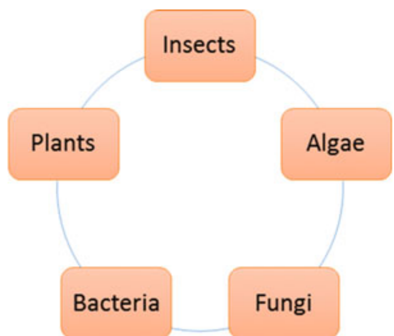

Fig. 15.1 Natural sources of pigments

challenged the researchers to explore the colorful microbial world. Various groups of microbes are found to produce pigments as metabolite (Durán et al. 2002). Algae and cyanobacteria produce beta-carotene. Astaxanthin is produced by *Phaffia rohodozyma* and *Haematococcus pluvialis* and yeast by *Xanthophyllomyces*. Fungi like *Penicillium* (red color), *Monascus* (Monascus) pigment respectively. Actinomycetes like *Streptomyces* are seen to produce prodigiosin. Bacteria like *Sarcina*, *Cryptococcus*, and *Rhodotorula* also produce a variety of pigments. Bacterial pigments are found to have broad applications in food industry, textile, cosmetics, and pharmaceuticals.

A diverse group of microbial community is reported for the production of pigments as shown in the Table 15.1 (Joshi et al. 2003; Kamla et al. 2012).

15.2 Types of Pigments

Microbial colors (pigments) are classified on different criteria as origin, color, stability, and site of production.

15.2.1 On the Basis Origin

Various microbes are seen to produce pigments. Thus can be classified into the following:

Bacterial pigments: Many bacterial genera are seen to produce impressive colors as one of their metabolites. It includes bacteria like *Flavobacterium* (zeaxanthin), *Rhodococcus* (canthaxanthin), *Chromobacterium* (violacein), *Staphylococcus* (staphyloxanthin), etc.

Fungal pigments: Fungi saprophytic eukaryotes are also found to produce pigments. The colorful world of fungi make them attractive when cultured in vitro. It includes *Monascus* sp. (Monascus), *Penicillium* sp. (Arpink), *Cryptococcus* sp. (melanin), *Rhodotorula* sp. (ß-carotene), etc.

Algal pigments: Autotrophic eukaryotes carry on photosynthesis with the aid of pigments produced in their cells. It includes *Dunaliella* (ß-carotene), *Rhodophyta* (phycocyanin and phycoerythrins), *Chlamydomonas* (lutein), etc.

15.2.2 On the Basis of Color

Diverse groups of microbes are producing various colored compounds as their metabolite. Pigments are classified as follows:

Table 15.1 Pigment-producing microbes

Microorganism(s)	Pigments/molecule	Pigments/ molecule
Bacteria		
Agrobacterium aurantiacum	Astaxanthin	Pink-red
Paracoccus carotinifaciens	Astaxanthin	Pink-red
Bradyrhizobium sp.	Canthaxanthin	Dark red
Flavobacterium sp., *Paracoccus zeaxanthinifaciens*	Zeaxanthin	Yellow
Achromobacter	–	Creamy
Bacillus	–	Brown
Brevibacterium sp.	–	Orange-yellow
Corynebacterium michigannise	–	Greyish to creamish
Corynebacterium insidiosum	Indigoidine	Blue
Rugamonas rubra, *Streptoverticillium rubrireticuli*, *vibrio gaogenes*, *Alteromonas rubra*	Prodigiosin	Red
Rhodococcus maris	–	Bluish-red
Xanthophyllomyces dendrorhous	Astaxanthin	Pink-red
Haloferax alexandrinus	Canthaxanthin	Dark red
Staphylococcus aureus	Staphyloxanthin Zeaxanthin	Golden yellow
Chromobacterium violaceum	Violacein	Purple
Serratia marcescens, *Serratia rubidaea*	Prodigiosin	Red
Pseudomonas aeruginosa	Pyocyanin	Blue-green
Xanthomonas oryzae	Xanthomonadin	Yellow
Janthinobacterium lividum	Violacein	Purple
Algae	–	
Dunaliella salina	β-Carotene	Red
Chlorococcum	Lutein	
Haematococcus	Canthaxanthin	
Fungi	–	
Aspergillus sp.	–	Orange-red
Aspergillus glaucus	–	Dark red
Blakeslea trispora	β-Carotene	Cream
Helminthosporium catenarium	–	Red
Helminthosporium avenae	–	Bronze
Penicillium cyclopium	–	Orange
Penicillium nalgiovense	–	Yellow
Fusarium sporotrichioides	Lycopene	Red
Haematococcus pluvialis	Astaxanthin	Red
Monascus sp.	Monascorubramin Rubropunctatin	Red-orange
Monascus purpureus	Monascin and ankaflavin	Red-yellow

(continued)

Table 15.1 (continued)

Microorganism(s)	Pigments/molecule	Pigments/ molecule
Monascus roseus	Canthaxanthin	Orange-pink
Monascus sp.	Ankaflavin	Yellow
Penicillium oxalicum	Anthraquinone	Red
Blakeslea trispora	Lycopene	Red
Cordyceps unilateralis	Naphthoquinone	Deep bloodred
Ashbya gossypii	Riboflavin	Yellow
Mucor circinelloides, *Neurospora crassa*, and *Phycomyces blakesleeanus*	β-Carotene	Yellow-orange
Penicillium purpurogenum, *Paecilomyces sinclairii*	–	Red
Paecilomyces farinosus	Anthraquinone	Red
Yeast		
Cryptococcus sp.	–	Red
Saccharomyces neoformans var. *nigricans*	Melanin	Black
Phaffia rhodozyma	Astaxanthin	Pink-red
Rhodotorula sp., *Rhodotorula glutinis*	Torularhodin	Orange-red
Yarrowia lipolytica	–	Brown
Actinomycetes		
Streptoverticillium rubrireticuli	Prodigiosin	Red
Streptomyces echinoruber	Rubrolone	Red

Yellow (lutein, riboflavin, monascin).
Blue (indigoidine, pyocyanin).
Red (prodigiosin, carotenoid, lycopene).

15.2.3 Based on Solubility

Microbial pigments are soluble in specific solvents. Depending upon their solubility, they are classified as follows:

Water soluble: The pigments soluble in water are anthocyanins, xanthophylls, etc.

Fat soluble: The pigments soluble in fat are canthaxanthin, carotenes, etc.

15.2.4 Based upon the Site of Production and Accumulation

Bacterial pigments are one of various secondary metabolites produced in the cells. Depending upon their accumulation, they are classified as follows:

Intracellular pigments: These are produced inside the microbial cells and observed as colored colonies appearing on the surface of slant or streak (e.g., *Micrococcus luteus*, *Serratia marcescens*, *Rhodospirillum*, *Micrococcus roseus*, *Streptomyces albus*, *Chromobacterium*).

Extracellular pigments: These pigments are produced within the cell but are secreted out of the cell. As these pigments are water-soluble, they diffuse in the surrounding medium to impart color to the medium (e.g., *Pseudomonas aeruginosa*, *Pseudo fluorescens*).

15.3 Bacterial Pigments

Because of less generation time, simple culturing techniques, easy genetic modification, cost-effective culture media, low cost of production, simple extraction procedures, and broad range of sources, bacteria have attracted various researchers as a choice of microbe for pigment production (Ahmad et al. 2012; Rao et al. 2017). Pigment-producing bacteria are found in all habitats and niches as soil (rhizosphere, desert), water (fresh water, marine water, industrial effluents), and endophytes and also found in a wide range of physical parameters as aerobic, anaerobic, high temperatures, high salt concentration, and high saline content.

Bacteria produce a large number of secondary metabolites; among them are a variety of pigment – colored – compounds ranging from red, yellow, orange, blue, green, and purple. These pigments are reported to exhibit a wide range of biological properties which are exploited for the betterment of human kind. Researchers are attracted to explore different sources for the isolation of pigment-producing bacteria.

15.3.1 Carotenoids

Carotenoids are the organic polyunsaturated hydrocarbon (40 carbon) with conjugated double bonds, situated in chromatophores of cytoplasmic membrane. These pigments are produced by plants, algae, fungi, and bacteria. There are about 1100 carotenoids known to date. Basically they are two types of carotenoids on the basis of the presence and absence of oxygen in the hydrocarbon chain.

1. **Xanthophylls** (lutein, zeaxanthin) contain oxygen in the hydrocarbon.
2. **Carotenes** (ß-carotene, lycopene) are strict hydrocarbons without any substituents, even oxygen.
3. **Canthaxanthin** and **echinenone** contain C=O group in their structure.

Astaxanthin contains both –OH and C=O groups in its structure. Thus astaxanthin, zeaxanthin, and canthaxanthin are the types of carotenoids (Sathasivam and Ki 2018). Halophilic bacterium *Cytophaga-Flavobacterium-Bacteroides* produced red- to pink-colored pigment from Balearic Island in Mallorca, Spain (Oren

and Rodríguez-Valera 2001) and astaxanthin from *Agrobacterium aurantiacum* and *Paracoccus haeundaensis* (Misawa et al. 1995; Lee et al. 2004). Halobacteria and extremophiles found in marine waters with high salinity are also reported to produce a considerable amount of carotenoids, specifically bacterioruberin and its C50-related pigments (Rodrigo-Baños et al. 2015).

15.3.2 Prodigiosin

Pigment "prodigiosin" was first named for its isolation from bacteria *Bacillus prodigiosus* (which was later renamed as *Serratia marcescens*) (Gerber 1975). The same bacteria have a historic fame for the appearance of red color on "bleeding bread" report (Bennett and Bentley 2000; Williamson et al. 2007). Prodigiosin is found to be produced from bacteria from different sources such as fresh waters, marine water, and even terrestrial habitat (Dufossé 2006). Prodigiosin is a red-colored pigment with pyrrolylpyrromethane as the core structure (Montaner and Pérez-Tomás 2003; Williamson et al. 2007). These biological properties attributed by prodigiosin like antibacterial, antibiotic, antimalarial, immunosuppressive, antifungal, and anticancer activities make them one of the most powerful research tools (Montaner and Pérez-Tomás 2003). Besides *Serratia*, several other bacterial species of marine waters as *Streptomyces*, *Actinomadura*, *Pseudomonas*, and *Pseudoalteromonas* have also been reported to produce prodigiosin and related compounds. Some unrelated microbial strains, such as *Hahella chejuensis*, *Streptomyces griseoviridis*, and *Vibrio psychroerythrus*, are also found to produce prodigiosin (Gulani et al. 2012).

15.3.3 Violacein

Violacein a violet-colored pigment with an indole derivative, having prominent biological properties, which includes anticancer, antiviral, antibacterial, antiulcerogenic, antileishmanial, antipyretic, trypanocidal, and antinematode activities (Gauthier et al. 1975; Bilsland et al. 2018). Violacein was extracted for the first time from marine bacterium *Chromobacterium marinum* isolated from open ocean waters (Hamilton and Austin 1967). It was later reported by many researchers reported to be produced from marine bacterium which produced varied shades of pigments as pinkish beige with reddish-brown diffusible pigment, lemon yellow, bright red turning carmine in old cultures, and orange to greenish-brown (Bilsland et al. 2018). Cytotoxicity of violacein extracted from *Chromobacterium violaceum* was studied on cell lines as HL60 leukemia cells (Ferreira et al. 2004). Violacein is also found to be active against wild-type and drug-resistant strains of *Plasmodium falciparum* a malaria parasite (Gauthier et al. 1975). Music treatment on

Chromobacterium violaceum and *Saccharomyces cerevisiae* shown increased production of violacein and alcohol, respectively (Chandra et al. 2018).

15.3.4 Xanthomonadin

Xanthomonadin a membrane (cytoplasmic membrane)-bound pigment was isolated from *Xanthomonas juglandis*. These are yellow-colored pigments serving as useful chemotaxonomic and diagnostic markers (Poplawsky et al. 2000). A gene cluster of 23 Kb synthesizing xanthomonadin, sequenced from *Xanthomonas campestris* pv., was cloned (Poplawsky et al. 1993). The free radical scavenging property of xanthomonadin was studied and served to protect the bacterial membrane from oxidative damage (Rajagopal et al. 1997).

15.4 Physical Parameters Influencing Pigment Production

Pigments are secondary metabolites synthesized by bacterial cells and is seen to be influenced by various physicochemical parameters as pH, temperature, incubation time, salt concentration, environmental stress, aeration, light, carbon and nitrogen source, and the nature of medium. The optimization growth conditions of microorganisms, particularly physical and nutritional parameters, are of prime importance in the development of any pigment production process, owing to their impact on the economy and practicability of the process. Medium optimization and physical conditions have been customarily performed using the one-factor-at-a-time method. The disadvantages of such a classical method are that it is time-consuming, laborious, and expensive; in addition, it fails to resolve the combined effect of different factors (Malik et al. 2012a, b; Kim and Seockmo 2018).

15.4.1 Temperature

Bacteria are found in a diverse range of temperature from psychrophilic to mesophilic to thermophilic. Pigments are naturally produced as secondary metabolites by bacteria in a wide range of temperature. The growth of *S. marcescens* MBB05 and the highest pigment-prodigiosin production are 151.0 mg/mL at 30 °C (Bhathini et al. 2017), while the intensity of pigment produced by *Cryptococcus* sp. was found to increase after preserving them in broth at 40 °C in the refrigerator, thus indicating the effect of lower temperature (Samyuktha and Mahajan 2016). Xanthophyll pigment by *Salinicoccus* sp. M KJ997975 was optimum at a pH of 7 and at a temperature of 30 °C in the presence of light and under shaking conditions, thus showing the effect of cultural conditions on the growth and

pigment production (Bhat and Marar 2015). The growth of *Monascus* sp. entails 25–28 °C for the production of pigment, whereas *Pseudomonas* sp. requires 35–36 °C for its growth and pigment production (Ibrahim et al. 2014).

15.4.2 pH

pH as temperature is another physical parameter which influences the growth and kind of pigment by microorganisms. A slight change in the pH is seen to alter the shade of the pigment, and also different colored pigments are seen to be produced at different pH. *S. marcescens* MBB05 was observed for prodigiosin production on a wide range of pH. Pigment production was seen at all ranges from acidic-neutral-alkaline pH, but was seen to be highest at 164.4 mg/mL at alkaline medium with pH of 8.0 to 9.5 (Bhathini et al. 2017). The yield of astaxanthin from Phaffia rhodozyma was 325 to 212 μg/g astaxanthin at a pH of 6.5 to 3.5 (Bennett and Bentley 2000). Mesophilic marine isolates of *Aquisalibacillus elongatus* MB592, Salinicoccus sesuvii MB597, and Halomonas aquamarina MB598 produced a significant amount of pigment at neutral pH (Fariq et al. 2019). The combined effect of pH and nitrogen sources affect significantly the production of pigment by *Monascus purpureus* (Patrovsky et al. 2019).

15.4.3 Light

Like other parameters, light also greatly influences the pigment production. According to a very old study with acid-fast organisms, it was observed that there was intense production of pigment when bacterial culture was incubated in the presence of light, whereas the same were depigmented when incubated in the dark (Baker 1938). This observation was supported by a pretty old reference from the year 1887. Further the experiment on many bacterial isolates with different sources of light as 100 watt Mazda lamp, UV light, and sunlight concluded that shorter light rays affect pigmentation. Pigmentation can be induced by incubating the bacterial isolates in light. Colored lights have its own effect on growth and pigment production by microbes, and a variety of pigments are produced at different concentration of *Spirulina platensis* when cultured in white-, blue-, yellow-, and red-colored light (Chainapong et al. 2012). A considerable amount of anthocyanin pigment was produced in a bubble column bioreactor in 10 days of incubation with continuous irradiation (Zhong et al. 1991), thus proving that not only different colors have influence on pigment production but the exposure time also plays a significant role.

15.4.4 Incubation Time

The growth of pigmented bacteria is seen after a quite sufficient incubation. Most of the studies showed the growth of pigment bacteria between 36 h and 96 h of incubation. Also some of the studies showed different pigment formations from the same bacterial isolate at different incubation time. Besides the different shades of the same color, pigment is also seen at various incubation time. The reason for all the abovementioned variations is not properly studied and becomes a challenge to the researchers. Mycobacterial species as *M. smegmatis* and *M. goodie* produced pigments only after prolonged incubation on agar plates of 7–10 days (Saviola 2014), which, of course, was due to the production of acids in the agar media. *Micrococcus* spp. showed maximum growth and pigment yield of 0.6289 mg at pH 9 after 96 h of incubation time (Athira et al. 2016).

15.4.5 Aeration and Agitation

Bacteria have diverse requirements of oxygen in terms of the production of metabolites. Bacterial cultures in broth show quicker and more production of pigments when aerated and agitated, which may be because of the proper distribution of nutrients throughout the media and oxygenation of media, of course with aerobic microorganisms. But when bacteria are grown on agar medium and aerobically, time taken for pigment production was prolonged. Bacterial cultures were tested for pigment production with and without oxygen supply and was reported that aerobic microorganisms produce orange-colored pigments (Baker 1938). The effects of pH, temperature, aeration rate, initial sugar and ammonium sulphate concentrations, and activator (cotton seed oil and Tween 80) addition on the growth and carotenoid production properties of *Rhodotorula mucilaginosa*, a soil yeast was investigated in a batch system with the result of total carotenoids concentration and carotenoids production yield were significantly increased with increasing aeration rate up to 2.4 vvm (Aksu and Eren 2005).

15.4.6 The Type of Fermentation

Standardization of media and fermentation conditions are the prime conditions studied by researchers for the maximum production of pigments. Studies showed that the selection of the type of fermentation affects greatly on the pigment production. It was reported that solid-state fermentation yields threefold more pigmentation than in the liquid-state of submerged fermentation (Bhathini et al. 2011). Pigment colors produced by microbes vary with the substrates added in the fermentation

media. The addition of all 20 amino acids in the media showed different colored pigments as yellow, orange, and red (Jung et al. 2003).

15.4.7 Salt Concentration

The effect of salt concentration (NaCl) was also found to be one of the important parameter influencing the pigment production. When bacterial isolates were inoculated in media with varied concentration of NaCl ranging from 0.5%, 1%, 2%, 4%, 6%, to 8%, the growth of bacteria was seen at all salt concentrations (Samyuktha and Mahajan 2016). A remarkable production of pigment was observed with sodium chloride and magnesium sulphate, and enhanced pigment production with sodium acetate was found in *Haloferax mediterranei* ATCC 33500 (Fang et al. 2010).

15.4.8 Environmental Stress

Bacteria produce pigments as secondary metabolites constitutively and under environmental stress. Studies showed that the species of *Synechococcus* produced carotenoids at low iron stress. Similarly, *Acinetobacter wolfii* was used for the production of pigment by exposing them to methanol. *Vibrio cholera* also was found to produce pigment under hyperosmotic stress and in acidic conditions. *Mycobacteria* also produced pigment at acidic stress at pH 5.0–6.0 (Saviola 2014).

15.4.9 The Effect of Music and Sound Waves

Music not only influences the human mind by releasing stress but also has been seen to have soothing effects on animal behavior. Recent studies revealed that sound waves have a remarkable effect on bacterial growth and also on the production of their metabolites. And this breakthrough became an interesting tool to investigate the factors to manipulate the microbial metabolites for commercial purpose. Many studies have been carried out on the effect of sonic range of sound on the growth and production of metabolites. Sonic stimulations of 300 Hz sound at different levels on *Chromobacterium violaceum* were studied. This resulted in the enhanced bacterial growth and significant production of quorum-sensing regulatory pigment – violacein (Fang et al. 2010 and Sarvaiya et al. 2016). A similar kind of study was conducted on marine bacterium *Brevibacterium* sp. And it was found that the growth of bacterium and the production of yellow-colored pigment were enhanced (Sarvaiya et al. 2016). The effect of sonic sound at varied parameters like the frequency of sound, distance between the source and bacterial culture, and time of exposure was analyzed to check its effect on the growth and pigment production of a marine

bacterium *Kocuria flava*. Remarkable increase in the yield (from 18.18 μg/mL to 86.33 μg/mL) of pigment was observed, at optimized conditions of 24 kHz frequency for 20 min. Bacterial pigments (prodigiosin and violacein) was found to be produced under the influence of audible sound by *Chromobacterium violaceum* and *S. marcescens*. These two bacteria were also found to show enhanced antibiotic susceptibility (3.81–18.69%) influenced by the sonic sound. These sound waves seem to increase the membrane permeability of the bacteria (Sarvaiya et al. 2016).

15.5 Applications of Bacterial Pigments

Colors are an inseparable component of human life. Natural sources of colors are always preferred for their safe application. Bacterial pigments due to their versatile nature have a wide range of applications in various fields (Fig. 15.2) (Venil et al. 2014).

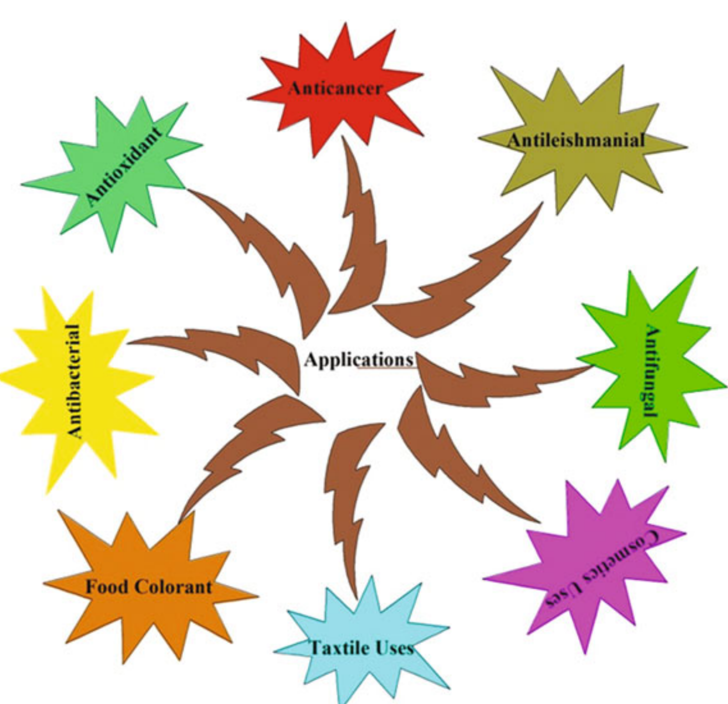

Fig. 15.2 Applications of bacterial pigments in various industries

15.5.1 Pigments in Cosmetics

The human skin is a first line of defense, an anatomical barrier for various pathogens. Overexposure to UV radiations on the skin often leads to skin damage resulting in wrinkle formations, coarseness, laxity, and mottled pigmentation (Pallela et al. 2010). Pigment melamin present in the skin often protects the skin from the continuous exposure to UV radiations which causes skin damage and sunburns (Ahmad et al. 2012; Venil et al. 2013a, b). For such deformations and damages, a mechanism of photoprotection is well developed by nature in terms of melanins (humans, animals, some bacteria). Besides many such photoprotective compounds are diversely present in nature – scytonemins (exclusively in cyanobacteria), mycosporine-like amino acids (MAAs, in cyanobacteria, algae, and animals), and mycosporines (in fungi) (Masuelli et al. 2016; Krishnan et al. 2017; Barretto and Vootla 2018). Thus, studies reveal that astaxanthin can be best used in cosmetics (as topical and oral use) to reduce hyperpigmentation of the skin, inhibit melanin synthesis, and improve texture and condition of the skin.

15.5.2 Pigments in Food Industries

Colors are the main features in food for the choice by the consumer, being attractive and also appearance and texture enhancer of food. The use of colors in food started in the ancient times; in the course of time, attention is grabbed toward the usage of natural colors to keep oneself away from allergic and other side effects of synthetic colors. Supplementation of bacterial pigments canthaxanthin from *Bradyrhizobium* sp. in poultry feeds (yellow pigment known as zeaxanthin) is seen to enhance the skin color in animals and yolk color in eggs. The pigment canthaxanthin was extracted from *Bradyrhizobium* sp., and gene sequencing was carried out (Poplawsky et al. 1993). Extreme halophile *Halobacterium* isolated from salt farm was found to produce keto-carotenoid (Rajagopal et al. 1997). Due to the deposition of minute crystals of the pigments in the eyes of animals found after the overdosage of the feed supplemented with the pigments, there was a pressure on the limit to use the aqua feed. Biosynthesis of astaxanthin from extreme halophile halobacterium was published by Yokoyama et al. in 1994 (Kumar et al. 2015). Astaxanthin is one of the ten carotenoid pigments that can be directly extracted from sunflower oil instead of organic solvents, which was assumed to help in the assimilation of pigments from animals (Dufossé 2006). Melanin-mediated silver nanostructures are used in food packaging industries (Woodhams et al. 2018).

15.5.3 Pigments in Textile Industries

Colored fabrics are the attraction of all time generation. Coloration to fabrics is an age-old practice using both natural and synthetic colors. Synthetic colorants are popular to give numerous shades, stability, and cheaper prices, and thus are more in use. However due to a few challenges with the use of synthetic colors, such as recovery of colors from nonrenewable resources, environmental toxicity, and human health issues, the need to search natural sources for colors has emerged. Pigments from plant sources were used prior to synthetic colors but was found to be less ecofriendly with very low yields. Thus microbial sources (fungi, bacteria) are explored to meet the demand of colorants used in fabrics. The problems faced with plant colors were also overcome with the microbes such as stability of colors, variety of colors, and increase yield by fermentation technology. Moreover, these microbial colors are also exhibiting antimicrobial activity which was another benefit to the textile industries. Prodigiosin a deep red pigment extracted from marine bacterial isolate *Vibrio* sp. (related to *Vibrio gazogenes* ATCC 29988 T) was used to dye fabrics (Alihosseini et al. 2008). The stability of colored fabric with prodigiosin was tested at various pH and temperatures with the result of less stability at acidic pH and high temperature. Also the colored fabric was antimicrobial in activity against *E. coli* and *Staphylococcus aureus*. This pigment could dye wool, silk, nylon, and acrylic fabrics (Malik et al. 2012a, b). Indole a blue-colored pigment extracted from *Pseudomonas* sp. HOB1 was successfully used to color cotton fabrics (Chainapong et al. 2012).

15.5.4 Pigments in Therapeutics

Bacterial pigments are versatile molecules having a broad range of therapeutic applications and thus are a boon to human race. They affect in many ways the human host cells as well as microbial cells. Mechanisms of action of many bacterial pigments are still to be studied and understood properly. This is because the structure and exact chemical composition of many of the pigment are yet to be recorded.

15.5.4.1 Bacteriostatic and Antibacterial Properties of Pigments

The molecule that controls the growth of bacteria by inhibiting their reproduction is bacteriostatic in nature. These molecules may not be bacteriocidal, but are reported to be bacteriostatic in activity. A red pigment was isolated initially from *Bacillus prodigiosus*, renamed as *Serratia marcescens* (Gerber 1975; Darshan and Manonmani 2015). Prodigiosin extracted from endosymbiont bacteria *Serratia marcescens* IBRL USM 84 isolated from the surface of a marine sponge *Xestospongia testudinaria* was a significant antibacterial against gram-positive

than gram-negative bacteria. Agitation of the culture media during growth enhanced the pigment production at 25 °C (Darah Ibrahim 2014). Various other bacteria, such as *Pseudomonas* sp., *Vibrio* spp., *Alteromonas* sp., *Rugamonas* sp., and *Streptoverticillium* sp., were also reported for the production of prodigiosin from various sources. Prodigiosin is reported to be bacteriostatic against *B. subtilis*, *E. coli*, *E. aeruginosa*, *S. aureus*, and *P. aeruginosa* (Darshan and Manonmani 2015). The emergence of multidrug resistance of bacteria has created a serious issue worldwide. Extensive research is going on to combat this problem. Microbial pigments exhibit antimicrobial property agents against a wide array of pathogens. Pigments such as carotenoids, melanins, flavins, quinones, monascins, violacein, and indigo have been reported as good antimicrobial agents (Abraham and Chauhan 2018). Prodigiosin isolated from *Serratia marcescens* was reported to be antibacterial against both gram-positive and gram-negative pathogenic bacteria. The activity was more in gram-positive bacteria like *S. aureus*, *S. saprophyticus*, *B. subtilis*, *Enterococcus avium*, and *S. pyogenes* than in gram-negative bacteria like *E. coli*, *P. aeruginosa*, *Aeromonas hydrophila*, *P. mirabilis*, and *K. pneumonia* (Darshan and Manonmani 2015; Srilekha et al. 2017). Various pathogenic strains such as *Acinetobacter anitratus*, *Agrobacterium tumefaciens*, *B. licheniformis*, *B. cereus*, *B. subtilis*, *B. thuringiensis*, *Erwinia* spp., *E. coli*, MRSA, *S. epidermidis*, and *S. aureus* were susceptible for prodigiosin (Venil et al. 2013a, b; Ibrahim et al. 2014). Violacein a violet-colored pigment synthesized by *Chromobacterium violaceum* was antibacterial against *S. aureus* MRSA (Venil et al. 2013a, b). Monascus pigment extracted from the fungus *Monascus purpureus* and *Monascus M3428* showed antimicrobial activity against gram-positive bacteria than gram-negative bacteria (Kim and Seockmo 2018). Staphyloxanthin showed antibacterial activity against pathogens like *E. coli*, *S. aureus*, and *Candida albicans* (Barretto and Vootla 2018). Like many other pigments extracted from bacterium exhibiting antibacterial activity against many human pathogens, violacein, a violet pigment produced by *Chromobacterium violaceum*, also showed antibacterial activity against methicillin-resistant *S. aureus*, making it more significant (Aruldass et al. 2018).

15.5.4.2 Antiviral Properties of Pigments

Viruses are the notorious particles causing serious harm and many diseases to human. Drug design for viral diseases is a crucial aspect as they often mutate. Antiviral molecule is the one which inhibits viral replication. This property is attributed by bacterial pigments. Monascus pigment derivatives from KCCM10093 seem to be suppressing chronic liver diseases caused by hepatitis C virus (HCV) (Darshan and Manonmani 2015). Violacein was reported to be antiviral against HSV (herpes simplex virus) and poliovirus after infection of HeLa cells (Durán et al. 2007). At concentrations of about 0.25 μg/ml (0.73 μM), violacein inhibited HSV at 62%, and, at 0.063 μg/ml (0.18 μM), it inhibited 56% of poliovirus-infected HeLa cells.

15.5.4.3 Antioxidant Properties of Pigments

A free radical is any atom having an unpaired electron in its outer orbital which is highly unstable and reactive. The occurrence of such free radicals in the body increases the probability of the occurrence of many chronic diseases like cancer, diabetes, and cardiovascular and autoimmune disorders (Abraham and Chauhan 2018). Thus free radicals are always in search of a molecule which can accept or share the pair of electron, thus damaging the host cells. Antioxidants are the free radical-scavenging molecules which donate electrons, neutralize them, and thus inhibit cellular damage. Various microbial pigments are antioxidant in nature such as carotenoid and naphthoquinone. Violacein was reported for anti-scavenging activity (Venil et al. 2013a, b). Staphyloxanthin extracted from *Staphylococcus gallinarum* KX912244 is reported for in vitro antioxidant activity with IC50 value of 54.22 μg/ml (Barretto and Vootla 2018). Various pigments isolated from halophilic bacteria were tested for the antioxidant activity on DPPH and were seen to be effective (Krishnan et al. 2017). Torularhodin and torulene – "non-familiar" carotenoids – are studied and have shown to possess provitamin A and antioxidant that helps the stabilization of membranes under stress conditions. Torularhodin is a unique carotenoid with carboxylic acid and shows considerable antioxidant activity (Sakaki et al. 2002). Research shows that torularhodin from yeast was found to protect against oxidative stress (Zoz et al. 2015). Pigment produced by bacterial isolate *Chryseobactreium* sp. kr6 exhibited antioxidant activity by scavenging ABTS radicals (Jiménez et al. 2018).

15.5.4.4 Anticancer Properties of Pigments

Cancer is one of the serious and deadly diseases troubling to man, due to the lack of proper treatment available,which can save human race by detecting and treating it at early stage. Researchers are making efforts to find a successful treatment against diseases. Studies revealed that microbial pigments demonstrate anticancer properties which have laid foundation to treat cancer. Pigments such as prodigiosin extracted from *Pseudoalteromonas* sp. 1020R exhibited cytotoxic activity against U937 leukemia cells. Similarly, melanin from S*treptomyces glaucescens* NEAE-H was reported for anticancer activity against skin cancer cell line (Rao et al. 2017). Cytotoxic effects of prodigiosin were seen in tumor cell lines and also in human cancer cells (Venil et al. 2013a, b). Violacein an indole, purple-colored pigment produced by *Chromobacterium violaceum* and *Janthinobacterium lividum*, have been reported to exhibit anticancer activity, i.e., head and neck cancer (Masuelli et al. 2016). The pigment staphyloxanthin extracted from *Staphylococcus gallinarum* KX912244 isolated from the gut of the insect *Bombyx mori* exhibited cytotoxic activity against four different cancer cell lines (Dalton's lymphoma ascites, Ehrlich ascites carcinoma, lung carcinoma, and mucus skin myeloma) (Barretto and Vootla 2018). Undecylprodigiosin extracted from *Streptomyces* spp. isolated from

leaf litter soil sample exhibited remarkable cytotoxic effect with IC_{50} value of 145 μg/ml against HeLa cell lines (Abraham and Chauhan 2018). Pigments like prodigiosins (PGs), characterized by a common pyrrolylpyrromethane have antitumor and immunosuppressive activity due to the possible mechanism of action of pH modulators: (ii) PGs as cell cycle inhibitors, (iii) PGs as DNA cleavage agents, and (iv) PGs as mitogen-activated protein kinase regulators (Tomás et al. 2003).

15.5.4.5 Pigments as Bioindicators

Any species or biomolecule that revels the existence of certain specific reactions, pathogens, or diseases are called bioindicators. Besides antibacterial, antiviral, antimicrobial, anticancer activities, and microbial activities, pigments also are seen to be bioindicators for various diseases. Studies showed that carotenoid lipid-soluble molecules were found to have a defensive role against oxidative damage to the cells, thus inhibiting cancer cell growth. Recent studies showed low levels of a-carotenoid, ß-carotenoid, and lycopene were found with low counts of CD-4 helper cells indicating them as bioindicator of HIV Sivasubramanian and Vijayapriya 2011). The pigment phycoerythrin is used to determine the rate of peroxyl radical scavenging in human plasma (Narsing et al. 2017). *Pseudomonas* which is a pathogenic bacterium and also drug-resistant and opportunistic pathogen causing nosocomial infections produces a secondary metabolite-soluble green pigment pyocyanin which is antifungal in action and acts as a bioindicator for the identification of a contaminant in hospital environment (Sudhakar and Karpagam 2011).

15.5.4.6 Inhibition of Cholesteryl Ester Transferase Protein

CETP is a molecule which increases LDL and reduces HDL by transferring cholesteryl ester and triglycerides from HDL to TG proteins. Inhibition of CETP helps to manage cholesterol levels. Fourteen derivatives of orange pigment (monascorubrin and rubropunctatin) were reported to inhibit CETP and thus can be effective in controlling cholesterol levels (Kim and Seockmo 2018). Antidiabetic property of carotenoids like astaxanthin, lycopene, zeaxanthin, and lutein leads to the prevention from diabetic retinopathy and thus can be safely consumed in diet (Sathasivam and Ki 2018).

15.5.5 Miscellaneous Activities

Pigments like lutein and zeaxanthin are also used as nutraceuticals and as dietary supplements for most death-causing diseases worldwide – cancer, cardiovascular diseases, and age-related macular and cognitive function degeneration (Barretto and Vootla 2018). Pigments like phycobiliproteins extracted from cyanobacterial species

are also shown to be effectively used as fluorescence probes as protein markers for gel electrophoresis (Sonani et al. 2016). Cell-free extract of pink-colored pigment extracted from *Methylobacterium* isolated from the phyllosphere of some commonly found plants like pigeon pea, sugarcane, potato, mustard, and radish are seen to enhance seed germination in wheat *Triticum aestivum* (Meena et al. 2012). Pigments like monascin and rubropunctatin isolated from *Monascus purpureus* exhibited teratogenic effects on chick embryos (Martínkovå et al. 1999).

15.6 Microbial Enzymes in Degrading Pollutants

Conserving nature for sustenance has become the need of time. Emerging industries has led to the accumulation of hazardous compounds in the environment such as heavy metals, dyes, and aromatic compounds. Industrial effluents release many such pollutants in the natural water bodies and soil, thus affecting the life cycle. Biological methods referred to as green technology are the fields of interest for environmental scientists to clean up such polluted areas. Bioremediation methods are economically sound and further don't add up the pollutants to the environment and hence are preferred over other treatment methods. The widespread use of herbicides, fungicides, insecticides, plasticizers, and hydraulic and heat transfer fluids have contributed large groups of halogenated organic compounds to environmental pollution. Microbial enzymes – oxygenases – degrade these halogenated compounds effectively (Karigar and Rao 2011). Many such bacteria are able to metabolize various organic pollutants; however, a single bacterium doesn't have the enzymatic capability to degrade all kinds of organic compounds. Microbial consortia having powerful biodegradable potential is definitely the key solution to the problem. Ligninolytic enzymes like laccase, lignin peroxidase, manganese, and peroxidase produced by bacteria *Paenibacillus* sp., *Bacillus* sp., and *Streptomyces* sp. detoxify the lignocellulosic waste from the environment (Kumar and Chandra 2020).

Microbial pigments not only are effective in therapeutic but also show promising results in degradation of various pollutants. Pigmented bacteria in consortia are seen to be utilizing hydrocarbons from crude oil-contaminated soil (Varjani and Upasani 2013). Long-term exposure to polycyclic aromatic hydrocarbon leaves various health effects as inflammation of the skin, cataracts, kidney and liver damage, and jaundice and the breakdown of red blood cells. Pigmented bacteria *Pseudomonas*, *Microbacterium*, and *Paracoccus* are found to be degrading naphthalene, phenanthrene, m-cresol, fluorene, fluoranthene, chrysene, and pyrene (Zhang et al. 2004). Construction of wetlands to remediate the industrial effluents is a promising approach. The effect of textile effluents in the water body on flora and fauna are of serious concern, and thus the detoxification of industrial effluents by applying beneficial bacteria in the wetland is the effective way of bioremediation. Effluent-degrading endophytic bacteria like *Microbacterium arborescens* TYSI04 and *Bacillus pumilus* PIRI30 gave remarkable results (Shehzadi et al. 2014).

Azo dyes released by most industries like textile, paper, food, leather, cosmetics, and pharmaceuticals pose serious environmental threat. Existing effluent treatments are not effective enough to remove these azo dyes completely as these recalcitrant are stable and resistant for complete degradation. Microbial enzymes such as azoreductase and laccase effortlessly degrade the synthetic azo dyes from textile industries (Sarkar et al. 2017). Biological approach such as bacterial decolorization and degradation gained momentum as they are ecofriendly, inexpensive, and effective (Saratale et al. 2011). The degradation of pollutants and dissolved oxygen are significantly seen as parameters for photosynthetic bacteria wastewater treatment (Meng et al. 2017). Oil spillage in oceans are another often-encountered serious issue which pose threat to marine life. Microbial consortia containing *Cellulomonas* spp., *Bacillus marisflavi*, *Dietzia maris*, and *Halomonas eurihalina* were found to metabolize diesel even at saline conditions (Le Borgne et al. 2008).

15.7 Conclusions

Colors are the important and attractive component used in the day-to-day life. Biocolors are preferred over synthetic ones for their easy accessibility, nontoxicity, biodegradability, and wide applications. Among natural pigments, microbial pigments are still more preferred for their easy and fast growth in the cheap culture medium and can be manipulated, and colors of variant shades can be obtained. Bacterial pigments are gaining the attention of researchers due to their effortless accessibility from natural resources, simple culturing techniques, easy extraction of pigments, and wide range of applications. The production of pigments can be enhanced by media optimization, physical parameter standardization, mutation, and gene cloning. Wide ranges of bacterial pigments are produced by diverse groups of bacteria as secondary metabolite with numerous applications which are further exploited for the betterment of mankind. Bacterial pigments exhibit various activities in therapeutics such as antibacterial, antiviral, antioxidant, antiprotozoal, antileismaniasis, antimalarial, and antidiabetics and cholesterol-lowering and anticancer properties. In addition, they also act as bioindicators of various diseases. Besides their applications in therapeutics, they are also used effectively on food industry as food colorants. Textile industries also exploit microbial pigments with efficacy. Many bacterial pigments showing radical scavenging activities are preferably used as additive in cosmetic industries (antiaging creams, sunscreens, fairness creams). As the pigment production is seen to be significantly influenced by various parameters, a versatile molecule can be synthesized by parameter optimization. However, understanding the role of factors influencing on pigment production, efforts can be made to enhance the quality and quantity of pigments by employing advanced techniques. Besides the promising role of pigmented bacteria in various therapeutic areas, they significantly produce a wide range of enzymes which not only gives them the adaptability to survive in extreme environment but also metabolize and degrade synthetic organic pollutants. Thus colorful bacterial community from

various natural sources can be further explored for the novel pigment-producing isolates which are active at various parameters expanding the area and clean up the environment ecofriendly.

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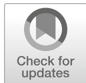
Chapter 16
Bioinoculants for Rapid Production of Vermicompost



Veeresh Santhebennur Jayappa, Keerthi Shivanand, and Paramesha Mahadevappa

Abstract Day by day underutilized agricultural waste is increasing; a major part of agricultural waste is burned in the agricultural land itself. In the meantime, there is more demand for organic fertilizer. In this chapter more emphasis is given to various bio-inoculants used for conventional compost and vermicompost production with less duration. A number of studies have been done for the production of vermicompost from agricultural waste, agro-based industrial waste, and also from the municipal waste. A good number of experiments have been done for the rapid production of vermicompost by altering feed, rearing structures, and opting for better species of earthworms. There were only a few studies done for the utilization of bio-inoculants for the production of compost and vermicompost. Few studies showed that bio-inoculants increase the rapid degradation of biowaste and improve the texture of compost, micro- and macro-nutrient content, and also microbial density and diversity. In this chapter the influence of bio-inoculants on specific microorganism growth, enzyme production, and quality parameters of vermicompost was discussed briefly. There are good opportunities to explore specific interactions of microorganisms, enzymes, and bio-inoculants during vermicompost production.

Keywords Vermicompost · Solid waste · Organic farming · Jeevamrutha · Biodynamic

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16.1 Introduction

Aristotle called earthworms the "intestines of the Earth" by observing barrows made by earthworms on the surface of the Earth. Earthworms have a major role in generation, improving fertility and enhancing the water retention capacity in the soil. Earthworms create favorable environmental conditions to live in and reproduce diverse microorganisms; hence, these are also called "ecosystem engineers."

In Latin vermis means worms; the process of converting organic materials into a valuable bio-fertilizer by using earthworms is known as vermicomposting. Earthworms digest partially decomposed organic materials and produce nutrient-rich castings; this process is called vermicomposting, and the aim of production or multiplication of earthworms is called vermiculture. Both vermicomposting and vermiculture are slightly different in the process of production; those who want bio-fertilizer opt for vermicomposting process, and those who want more quantity of earthworms than fertilizers opt vermiculture. In the process of vermiculture, continually reproduce worms in order to have a more number of earthworms so that sufficient enough to market to customers who use them as fish feed, medical treatment purpose or nutrient meal etc.

Conventionally, earthworms have been used for decomposing organic wastes to increase the soil structure and fertility. The main aim is to produce compost with less time and good quality. Worldwide farmers and gardeners are producing, marketing, and using vermicompost as an excellent soil conditioner. The culturing of earthworms to treat organic waste and utilize castings has become a booming area for solid waste management. Earthworms are now produced in large quantities for bioremediation to aerate, sanitize, and deodorize contaminated land. Worldwide millions of tonnes of earthworms are used for waste conversion purposes. The resultant vermicompost is odorless and destroys all pathogens. Vermicomposting and vermiculture emerged as a significant method of bioremediation and as natural bioreactors. The vermicomposting method yields organic fertilizers, permits safe disposal of particular organic wastes, and reduces the land requirement for landfill.

Based on the goal of production of fertilizer and earthworms, if they want to produce a large number of earthworms, then we have to keep worm density high and make environmental condition to enhance reproductive rate, and if the aim is to produce vermicompost, then they have to maintain low worm density in all period of production.

Earthworms are found in warm soils and many tropical soils. More than 7000 species derived from 23 families were reported across the globe. Pechenik (2009) documented that the earthworms morphological characteristics found to be largely variation like size ranges from an inch to two yards, and live seasonally at all depths of the soil.

16.2 The State of Bio-Compost and Chemical Fertilizers

Agro-based industries, like coir industry, pulp and paper mill, dairy industry, biscuit industry, beverage industry, oil refineries, aromatic oil extraction units, etc., generate a large quantity of nutrient-rich organic waste; these fertilizer substrates are conventionally dumped into waste lands or buried or burnt. These conventional methods of disposal of valuable materials cause environmental pollution and degradation. Without composting or partially decomposing organic wastes, their application to agricultural land leads to decrease soil fertility and causes toxicity to growing crop.

Animal waste is well thought-out as an essential raw material for fertilizers in crop fields and add-on organic fertilizer in getting a better quality of soil, but at the same time, it acts as a source of environmental pollutant. Wherever there are large livestock-rearing centers, local lagoon spills lead to eutrophication of water bodies as well as destruction of fish population. Livestock wastes also extensively contaminate groundwater by increasing microorganisms and nitrates. Still we cannot convert livestock waste into fully nutritious fertilizer with a hundred percent utilization. We are much worried about the reduction of odor produced from the livestock waste. The US Geological Survey reported that wherever there are centers of livestock rearing, they can trace the presence of nitrogen and phosphorus in higher concentration in the streams. Garg et al. (2006) coined that the outbreak of *Pfiesteria piscicida from the source of animal waste causes* eutrophication of water bodies as well as devastation of fauna.

Overuse of chemical fertilizers day by day increasingly remove fertile soil from the agricultural land, and the meantime leaching activity also pollutes the surrounding environment. Increased use of chemical fertilizer and reduction of microbial and macro-fauna in the agricultural land increase pests and diseases to crops. To avoid pests and disease, we have to use highly poisonous chemicals. The residue absorbed by the crops, soil, and water leads to health problems in the surrounding environment (Malathi 2001). Asha et al. (2001) reported that the issues due to the use of chemical fertilizers reduce the rate of germination and seedling growth and increase vulnerability to diseases. From agricultural land, excess used chemical fertilizer like phosphate and nitrogen leached as nitrate into the groundwater as well as near surface water bodies. These chemical fertilizers can cause respiratory illness, cancer and blue baby disease in human beings. Addiscot in 1996 reported the eutrophication of water bodies due to imbalance in the use of chemical fertilizers in agriculture.

The world production, import, exports, and consumption of conventional fertilizers in 2001–2002 are about 1457.79, 605.01, 5974, and 1377.31 megatonne, respectively (Anonymous: Fertilizer Marketing News, June-2005). In India in 1950–1951, the fertilizer consumption is 0.066 (million tonnes), and the number of times it increased during 1950–1951 was nil. In the period of 2003–2004, fertilizer consumption was about 16.80 (million tonnes), and the number of times it increased during 1950–1951 to 2003–2004 is about 254.5. Karnataka consumption of N, P2O5, and K2O was 369.74 million tonnes in 2002–2003 (provisional) (FAI, Fertilizer statistics).

16.3 Farmers' Friend: Earthworm

Earthworms can be seen in all the types of soils, where there is sufficient food supply and moisture condition (Julka 1988). Gates (1972) and Julka (1988) reported that earthworms are found in grasslands, forests, orchards, gardens, cultivated fields, greenhouses, and plant nurseries. Less number of cavernicolous earthworms has been documented by Stephenson in 1924. Julka (1988) also reported a few species living under the snow on high mountains. More earthworm species reported from neutral soils compared to acidic conditions (Satchell 1995).

In recent decades earthworm numbers and biomass are declining due to improper agricultural activities like irrigation, fertilization, drainage, tillage, lime use, stubble retention, crop rotation, slurry application, stocking rate, and pesticide use (Lee 1985; Lavelle et al. 1989; Fraser 1994). Kale and Seenappa (1997) reported that in subtropical and tropical agricultural fields, there is a wider variation in earthworm species than the species richness. The study on the vulnerability of earthworms in agro-climatic regions of India was made by Julka in 2001. The functional role of earthworm in Indian agro-ecosystems has also been revived (Kale 2001).

The world literature on earthworms in agricultural soils is enormous. In the global scenario, a number of reports have been made about the improvement of soil structure due to the activities of native earthworm species (Hindell et al. 1994; Hirth and Mckenzie 1994), availability of nutrient (Barley 1959), mixing of soil, use of lime and surface organic matter (Barley 1959; Baker et al. 1993), reduction of root diseases (Stephens et al. 1993), and plant yield and quality (Abbott and Parker 1981; Shwetha et al. 2007). The role of earthworms in the process of nutrient cycling was documented by Lavelle et al. (1998).

16.4 The Scenario of Vermicomposting

The degradation of organic waste by earthworm consumption is known as vermicomposting. The process of vermicomposting is shown in the flowchart (Fig. 16.1).

Finally, there are three phases in the process of vermicomposting.

Phase 1: Collection of the waste; separation of metal, glass, ceramics, etc.; and storage of segregated organic waste.

Phase 2: Introduction of earthworms to partially decomposed organic waste beds.

Phase 3: After the organic waste was consumed by the earthworms, they start producing casting called vermicompost. Full organic beds converted into vermicompost, earthworms, cocoons, vermicompost, and undigested materials are separated.

Excess liquid leached from the vermi bed is called vermiwash. It also contains coelomic fluid extracted from earthworms. This extraction has enzymes, and it helps

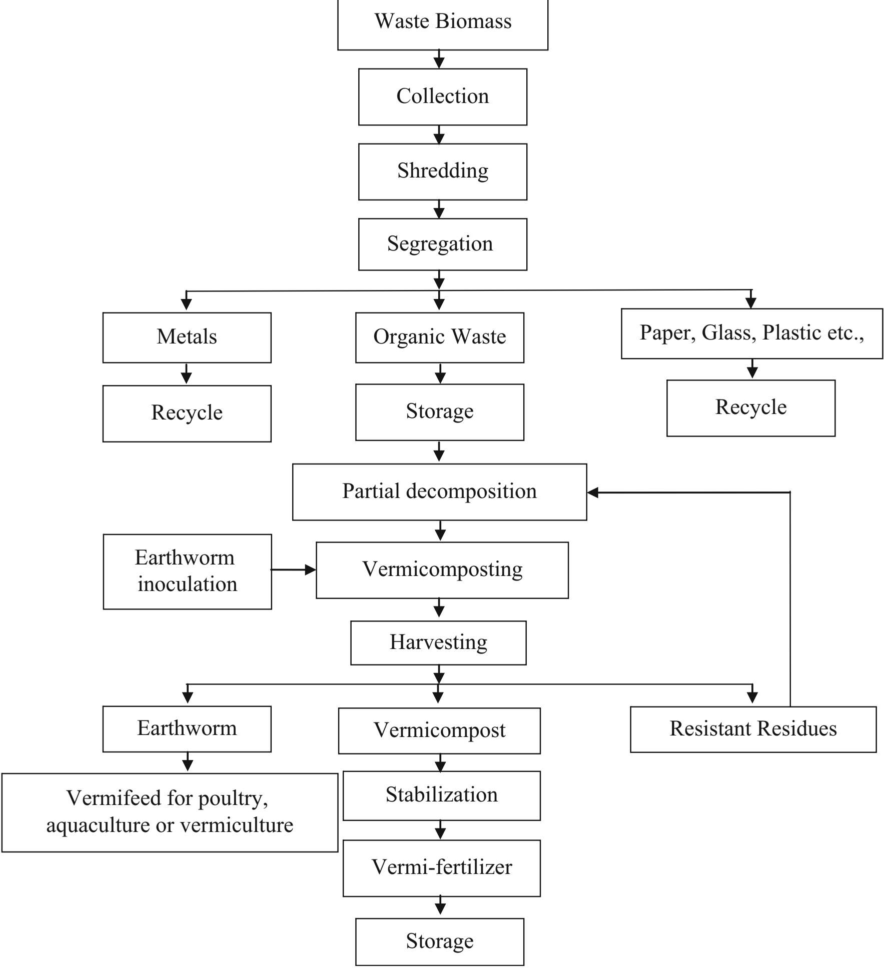

Fig. 16.1 Vermicomposting process flowchart

in pest resistance, stimulates the growth, and increases the yield of crops. It is now commercially sold as foliar fertilizer.

There are several vermicomposting methods being developed, varying in the design and construction of the vermicomposting pits, the species of earthworms used, the nature of the feeding material, the bedding material for the earthworms, and so on.

Julka and Paliwal (1993) reported that based on the local climatic conditions, earthworms can be cultured in both indoor and outdoor. More number of reports are available either on vermicomposting experiments conducted in semi-field conditions or in laboratories (Parlekar et al. 1992). Kumar and Singh (2001) used the outdoor field bed and the heap experimental method, and Biradar et al. (2000) used the

outdoor pit method. Kulkarni (1997) made a study on the production of vermicompost in the field, and observation was made under the influence of seasonal variations.

The overall theory relies on the management of organic waste with no secondary ill effect. The only known attempt of microbial inoculation and combined compost methods were followed by Ndegwa and Thompson (2001), Cristina et al. (2008), and Alidadi et al. (2005). Firdous et al. (2019) reported that during banana leave vermicomposting, microorganisms were isolated and also enzyme activity was analyzed. They also reported the presence of protease and catalase activity in the composting process. In nature primary decomposers are the microorganisms that enhance the process of composting. Microorganisms initiate enzymatic activity and digestion of soil organic matter. In the final stage of the vermicomposting process, stabilization of compost is completed by the microorganisms through enzymatic activity (Arslan et al. 2008).

In the way of development of new organic waste management strategies, it is quite essential to develop an effective method of composting using earthworms and microbial inoculants.

16.5 Production of Vermicompost from Diverse Bio-Waste

Many researcher utilized various species of earthworms, and the different organic wastes include horse waste (Hartenstein et al. 1979), activated sludge (Kale et al. 1982), pig waste (Chan and Griffiths 1988), cow slurry (Hand et al. 1988), sewage sludge (Benitez et al. 1999), brewery yeast and paper mill sludge (Butt 1993), vine fruit industry bio-waste (Atharasopoulous 1993), mango leaves and rice stubbles (Talashilkar et al. 1999), poultry dropping (Ghosh et al. 1999), crops waste (Bansal and Kapoor 2000), vegetable waste (Narayana 2001), water hyacinth and paper waste (Gajalakshmi et al. 2001), textile mill sludge (Kaushik and Garg 2004), cattle dung (Gunadi and Edwards 2003), goat waste (Loh et al. 2004), etc. The study on converting municipal solid waste to vermicompost by earthworm species *E. fetida* (exotic) and *P. excavates* (local) was evaluated by Kaviraj and Sharma (2003). Mba (1996) reported the partial detoxification of toxic cassava peel wastes into valuable vermicompost by using *Eudrilus eugeniae*. Few reports also stated that bitter cassava root peels are toxic to soil invertebrates and it also reduces the plants' root growth. Gajalakshmi and Abbasi (2004) through the “high-rate” reactors converted *Azadirachta indica* residue into vermicompost by using earthworms (*E. eugeniae*). Zharikov et al. (1993) used the low-quality bacterial preparations produced from the microbiological industries and used husks and sewage sludge for the production of vermicompost.

Edwards (1988) documented the conversion of animal excreta, agro-industrial wastes, and sewage sludge into vermicompost. Frank et al. (1983) found that the sludge is not suitable for direct use in the agricultural land, but it is possible for use when it is converted into vermicompost. In many countries like Australia and

New Zealand, earthworms are used to compost toilet waste for the production of vermicompost by using various designs of vermi reactors. The most improved method is the Dowmus composting toilet (Edwards 1998). In a similar study also made in Belgaum City of Karnataka, a colony of 500 homes generated solid waste, and the sewage was used for vermi culture at the Indian Aluminium Co. Ltd. site by using vermi filter created for treating up to 100 m^3 per day, and the treated water was utilized for garden irrigation (White 1996).

In a comparative study on the production of vermicompost from cattle waste and goat waste by *Eisenia foetida*, results showed that the biomass gain and cocoon production were less in goat waste compared to that of the cattle waste. Kale et al. (1982) made a study on the production of vermicompost from different organic wastes such as cow dung, biogas sludge, sheep dung, horse waste, poultry manure, and sand as control by using *Perionyx excavatus*. The study also reported that earthworms consumed cow and horse waste when it was fed, but sheep waste was consumed 3–4 days after it was added. Gunadi and Edwards (2003) studied the *E. foetida* growth, fecundity, and mortality rate in different varieties of wastes such as supermarket waste, cattle manure, and pig manure for more than 1 year. They observed that the growth of *E. foetida* in cattle waste is less compared to pig waste, and also the subsequent substrate supply extended the earthworms' fecundity rate. They also reported that after 60 weeks of vermicomposting, there was a tendency of decreasing the earthworms' weight. Singh et al. (2004) studied the requirement of optimum moisture for *P. excavatus* to convert organic waste into vermicomposting. They found that the requirement of optimum moisture content is 80% for the vermicomposting process, and it also positively reflected the maximum earthworm reproduction.

16.6 Suitable Earthworm Species for Vermicomposting

Worms which live on the surface of the Earth are called epigeic earthworms. Important epigeic earthworm species include *Eudrilus eugeniae*, *Eisenia foetida*, and *Perionyx excavates*, and they are used for vermicomposting. For vermicomposting and vermiculture, earthworms must have the following important characteristics:

- The earthworm, supposed to have the capability of high biomass consumption, less time to convert it into body protein and excrete castings, and it must have a higher growth rate.
- The earthworm must have a resistance to various environmental factors such as temperature, light intensity, moisture, and varied carbon and nitrogen ratios in feed substrates and must feed on diverse organic residues.
- The earthworm must have less hatching intervals and reproduce more numbers of cocoons, enabling a rapid increase in population. When the earthworm population

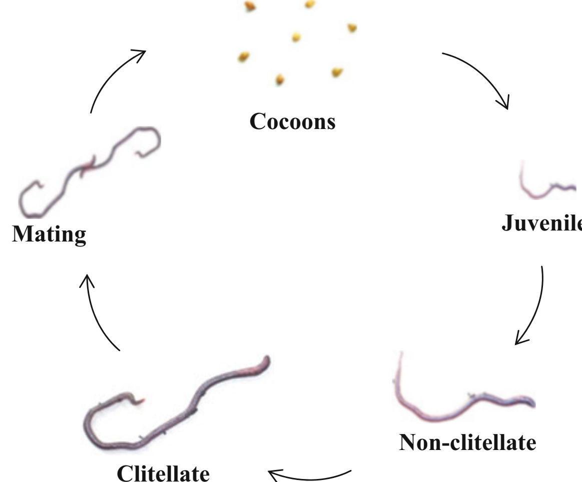

Fig. 16.2 The life cycle of an earthworm

is more, there will be an increasing rate of conversion of organic waste to vermicompost.

- The earthworms must have less period in reaching the mature/adult phase in their life cycle.
- Using different species of earthworms has more efficiency than using a single species.
- The worm should be resistant to diseases.

Africa distributed a number of organisms worldwide; among them is the earthworm species *Eudrilus eugeniae*. However, this species of earthworm is commonly used in the fish bait market of various countries like the USA, Europe, Canada, and Asia. *Eudrilus eugeniae* is commonly called as African night crawler. This species is the second widely accepted for the production of vermicompost in the world. Its growth rate is higher than other species and gains increasing biomass at the rate of 12 mg per day and maximum body weight up to 4.3 mg per individual. Juveniles mature after 40 days of egg rupture, and in the next 7 days, each individual commences cocoon hatching (on average one cocoon per day). One to 3 years is the lifespan of the earthworms in the laboratory condition. The general life cycle of the earthworm is depicted in Fig. 16.2. In some places like the tropical and subtropical regions, *Eudrilus eugeniae* species which has less low-temperature tolerance is widely used than the *Eisenia foetida*, a low-temperature-tolerant species.

The earthworm digestive system consists of a pharynx, esophagus, gizzard, and an anterior intestine. The role of anterior intestine and posterior intestine is to produce enzymes and absorb nutrients, respectively. During organic waste digestion in the digestive system, there is a faster increase in the number of microorganisms of up to a thousand times.

Vermicompost provides suitable forms of plant nutrients like nitrogen, phosphorus, potassium, and calcium that can easily be absorbed by the plants than those

forms existing in the raw organic matter (Ndegwa and Thompson 2001). Tomati et al. (1987) reported that plant growth regulators are also present in vermicompost.

16.7 The Influence of Vermicasts on Soil Microbial Activity

Earthworms have direct and indirect activities such as (1) comminuting, casting, and burrowing; (2) grazing; and (3) dispersal impact on soil microflora and faunal population. Through these activities, the substrate's biological and physicochemical changes take place. These activities cause drastic change in microbial communities, structure, diversity, and density in the drilosphere (Brown 1995). Marinissen and De Ruiter (1993) observed the regulating processes of nutrient cycling through earthworm activities; it enhances the nutrient content as well as converts it into an available form for microorganisms and plants. In the process of organic waste decomposition, microorganisms are the primary responsible agents (Brown et al. 2000).

Vermicompost is rich in microbial density and diversity (Edwards 1998). Edwards and Bohlen (1996) observed that soil microorganisms are influenced by burrowing and casting activities of earthworms, and Lee in 1985 reported that the earthworm nutrient-enriched casts act as good supporting media for microbial growth. A number of literature reported the studies made on earthworm gut microbial communities (Karsten and Drake 1997). Reyes and Tiedje (1976) found that gram-negative bacteria are general inhabitants in the intestinal canal of earthworms. In the gut of the earthworm species *Eisenia lucens* and *Pheretima*, *Vibro* sp. and *Aeromonas hydrophila* were reported (Marialigeti 1979). Daane et al. (1998) in their study stated that the scanning electron microscopy evidenced that there were several rod-shaped bacteria in the egg capsule of *E. fetida* and also confirmed mutualism. Sampedro and Whalen (2007) also observed that the earthworm's gut, burrows', and casts' microbial communities differ from those of the soil, which establish the relationship that either good or poorer conditions affect microorganism stimulation or depression during the decomposition of soil organic matter.

One of the most important aspects of the drilosphere are the middens built on the surface of the soil, covering the burrows entrance by the earthworm *Lumbricus terrestris*. Hamilton and Sillman (1989) reported that the middens are made up of vermicasts and plant litter and they contain higher nutrients compared to parent soils (Wilcox et al. 2002). Middens also support a more diverse microfauna community (Schrader and Seibel 2001). Bohlen et al. (2002) observed that earthworm middens contain more microbial activity and nutrient dynamics and it also affects soil microbial communities. The earthworm ecology gains more importance because earthworms establish relationships with microorganisms during the decomposition of organic matter. This relationship is remarkable because both earthworms and microbial communities were detritivores and they compete with each other for the same resource pools (Scheu and Falca 2000).

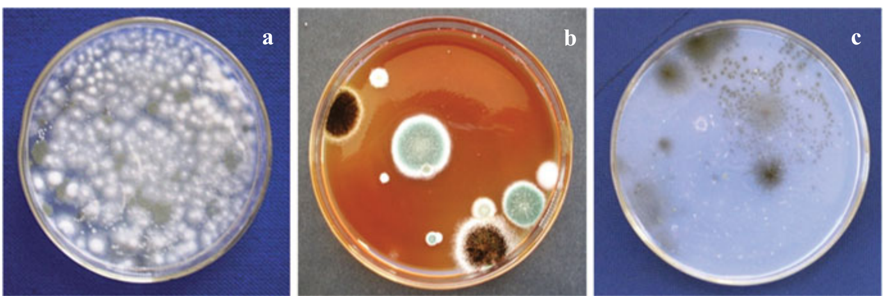

Fig. 16.3 Colonies of bacteria (**a**), fungi (**b**), and actinomycetes (**c**) isolated during vermicomposting process

Several experiments proved that earthworms consume microorganisms as a food. Fungi and protozoa communities were the major sources of nutrients compared to bacteria and algae communities. Earthworms grow and reproduce best under diverse microbial communities than in sterile conditions like individual cultures of certain fungi, bacteria, and protozoa communities. Allievi et al. (1987) studied the modification of microflora during rabbit manure vermicomposting; the results showed that the actual vermicomposting brought out minor change in microbial colonies similar to extended maturation of rabbit manure. However, few studies also proved that the definite nutritional exchanges were observed between microorganisms and *E. fetida*. The earthworms feed directly on the cells of certain microorganisms, and few species were found to be toxic to *E. fetida*. Hand et al. (1988) reported that the seeding of *Acinetobacter calcoaceticus* to vermiculture beds showed the stimulation of earthworm growth and consumption of the substrate, and Aquino et al. (1994) also reported that no difference was observed for *Acetobacter diazotrophicus* inoculations on the rate of earthworm reproductively.

The diverse group of microorganisms comprising the population densities of bacteria, fungi, and actinomycetes successively or independently acts on the organic constituents (Fig. 16.3).

Inoculating effective lignocellulolytic microorganisms, particularly fungi, accelerates the composting process. The high density of such fungi reduces the period of decomposition. There are many fungi such as *Phanerochaete chrysosporium*, *Pleurotus* spp., *Trichoderma* sp., *Paecilomyces fusipus*, and *Trichuris spiralis* that can be used as decomposing fungi. Generally, the compost is low in nutrient status. The microorganisms that are establish during the process of decomposition are diazotrophs that fix atmospheric nitrogen and phosphate solubilizers that increase the available phosphorus by producing different types of organic acids such as gluconic, citric, acetic, ketoglutaric acids, etc. A group of fungi, bacteria, and actinomycetes utilize the soluble constituents during the decomposition process and produce certain metabolites that have the property of plant growth stimulation (hormones) and antagonistic to other harmful microorganisms (bio-control agents). Earthworms have a major role in the degradation of organic matter through the process of conditioning the substrate and altering the biological activity, but at the

same time, microbes also play a very important role by making biochemical changes in organic substrates (Aira et al. 2002).

16.8 Methods of Rapid Vermicomposting

Several researchers coined the different methods of vermicomposting by changing the vermiunit's physical, chemical, and biological characteristics in order to increase nutrient content, stabilization period, and rapid vermicomposting process. It was observed in a study that the temperature is a key factor for the mineralization and decomposition of vermicompost. This study proved that the fastest stabilization process occurred at 25°C; higher temperature increases microbial activity as well as earthworms' growth during 30-day period. The same study also gives the evidence of bacterial alpha diversity that exhibited positive response to a higher temperature with diverse principal genus in the end vermicompost. This study concluded that 25°C is the most favorable temperature for vermicomposting of dewatered sludge with the use of *E. fetida*.

Gajalakshmi et al. (2001) reported that in the usual vermicomposting unit, nearly 50% of the bed volume consists of successive layers of organic substrates and another 50% leave space for the unit. Hence, this conventional method was not able to compost 100% of the organic wastes, and also less quantity of organic waste can be accommodated on the vermi bed. To utilize the full space of the vermicompost unit, they developed an adapted vermi-reactor or vermiunit method by adding more layers of organic waste and covering a thick moistened cotton cloth on the top layer. This will provide both shade and moisture to the earthworms. Hence, earthworms consume the top layer of organic waste also. This method increases the efficiency of converting organic waste into vermicompost.

Tiwari et al. (1989) stated that the addition of appropriate ligninolytic and cellulolytic strains speeds up the rate of composting and it will also increase the nutrient content in the finished compost. Manna et al. (2003) stated that because of the lignin recalcitrant characteristic, it will take more time to decompose. Chahal (1994) noted that the white-rot fungus *Phanerochaete chrysosporium* is referred as an effective lignin-decomposing fungi and it also increase the rate of decomposition. Anand et al. (2006) found that *Trichoderma viridae* has the ability to bio-accumulate copper and also produce cellulase enzyme. This method is referred to be good for composting (Bhardwaj and Gaur 1985). The final compost content of nitrogen can be increased by adding N-fixing bacteria during the composting process (Kaushik and Garg 2004), and it also depends on the nature of organic wastes utilized (Beauchamp et al. 2006). Hendrikson (1990) found that the microbial population increased in vermicastings instead of being destroyed during the organic substrate passage through the earthworm's gut.

Pramanik et al. (2007) reported that the production of vermicompost using cow dung alone as feed showed good nutrient content, production of enzymes, and increased microbial population. Vermi bed modification by adding lime does not significantly affect nutrient content and enzymatic production during vermicompost production. However, the combination of lime and microorganisms showed a significant effect on vermicompost quality. *T. viridae* was not affected by the presence and absence of lime, but *B. polymyxa* required lime addition. If *P. chrysosporium* is inoculated in the vermi bin, it did not require lime addition to increase the nutrient value of vermicompost, and it also worked as a sink for enzymes present in the vermicompost.

The correct earthworm species selection is more important particularly when doing vermiculture (Appelhof et al. 1996). Ismail (1997) referred that the C:N ratio of feed organic material should not be more than 40. If we use higher C:N ratio, substrates such as paper and soaked cardboard will increase the body weight of earthworms. Kale (1995) reported that the if we want earthworms to grow faster and produce more cocoons, then we have to maintain the favorable environment such as selecting slightly dark place with humidity, bed moisture of 40–50%, temperature at 20–30 °C, and neutral pH, and feeding substrate should be partially decomposed with rich in nitrogen.

Loh et al. (2004) reported that the rapid earthworm growth and cocoon production can be achieved by top feeding of the slurry compared to adding of the slurry to the feeding substrate. This study also shows a higher earthworm growth and cocoon production seen in cattle manure application than goat, but cocoons hatchability was not affected by different manure treatments. Jain et al.'s (2003) laboratory study reported that better performance can be achieved with modification of vermiunit and higher vermicompost mg per liter per day. This study also documented that the size of the vermi reactor is only responsible for higher-quantity vermicompost production.

Veeresh et al. (2010) made an experiment for the rapid production of vermicompost from sugar factory and paper mill sludge by inoculating microbial biodynamic consortium (*Jeevamrutha*). In this study, they compared the influence of the inoculation of *Jeevamrutha* and cow dung on microbial activity. They found higher actinomycetes and bacterial densities in the *Jeevamrutha*-treated group, and fungal density was higher in the cow dung-treated treatments. In addition to this, they also recorded the microbial density, and the microbial culture-treated groups showed the significant relationship. The abovementioned study results showed that the inoculation of microbial consortia and cow dung to feeding organic substrates significantly increases the microbial density during decomposition. This study evidenced that the inoculation of microbial culture to the decomposing substrates reduces the period of decomposition as well as process of vermicomposting.

The process of conversion of agro-industrial waste to manure through rapid vermicomposting revealed that the inoculation of a biodynamic consortium to organic substrates enhanced the bacterial and actinomycetes densities than treatments received from cow dung and control groups. However, mixing cow dung with decomposing materials exhibited the highest fungal density compared to other

treatment groups. When *Jeevamrutha* was combined with earthworms, there was a significant improvement of microbial and bacterial density, which was significantly correlated with the actinomycetes population. Finally, this study concludes that *Jeevamrutha* and cow dung are better microbial consortia to enhance the microbial density and vermicomposting than if none were to be used.

16.9 Conclusion

The inoculation of diversified microorganisms during the degradation of biomass hastens the process of degradation and enriches the compost nutrient content. Various biodynamic preparations were traditionally done across the world, but the standardization of process is in the infant stage. There is an ample amount of opportunity for researchers for the characterization of biodynamic preparations as well as recognition of the role of each microbial community and enzyme activities during the process of biomass degradation.

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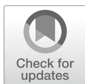
Chapter 17
Microbial-Mediated Mechanism to Improve Rock Phosphate Solubilization and Its Agronomic Implications



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Abstract The regulation of plant metabolism, development, and growth depends upon different nutrient requirements, and phosphorus (P) plays an essential role in it. Though there is a huge presence of P in soil, the bioavailability of the element is very less for plants due to its chemical fixation in soil environment. Application of rock phosphate in cooperation with solubilization by microbes may help to enhance the phosphate level in soil. This technology not only remunerate the expensive industrial manufactured fertilizers but also helps to mobilize the fertilizers added to the soil. The phosphate-solubilizing microorganisms predominantly belongs to the genera of *Aspergillus*, *Actinomycetes*, *Aereobacter*, *Agrobacterium*, *Bacillus*, *Enterobacter*, *Erwinia*, *Micrococcus*, *Penicillium*, *Pseudomonas*, *Rhizobium*, and *Trichoderma*. The solubilization mechanisms used by these PSMs were several such as (i) lower pH by acid production, (ii) chelation of ion, and (iii) exchanging reactions within the broth. These qualities put froth an ideal technology for the development of potent microbial inoculum for ultimate utilization in agricultural systems. The potentiality of PSMs as rock phosphate solubilizers to enhance crop production and the detailed mechanisms are discussed.

Keywords Rock phosphate · Microbial solubilization · Soluble P · Enhanced crop

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17.1 Introduction

Phosphorus (P) is considered an essential macronutrient for plant growth and improvement after nitrogen (N). It plays an essential role in plant metabolism, i.e., cell cycle regulation, division of the cell, plant development, photosynthesis, breaking of sugars, transportation of nutrient within the plant, regulation of metabolic pathways, and genetic character transfer from one generation to another (Hamdali et al. 2008; Sharma et al. 2013). Although there is considerable level of P in the soil system, a large portion of this is not readily available to plants due to complex formation with cations like iron, aluminum, and calcium (Son et al. 2006). In order to fulfil the P requirement of plants, the commercially available chemical fertilizers can be applied to increase the crop productivity. However, the high-cost production and the fertilizers being chemically processed with insoluble mineral phosphate (high-grade ore) with the treatment of sulfuric acid in high temperature makes it a polluting constituent (Shigaki et al. 2006; Vassilev et al. 2006). With the application of phosphorus-based fertilizers to the soil, the initiation of reactions between P, soil, and nonphosphatic fertilizer components takes place, which results in the formation of insoluble P and makes it unavailable for plant growth regulation (Ivanova et al. 2006).

Furthermore, the soluble P gets rapidly washed away, responsible for the eutrophication of freshwater and pollution in groundwater used for drinking (Shigaki et al. 2006). Recently the focus is shifted toward the microbial-mediated rock phosphate solubilization techniques and its application in agricultural field. The phosphate-solubilizing microorganisms (PSM) converts the insoluble phosphates to a soluble form by acidification, chelation, and exchange reaction (Coutinho et al. 2012). Microbial activities play a prime role in the increment of bioavailability of minerals that are the main part of plant growth regulations and eventually reduce the demand of unsustainable chemical fertilizers (Kennedy et al. 2004). Therefore, this process not only seems a cheap alternative to the use of expensive chemical fertilizer but also enhances the mobilization of fertilizers added to the soil.

17.2 Availability of Rock Phosphate

The distribution of rock phosphate reserves can be found in sedimentary and ingenious deposits around the world. The phosphate reserves around the world were approximate 68 billion metric tons according to the US Geological Survey of 2017 (Kohn et al. 2018). Morocco contained approximately 70% of the total reserves of phosphate rock which is 50 billion tons. After that China comes in the second position, having 5% of the total reserves, which indicates 3.1 billion tons. However China is the largest producer of phosphate at 138 million metric tons. The third largest reserves of phosphate are Syria and Algeria which contributes 3% to the world's phosphate reserves, i.e., 1.8 and 2.2 billion metric tons, respectively. Russia,

South Africa, the USA, Egypt, and Jordan each contribute to 2% of world's phosphate reserves. The 1% of world's phosphate reserve belongs to Peru, Saudi Arabia, Senegal, Australia, and Iran, respectively, and other countries account for less than 1% of the total reserves which contribute to the global phosphate production.

In comparison to other producers all over the world, the contribution of India in the world market doesn't show any profitable numbers. According to the NMI data based on the UNFC system, India provides 312.67 million tonnes of phosphate, while 266.87 million tons of phosphate comes from the remaining resource categories. The reserves constitute 45.80 million ton only. The states like Jharkhand, Rajasthan, Madhya Pradesh, Uttar Pradesh, and Uttarakhand contribute 34%, 31%, 19%, and 8% each, respectively, to the total reserves in India. Meghalaya and Gujarat also constitute less quantities of resources. According to the Indian Minerals Yearbook (2016), if the gradation of phosphorus is taken in account, the low-grade phosphorus is 37%, followed by beneficial (29%), blendable (11%), chemical fertilizer and soil reclamation (8% each), and the remaining unclassified and not-known grades (about 7%) (Indian Minerals Yearbook 2016).

The major mineral of rock phosphate is apatite, and the most common is fluorapatite-$Ca_5(PO_4)_3F$ or $Ca_{10}(PO_4)_6(F,OH)_2$. As the mineral is insoluble in nature, it cannot be used as fertilizer directly. Some of the soil's physicochemical factors such as pH, reaction time, and Ca^{2+} or $H_2PO_4^-$ absorption ability may affect the rock phosphate solubility. The level of solubility is determined from the degrees of fitness and the origin of phosphate. Compared to phosphates of igneous or metamorphic origin, the sedimentary RP origin may be more soluble (Nahas 1996). The most important factor is the soil pH which affects the efficacy of the RP (Ma and Rao 1999), as the increment in soil acidity results in higher rate of dissolution and effectiveness. The immobilization of Pb in Pb-contaminated soil has been reported by several studies after the application of phosphoric acid to RP (Yang et al. 2001; Cao et al. 2003; Yoon et al. 2007; Cao et al. 2009). However, the acidic water solubilization of P source exhibits a great disadvantage due to the low pH results in potential leaching of both the applied P and Pb in the soil. Therefore the application of P-solubilizing microorganisms to act on RP to enhance solubilization could be a potential approach than acidification.

17.3 Phosphate-Solubilizing Microorganisms (PSMs) Accountable for RP Solubilization

The solubilization of insoluble mineral phosphate into ionic forms could be possible by using soil microorganisms from different ecological niches which can be utilized by the crop plants. From the soil and rhizosphere samples, many PSMs like bacterial, fungal, yeast, and actinomycete species have been isolated, identified, and characterized (Kucey et al. 1989). Bacterial species acts as a potent culture in comparison to

fungus to solubilize phosphorus (Alam et al. 2002). PSMs are present ubiquitously in nature, and their broad range of variation is observed in different soil environments (López-Arredondo and Herrera-Estrella 2012). In the whole microbial population, the presence of PSB is 1–50%, and PSF are found to be 0.1–0.5%, showing higher rate of P solubilization.

17.3.1 Bacteria

The potent bacterial strain can be isolated by a series of experiments on bacterial species that help to solubilize efficiently the insoluble inorganic phosphate compounds, like tricalcium phosphate, dicalcium phosphate, hydroxyapatite, and rock phosphate (Goldstein 1986). The bacterial genera having solubilizing properties are found to be *Achromobacter*, *Aereobacter*, *Agrobacterium*, *Bacillus*, *Burkholderia*, *Flavobacterium*, *Micrococcus*, *Pseudomonas*, and *Rhizobium*, and they increase the accessibility of soluble phosphate and enhance biological nitrogen fixation, resulting in enhanced plant growth (Kucey et al. 1989; Ponmurugan and Gopi 2006). With the incorporation of *Pseudomonas* spp., the nodule number, its dry weight, yield components, grain yield, nutrient availability, and uptake in soybean crop have been enhanced (Son et al. 2006). PSB supports the enhancement in seedling length of *Cicer arietinum* (Sharma et al. 2007), while the inoculation of PSM along with PGPR showed reduction in P application by 50%, and the corn yield has not been affected (Yazdani et al. 2009). Below is the list of a few bacterial species studied for their P-solubilizing potential:

Gram-positive bacteria: *Bacillus brevis*, *B. cereus* var. *albolactis*, *B. circulans*, *B. coagulans*, *B. firmus*, *B. megaterium*, *B. megaterium* var. *phosphaticum*, *B. mesentricum*, *B. mycoides*, *B. polymyxa*, *B. pumilus*, *B. pulvifaciens*, *B. sphaericus*, *B. subtilis*, *Clostridium* sp., *B. licheniformis*, *B. amyloliquefaciens*, and *A. atrophaeus*.

Gram-negative bacteria: *Acetobacter diazotrophicus*, *Achromobacter* sp., *Aerobacter aerogenes*, *Agrobacterium radiobacter*, *Agrobacterium* sp., *Alcaligenes* sp., *Arthrobacter mysorens*, *Bradyrhizobium* sp., *Brevibacterium* sp., *Burkholderia cepacia*, *Citrobacter freundii*, *Enterobacter aerogenes*, *Enterobacter agglomerans*, *Enterobacter asburiae*, *Enterobacter cloacae*, *Escherichia freundii*, *Escherichia intermedia*, *Erwinia herbicola*, *Flavobacterium* sp., *Gluconacetobacter diazotrophicus*, *Micrococcus* sp., *Mycobacterium* sp., *Nitrosomonas* sp., *Pseudomonas calcis*, *P. cepacia*, *P. fluorescens*, *P. putida*, *P. rathonia*, *P. striata*, *P. syringae*, *Serratia marcescens*, *S. phosphaticum*, *Thiobacillus ferrooxidans*, *T. thiooxidans*, *Rahnella aquatilis*, *Rhizobium meliloti*, *Xanthomonas* sp., *Azotobacter chroococcum*, *Kluyvera ascorbata*, *Azospirillum brasilense*, *A. lipoferum*, and *Acinetobacter calcoaceticus*.

17.3.2 Fungi

After bacterial species, fungal strains such as *Aspergillus* and *Penicillium* are recognized to be the most potent P solubilizers (Whitelaw 2000). Mycorrhizal fungi and phosphate-solubilizing microorganisms are identified to process the bio-available P (Fankem et al. 2006). To solubilize the phosphate rocks, a nematophagous fungus *Arthrobotrys oligospora* has also been identified as a potent species (Duponnois et al. 2006). These fungal species have the capability in converting the insoluble P into its soluble form, produce plant growth promoting substances, and also protect plants from soil pathogens. A fungus does not lose its solubilizing capability with repeated subculturing under laboratory conditions like that of the PSB (Sperber 1958; Kucey 1983). Moreover, the fungus has the capability to traverse longer distance in the soil than bacterial species making them suitable for P solubilization (Kucey 1983). Generally in PSF the rate of acid production is higher in comparison to bacteria, which indicates the higher P-solubilizing activity (Venkateswarlu et al. 1984). Duponnois et al. (2006) have also reported nematophagous fungus, i.e., *Arthrobotrys oligospora*, which shows a significant rate of RP solubilization (Meena et al. 2013, 2016; Bahadur et al. 2014; Maurya et al. 2014; Jat et al. 2013; Kumar et al. 2015; Ahmad et al. 2016; Parewa et al. 2014; Prakash and Verma 2016). Mycorrhizal fungi are another group of microorganisms playing an important role in P acquisition (Fankem et al. 2006). Below are the list of fungi studied for having potential P dissolution phenotype.

PSF: *Achrothecium* sp., *Alternaria tenuis*, *Aspergillus aculeatus*, *A. awamori*, *A. carborundum*, *A. flavus*, *A. foetidus*, *A. fumigatus*, *A. japonicus*, *A. nidulans*, *A. nidulans* var. *acristatus*, *A. niger*, *A. rugulosus*, *A. terreus*, *A. wentii*, *Cephalosporium* sp., *Chaetomium globosum*, *Cladosporium herbarum*, *Cunninghamella* sp., *C. elegans*, *Curvularia lunata*, *Fusarium oxysporum*, *Helminthosporium* sp., *Humicola lanuginosa*, *H. inslens*, *Mortierella* sp., *Micromonospora* sp., *Mucor* sp., *Myrothecium roridum*, *Oidiodendron* sp., *Paecilomyces lilacinus*, *P. fusisporus*, *Penicillium aurantiogriseum*, *P. bilaji*, *P. digitatum*, *P. funiculosum*, *P. lilacinum*, *P. oxalicum*, *P. pinophilum*, *P. rubrum*, *P. rugulosum*, *P. simplicissimum*, *P. variabile*, *Phoma* sp., *Populospora mytilina*, *Pythium* sp., *Rhizoctonia solani*, *Rhizopus* sp., *Sclerotium rolfsii*, *Torulaspora globosa*, *Torula thermophila*, *Trichoderma harzianum*, *T. viridae*, *Schwanniomyces occidentalis*, *Emericella rugulosa*, *Penicillium camemberti*, and *Colletotrichum* sp.

Yeast: *Pichia fermentans*, *Schizosaccharomyces pombe*, and *Yarrowia lipolytica.*

17.3.3 Other Soil Microorganisms

17.3.3.1 Mycorrhizae

The most essential component for sustainable soil-plant systems among the microbial community is identified to be the Arbuscular mycorrhizal fungi (AMF) (Schreiner et al. 2003). It has a positive response on nitrogen (Barea et al. 1991), phosphorus (Bolan 1991), and other micronutrient (Bürkert and Robson 1994) uptake; responsible for soil aggregation (Tisdall 1994) for the plant system; and has also analogist impacts against some plant pathogens (Duponnois et al. 2005). Moreover, research in this field has shown that in comparison to non-inoculated plant, the AMF, in symbiotic association with the host, uses additional soluble phosphate from rock phosphate (Antunes and Cardoso 1991; Guissou et al. 2001). The insoluble mineral phosphate solubilization by organic acid produced by AMF has been proved by many evidences (Lapeyrie 1988).

17.3.3.2 Actinomycetes

In recent years the actinomycetes attracted the research interest globally due to its P-solubilizing property, because this community of soil microbes has better tolerance toward extreme environment like drought, temperature, etc. and also has the ability to produce benefits such as antibiotic and compounds like phytohormone production which is ultimately responsible for plant growth regulation (Fabre et al. 1988; Hamdali et al. 2008). The major area of rhizoplane and rhizosphere are occupied by the actinomycetes (Solans and Vobis 2003; Frioni 2006) and have a key role in the regulation of soil nutrient cycle (Elliott and Lynch 1995). Below are the list of few actinomycetes known to have an active role in P solubilization.

Actinomyces coelicolor, *Actinomyces* sp., *Agromyces soli*, *Angustibacter luteus*, *Isoptericola hypogeus*, *Isoptericola variabilis*, *Kocuria flava*, *Kocuria palustris*, *M. kitamiense*, *Microbacterium yannicii*, *Microbacterium aurantiacum*, *Micromonospora* sp., *Nocardia* sp., *S. cinereorectus*, *S. cinnabarinus*, *Streptomyces* sp., *Streptomyces violascens*, *S. noboritoensis*, *Streptoverticillium* sp., and *Thermoactinomycetes* sp.

17.3.3.3 Cyanobacteria

Similar to bacteria, the *Cyanobacteria* sp. has also the property to mobilize bound phosphates like $FePO_4$, $Ca_3(PO_4)_2$, $AlPO_4$, and $(Ca_5(PO_4)_3OH)$. The bound P seems to be released by different mechanisms like organic acid production, chelation, dissimilatory reduction, and enzymatic solubilization or might be involving more than one mechanism (N. Kishore et al. 2015). These are few *Cyanobacteria* being studied for their ability to solubilize bound P: *Anabaena*, *Calothrix braunii*,

Hapalosiphon fontinalis, *Nostoc* sp., *Scytonema*, *Scytonema cincinnatom*, *Tolypothrix*, *Tolypothrix tenuis*, *Tolypothrix ceylonica*, *Westiellopsis prolifica*, and *Phormidium* sp.

17.3.3.4 Protozoa and Other Mesofauna

Protozoa present in the soil are dependent upon the ingestion of some bacterial species like *Aerobacter*, *Bacillus*, *Agrobacterium*, *Escherichia*, *Pseudomonas*, and *Micrococcus* and to their protoplasm. They are well known to have the capability to assimilate soluble minerals and increase P bioavailability. They represent a major mechanism for regulating fungal and bacterial members in the soil and ultimately influences the soil P cycle (Alphei et al. 1996).

Mesofauna are responsible for enhancing P cycling and availability in a range of soils; however, they are difficult to handle practically; therefore, they are applied in agricultural soil directly for nutrient availability (Lopez-Hernandez et al. 1993). Several PSMs present in the soil don't have enough population to compete with other bacterial community that are already established in rhizospheric zone. In consequence, the amount of P released by them is generally inadequate for proper in situ plant growth. Therefore, the application of potential microorganism at higher doses than that of the normally available microbial community can be improve methods for plant growth improvement.

17.4 The Mechanism of Rock Phosphate Solubilization

Phosphate-solubilizing microorganisms (PSMs) are able to solubilize insoluble inorganic phosphate compounds, like tricalcium phosphate, dicalcium phosphate, hydroxyapatite, and rock phosphate through various mechanisms (Goldstein 1986).

In general the accepted major mechanism for mineral phosphate solubilization is the synthesis and secretion of organic acids by soil microorganisms (Halder et al. 1990) either by (i) decreasing the level of pH, (ii) by enhancing the cation chelation that are bound to P, (iii) by competing with P for adsorption sites on the soil, or (iv) by the formation of soluble complexes with metal ions association with insoluble P (Ca, Al, Fe) and thus releasing P (Omar 1998). The conceptual diagram of RP solubilization is presented in Fig. 17.1.

Many studies have been done to investigate the ability of phosphate in microbes in solubilizing insoluble rock phosphate in a pure liquid culture medium (Gupta et al. 2010; Bhattacharjya et al. 2019). The bio-solubilization of inorganic rock phosphate in liquid medium by microbial species is often due to the excretion of organic acids (Table 17.1). Microorganism-secreted organic acids are rich sources of H^+ ion; this ion dissolves the mineral phosphate and converts it into bioavailable phosphate for the utilization of green plant. Panhwar et al. (2013) and Gomes et al. (2014) found a significant negative correlation between the final culture medium pH and the

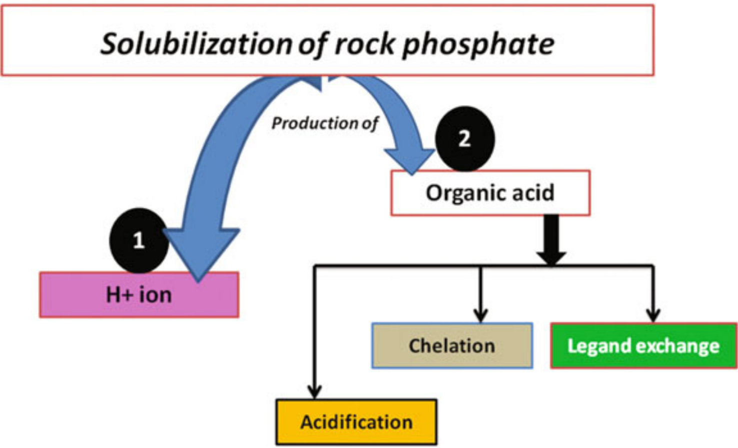

Fig. 17.1 The mechanism of rock phosphate solubilization (Arcand and Schneider 2006)

Table 17.1 Organic acids produced by PSMs responsible for rock phosphate solubilization

Organism	Organic acids	References
Rock phosphate-solubilizing bacteria		
Pseudomonas trivialis	Oxalic, gluconic, lactic, 2-ketogluconic, formic	Vyas and Gulati (2009)
Citrobacter sp.	Acetic, pyruvic, gluconic	Patel et al. (2008)
Fluorescent *Pseudomonas* sp.	Oxalic, gluconic, lactic, formic	Vyas and Gulati (2009)
Rock phosphate-solubilizing fungi		
Aspergillus flavus, *Aspergillus candidus*, *Aspergillus niger*, *Aspergillus terreus*, *Aspergillus wentii*, *Fusarium oxysporum*, *Penicillium chernestinum*, *Trichoderma isridae*, *Tritirachium* species	Acetic, citric, fumaric, gluconic, glutaric, lactic, maleic, malic, tartaric	Akintokun et al. (2007)
Penicillium bilaii	Citric, oxalic	Cunningham and Kuiack (1992)
Arbuscular mycorrhizal fungi	Citric, malonic, fumaric, malic, oxalic, tartaric, gluconic	Ouahmane et al. (2007)

concentration of soluble phosphate. This negative correlation indicates that rock phosphate solubilization is a consequence of the decrease in pH of the medium due to the production of organic acids. Yadav et al. (2017) also reported that organic acids could be generated in the cellular level to facilitate P solubilization from mineral phosphates in elevated quantity by the supplementation of both protons and metal-complexing organic acid anions, which might influence the microbial community in turn.

17.5 Agronomic Implications of Microbial-Mediated RP Solubilization

Due to the insolubility of rock phosphate, it becomes insufficient for the crop to uptake required the amount of soluble P. The PSM utilization can improve the crop yields up to 70% (Verma 1993). Several authors reported enhanced plant growth and higher uptake of P through the application of rock phosphate with PSMs to soils (Rachewad et al. 1992a, b). It has also been reported that the combined incorporation of PSB and arbuscular mycorrhiza results in higher uptake of both the native P from soil and the P from rock phosphate (Goenadi and Sugiarto 2000). In alfalfa plant the accumulation of N and P with increased biomass was seen, with the inoculation of AM fungi added with RP, in field conditions (Barea et al. 2002a, b). The application of potent PSMs increases the accessibility of soluble P and enhances plant growth by improving the biological nitrogen fixation (Kucey et al. 1989; Ponmurugan and Gopi 2006). Abbasi et al. (2013) stated that the combination of organic waste, PSB with RP, has shown significant increase in agronomic effectiveness of RP and improved soil fertility. The application of RP with these combinations would display favorable results, which will minimize the utilization of high-cost P fertilizers.

17.6 Future Prospects

The application of RP as a P fertilizer shows greater potential for becoming extensive once the bioavailability of P from RP sources can be enhanced to a range, in which there should be a significant quantity of crop yields and plant tissue P content. In the establishment of sustainable soil management systems, there is every reason to believe that the PSM as biofertilizers will likely to improve their utilization. The main focus of the agricultural produce is the consumers' health and the quality and nutritional value of those products. So in order to reach the consumer requirements, the employment of PSM as biofertilizers can be a preferable option to increase food production without imposing any health hazard and also to conserve the environment. Further research is required for the comprehensive study of in vitro RP solubilization to elucidate the exact biochemical mechanism involved in the release of P from insoluble RP in liquid broth, continue gaining an understanding about PSM, and transfer this knowledge into a form that can easily be utilized by farmers.

17.7 Conclusion

Phosphorus is one of the essential mineral macronutrient for the maintenance of plant growth regulation. Farmers need to apply chemical phosphorus fertilizer in a timely manner for the proper growth and to avoid P deficiency of plants. The efficacy

of phosphate fertilizers is scanty due to their poor mechanism of fixation in both acidic and alkaline soils, and it requires a greater input which can't be afforded by the farmers of the developing nations. The appliance of RP as a phosphatic fertilizer has the probability to become well-known once the bioavailability of P from RP sources can be improved to an extent so that the total crop yields and P content in plant tissue are increased significantly. The biological mechanism for microbe-induced RP solubilization represents innovative solutions for creating P from RP highly accessible to plant. Technologies show the potential to enhance dissolution of indigenous sources of RP and transform these resources into more agronomically effective P fertilizers. Principal mechanisms of RP solubilization is the lowering of pH by organic acids produced from microbes. More study is required to enhance the performance of phosphate-solubilizing microbes (PSMs) as microbial inoculants to increase the plant's nutrition. PSM technology incorporated with RP can contribute to low-energy input farming systems and a cleaner environment. Although there an undoubtable potential clearly exists for developing such kind of inoculants with RP, their widespread application remains limited by a poor understanding of population dynamics and microbial ecology in the soil and by the inconsistent performance over an extent of environments. Therefore, there is a need to develop microbial-mediated RP solubilization technologies specific to various regions, and approach should be taken to make the farmers understand in a short duration of time.

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