Chapter 19
Microbial Degradation of Petroleum Hydrocarbons: An Overview

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Abstract Petroleum hydrocarbon contamination is severe in both terrestrial and aquatic environments. Hydrocarbon contamination affects both vertebrates and invertebrates in the environment under threat by oil spills. Several microbes participate in the decomposition of petroleum hydrocarbons (PH) under natural conditions. Microbes' ability to remove PH has been studied extensively and exploited in the restoration of various spots affected by liquid hydrocarbon spills. Microbes involved in the decomposition of petroleum products consist of several bacteria and fungi. Almost 175 genera of bacteria, haloarchaeal bacteria, and eukarya are capable of degrading hydrocarbons into carbon dioxide and water. A consortium of microbes, rather than single species, is involved in degrading petroleum products as hydrocarbonoclastic microbes rarely function alone. Microbes isolated from terrestrial and marine environments contaminated with PH have proven to be excellent biodegraders of PH. Some of the Gram-negative hydrocarbonoclastic bacteria are *Pseudomonas aeruginosa*, *P. fluorescens*, *Vibrio*, *Haemophilus* spp., *Marinobacter*, *Cycloclasticus*, *Pseudoalteromonas*, *Marinomonas*, *and Halomonas*, whereas *Mycobacterium* spp., *Rhodococcus* spp., *Paenibacillus* spp., and *Bacillus subtilis* are some of the Gram-positive hydrocarbonoclastic bacteria. Hydrocarbonoclastic fungi include *Phanerochaete chrysosporium*, *Bjerkandera adusta*, *Penicillium* sp., *Aspergillus* sp., and *Pleurotus ostreatus*. It has been observed that bacteria, fungi, and microalgae are in close association while degrading PH, and it has been observed that the close association of bacteria and microalgae improves the degradation of PH. Enzymes produced by microbes which decompose PH comprise bacterial oxygenases and fungal exoenzymes such as lignin-modifying peroxidases like lignin peroxidases (LiPs), manganese peroxidases (MPs), and monocopper oxidases like laccases and epoxide hydrolases. Biodegradation of PH depends on various abiotic factors such as pH, temperature, oxygen, salinity, pressure, nutrients such as nitrogen and phosphorous, and their physical state. This review focuses on the microbes capable of degrading PH, the microbial processes involved in the degradation of

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PH, factors affecting the bioremediation, case studies involving bioremediation, and the recent strategies employed in in situ removal of PH.

19.1 Introduction

The need for oil as a fuel accelerated with the onset of the industrial revolution and has continued unabated in the recent years. Though oil as a fuel has led mankind to obtain unimaginable wealth and unparalleled technological development, human beings have started to understand the downside to the indiscriminate use of gasoline and derivatives. Long after the use of petroleum by-products ceases, mankind will continue to pay a huge price for its insatiable thirst for fossil fuels. Because of the ubiquity of vehicles and the usage of crude oil and it's by-products in all aspects of human life, contamination with PH is very common.

PH pollution occurs not only from the source, i.e., oil rigs, but also from other anthropogenic causes such as the oil spills from ships transporting oil across the ocean, oil spills from engines of motorized vehicles, and seepage from underground oil storage units. Therefore, the sources of PH contamination are extensive, and the threat to the environment is dire and must be mitigated. PH contamination affects living organisms in various ways. Oil spills on agricultural land leads to reduction in plant growth by affecting the germination process. It also affects soil aeration by displacing air from the pore space thus affecting its fertility.

Destructive effects of petroleum can be lethal or sub-lethal to plants and microbes. The rates of recovery from the effects of oil spills vary; wetland plants and mangroves take as much as 5 years and 20 years, respectively, to recover, while microalgae of the water column require only few weeks. High concentrations can be fatal to aquatic bacteria and algae. Invertebrates such as zooplankton and intertidal mollusks take few weeks to 10 years, respectively, to recover from the effects of polycyclic aromatic hydrocarbons (PAHs). Oil spills affect invertebrates both at the community and population levels. The early stages of vertebrates such as fish are more susceptible to oil as they do not have the means to escape. Movement of large quantities of crude oil into shallow waters can lead to heavy loss of adult fish. Many sublethal effects of PAH on fish have been recorded. It includes noncancerous lesions and cancerous tumors. PAHs can be lethal to adult reptiles, amphibians and their eggs. Sublethal and lethal effects can be seen on amphibian larvae. In marine oil spills, birds which spend more time on water are very vulnerable and often die to the ingestion of oil and coating of their feathers by spilled oil. Swallowed oil also causes other nontoxic effects in birds. Oil spills affect the local indigenous population more than others. Birds with a long life span and low procreative rate suffer most and take a long time to convalesce from oil spills. PAH fraction of petroleum has been found to be the reason for the toxicity of oil spills among birds. Animals with fur are more likely to be killed and animals which use fat as insulators from cold are occasionally destroyed by crude oil spills. Though the hydrocarbon ingestion can be cleared by metabolism, it causes nontoxic effects. Laboratory studies

have shown that various fractions of petroleum can cause cancer in animals. In short, oil spills affect invertebrates and vertebrates by different means such as contact, toxicity, and alteration of the habitat. Different fractions of petroleum cause different changes in animals; individual PAHs are toxic, whereas incompletely processed PAHs with an alkyl radical induce DNA impairment, growth anomalies, and malignant and nonmalignant variations in tissues. In human beings, PH cause liver damage and skin problems (Hoffman et al. 2002).

It is easy to understand how far reaching the effects of petroleum contamination are and the importance of the removal of these recalcitrant pollutants from the environment. Though several methods are available, none are cheaper, economic and environment friendlier than the use of the naturally occurring microorganisms. There are abundant studies which show the abilities of numerous naturally occurring microorganisms to degrade petroleum and its various fractions. Therefore it is wiser to augment and exploit their biodegradative potential for our purposes.

19.2 Role of Microbes in the Biodegradation of Petroleum Hydrocarbons

Several microbes degrade petroleum hydrocarbons. Plenty of scientific articles on the microbes involved in the biotransformation of PHs are available. Primary role in the biodegradation of PH is played by fungi and bacteria. Between them, bacteria are more versatile and hence play a major role.

19.2.1 Bacteria and Fungi Involved in the Biodegradation of Petroleum

Bacteria belonging to several genera are involved in the biodegradation of PH, and some of them are reviewed here based on published reports. According to the review by Chikere et al. (2011), bacteria belonging to the 20 different genera are of the most chief petroleum-degrading bacteria in the terrestrial environment. It comprises of Gram negatives such as *Achromobacter*, *Acinetobacter*, *Alcaligenes*, *Burkholderia*, *Collimonas*, *Flavobacterium*, *Pseudomonas*, *Ralstonia*, *Sphingomonas*, and *Variovorax*. *Bacillus*, *Arthrobacter (pleiomorphic*), *Corynebacterium*, *Dietzia*, *Gordonia*, *Micrococcus*, *Mycobacterium*, *Nocardia*, *Nocardioides*, *and Rhodococcus* are the Gram-positive bacteria seen in this category. The same study also has listed the fungi belonging to the following genera as common fungi isolated from soil which are capable of degrading PHs: *Aspergillus*, *Penicillium*, *Fusarium*, *Trichoderma*, and *Candida* which are *Ascomycetes*; *Cunninghamella* and *Mucor* belonging to the *Mucorales*; and *Phanerochaete*, *Sporobolomyces*, and *Rhodotorula* – basidiomycete fungi. Hyphomycete and zygomycete fungi of the terrestrial

environment are capable of metabolizing n-alkanes and di- and tricyclic polyaromatic hydrocarbons (Launen et al. 1995). Filamentous fungi are preferred for the bioremediation of flare pit soils contaminated with PHs as they are capable of accessing hydrocarbons by the movement of their hyphae without being restricted by hydrophobic surroundings, penetration of anoxic petroleum containing soil aggregates, and their ability to tolerate osmotic pressure and arid conditions and for the enzymes in the early stages of PH biodegradation (April et al. 1999). Several Gram-negative rods like *Pseudomonas fluorescens*, *P. aeruginosa*, *Alcaligenes* sp., *Acinetobacter lwoffii*, and *Flavobacterium* sp. from unhygienic tropical streams were good degraders of PHs. Gram-positive rods and cocci such as *Bacillus subtilis*, *Bacillus* sp., *Micrococcus roseus*, and *Corynebacterium* sp. from the same site were found to be adept in breaking down PHs (Adebusoye et al. 2007). Some 22 different genera of bacteria and fungi belonging to 14 different genera were listed by Bartha and Atlas (1977) as capable of utilizing PHs, and they were isolated from the aquatic environment. As high as 130 different bacterial strains were isolated from Bombay, India and were found to be excellent biodegraders of crude oil (Rahman et al. 2002). In hypersaline environments contaminated with PHs, the dominant bacteria capable of aerobic degradation observed in various studies are *Halomonas*, *Alcanivorax*, *Marinobacter*, *Haloferax*, *Haloarcula*, and *Halobacterium* (Fathepure 2014). Fungi such as *Fusarium lateritium* and *Drechslera* sp. are capable of metabolizing crude oil at a moderately saline environment with a salinity of 5–10 ppt (Obuekwe et al. 2005). Halophilic microbial consortium capable of degrading 5-ring polyaromatic hydrocarbons was isolated from the beach polluted due to the Prestige oil spill by Vila et al. (2010). Pyrene-degrading uncultured *Gordonia* sp. was the pivotal organism in the bacterial consortium isolated form waters polluted by oil spills (Gallego et al. 2014).

19.2.2 Ligninolytic and Non-ligninolytic Fungi Capable of Degrading Petroleum Hydrocarbons

Fungi capable of degrading PHs are of two types, namely, ligninolytic (producing lignin-modifying peroxidases like LiPs, MnPs, and copper-containing laccases) and fungi which produce cytochrome P450 monooxygenases like enzymes for degrading wood. Ligninolytic brown rot fungi such as *Laetiporus sulphureus* and *Flammulina velutipes* are known to degrade PHs like phenanthrene, fluoranthene, and fluorene. Unlike many bacteria, most fungi co-metabolize PHs into smaller compounds with the help of monooxygenases and also can lead to complete mineralization with the release of CO_2 (Cerniglia and Sutherland 2010).

Microbes involved in PH degradation were studied by culture-dependent methods and by PCR amplification of the universally conserved gene for 16S rRNA. Recently metagenomics is used to learn about the noncultivable microbes and proved to be an efficient way of evaluating the microbes engaged in the breakdown of PHs. Be it fungi or bacteria, microbes with biodegradation potential are

often isolated from contaminated sites as such indigenous microbes would have adapted to that environment and hence are more efficient than allochthonous microbes. Some of the fungi and bacteria involved in bioremediation of PHs and their sources are given in Tables 19.1 and 19.2.

Table 19.1 Microorganisms capable of degrading petroleum hydrocarbons

Strains	Source	Fraction degraded	References
Leclercia adecarboxylata	Soil polluted with oily sludge, Digboi oil refinery, Assam, India	Polycyclic aromatic hydrocarbons	Sarma et al. (2004)
S. marcescens *Bacillus pumilus* *B. carboniphilus* *B. megaterium* *B. cereus*	Naphtha-transporting pipeline, India	Naphtha	Rajasekar et al. (2010)
Serratia marcescens *Bacillus* sp. *B. cereus* *B. subtilis* *Pseudomonas aeruginosa* *Klebsiella* sp. *Pseudomonas* sp. *B. litoralis*	Petroleum-transporting pipeline, India	Diesel	Singh (2012)
Micrococcus sp. *Pseudomonas* *Acinetobacter* *Proteus* *Bacillus* *Actinomyces* *Corynebacterium* *Enterobacter* *Brevibacteria* *Citrobacter*	Nigeria	Crude oil	Adoki and Orugbani (2007)
Sulfate-, nitrate-reducing bacteria and fermenting bacteria	Sediment, Southeast Louisiana, USA	Crude oil, BP oil spill	Boopathy et al. (2012)
Alcanivorax	Marine oil spills, Japan	alkane	Harayama et al. (2004)
Cycloclasticus	an oil-coated grain of gravel, Japan	various aromatic hydrocarbons	Harayama et al. (2004)
Pseudomonas putida *Sphingomonas* sp.	Oil refinery sludge, Spain	Polycyclic aromatic hydrocarbons	Pizarro-Tobías et al. (2015)
Aspergillus sp., *Penicillium* sp.	Soil contaminated with oil spills, Madurai, India	Petroleum	Vanishree et al. (2014a, b)
Pseudomonas aeruginosa *Bacillus subtilis*	Soil from gasoline stations, Madurai, India	Petrol	Darsa and Thatheyus (2014) and Darsa et al. (2014)

Table 19.2 Offshore oil-degrading microorganisms

Strain	References
Penicillium *Aspergillus* *Fusarium* *Geotrichum* *Gliocladium* *Mucor*	Bartha and Atlas (1977) and Jenkins and Adams (2011)
Acremonium *Aspergillus* *Aureobasidium*, *Pichia* *Candida* sp., *C. maltose*, *C. tropicalis* *C. apicola* *Cladosporium* *Debaryomyces* *Monilia* *Mortierella* *Rhodotorula* *Saccharomyces* *Torulopsis* *Trichoderma* *Verticillium*	Boguslawska-WsE and Dbrowski (2001)
Vibrio sp., *Achromobacter*, *Acinetobacter* *Alcaligenes*, *Actinomycetes*, *Arthrobacter* *Bacillus*, *Cycloclasticus* *Coryneforms* *Chromobacterium* *Flavobacterium*, *Micrococcus* *Microbacterium*, *Mycobacterium* *Nocardia* *Pseudomonas*, *Sarcina* *Serratia*, *Streptomyces*, *Xanthomonas*	Bartha and Atlas (1977), Singh (2006) and Giebel et al. (2011)
Aspergillus sp., *Penicillium* sp.	Swannell et al. (1999)

19.3 Microbial Processes Observed in the Biodegradation of PH

Biodeterioration of PH can occur in both aerobic and anoxygenic metabolism; but aerobic metabolism is more common method of biodegradation. The most easily biodegradable fraction of PHs is *n*-alkanes, and the least biodegradable are the PAHs and asphaltenes. Commonly, there are three stages in the transformation of petroleum compounds by microbes: absorption of petroleum compounds to the surface of the microbes, followed by the transfer of these compounds to the cell membrane and finally the degradation of these xenobiotics inside the microbial cell (Atlas 1995).

The involvement of oxygen is seen in the aerobic pathway, and the enzymes such as mono- and dioxygenases add oxygen to the hydrocarbon (Widdel and Rabus 2001). Alkanes are converted to fatty acids by the catalytic action of microbial enzymes such as oxygenases and dehydrogenases. The fatty acids are then gradually mineralized to acetyl-CoA and finally into CO_2 and H_2O through the Krebs cycle. Enzymes such as alkane monooxygenase and dehydrogenases (fatty alcohol and fatty aldehyde) are vital in the efficiency of the biodegradation (Singh 2006). Several studies describe the degradation of alkanes in the presence of oxygen by bacteria as follows: the degradative pathway starts with oxidative attack at the terminal methyl group of alkanes forming fatty alcohol, fatty aldehyde, and fatty acid. Carboxylic acid through ß-oxidation can join with CoA to produce acetyl-CoA which passes into the tricarboxylic acid (TCA cycle) (Hua et al. 2011; Jiang et al. 2011). Microbial degradation of petroleum hydrocarbons by aerobic metabolism is dealt with comprehensively illustrated and explained in a review by Sierra-Garcia and de Oliveira (2013) and Gkorezis et al. (2016).

19.3.1 Enzymes and Genes Involved in the Oxygenic Degradation of PHs

Methane monooxygenases (MMO) are the first line of enzymes in the degradation of shorter (with one to four carbons)-chain length n-alkanes. There are two diverse forms of monooxygenases in the MMO enzyme family: sMMO-soluble (not membrane bound) di-iron methane monooxygenase and pMMO, methane monooxygenase which contains copper and is membrane bound. The alpha subunit of sMMO is encoded by *mmoX* and *pmoA* gene codes for the alpha subunit of pMMO. sMMO catalyzes the co-oxidation of a wide category of hydrocarbons. But pMMO acts mostly against short-chain length alkanes and alkenes (up to five carbons).

Butane monooxygenases (BMOs; also called as propane monooxygenases) catalyze the biotransformation of gaseous alkanes (Kotani et al. 2003). Enzymes such as sMMO and pMMO are seen in *Methylosinus trichosporium* and *Methylococcus capsulatus* (Baik et al. 2003; Lieberman et al. 2003). Cytochrome P450-1, cytochrome P450-2, and cytochrome P450-3 are some enzymes of the cytochrome P450 family of enzymes in *Alcanivorax borkumensis* involved in the degradation of alkanes (Schneiker et al. 2006). *alkB1* (codes for AlkB1), *alkB2* (codes for AlkB2), *p450-1* (codes for cytochrome P450-1), *p450-2* (codes for cytochrome P450-2), and *p450-3* (codes for cytochrome P450-3) are some of the genes coding for enzymes which catalyze the degradation of alkanes in *Alcanivorax hongdengensis* (Wang and Shao 2012). *alkB1* (AlkB1 – oxidizes n-alkanes with 16–24 carbon chains) and *alkB2* (AlkB2 – oxidizes n-alkanes of 12 to 20 carbon chains) are also seen in two separate strains of *Pseudomonas aeruginosa* and *Alcanivorax borkumensis* (Marin

et al. 2003; van Beilen et al. 2004). The enzyme AlkB has been isolated from a strain of *Gordonia* spp. (Lo Piccolo et al. 2011). An extensive list of alkane degrading enzymes and genes is reviewed by Gkorezis et al. (2016). The site of various enzymes in Gram-negative bacteria is illustrated by Van Hamme et al. (2003).

19.4 Degradation of Petroleum Hydrocarbons Under Anoxygenic Conditions

Though comparatively slower than the aerobic degradation pathways, anaerobic degradation pathways are significant because of the richness of anaerobic electron acceptors in the natural environment than dissolved oxygen. Also, less human intervention is required for anaerobic degradation systems. As a result, it would be worthwhile to pay more attention to understand and exploit anaerobic PH degradation in the future (Harayama et al. 2004).

Several investigators have observed that, in the absence of oxygen, various classes of PH are degraded by microbes with nitrate, ferrous iron, or sulfates the electron acceptors (So et al. 2003; Widdel and Rabus 2001).

Sulfate-reducing bacterium which closely resembles *Sulfococcus*, strain Hxd3, isolated from soil was found to be able to degrade alkanes to produce carbon dioxide under anaerobic condition which facilitated sulfate reduction. This strain can transform alkanes through subterminal carboxylation. Strain AK-01, isolated from estuarine sediment, is also a sulfate-reducing bacterium capable of degrading alkanes. Apart from sulfate reducers, denitrifiers adept in anoxygenic alkane breakdown were reported (Wilkes et al. 2002).

19.5 Pathways of Decomposition of n-alkanes Under Anoxygenic Conditions (Rabus et al. 2002; Kniemeyer et al. 2003)

n-alkanes are degraded by sulfur-reducing bacterium, strain Hxd3 under anaerobic conditions in the following manner: As the first step, the alkyl chain is carboxylated at the C3 position leading to the formation of intermediates. Next, removal of two carbon atoms from carbon 1 and carbon 2 to produce fatty acid which was mineralized to carbon di oxide by β-oxidation or gets assimilated into the microbial cell. (b) The strain HxN1 which is a denitrifier converts n-alkane and fumarate to (1-methylalkyl) succinate which was then changed to CoA-thioester. CoA-thioester undergoes reorganization to produce to form an intermediate which undergoes decarboxylation to form 4-methylalkyl-CoA. 4-Methylalkyl-CoA undergoes β-oxidation and is further degraded. This pathway leads to regeneration of fumarate that is recycled to activate n-alkane.

19.5.1 Mechanisms of Biodegradation of Petroleum Hydrocarbons by Non-ligninolytic Fungi

Most central paths for the breakdown of PAHs by non-ligninolytic fungi involve cytochrome P450 monooxygenase enzymes. *Cunninghamella elegans* and *Pleurotus ostreatus* (ligninolytic fungi) degrade PAHs in the following way: (i) cytochrome P450 monooxygenase catalyzes the formation of less stable arene oxide, and (ii) transformation of arene oxide into trans-dihydrodiol with the help of epoxide-hydrolase enzyme. Through other enzyme-independent pathways, arene oxide can be reorganized into phenol derivatives and later joined to sulfate, xylose, glucuronic acid, or glucose. *C. elegans* converts fluoranthene into fluoranthene dihydrodiol intermediates. Whether the intermediate metabolites produced are less toxic or more toxic than the parental molecule depends on the fungal enzymes involved in the catalysis. Oxidation of PAHs mediated by cytochrome P450 monooxygenases produces highly toxic and carcinogenic epoxides and dihydrodiols. On the other hand, PAH oxidation by peroxidase leads to the formation of the less toxic quinine. Therefore, it is obvious that it is wiser to pursue a strategy involving the ligninolytic enzymes for the decontamination and detoxification of sites with PAHs contamination (Jerina 1983; Sutherland et al. 1995; Tortella et al. 2005; Cerniglia and Sutherland 2010).

19.5.2 Mechanisms of Biodegradation of PHs by Fungi Capable of Degrading Lignin

Ligninolytic white-rot fungi are common. They produce extracellular lignin-degrading enzymes such as LiPs, MPs, and laccases. These two peroxidases and laccases (phenol oxidase enzymes) produced for the degradation of wood lignin can also metabolize PAHs (Hammel 1995; Cerniglia and Sutherland 2010). Unlike bacterial intracellular enzymes, the fungal extracellular enzymes move toward the immobile PAHs. Therefore, they are considered as better in the initial attack against PAHs in soil than the bacterial enzymes. Also, ligninolytic fungal enzymes have broad substrate specificity than their bacterial counterparts and can degrade a broad category of substrates, including the most unmanageable (Tortella et al. 2005; Cerniglia and Sutherland 2010). The transformation of PAHs by ligninolytic fungi is as follows: oxidation of PAH ring by hydroxyl free radicals produced by ligninolytic enzymes results in the production of PAH-quinones and acids (Sutherland et al. 1995).

Bezalel et al. (1997) reported that cytochrome P450 monooxygenases and epoxide hydrolases together are used by ligninolytic fungi to metabolize PAHs. The authors also have deduced the process of degradation of phenanthrene by *Pleurotus ostreatus*. Andersson et al. (2003) reported the degradation of soil artificially spiked with a variety of PAHs like fluorene by *Antrodia vaillantii* and *Pleurotus ostreatus*.

Increased degradation of PAHs was observed although the degradation processes by *Pleurotus ostreatus* resulted in toxic metabolites and reduction of the local microbial population which could have been the reason for the lack of complete mineralization of PAH (Tortella et al. 2005; Cerniglia and Sutherland 2010). But, *Antrodia vaillantii* did not produce any toxic metabolites while degrading in spite of having a similar degradative pathway. The white-rot fungus *Phanerochaete chrysosporium* also oxidized a variety of PAHs like pyrene, anthracene to their equivalent quinines with the help of LiPs and MPs (Bogan et al. 1996). Complete decomposition of high molecular weight PAHs by *Phanerochaete chrysosporium* has also been reported (May et al. 1997). Soil fungi such as *Aspergillus* sp., *Trichocladium canadense*, and *Fusarium oxysporum* can degrade both high and low molecular weight PAHs under microaerobic and near anoxic conditions (Silva et al. 2009). Extensive degradation of high molecular weight PAHs was reported in *T. canadense*, *Aspergillus* sp., *Verticillium* sp., and *Acremonium* sp. These studies prove that fungi are similarly worthy candidates for the biotransformation of sites polluted with PHs along with their bacterial counterparts.

19.6 Factors Affecting the Microbial Biodegradation of PHs

There are three crucial interconnected categories consisting of parameters that command the level of PHC "exposure" to biodegradation: (a) *microbial properties*, (b) *environmental factors*, and (c) *properties of the petroleum hydrocarbon* (Gkorezis et al. 2016).

The diesel biodegradation in a high-altitude alpine glacier shows the role of several factors illustrated above. The addition of nutrients is one of the chief factors that affect the rate of degradation of hydrocarbons. Addition of N-P-K fertilizers to alpine soils in high-altitude glaciers leads to higher degradation of diesel (Margesin and Schinner 2001). However, in the second year, the degradation efficiency was low, and it was concluded that both acidification and aging of the diesel could have contributed to the low rate of degradation. *Gamma-* and *Betaproteobacteria* were noticed in higher abundances in polluted soils than in pristine soils (Labbé et al. 2007). There was upregulation of genes of enzymes involved in biodegradation such as *alkB*, *xylE*, and *ndoB* in *Pseudomonas* in polluted soils than unpolluted virgin sites. Such upregulation was also seen in the case of *alkM* genes in *Acinetobacter* isolated from polluted soils (Margesin et al. 2003). The effect of temperature on biodegradation could be observed as the degradation rates were increased during the summer seasons due to high metabolic rates in the summer season. It was also observed that the lower temperature reduces the bioavailability of the PHs. The effect of temperature on the bacterial communities was visible by the fact that *Proteobacteria*, particularly *Gammaproteobacteria*, are best-adjusted bacterial group in cooler environments with hydrocarbon pollution. Uhlik et al. (2012) also observed the dominance of *Proteobacteria* in sites with chronic contamination of

aromatic hydrocarbons. The *Proteobacteria* dominance increased with the addition of biphenyl, naphthalene, or benzoate.

The composition of the crude oil also affects the biodegradation efficiency. Compared to low molecular weight PAHs, fungal degradation of heavier aromatics, resins, and asphaltenes is tough and sluggish (Atlas 1981; Leahy and Colwell 1990). Change in microbial community in response to pollution also affects the rate of degradation. The increase in the obligate hydrocarbonoclastic bacteria (OHCB) at the time of PH pollution is important as they are capable of breaking down hydrocarbons which are useless for other microbes (Yakimov et al. 2007). *Alcanivorax borkumensis* SK2 is a model OHCB, and it is very versatile in being capable of degrading even longer alkanes and long-chain isoprenoids and produces biosurfactants and exopolysaccharides. The adaptation of the strain SK2 to degrade PHs is very obvious by the presence of three P450 cytochromes and two alkane hydrolases (AlkB1, AlkB2) for the degradation of PHs. This strain also lacks the genes necessary for glucose metabolism highlighting its specialized adaptations to degrade PHs (Schneiker et al. 2006). Ghosal et al. (2016) have detailed the role played by various factors that affect the efficiency of PH degradation. Apart from pH, oxygen, nutrients, and bioavailability of the hydrocarbons, the production of toxic metabolites during biodegradation also plays a major role.

19.7 Case Studies

Though there are numerous bacteria and fungi showing enormous potential as biodegraders of petroleum in vitro, their value can be tested truly only when they are applied for the decontamination of sites with petroleum spillage. Such in situ decontamination of polluted sites reveals how efficient the microbes are in real-world situations when they are exposed to a plethora of interdependent factors. Let us review some of these case studies where in situ bioremediation was undertaken.

19.7.1 Bioremediation of Alpine Skiing Area Polluted with PHs

Margesin and Schinner (2001) demonstrated that biodegradation is possible even in extreme environments like the alpine glaciers which are at 3000 m from sea level. Field incubated mesocosms (lysimeters) were used with and without the addition of fertilizers (N, P, K). For three seasons, the alpine soil contaminated with diesel oil was monitored along with the soil leachate. After 780 days, there was approximately 70% and approximately 50% reduction in the soil with fertilizers and soil without fertilizers, respectively. This significant reduction of diesel oil was much better in fertilized soils than in unfertilized sites with significant enhancement of

microbial numbers and other biological parameters such as soil respiration and catalase and lipase activity. However, the desired cleanup of the site could not be achieved because of the rapid decline in the biostimulatory effect of the autochthonous soil microbes. This study is an example that significant decontamination of PHs by bioremediation is possible even in unfavorable environments.

19.7.2 In Situ Bioremediation of Prestige Oil Spill (Medina-Bellver et al. 2005)

In November 2002, several hundred kilometers of the Spanish coastline was contaminated with heavy fuel following the sinking of Prestige, an oil tanker carrying 17,000 tonnes of oil. Samples were collected in the subsequent months (December 2002 and February 2003), from the Galician coast in order to evaluate the ability of the indigenous population for in situ biodegradation. Their results showed that indigenous bacteria were capable of transforming the crude oil (naphthalene, anthracene, phenanthrene, pyrene, and undecane) into inorganic carbon. Nitrogen and phosphorous are the restrictive factors in the bioremediation of marine oil spills. This shows the significance of adding nutrients to increase the rate of bioremediation.

19.7.3 Bioremediation of Acidic Oil Sludge, Digboi Refinery Premises, Assam, India

The in situ degradation potential of *Candida digboiensis* TERI ASN6, isolated from the premises of Digboi refinery, Assam, northeast India, has been demonstrated. Thousands of tonnes of acidic oil sludge with highly acidic pH (pH 1–3) had accumulated in the premises due to primeval wax refining techniques. In the laboratory, *Candida digboiensis* TERI ASN6 was found to breakdown acidic petroleum hydrocarbons under acidic conditions (at pH 3). Since the addition of nutrients was able to increase the efficiency of the bioremediation of the acidic sludge by *C. digboiensis* TERI ASN6, this type of treatment was selected for complete in situ bioremediation of the refinery premises. Under laboratory conditions, in minimal medium, the yeast strain degraded alkanes like eicosane and heneicosane at pH 3 in a fortnight. The in vitro treatment of soil with total petroleum hydrocarbons (TPH) by the novel acidophilic yeast strain with nutrients resulted in the significant removal of TPH when compared with the untreated soil. Subsequently, this treatment was tried out on the contaminated area of nearly 3500 m^2 of the factory which resulted in the reduction of TPH to 7.96 g kg^{-1} soil in nearly 6 months (75 days). This novel yeast's ability to withstand highly acidic conditions is pertinent to decontaminate sites with low pH and also underscores the need to isolate indigenous microorganisms for use

in bioremediation. Microorganisms isolated from contaminated sites are more robust and are more suited and acclimatized to that particular condition as they are autochthonous (Sood et al. 2010).

19.7.4 Bioremediation of Weathered Drill Wastes Severely Contaminated with TPHs

Heavy contamination with TPH and BTEX (benzene, toluene, ethylbenzene, and xylene) (51.2–95.5 mg kg^{-1}) of weathered drill wastes was decontaminated with the help of a combination of indigenous bacterial and fungal consortium. It was carried out in 3 years in three stages such as initial remediation, basic biotransformation, and inoculating a biopreparation. The microbial consortium consisted of 14 nonpathogenic indigenous bacteria (mostly *Actinomycetes*) and 5 nonpathogenic fungi. Bacterial consortium was found to be able to degrade 63–75% of nC(9)-nC(20), 36–51% of nC(21)-nC(36), 36% of BTEX, and 20% of PAHs, whereas adding fungi increased the efficiency of biodegradation of these contaminants and they were finally reduced to soil standards. The in situ bioremediation was efficient with the soil becoming nontoxic at the end of 3 years (Steliga et al. 2012).

19.7.5 In Situ Bioremediation of Soil Polluted with Oil Sludge (Mishra et al. 2001)

Oil refinery soil polluted with oil sludge was evaluated for indigenous hydrocarbon degrading capacity. It was observed that the autochthonous bacteria in the soil capable of degrading hydrocarbons were very little (10^3–10^4 CFU/g). Therefore, it was decided to stimulate bioremediation by adding *Acinetobacter baumannii* and nutrients to the contaminated site. Before the full-scale study, a feasibility study was undertaken with six different treatments. Among the treatment procedures, it was observed that the addition of nutrients and the addition of a bacterial consortium with *Acinetobacter baumannii* bring about the maximum biodegradation of TPH in 4 months. Hence, biostimulation with *Acinetobacter baumannii* and nutrients was taken up for the comprehensive study. Plot A and plot B were spiked with the bacterial consortium along with nutrients, while plot C was kept as control. In plot A and plot B, 92% and 90% of the TPH was removed in 12 months compared to 14% removal of TPH in the control. In 365 days, the alkane, aromatic, and NSO (nitrogen-, sulfur-, and oxygen-containing compound) and asphaltene portions of TPH in plot A were reduced by 94%, 91%, and 85%, respectively. In plot B also, more than 90% of the alkanes and aromatics were removed. The NSO plus asphaltene fractions were reduced by 63.5% within a year (345 days). In the control, plot C, only 17% of alkane and 12.9% of aromatic fractions were removed, whereas the removal

of NSO plus asphaltene fraction was minimal (5.8%). Thus there was almost complete removal of TPH from the contaminated soils and the introduced *Acinetobacter baumannii* strains were found to be stable even after a year of addition. Moreover, within a year, there was significant improvement of the physicochemical characteristics in the soil at the refinery sites under biodegradation.

19.7.6 Bioremediation and Rhizoremediation of PH Contaminated Sites in Spain (Pizarro-Tobías et al. 2015)

An in situ bioremediation and rhizoremediation was tried out in petroleum-contaminated oil refinery in Murcia, Spain. Three types of treatments, namely, microbial consortium containing rhizobacteria which are excellent plant growth promoters, PAH-degrading bacteria, and the combination of these microbial consortia with pasture plants, were employed on selected sites where the TPH contamination was 30,000 ppm. The study showed that rhizoremediation favored the growth of autochthonous petroleum-degrading microbes. Toward the end of the treatment duration, there was significant decrease of TPHs in the soil. The microbial consortia used contained several *Pseudomonas putida* strains and *Sphingomonas* strains. Also bacteria belonging to the phyla *Actinobacteria*, *Bacteroidetes*, *Gemmatimonadetes*, and *Proteobacteria* were involved in the in situ biodegradation.

19.8 Recent Strategies Employed in In Situ Removal of PHs

Rhizoremediation or rhizodegradation is a recent strategy to decontaminate terrestrial ecosystems polluted by oil spills. The symbiotic relationship between plants and bacteria increases the growth and survival of the plants in addition to improving the decontamination of polluted sites. As a substitute, the addition of plants can significantly increase biodegradation rates and effects as plants offer a niche for microorganisms, advance soil permeability (thus allowing the bulk transmission of substrates and electron acceptors), and interchange restrictive nutrients with their microbial equals. In exchange, plant-associated microbes advance plant development by decreasing soil noxiousness through pollutant elimination, generating plant growth promoting substances, releasing appropriated plant nutrients from soil, fixing atmospheric nitrogen for nitrogen assimilation, and largely creating the basics of nutrient cycling. For practical purposes, the joint deeds of plants and their associated microorganisms are beneficial for restoration of PAH-polluted soil as it is economical and successful in diverse settings. Several successful experiments on the removal of PHs have been carried out by rhizoremediation. *Zea mays* was employed in conjunction with *Pseudomonas* sp. and *Pseudomonas putida* for the successful bioremediation of

phenanthrene/pyrene (Chouychai et al. 2009, 2012). *Zea mays* was also employed along with *Gordonia* sp. for removing diesel by Hong et al. (2011). *Pantoea* sp. strain BTRH79 and the plant *Lotus corniculatus* were able to successfully remove diesel oil (Yousaf et al. 2010).Yu et al. (2011) employed the bacterium *Acinetobacter* sp. along with the plant *Lolium multiflorum* for the removal of various PAHs.

19.9 Conclusions

It has been long known that indigenous microbes in the polluted sites are capable of removing the pollutants as part of their metabolism. For decades, this microbial metabolism has been exploited for the noble purpose of mitigating the ever increasing pollution of the environment. Though many of the studies are lab-scale degradation experiments, it is necessary to proceed to the field level or in situ studies to test their efficacy under more complex, real-world situations. As more and more success stories of in situ bioremediation of PHs emerges, it gives hope that that these tenacious pollutants might be removed from the environment and restoration of pristine conditions is possible. More research is required to overcome bottlenecks and to discover better and novel microorganisms capable of degrading PHs. As research suggests, microbial consortium is preferred over a single microbe as the degradation efficiency is better in the former. The catabolic properties of microbes can be enhanced or modified by gene manipulation, and such genetically modified organisms also can be used in the fight to remove PH contamination. The need of the day is the development of cheaper and efficient ways to remove pollutants from the environment. Microbial bioremediation has given a glimpse of this, and more research is required to revolutionize this field and to properly harness the abilities of these incredibly versatile microbes.

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