Vivek Kumar · Manoj Kumar
Ram Prasad *Editors*

Microbial Action on Hydrocarbons

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Preface

Hydrocarbon-based products are chief source of energy for automobiles, industry, and everyday life. Leakages and unintentional and inadvertent spills occur regularly during the exploration process, production process, refining process, transportation, and stowage of hydrocarbon and hydrocarbon products. The amount of raw crude oil waste was estimated to be around 600,000 metric tons per year with a range of uncertainty of 200,000 metric tons per year. Discharge of petroleum hydrocarbons into the nature whether unintentionally or owing to human activities is a foremost cause of soil, water, and air pollution. Contamination of soil with petroleum hydrocarbons triggers the widespread damage of local ecosystem, since buildup of pollutants in the plants and animals' tissue may initiate mutations or mortality. Technology usually employed for remediation of soil includes several methods such as mechanical, covering, evaporation, dispersal, and washing. Conversely, these know-hows are not cost-effective and may lead to the incomplete breakdown or disintegration of hydrocarbon contaminants.

To overcome the side effects of the abovementioned approaches, the application of potential microbes has been suggested, and this tactic is known as bioremediation. The procedure of bioremediation, defined as the application of effective microbes to depollute or eradicate the pollutants due to their varied metabolic proficiencies, is a developing scheme for exclusion, detoxification, and degradation of various ecological pollutants which also comprise the hydrocarbon products. Furthermore, bioremediation expertise is believed to be harmless, ecofriendly, and comparatively economical. Biodegradation by naturally occurring microbial populations represents one of the prime mechanisms by which the hydrocarbon and other petroleum contaminants can be separated from the environment in a cheaper way compared to other remediation technologies.

Accomplishment of oil spill bioremediation relies on someone's ability to create and maintain such conditions that support enhanced hydrocarbon biodegradation rates in the contaminated environment. Numerous scientific research articles have covered several factors that manipulate the hydrocarbon biodegradation process. One significant requirement is the existence of microbes having the proper metabolic competences. In the presence of these potential microbes, and by guaranteeing

the sufficient oxygen and nutrient concentrations, the ideal growth rates and hydrocarbon biodegradation can be continual at a pH between 6 and 9. Further to sustain the biodegradation process, the chemical and physical features of the hydrocarbon and its surface area are also imperative in determining the success of bioremediation. To achieve the process of bioremediation of spill, there are two foremost tactics to petroleum spill bioremediation: (1) bioaugmentation process, in which the known petroleum degrading bacteria are added to complement the prevailing microbial population, and (2) biostimulation process, in which the autochthonous petroleum degraders' growth is encouraged by the addition of suitable nutrients or by other growth-restrictive co-substrates.

Maximum existing research studies have focused on assessing the hydrocarbon bioremediation affecting factors or examining different hydrocarbon products and approaches through laboratory studies only. Merely very few numbers of field trials and large-scale tryouts have delivered the conclusive and substantial demonstrations of this microbial technology. The current understanding of petroleum bioremediation is also limited because most of these field studies have been carried out on the appraisal of bioremediation technology dealing with large-scale marine shoreline oil spills. All over the globe, various petroleum hydrocarbon-contaminated environments are categorized by low or high temperatures, high salt concentrations, acidic or alkaline pH, or varying pressure. Hydrocarbon-degrading microbes adapt themselves to grow and flourish in these environments and play a significant role in the natural remediation of extreme habitats. On the other hand, our knowledge of bioremediation aspects of barophilic, acidophilic, or alkaliphilic microbes is limited.

This book entitled *Microbial Action on Hydrocarbons* attempts to undertake bioremediation problems of hydrocarbons especially the raw petroleum oil and its obstinate aromatic parts in soil, water, and air, although the primary focus of this book is to establish the methods, mechanism, extent, and efficacy of bioremediation process. To understand better the process of biodegradation, it is also essential to understand the exact chemical composition of original petroleum hydrocarbon product at the place of spill and the native microbial populations. This edition introduces the reader to biodegradation technology, which is a key process in the bioremediation of hydrocarbon-based contaminants at spill places. Furthermore, this book also provides a rationalized information related to microbial bioremediation of petroleum hydrocarbon contaminants toward enhanced understanding in biodegradation challenges. We wholeheartedly thank all the contributors who have proficiency in this field of research for their cutting-edge, timely chapters and their help in making this book a successful attempt.

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About the Editors

Dr. Vivek Kumar is an Associate Professor at the Himalayan School of Biosciences, Swami Rama Himalayan University, Jolly Grant, Dehradun, India, and is leading the Plant-Microbe Interaction and Microbial Ecology research group. He obtained his master's and doctoral degrees from CCS Haryana Agricultural University, Hisar, Haryana, India. He is an editor and reviewer of several respected international journals. He has published over a hundred research papers, book chapters, and review articles. He has also authored one practical manual and edited several books with Springer Nature. Dr. Kumar also served as a microbiologist for 8 years at the Department of Soil and Water Research, Public Authority of Agricultural Affairs and Fish Resources, Kuwait. He has been credited with the first reporting and identification of Pink Rot inflorescence disease of the date palm in Kuwait caused by *Serratia marcescens.* He has also organized a number of conferences/workshops as convener/organizing secretary.

Dr. Kumar's research areas include environmental microbiology, plant-microbe interactions, and bioremediation. In 2002, he was awarded "Young Scientist Award" in "Agricultural Microbiology" by the Association of Microbiologists of India (AMI).

Dr. Manoj Kumar is currently working as an Associate Professor at the Centre for Life Sciences, Central University of Jharkhand, Ranchi, India, and is leading the Plant Developmental Biology research group. He pursued his PhD in Plant Biotechnology at the prestigious Jawaharlal Nehru University and was then awarded two consecutive postdoctoral fellowships: DBT-PDF from IISc Bangalore in 2005 and then NRF-PDF from the University of Pretoria. His research interests focus on plant biotechnology, and he is affiliated with Ton Duc Thang University, Vietnam. Until recently, he was a coordinator of the Bio-resource Chapter (Northern India) and served on the editorial boards of five international journals.

Dr. Kumar has published several research papers, books, and review articles. He has also guided several research projects and collaborated internationally.

Dr. Ram Prasad is an Assistant Professor at the Amity Institute of Microbial Technology, Amity University, Uttar Pradesh, India. His research interests include plant-microbe interactions, sustainable agriculture, and microbial nanobiotechnology. Dr. Prasad has published more than a hundred research papers, reviews, and book chapters and has five patents issued or pending and edited or authored several books. Dr. Prasad has 12 years of teaching experience and was awarded the Young Scientist Award (2007) and Prof. J.S. Datta Munshi Gold Medal (2009) by the International Society for Ecological Communications, FSAB Fellowship (2010) by the Society for Applied Biotechnology, Outstanding Scientist Award (2015) in the field of Microbiology by Venus International Foundation, BRICPL Science Investigator Award (2017), Award for Excellence in Research from 6th Academic Brilliance Awards-2018, and the American Cancer Society UICC International Fellowship for Beginning Investigators (USA, 2014). In 2014–2015, Dr. Prasad served as a Visiting Assistant Professor at the Department of Mechanical Engineering at Johns Hopkins University, USA, and since 2017 has served as a Research Associate Professor at the School of Environmental Science and Engineering, Sun Yat-Sen University, China.

Chapter 1
Microbial Consortia and Biodegradation of Petroleum Hydrocarbons in Marine Environments

J. Paniagua-Michel and Babu Z. Fathepure

Abstract The pollution of petroleum hydrocarbons in the sea is an increasingly widespread international problem that threatens the environment and human health. At present, there are important advances in relation to innovative and effective technologies for the elimination of oil contaminants from the marine environment. The main advantages of microbial remediation lie in its low cost and high efficiency in a sustainable manner. Numerous laboratory-scale studies and field application of microorganisms to clean up hydrocarbon-impacted marine and coastal environments have clearly demonstrated the viability of bioremediation technologies under various environmental conditions. In addition, due to the complex mixture represented by petroleum hydrocarbons, a consortium of taxonomically diverse species with broad enzymatic capabilities is required, because a single species can metabolize only a limited range of hydrocarbon substrates. Because, in natural environments, most of the microorganisms (>99%) coexist in the form of microbial consortia, there are major expectations on the uses of consortia of microorganisms to perform the degradation of complex molecules present in petroleum hydrocarbons. The members of the microbial communities acting together may exhibit the ability to secrete biosurfactants leading to the enhanced solubilization and removal of hydrophobic hydrocarbons. Recent reports have evidenced that halophilic bacteria and archaea have the capacity not only to cope with high-salinity stress but also be able to metabolize n-alkanes and PAHs suggesting their key role in mitigating vast areas of highly saline coastal habitats impacted by petroleum compounds that pose threat to both terrestrial and marine ecosystems. In this chapter, we report on recent developments on the biodegradation and bioremediation of petroleum hydrocarbons by microbial communities in marine and other high-salinity environments,

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and molecular mechanism of hydrocarbon degradation in halophiles has been described.

1.1 Introduction

The anthropogenic and global pollution of marine and coastal environments by petroleum hydrocarbons is considered as one of the major environmental problems and a negative consequence of industrialization worldwide. The major pollution accidents in marine environments are attributed to crude oil, which represents a constant destructive agent to marine habitats with serious consequences to the human health due its carcinogenic, neurotoxic, and mutagenic effects to both aquatic and terrestrial organisms (Das and Chandran 2011; Shen et al. 2017) as well as the respective socioeconomic implications (Chronopoulou et al. 2014). One of the most recent and catastrophic accidents of the Deepwater Horizon rig released about 700 million liters of crude oil and a massive formation of oil slick that impacted the ecology of the shoreline. Such accidents and constant coastal oil spills are deleterious agents to marine ecosystems, viz., vulnerable coastal zones, salt marshes, estuaries, and other productive coastal lagoons (Coulon et al. 2012). Crude oil composition is a mixture of molecular species represented by heteroatomic and non-heteroatomic hydrocarbons and classified into different hydrocarbons (saturated, aromatic, resins, and asphaltenes) (Prince and Walters 2007). The complex chemical components of petroleum, such as the lipophilic nature of PAHs, represent a high potential for biomagnification through marine and aquatic organisms and their respective food chain (Patowary et al. 2016). Studies concerning petroleum hydrocarbons in the marine environment have been under intense research. However, little knowledge is reported concerning the development and applied bioremediation of petroleum pollutants (crude oil and PAHs). The potential application of marine microbial consortia in the mitigation of pollution by petroleum hydrocarbons is considered an effective strategy to achieve eco-friendly bioremediation leading to sustainability in fragile marine environments and, presently, the only option for high biodiversity and sensitive environments. Among the main physical and chemical technologies applied for the remediation of the environment, biological methods, such as bioremediation, are selectively preferred because of its low cost and sustainability, besides safety and efficiency.

The ability of microbial consortia to biodegrade petroleum pollutants such as phenols, benzoates, monoaromatics (benzene, toluene, ethylbenzene, xylenes), polyaromatics (phenanthrene, naphthalene, etc), aliphatics and other components in crude oil represents a promissory blue technology to be used in the bioremediation of coastal and impacted seawater bodies. In a bioremediation process of a complex mixture such as in the case of petroleum hydrocarbons, a microbial consortium composed of diverse taxonomic microorganisms is preferable since the chances of finding a microorganism capable of degrading a mixture of different contaminants are almost impossible (Safonova et al. 2004).

Here we focus on recent progress on biodegradation and bioremediation of petroleum hydrocarbons by microbial consortia from marine environments including aspects of the effect of saline and hypersaline environments on the biodegradation efficiency of petroleum contaminants by microbial components structured in consortia.

1.2 Blue Technologies to Recover Oil-Polluted Areas

Nowadays many innovative technologies are under development against oil spills, aiming the restoration of polluted seawater environments, based on the biochemical capability and enhancement of indigenous microbiomes to degrade oil spills (Shen et al. 2017). Among the existing technologies applied for this purpose (physical and chemical methods), bioremediation is considered the ultimate fate, when hydrocarbon enters the marine environment (Divya et al. 2015). This blue technology, or technology for the ocean (marine bioremediation), is a socially acceptable and a noninvasive approach to minimize and to restore the environmental quality of sites impacted by deliberated point source and nonpoint source oil spills (Paniagua-Michel and Rosales 2015). This technology, and their respective biostimulation and bioaugmentation, has proven to be suitable alternative and in some cases the only viable option that can be implemented on a large scale in ocean environments (Snape et al. 2001; Hassanshahian et al. 2014). At sea, microorganisms play an important function in degrading petroleum hydrocarbons. The oceans harbor the ancient forms of life of all and different taxa of microorganisms (Prince 2010) which have evolved through time and that have been developing at different scales and before the human presence to coexist with hydrocarbons. Even that, an important number of marine microorganisms is biochemically equipped to biosynthesize and to flourish on hydrocarbons as their preferred carbon substrata. Hence, spilled hydrocarbons on the world's oceans from natural percolates have been contributing for millennia of years to nourish coexisting species (600,000 tonnes, 700 million liters per year) (National Research Council 2003).

When oil components reach the shore, they can remain for long periods and are harmful to marine organisms (Chronopoulou et al. 2014; Atlas and Bragg 2009). However, many microbes have developed pathways for hydrocarbon metabolism, which contribute to the removal of hydrocarbon pollutant components (Yakimov et al. 1998, 2007; McGenity 2014). Therefore mentioned properties of marine microorganisms are being harnessed in the approaches of biodegradation and bioremediation of pollutants in fragile and sensitive environments such as the marine ecosystem. This technology leads to complete mineralization, is eco-friendly and cost-efficient, and may lead to sustainable solutions. Because of the efficient conversion of complex organic pollutants, the final step of bioremediation is the mineralization of hydrocarbon contaminants into innocuous products such as water, inorganic products, cell protein, and carbon dioxide (Olajire and Essien 2014). In marine and aquatic environments, such as in estuarine, coastal lagoons, bays, and

deep-sea areas, degradation of petroleum is dependent on nutrient concentrations, mainly nitrogen and phosphorus as well as of salinity, temperature, pressure, pH, and oxygen levels which are main factors influencing the kinetics of biodegradation. In principle, microbial degradation is the current strategy applied after an oil spill, in which consortia of microorganisms effectively degrade aliphatics, monoaromatics, and light PAH compounds. Other compounds such as complex compounds of high-molecular composition (aromatics, asphaltenes, and resins) have been reported as recalcitrant or degrade slowly. Successful examples of bioremediation in an oil-polluted coastal marine site of Kuwait were reported by using microbial consortia with dominating taxa represented by cyanobacteria, viz., *Lyngbya*, *Phormidium*, and *Microcoleus*. The mat components achieved successful oxidation and removal of n-alkanes (Al-Hasan et al. 1998). The complementary action of the different taxa of consortia may play simultaneously functional roles during biodegradation and removal of pollutants. For instance, phototrophs (*Cyanobacteria* and microalgae) in consortia by means of photosynthesis provide molecular oxygen, a key electron acceptor to the pollutant-degrading heterotrophic bacteria at the beginning of the degradation stages (Cerniglia 1992). In turn, bacteria support photoautotrophic growth of the partners by providing carbon dioxide and other stimulatory compounds (Subashchandrabose et al. 2011), as exemplified in Fig. 1.1. Moreover, other important roles of cyanobacteria and algae contribute to combine biosorption and transformation processes to mitigate and to remove xenobiotics from the environment. Thus, the shared phototrophic and heterotrophic capabilities contribute to the biodegradation potential of all consortium structures aiming the removal of pollutants (Rosales and Paniagua-Michel 2014; Safanova et al. 2004).

Nevertheless, the oil composition determines the rate of biodegradation; thus the saturated components are fast degraded followed by the aromatic (Sugiura et al. 1997). Because of the complex mixture and structural components that represent crude and refined oils, their biodegradation is challenging because they display selective actions on specific molecules (Prince and Walters 2007). That is why the shared functions of several species with different enzymatic composition lead to an

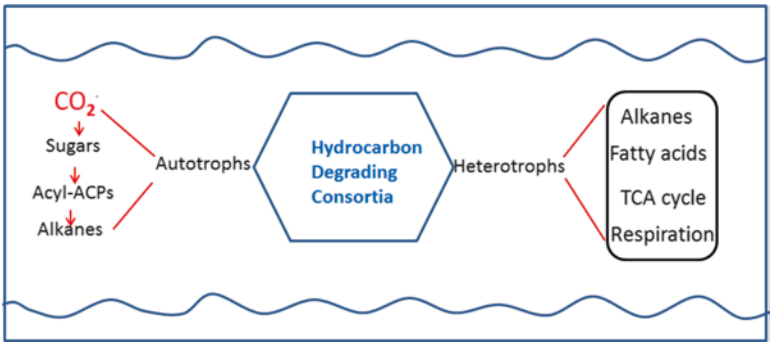

Fig. 1.1 Diagrammatic representation of hydrocarbon biodegradation processes by microbial consortia in marine environments

increase in the kinetics of the petroleum biodegradation in marine environments (Olajire and Essien 2014), because single species are able to metabolize a narrow spectrum of substrates from oil hydrocarbons. That is why microbial consortia are preferred to stimulate the bioremediation and biodegradation of dispersed oils from coastal and marine environments.

1.3 Consortia vs Single Organisms in Biodegradation of Petroleum Hydrocarbons

Because pollutants of petroleum hydrocarbons in marine environment do not occur as simple molecules but instead are complex mixtures, the need of simultaneous or complementary action of microorganisms to achieve an efficient biodegradation and bioremediation of these major pollutants is becoming a priority issue. Nature is considered a universal reservoir of microbial consortia capable of performing different and complicated environmental functions that individual populations and monocultures cannot perform (Brenner et al. 2008). In nature, microbial consortia represent 99% over the existing microorganisms (Ding et al. 2016). The need for microbial consortia to degrade complex molecules present in the pollutants, viz., crude oils and refined products, has led to the reassessment of the many and diverse functional roles of these communities. Microbial consortia are defined as a group of different species of microbial populations interacting and functioning together as a community (Bao et al. 2012). However, the majority of microorganisms in natural environments develop as part of complex and dynamically changing microbial consortia but not in isolation. Its structural arrangement benefits their community and allows to gain fortitude and stableness to environmental changes and to generate robustness during development to all the components of a consortium. In this mutual structure, communities withstand the action or effect of attacks of species invasions (Burmolle et al. 2006; Brenner et al. 2008). Therefore, consortia organization could have all the functions to weather oscillating conditions of nutrient concentrations, due to the different and diverse metabolic capabilities of species acting in concert with their respective shared functions, metabolites, and quorum sensing within the community (Brenner et al. 2008).

Bioremediation of complex hydrocarbon mixture in order to be successful usually entails the involvement of microbial consortia, because individual strains of microorganism are lacking of the enzymatic machinery required to metabolize most of the hydrocarbon substrates of the crude oil and succeed only in a narrow range of hydrocarbon compounds (Patowary et al. 2016). Therefore, mixed populations harboring robust and diverse sources of active enzymes are responsible to increase the rate and extent of petroleum biodegradation further (Priya et al. 2015). Recent results have evidenced that a well-combined microbial consortium will share complementary catabolic pathways, specifically directed to degrade hydrocarbons (McGenity et al. 2012). Furthermore, a more effective degradation action can be

achieved by complementary performance of single strains, viz., co-metabolic turnover reactions (Deppe et al. 2005). These advantages, as in the case of improved biodegradation of hydrocarbons in bacterial co-cultures with microalgae, need to be more developed and applied in the remediation of petroleum contamination (Fig. 1.1). Degradation of capabilities of bacteria, microalgae, and fungi acting simultaneously and in concert as in consortia structure has been demonstrated recently on bioremediation studies of low-molecular-weight PAHs (naphthalene, fluorene, and phenanthrene) from marine environments.

Generally, aromatic-degrading microorganisms are able to use either monoaromatic hydrocarbons, hydrocarbons with two to three rings, or hydrocarbons with three to four rings.

However, in the case of five or higher fused benzene rings, viz, BaP, still undergoes biodegradation limitations (Juhasz and Naindu 2000). Contrary to the many factors involved during growth of microorganisms in consortia on hydrocarbon substrates, inhibitory or blocking factors like the substrate recalcitrance and the poor solubility of hydrophobic organic compounds in aqueous systems are limiting factors for efficient biodegradation initiatives (Patowary et al. 2016; Joutey et al. 2013). For instance, aromatic rings are hardly broken down by alkane degraders, and alkanes are toxic substrate for PAH degraders. Recent findings (Malik and Ahmed 2012) on the biodegradation capabilities of a bacterial consortium isolated from an oil field show higher removal efficiencies of alkanes (>90% for tridecane, C13), in second place pentadecane (C15, at 77.95%), and 74% for octadecane (C18), and the remaining alkanes showed 56–69%. A remarkable feature was that the monoaromatics, such as xylene, toluene, and benzene, that exhibited high evaporation rates after 4 days of incubation, contrary to 46.17–55.3% shown after 24 days by polyaromatic components represented by anthracene, phenanthrene, and pyrene. The role of different cyanobacteria and microalgae species also have been shown to degrade naphthalene (Cerniglia et al. 1980).

Results obtained by Deppe et al. (2005) reported that a consortium of non-culturable strains was essential in the degradation of unprocessed oil. Hence, linear alkanes as well as isoprenoids represented by phytane and pristine besides ethylbenzene, m-xylene/p-xylene, and naphthalene were also biodegraded. In principle, a selective preference for metabolism of C8–C15 n-alkanes was observed in the first place and secondarily the C16–C36 n-alkanes. In this way, the degradation pattern of n-alkanes was performed at low temperatures (4° C), which corroborates former researches undertaken at that temperatures, mainly the solubility of single compounds that determines their bioavailability (Deppe et al. 2005). The abundance of *Marinobacter hydrocarbonoclasticus* is associated to its property of elevated hydrocarbonoclastic specialization in bacteria from marine environment, which is ubiquitous, ca. 90% of microbial community in events of oil spills (Yakimov et al. 2002). The high dominance of these bacteria and their physiological and metabolic functions to degrade unprocessed oil compounds make an important contribution to the overall degradation potential of the consortium (Deppe et al. 2005).

During the last decade, interest in engineering microbial consortia has emerged, because of their unique metabolic capabilities and robustness, and therefore, they

can perform specialized and challenging tasks that in the case of individual populations are more complicated or even unfeasible.

Recent applications of synthetic biology on the constructed synthetic microbial consortia are highly promissory, because it might allow expanding current microbial capabilities and new optimal features for practical applications on the hydrocarbons' bioremediation from fragile and sensitive environments (Ding et al. 2016). Despite the advantages of these life forms, some challenges of naturally occurring microbial consortia remain, such as long operation cycle and poor stability and controllability, which interfere their practical applications (Ding et al. 2016).

Nevertheless, it remains a great challenge to realize such multispecies cultures in crude oil and petroleum-refined products' biodegradation and bioremediation.

1.3.1 Microbial Consortia and Biosurfactant-Assisted Biodegradation

The distribution of hydrocarbon-degrading microorganisms in the sea concomitantly to the natural biodegradability of hydrocarbons in the environment is very critical for the development of bioremediation strategies. The ability of structural components and specific properties of microbial community equipped with biochemical reservoirs are key factors that determines its potential to produce degradative enzymes, growth factors, and biosurfactant metabolites. Microbial origin surfactants almost totally have a lipid origin and are usually classified as natural lipids, fatty acids, lipopolysaccharides, glycolipids, phospholipids, and lipopeptides (Paniagua-Michel et al. 2014). Bio-surfactants are characterized as amphipathic compounds, and their capabilities to uptake hydrocarbons inside the cells are a condition depending on its hydrophilic and a hydrophobic domain. Bio-surfactants are especially interesting molecules because they are coadjuvants in oil cleaning and recovering tasks, as well as in oil emulsification, and in separating oil-in-oil emulsions (Paniagua-Michel 2017). These attractive metabolites from microbial consortia are preferred because of its low toxicity and high biodegradability in comparison to synthetic counterparts. That is why its surfactants and surface-active property are more suitable for sustainable hydrocarbon bioremediation. Moreover, microorganisms from marine sources usually show higher yields when compared to organisms from land or freshwater species (Maneerat 2005). Despite several microorganisms (yeast, algae, and protozoa) have shown a promissory potential for hydrocarbon bioremediation, the effectiveness of microbial consortia has been demonstrated in the mitigation and bioremediation of hydrocarbons from marine effluents and environments. The capability of biosurfactant production by bacteria and microalgae plays an important role in reducing the tense-active compounds and the required micelle concentration in hydrocarbon mixtures. Hence, microemulsions emerge together with micelles and contribute to disaggregate and to dissolve hydrophobic hydrocarbons and their uptake by microorganisms (Banat et al. 2014). The advantages of combining bacterial consortia in the biodegradation of petroleum

hydrocarbons were reported by Patowary et al. (2016) based on the intrinsic property of the bacteria in reducing the surface tension of hydrocarbons by biosurfactant production. Hence, the advantage of combining hydrocarbon-degrading bacterial strain (*B. pumilus* KS2 and *B. cereus* R2) isolated from crude oil fields (Assam, India) represented a higher advantage when compared to single strains. The bacterial individual strains of the consortia also exhibited higher degradation levels of crude oil fractions of hydrocarbons, as in the case of the presence of members of recalcitrant PAHs. Despite the high potential and capability of microbial consortia, the need for field trials for the removal of hydrocarbons from impacted marine sites still is a challenging issue deserving for more research.

1.4 Biodegradation of Aliphatic and Polycyclic Aromatic Hydrocarbons Under High-Salinity Marine Environments

There has been considerable progress on the biodegradation of petroleum compounds by aerobic and anaerobic microorganisms in nonsaline soil, freshwater, and low salinity marine environments (Cao et al. 2009; Harayama et al. 1999; Van Hamme et al. 2003). However, little is known about the fate of hydrocarbons in hypersaline environments (Castillo-Carvajal et al. 2014; Fathepure 2014; Le Borgne et al. 2008; Martins and Peixoto 2012; Oren 2017). Many high-salinity environments such as nearshore oil production sites, salt marshes, sabkhas, and other coastal flats, especially in arid and semiarid regions of the world, are not only highly saline but also contaminated with numerous petroleum compounds (Al-Mailem et al. 2010, 2013; Erdogmuş et al. 2013; Tapilatu et al. 2010; Zhao et al. 2017). Contamination of these ecosystems is a serious concern due to the persistence and high toxicity exhibited by petroleum hydrocarbons. Although microorganisms hold great promise and have a huge potential in bioremediation of hydrocarbon-contaminated material, similar potential at elevated salinities cannot be realized due to inhibition of microbial activity by salt. Therefore, bioremediation of hypersaline environments can only be achieved using salt-loving and salt-tolerant microorganisms capable of degrading petroleum compounds. In the last two decades, there have been several reports on the isolation and hydrocarbon degradation by bacteria and archaea over a broad range of salinity (3–32% NaCl). However, organisms belonging to only few genera have been found to be capable of degrading hydrocarbons under elevated salinity (from 10% and up to even saturation). For example, members of the genera *Alcanivorax*, *Marinobacter*, *Halomonas*, *Halobacterium*, *Haloferax*, *Halococcus*, *and Haloarcula* have been repeatedly isolated or found in some of the most saline environments (Castillo-Carvajal et al. 2014; Fathepure 2014; Le Borgne et al. 2008; Martins and Peixoto 2012; Oren 2017; Sorokin et al. 2012). *Halomonas* and *Marinobacter* have been isolated from marine and nonmarine hypersaline environments. Many studies show that *Halomonas* spp. degrade

mainly phenolics and aromatic acids and to a lesser extent aliphatic and PAHs. On the other hand, *Marinobacter* spp. degrade both aliphatic and PAHs and to a lesser extent phenolics and aromatic acids. *Alcanivorax* have relatively low salt tolerance. They are n-alkane-utilizing specialists, mostly isolated from marine environments (Sorokin et al. 2012). The archaea, *Halobacterium*, *Haloferax*, *Haloarcula*, and *Halococcus*, have been isolated from hypersaline environments including solar evaporation ponds, salt marshes, sabkhas, and other coastal flats (Al-Mailem et al. 2010, 2013; Erdogmuş et al. 2013; Tapilatu et al. 2010; Zhao et al. 2017). These archaea mainly degrade aliphatic, monoaromatic, and PAHs and to a lesser extent phenolics and aromatic acids.

Most studies to date merely provide cursory information on isolation and characterization of hydrocarbon degradation by bacteria, archaea, and some eukaryotes in saline environments, but little efforts have been made to explore their physiology, biochemistry, and genomics in greater depths (Oren 2017). In this section, we provide an updated overview on degradation potential of aliphatic and polycyclic aromatic hydrocarbons (PAHs) at salinity >10% as they represent the most toxic, persistent, and/or abundant fraction in crude oil. For additional information on halophilic and halotolerant organisms and their hydrocarbon degradation potential, reader can refer to some of the recently published excellent reviews (Castillo-Carvajal et al. 2014; Fathepure 2014; Le Borgne et al. 2008; Margesin and Schinner 2001; Martins and Peixoto 2012; Oren 2017; Sorokin et al. 2012).

1.5 Degradation of Aliphatic Compounds by Bacteria and Archaea

Alkanes are major fraction of crude oil; hence their degradation constitutes an important aspect of bioremediation of contaminated sites. Ward and Brock (1978) were among the first few to study the biodegradation of aliphatic hydrocarbons in the presence of high salt ranging from 3% to 28% in samples collected from and around the Great Salt Lake, Utah. These studies reported decreasing rates of hexadecane degradation with increasing salinity up to 17%, and the degradation was severely inhibited at salinity above 20%. In 1992 Gauthier and coworkers (Gauthier et al. 1992) have successfully isolated *Marinobacter hydrocarbonoclasticus* from contaminated sediments in the Mediterranean Sea and showed the utilization of aliphatic compounds such as tetradecane, hexadecane, eicosane, heneicosane, and pristane in the presence of 4.6–20% NaCl. In addition, the organism also degraded an aromatic compound, phenanthrene, as the sole source of carbon and energy. Later studies by Fernandez-Linares et al. (1996) showed that *M. hydrocarbonoclasticus* degraded eicosane at higher rate with increasing salinity from 1.2% to 14.5%. These results contradicted earlier observations by Ward and Brock (1978) where degradation rate decreased with increasing salinity. Huu et al. (1999) have reported the isolation of another closely related *Marinobacter* species, *M. aquaeolei*, from an

oil-producing well in Vietnam that degraded n-hexadecane and pristane as the sole sources of carbon in the presence of 0–20% NaCl. Zvyagintseva et al. (2001) have isolated *Rhodococcus erythropolis* and *Dietzia maris* from an oil-polluted soil that are shown to degrade n-alkanes and iso-alkanes with chain length C_{11}-C_{30} and C_{14}-C_{18}, respectively, at NaCl concentrations up to 10%. Abed et al. (2006) have reported the degradation of pristane and n-octadecane at salinity ranging from 5% to 12% by cyanobacterial mat community from the coastal flats of the Arabian Gulf. Al-Mueini et al. (2007) have isolated an extremely halophilic actinomycete, *Actinopolyspora* sp. DPD1 from an oil production site in the Sultanate of Oman. The actinomycete degraded pentadecane, eicosane, and pentacosane in the presence of 25% salt. In this study, the authors found that degradation rate decreased with increasing chain length. For example, complete degradation of pentadecane occurred in only 4 days, 80% of eicosane degraded in 10 days, 15% of pentacosane degraded in 15 days, and no triacontane was degraded even after 20 days of incubation. In addition, this organism also degraded fluorene. Biodegradation of fluorine by the actinomycete resulted in the production of novel metabolites suggesting a metabolic pathway that was not previously described. Sass et al. (2008) have isolated a strain (DS-1) closely related to *Bacillus aquimaris* from Discovery deep-sea hypersaline anoxic sediment that used n-dodecane and n-hexadecane as the sole sources of carbon in the presence of 12–20% NaCl. Mnif et al. (2009, 2011) have isolated *Halomonas* sp. strain C2SS100 and *Pseudomonas* sp. strain C450R that degraded crude oil in the presence of 10% NaCl. These organisms degrade hexadecane in crude oil as the sole carbon sources in the presence of 5–10% NaCl and produced an emulsifying agent that facilitates the assimilation of hydrocarbons. Dastgheib et al. (2011) have isolated a halotolerant *Alcanivorax* sp. strain Qtet3 (with 99.8% 16S rRNA gene similarity to *Alcanivorax dieselolei*) from hydrocarbon-contaminated soils in Iran. Strain Qtet3 degraded various n-alkanes (from C_{10} to C_{34}) with considerable growth on C_{14} and C_{16}. Strain Qtet3 completely degraded tetracosane ($C_{24}H_{50}$) as the sole source of carbon in 10 days in the presence of 0–10% NaCl. Also, the organism degraded phytane and pristine. In this study, the investigators tested the bioaugmentation potential of strain Qtet3. Results show that the strain could survive and degrade crude oil components in saline soil in the presence of 0–12.5% NaCl suggesting the ability of a marine hydrocarbonoclastic *Alcanivorax* sp. to bioremediate crude oil-contaminated saline soil. Al-Mailem et al. (2013) isolated two halophilic bacteria, *M. sedimentalis* and *M. falvimaris*, on the basis of their ability to use crude oil as the growth substrate from hypersaline sabkhas in Kuwait. The isolates grew on n-alkanes (C_9–C_{40}) and aromatic compounds such as benzene, biphenyl, phenanthrene, anthracene, and naphthalene as sole sources of carbon in the presence of 6–9% NaCl. Results also showed that though their optimal salinity for growth and hydrocarbon biodegradation is between 6–9% NaCl, they still grew and utilized crude oil in the presence of 17–20% NaCl. More importantly both the organisms were shown to possess nitrogen-fixing ability, which is important for the bioremediation of hydrocarbons without the addition of expensive fertilizers.

Studies also reported the ability of archaea to degrade aliphatic hydrocarbons at high salinity. Bertrand et al. (1990) were among the first to report the isolation of a

halophilic archaea strain EH4 (later identified as *Haloarcula vallismortis*; Tapilatu et al. 2010) from a salt marsh in Southern France that are shown to degrade various aliphatic and aromatic hydrocarbons such as tetradecane, hexadecane, eicosane, heneicosane, pristane, acenaphtene, phenanthrene, anthracene, and 9-methyl antracene in the presence of 20% NaCl. The organism's growth rate and the extent of eicosane degradation decreased with decreasing salinity. Growth occurred at maximum rate in the presence of 20% NaCl and not detected at <10% NaCl. Kulichevskaya et al. (1991) have isolated a halophilic archaea *Halobacterium* sp. H-352 (assigned to the genus *Halobacterium* based on phenotypic characteristics) from the Bondyuzskoe oil field water in Russia. This organism degraded n-alkane (C_{10}–C_{30}) in the presence of 30% NaCl. Interestingly, studies also have found hydrocarbon-degrading organisms in uncontaminated hypersaline environments. For example, Tapilatu et al. (2010) have reported the isolation of strains *Haloarcula* and *Haloferax* from a shallow crystallizer pond with no known contamination in Salins-de-Giraud, Camargue (France). These strains degraded heptadecane, eicosane, and phenanthrene at 22.5% NaCl. Al-Mailem et al. (2010) have isolated three extremely halophilic archaea, *Haloferax*, *Halobacterium*, and *Halococcus*, from a hypersaline coastal area of Arabian Gulf that degraded *n*-octadecane, phenanthrene, and benzene as the sole sources of carbon in the presence of 26% NaCl. Recently, Zhao et al. (2017) have isolated a halophilic archaeon *Halorientalis hydrocarbonoclasticus* strain 1M1011 from a saltern in Tianjin (China). The isolate degraded hexadecane as the sole source of carbon in the presence of 21% NaCl. To obtain greater insights into its ability to degrade hydrocarbons, complete genome of this organism was sequenced. Genome analysis predicted the presence of genes for alkane hydroxylase and beta-oxidation steps for hexadecane metabolism.

1.6 Degradation of PAHs by Bacteria and Archaea

Crude oil contains PAHs with two or more fused aromatic rings in linear, angular, or cluster arrangements. PAHs can enter the environment including hypersaline environments due to human and natural activities. The persistence of PAHs in the environment is of special concern due to their toxic, mutagenic, and carcinogenic properties (Cao et al. 2009; Gibbs 1997; Menzie et al. 1992). Generally, the persistence of PAHs in soil and water depends on the number of rings and environmental factors such as pH, temperature, and salinity (Kanaly and Harayama 2000). Although studies have reported on the degradation PAHs by non-halophiles, little is known about their fate in hypersaline marine and coastal environments (Ghosal et al. 2016). Reduced solubility of PAHs and oxygen at high salinity (Margesin and Schinner 2001; Whitehouse 1984) has been implicated for the low microbial diversity in these environments. Gauthier et al. (1992) have described the isolation of *M. hydrocarbonoclasticus* that can degrade a large number of aliphatic hydrocarbons as well as phenanthrene as the sole sources of carbon. Zhao et al. (2009) have described the degradation of phenanthrene in the presence of 0.1–15% NaCl by a

bacterial consortium developed from soil samples from the Shengli Oilfield, China. Complete degradation of phenanthrene occurred within 5–8 days in the presence of 5–15% NaCl, but no degradation occurred at 0.1 and 20% NaCl. Molecular analysis of the consortium indicated the presence several bacteria in the class *Gammaproteobacteria* with high sequence similarity with *Halomonas salina* (98.1%), *Chromohalobacter salexigens* (100%), *Alcanivorax dieselolei* (99.3%), *M. flavimaris* (99.5%), and *Idiomarina loihiensis* (99.5%) in the enrichment. Also, a member of the *alphaproteobacterium*, *Thalassospira xiamenensis* (98.6%), was found in the enrichment. Among the detected microorganisms, species of *Halomonas*, *Marinobacter*, and *Alcanivorax* have been shown to degrade both aliphatic and aromatic hydrocarbons at salinity. Dastgheib et al. (2012) have obtained a two-membered mixed culture (Qphe-SubIV) consisting of a *Halomonas* sp. and a *Marinobacter* sp. from oil-contaminated saline soil collected from five different regions in Iran. The enrichment degraded several PAHs such as naphthalene, phenanthrene, anthracene, fluoranthene, fluorine, pyrene, benz [a] anthracene, and benzo [a] pyrene in the presence of 1–15% NaCl. Based on substrate utilization pattern and detection of catechol dioxygenase gene in this study, it was hypothesized that perhaps the *Marinobacter* sp. was responsible for phenanthrene degradation, while *Halomonas* sp. utilized the toxic metabolites produced during phenanthrene degradation. Gao et al. (2013) have isolated *M. nanhaiticus* strain D15-8W from a phenanthrene-degrading enrichment culture developed from sediment from the South China Sea. The strain D15-8W degrades naphthalene, phenanthrene, and anthracene in the presence of 0.5–15% NaCl. Guo et al. (2015) have enriched a phenanthrene-degrading bacterial consortium (HF-1) from an oil-contaminated saline soil collected from Shengli Oilfield in China. The enrichment degraded phenanthrene as the sole source of carbon as high as 20% NaCl within 4 days. Zhou et al. (2016) described the halophilic *Thalassospira* sp. strain TSL5-1 that degrades pyrene at salinity ranging from 0.5% to 19.5%. Recently, Gomes et al. (2016) have isolated several bacteria phylogenetically related to *Idiomarina* sp., *Brevibacterium* sp., *Nitratireductor* sp., *Halomonas shengliensis*, *Modicisalibacter tunisiensis*, *M. flavimaris*, and *Bacillus flexus* from activated sludge and production water from Marine Terminal Almirante, Barroso, in Brazil. These bacteria efficiently degraded phenanthrene, pyrene, and benzopyrene and to a lesser extent naphthalene, phenol, and hexadecane in the presence of 9% NaCl. More recently, Pugazhendi et al. (2017) developed a highly enriched culture using a brine sample from a desalination plant in Saudi Arabia that degrades a variety of PAHs in the presence of low salinity (4% NaCl). The enrichment also degraded phenanthrene and pyrene up to 30% salinity. However, the rate of degradation retarded with increasing salinity, and no growth occurred above 30% NaCl. Addition of yeast extract improved the rate of phenanthrene degradation at 25–30% NaCl. High-throughput sequencing revealed that the consortium was mainly composed of *Ochrobactrum halosaudia* strain AJH1, *Ochrobactrum halosaudia* strain AJH2, and *Pseudomonas aeruginosa* AJH3.

Studies also show the ability of archaea to degrade PAHs in high-salinity environments. As mentioned above, strain EH4 (*Haloarcula vallismortis)* not only

degraded *n*-alkanes but also degraded a mixture of aromatic compounds such as acenaphthene, anthracene, and phenanthrene at >20% NaCl (Bertrand et al. 1990). Bonfá et al. (2011) have isolated several strains of *Haloferax* from water or sediment samples collected at five hypersaline locations such as salt marshes in the Uyuni salt flats in Bolivia, Cahuil marine salterns in Chile, Cabo Rojo marine salterns in Puerto Rico, sabkhas in the Persian Gulf, and the Dead Sea near Israel and Jordan. These isolates not only degraded a mixture of aromatic acids such as benzoic acid, *p*-hydroxybenzoic acid, and salicylic acid but also degraded a mixture of the PAHs including naphthalene, anthracene, phenanthrene, pyrene, and benzo[a] anthracene at high salinity (20% NaCl). Extremely halophilic archaeal strains of *Haloferax*, *Halobacterium*, and *Halococcus* isolated from a hypersaline coastal area of the Arabian Gulf degraded a variety of aliphatic and aromatic hydrocarbons including PAHs such as naphthalene, biphenyl, and phenanthrene at 23–26% salinity (Al-Mailem et al. 2010). Erdogmuş et al. (2013) showed the degradation of naphthalene, phenanthrene, and pyrene as the sole carbon sources in the presence of 20% NaCl by several archaeal strains including *Halobacterium piscisalsi*, *Halorubrum ezzemoulense*, *Halobacterium salinarium*, *Haloarcula hispanica*, *Haloferax* sp., *Halorubrum* sp., and *Haloarcula* sp. isolated from brine samples of Camalt Saltern in Turkey. Recently, Khemili-Talbi et al. (2015) have reported the isolation of *Natrialba sp.* strain C21 from oil-contaminated saline water in Ain Salah (Algeria) that are shown to degrade phenol, naphthalene, and pyrene as the sole sources of carbon at 25% salinity. This is the first report on the ability of a strain belonging to the genera *Natrialba* in the *Halobacteriaceae* family.

To date, studies have shown that both bacteria and archaea have the capacity not only to cope with high-salinity stress but also be able to degrade n-alkanes and PAHs suggesting their key role in mitigating vast areas of highly saline coastal habitats impacted by petroleum compounds that pose threat to both terrestrial and marine ecosystem.

1.7 Molecular Mechanisms of Degradation of Aliphatic and PAHs at High Salinity

In the last two decades, there has been increasing interest in understanding the fate of hydrocarbons in hypersaline environments, and a few reports dealing with mixed cultures as well as axenic cultures of bacteria and archaea have appeared in the literature that degrade petroleum hydrocarbons over a broad range of salinity. As indicated above, the main focus of these studies has been enrichment, isolation, and determination of hydrocarbon degradation potential of halophilic and halotolerant organisms, but much less emphasis has been placed on exploring the molecular mechanism of degradation and how they differ from mechanisms that non-halophiles use (Fathepure 2014; Guo et al. 2015; Oren 2017).

1.7.1 Aliphatic Compounds

Dastgheib et al. (2011) using degenerate primers were able to amplify two putative *alk* B genes that code for alkane hydroxylases that catalyze the hydroxylation of aliphatic hydrocarbons in *Alcanivorax* sp. strain Qtet3. The strain Qtet3 is capable of degrading a wide range of *n*-alkanes (C_{12} to C_{34}) in the presence of 0–15% NaCl. Nie et al. (2013) have analyzed the full genome of the alkane-degrading (C_{10} to C_{36} alkanes and propane*) Amycolicicoccus subflavus* isolated from an oily sludge at Daqing Oilfield, China. The organism is capable of growth in the presence of 1–12% NaCl. Among the n-alkanes, the organism grew rapidly utilizing C_{16} and C_{18} alkanes, while C_3 and C_{10} resulted in the lowest growth. A moderate growth occurred on C_{14} and C_{20} to C_{36} alkanes as carbon sources. Four types of alkane hydroxylase coding genes were detected in the genome. A quantitative real-time reverse transcription PCR was used to determine the expression of various alkane-degrading genes. Homologs of *Alk*B alkane hydroxylases were expressed by C_{10}–C_{36} alkanes suggesting the organism's potential to degrade medium- to long-chain alkanes. Similarly, two CYP153 genes that are induced by alkanes, C_{10} to C_{20} and C_{24}, were also found on the genome. In addition, *Lad*A and propane monooxygenase genes responsible for the oxidation of long-chain alkanes and propane, respectively, were also detected. Interestingly, analysis showed that key genes necessary for the degradation of aromatic compounds were missing in the genome. For example, genes for benzoyl CoA synthetase, phenol 2-monooxygenase, catechol 1,2-dioxygenase, and catechol 2,3-dioxygenase were not detected on the genome. Recently, Zhao et al. (2017) have predicted several genes needed for the metabolism of n-alkane in hexadecane-degrading *Halorientalis hydrocarbonoclasticus* strain IM1011 genome. Genome analysis showed the presence of several copies of alkane hydroxylases such as of luciferase-like monooxygenase/LLM class oxidoreductase (*LadA*) and cytochrome P450 was found in IM1011 genome suggesting the organisms ability to convert alkanes to corresponding alcohols by the addition of oxygen to the terminal methyl group (Rojo 2009; Wang and Shao 2013). Analysis also predicted the presence of genes necessary for converting alcohols to corresponding aldehydes and then to corresponding fatty acids, which enter beta-oxidation pathway for compete metabolism. These genes include alcohol dehydrogenase (four copies), aldehyde dehydrogenase/aldehyde ferredoxin oxidoreductase (eight copies), acyl-CoA synthetase/long-chain fatty acid-CoA ligase/AMP-dependent synthetase, and genes in the beta-oxidation steps. These studies clearly establish that halophiles have the necessary mechanism to degrade major constituents of crude oil in some of the most challenging environment.

1.7.2 PAHs

Recently, a few studies have reported that halophilic and halotolerant bacteria and archaea degrade PAHs using genes and enzymes similar to those found in non-halophiles. Tapilatu et al. (2010) have studied degradation of phenanthrene by *Heloferax* strain MSNA 14 isolated from a hypersaline pond in the Camargue, France. It was shown that the strain degraded 43% of phenanthrene in 30 days in the presence of 22.5% NaCl. Analysis of degradation products by GC-MS detected 2,2′-diphenic acid in the culture medium. It was hypothesized that degradation occurred by dioxygenation of phenanthrene at the C9-C10 position (k-region) yielding *cis*-9, 10-dihydrodiol, 9,10-dihydroxyphenanthrene, and then 2,2′-diphenic acid. A well-defined, two-membered consortium (Qphe-SubIV) containing strains of *Marinobacter* and *Halomonas* degraded phenanthrene at salinity up to 15%. Analysis showed accumulation of 2-naphthol and 2-hydroxy 1-naphthoic acid as major metabolic intermediates (Dastgheib et al. 2012) suggesting that the catabolic reaction starts with a dioxygenation at the C1 and C2 positions. Erdogmus et al. (2013) isolated several archaea in the genus *Halobacterium*, *Haloferax*, *Halorubrum*, and *Haloarcula* from a Saltern in Turkey. Using degenerate primers and PCR, gene that code for 1,2-CTD was amplified from all isolates, except from *Haloarcula* sp. (genus *Haloarcula*) when grown on PAHs such as naphthalene, phenanthrene, and pyrene as the sole sources of carbon at 20% salinity. In addition, specific activity for 1, 2-CTD could be detected in crude extracts from all isolates. Also, gene that code for 3, 4-PCD activity was amplified from *Haloferax*, *Halobacterium*, and *Haloarcula*. Interestingly, genes for *meta*-cleavage pathways such as 2,3-CTD and 4,5-PCD were not detected in the isolates. These results suggest that degradation of naphthalene, phenanthrene, and pyrene occurred by the *ortho*-cleavage of the beta-ketoadipate pathway. Similar pathway is also used by the newly isolated *Natrialba* strain C21 during degradation of naphthalene or pyrene as the sole source of carbon at 25% salinity (Khemili-Talbi et al. 2015). Measurement of ring-hydroxylating dioxygenase activity in crude extract of cells grown on naphthalene or pyrene showed activity for 1,2-CTD, but no activity was detected for 2,3-CTD, 3,4-PCD, and 4,5-PCD. Guo et al. (2015) have cloned and expressed two novel 2,3-CTD genes from a microbial consortium (HF-1) that degraded phenanthrene at 10–20% NaCl. BlastP analysis showed that the two dioxygenases showed highest similarity (93 and 95%) with proteins in *Marinobacter algicola*. Measurement of enzyme activity showed that both the enzymes had high salt tolerance and active at even as high as 30% NaCl. Recently, Wang et al. (2017) have identified naphthalene dioxygenase-like PAH-degrading genes in a bacterial consortium (CY-1) that degraded phenanthrene at 10% NaCl. The microbial community analysis by 454-pyrosequencing showed that the consortium was dominated by *Marinobacter* spp. (41% of total 16S genes). In this study, a 5.6 kb PAH-degrading gene cluster mainly containing the large subunit (coded by *pahAc*) component of PAH ring-hydroxylating dioxygenase is overexpressed in *E. coli* successfully. Measurement of PAH ring-hydroxylating dioxygenase activity showed high enzyme activity at

10–20% NaCl indicating a strong salt tolerance. Overall, these limited studies indicate that both bacteria and archaea degrade aliphatics and PAHs using pathways similar to those found in non-halophiles. However, studies exploring genomics and metagenomics of hydrocarbon-degrading organisms in high-salinity environment are needed to elucidate degradation pathways and to identify novel ones to realize their full genetic potential to survive, compete, and degrade hydrocarbons in hypersaline conditions.

1.8 Conclusions

The urgent need for bioprospecting initiatives for new microbial populations capable of biodegrading petroleum hydrocarbons and with co-metabolic properties is desirable, as in microbial consortia that house new enzymatic systems that can metabolize molecules contained in crude oil. The richest source of microbial biodiversity, namely, marine environments, could lead to more culturable and non-culturable strains with valuable catabolic pathways for major field applications of initiatives of oil pollutant bioremediation. Moreover, new technologies that provide better in-sights into microbial biodiversity and functionality of hydrocrabon degrading microbial consortia through omics and massive sequencing could help develop robust bioremediation initiatives in coastal and marine environments.

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Chapter 2
Biosurfactants in Improving Bioremediation Effectiveness in Environmental Contamination by Hydrocarbons

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Abstract Recent biotechnological advances currently evidence new surfactant production technologies. Biocompounds produced by fermentative processes appeared as an economic and sustainable alternative to many synthetic molecules. Thereby, biosurfactants have become a promising substitute due to their synthesis potential by a wide variety of microorganisms. Biosurfactants are a highly diverse group of structures, such as glycolipids, lipopeptides, polysaccharide-protein complexes, phospholipids, fatty acids, and neutral lipids. This diversity promotes many advantages compared to synthetic surfactants, thus making biosurfactants the most natural choice for technological advances associated with sustainable development. Such advantages include fermentative production viability by using renewable resources, effectiveness in small concentrations even under extreme conditions, selective and specific potential for several applications, lower toxicity, higher biodegradability, and better stability to physicochemical variations. Despite their benefits, biosurfactants are not widely used because of the high production costs. Hence, cost-effective substrates, optimized cultivation conditions, and mutant lineage development are imperative to make these biomolecules an economically competitive product to propose a widespread replacement of synthetic surfactants.

2.1 Introduction

Petroleum spills, including oil-based products, can cause considerable damage to the environment, generating an enormous public concern. Oil pollution demands fast and cost-effective solutions. It is estimated that about 0.40% of global oil

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production eventually reaches the oceans (Banat et al. 2000). Several accidents with oil spills in recent years have continuously shown the environmental damage from hydrocarbons (Montagnolli and Bidoia 2012).

The major oil spill sources can be traced down to all stages of petroleum processing (exploration, refining, and transport). Accidents are prone to occur in marine tanks, complex bed drilling, marine fleets, refineries, and associated leaks (El-Tarabily 2002; Mille et al. 2006). In Brazil, many accidents involving petroleum, including derivatives such as gasoline and jet fuel, have caused serious environmental problems. In 1998, 1.200 m^3 of diesel oil were released due to pipeline corrosion underneath a considerable area below the city of Cubatão-SP. Pipeline corrosion was also responsible for 1.300 m^3 of hydrocarbons spilled in Guanabara Bay in Rio de Janeiro, which had a history of being contaminated constantly by oil spills (Benincasa 2007).

The lubricating oils are an important petroleum-based product, reaching about 60% of petrochemical products. Its importance is mostly industrial but also due to the massive automobile applications (Mang and Gosalia 2017). However, this high consumption also means a serious potential for environmental problems (Rahman et al. 2002). Lubricants often leak chronically from storage tanks (Lopes et al. 2008). Thus, continuous and prolonged release sets up the scenario for a long-term and highly persistent pollution regarding lubricant oils, leading to the contamination of soils and groundwaters (Chavan and Mukherji 2008). In addition, used lubricant oil suffers structural changes caused by high pressures and temperatures inside automobile engines that affect the biodegradability (Lopes and Bidoia 2009).

The persistence of petroleum hydrocarbons in the environment depends on several factors, such as chemical structure, concentration, and dispersion (Atlas 1981; Mille et al. 2006; Prosser et al. 2016; Duan et al. 2017). Physicochemical treatments are applied in the event of the oil spill; however, these treatments are very expensive, and even more strategy might be necessary depending on the chemical agents chosen as dispersants or catalysts (Elanchezhiyan et al. 2016; Grote et al. 2018).

Alternatively, the conventional physical treatments can separate soil and contaminants without destroying or chemically modifying the oils. Most of the hydrocarbons are absorbed in the soil matrix, reducing the removal efficiency of any treatment. Biological processes, on the other hand, are promising clean decontamination technologies, as they combine simplicity and cost-effectiveness. Thus, among many novel strategies, bioremediation emerges as the least aggressive and the most suitable method for keeping the ecological balance (Montagnolli and Bidoia 2012).

The decrease of contaminant concentrations by biodegradation or other treatment does not always indicate a decreased toxicity. The incomplete degradation and consequent formation of toxic intermediate compounds (metabolites) can result in increased toxicity during remediation processes (Tamada et al. 2012). Therefore, the combination of chemical analysis and ecotoxicity assays is recommended to elucidate the risks associated with contamination. In this way, a detailed monitoring is crucial for better bioremediation strategies and the establishment of environmental safety standards (Al-Mutairi et al. 2008).

2.2 Bioremediation of Areas Impacted by Petroleum Hydrocarbon

The microbial ability to use petroleum hydrocarbons as a carbon source was first demonstrated by Zobell (1946), who also revealed that these organisms were widely distributed in nature. The author described 100 bacterial species, belonging to 30 genera capable of metabolizing petroleum. Later, Bartha and Atlas (1977) expanded this list into another 22 bacterial genera, 14 fungi, and 1 alga. Any efficient bioremediation proposal should demonstrate that the contaminant removal is primarily due to biodegradation rates. In other words, the biodegradation must be higher than natural attenuation degree of decontamination. Among the many difficulties in the development of bioremediation methods is the reliability of laboratory-scale experiments in comparison to field results (Juhasz et al. 2000; Simpanen et al. 2016).

There are also several strategies for improving natural attenuation processes. Many of them are extremely cost-effective compared to physical and chemical treatments. The most common approaches in bioremediation are biostimulation, bioaeration, bioaugmentation, land farming, composting, and phytoremediation (Wu et al. 2016; Agnello et al. 2016). The insertion of nutrients (biostimulation) and oxygen (bio-aeration) favors microbial metabolism when using pollutants as substrates (Seklemova et al. 2001). Another commonly used procedure known as bioaugmentation is the inoculation of an enriched microbial consortium in soil (Richard and Vogel 1999; Barathi and Vasudevan 2001; Agnello et al. 2016). Montagnolli et al. (2009) obtained biodegradability datasets of lubricating oils using respirometric flasks. Kinetic models have been applied to biodegradation curves, which demonstrated the rate of biodegradation of automotive lubricating oils compared to vegetable oils. It was observed that petroleum degradation tends to decrease slower and last longer. However, the influence of other factors in biodegradation had not been determined. To better establish the optimal conditions of biodegradation, it is important to know the key features of an impacted area, such as residual oil concentration, density of degrading microorganisms, and microbial biodegradation potential.

Generally, an accelerated biodegradation of hydrocarbons depends on the presence of specific microorganisms. In addition, the composition of the microbial population is directly affected by the environmental conditions and the type of hydrocarbons (Admon et al. 2001). However, these indigenous communities lack species with proper enzymatic mechanisms necessary for a rapid biodegradation, which results in long-term biodegradation processes if not bioremediated (Díaz-Ramírez et al. 2008).

In this regard, the bioremediation of soils contaminated with mixed hydrocarbons from petroleum sources is a challenge due to the poor bioavailability and complex chemical composition of these compounds (Lee et al. 2008; Sabate et al. 2004; Yu et al. 2018). The hydrocarbon concentration and the presence of oxidative metabolites with potential risks to the environment can also remain after treatment (Pagnout et al. 2006).

The chromatographic profile of lubricating oils (Lladó et al. 2012), for example, shows a considerable fraction of a nondegradable complex mixture (unresolved complex mixture, UCM). In fact, little is known about the composition of the UCM, although the major components of oils are characterized by their high resistance to biodegradation (Wang and Fingas 2003; Wang et al. 2016). Most of the petroleum-based products are composed of branched and cyclic aliphatic and aromatic compounds, including polycyclic aromatic hydrocarbons (PAH) (Nievas et al. 2008).

The PAHs are often found in the environment due to atmospheric deposition originated from natural sources such as burning biomass and volcanic activity or artificial sources including burned fuels and many environmental accidents from the petrochemical industry (Lors et al. 2010). The release of PAHs represents a great concern due to their toxic, mutagenic, and carcinogenic properties (Martins et al. 2013). Although these aromatic molecules can undergo chemical oxidation, photolysis, adsorption, and volatilization, however, the microbial degradation of PAHs is, in most cases, the main alternative of soil treatment (Yu et al. 2018, Liu et al. 2017) .

Several studies on petroleum hydrocarbon biodegradation have been adopting different methodologies (Bidoia et al. 2010; Cerqueira et al. 2014; Zhang et al. 2016), but they all indicate that degradation occurs at least in some specific fractions of these substances. There is no general rule in petroleum biodegradation patterns, as most cases shown with preferential remediation of the lighter compounds were observed, whereas, in other studies, it was directed toward the heavier hydrocarbons (Huang et al. 2004).

In aquatic environments, the biodegradation of pollutants is limited and depends on the bioavailability of nutrients (such as nitrogen and phosphorus) required for the onset of microbial growth. The use of soluble salts containing these elements is an effective way to recreate and optimize the biodegradation under laboratory conditions. Field results are often not the same, as in situ treatments yield unsatisfactory stirring of the medium as well as a much reduced dissolution of salts. As a viable alternative, biosurfactants can be associated with the nutrient solution. This is importantly aimed toward petroleum pollution, because the oil is emulsified by the action of biosurfactants and thus provides a rapid microbial growth (Thavasi et al. 2011; Bezza and Chirwa 2015a, b; Mnif et al. 2017).

2.3 Surfactants

Surfactants are amphipathic molecules, i.e., compounds which have polar (hydrophobic) and nonpolar portions (hydrophilic), shifting solubility of other molecules in aqueous solutions based on polarity. These molecules act on the water-oil interface, thus forming micelles in various shapes and sizes (Van Hamme et al. 2006). Surfactants are an important class of chemicals widely used in modern industry (Develter and Lauryssen 2010; Franzetti et al. 2010). In 2007, chemical surfactant production reached about 10 million tons (Van Bogaert et al. 2007). In this context,

the market share is led by cleaning detergents (up to 50% of surfactant production), generating a 60-billion-dollar revenue in 2004 (Scheibel 2004). It is known that almost all commercially available surfactants are now chemically synthesized from petroleum.

The conventional chemical surfactants derived from petroleum are subject to the availability of fossil fuels and pose potential threats to the environment due to their recalcitrant nature (Makkar and Rockne 2003; Aparna et al. 2012). Approximately, 0.57 tons of petrochemical intermediates are consumed for each ton of surfactant produced (Patel et al. 1999). By projecting these values to the global production of surfactants, it is estimated that 7.40 million tons of petrochemicals are destined annually for the production of surfactants (Reznik et al. 2010). Thus, there is a trend toward eco-friendly technologies mobilizing the search for novel biodegradable compounds and renewable substrates, including industrial waste (Marchant and Banat 2012; Sasayama et al. 2018).

2.4 Biosurfactants

The advances in surfactant technologies are within the scope of many biotechnological types of researches. There was a subtle increase in the number of patents involving biosurfactants at the beginning of this century. More than 70% of these were reported between 2000 and 2010. In contrast, most of the patent registrations for chemical surfactants were performed in the 1900s, with a sharp drop after 2000 (Müller et al. 2012).

Biosurfactants are a natural choice as substitutes to synthetic surfactants because they have several advantages, such as (1) viable fermentative production using renewable resources; (2) effectiveness in small quantities, even under extreme conditions; (3) selective and specific potential for various applications; (4) low toxicity; (5) high biodegradability; and (6) stability to pH, salinity, and temperature variations (Abdel-Mawgoud et al. 2010; Banat et al. 2000; Cameotra and Makkar 2010; Lovaglio et al. 2011; Hazra et al. 2011; Zhao et al. 2017).

The biosurfactants are produced by microorganisms to increase cellular access to hydrophobic substrates. This facilitates the metabolism and promotes the development of biomass, hence increasing biodegradation (Bordoloi and Konwar 2009; Singh et al. 2007). The major advantage of biosurfactants compared to synthetic surfactants lies in their structural diversity and environmental acceptability. Biosurfactants are biodegradable, biocompatible, and less toxic with higher specificity, the possibility of in situ production. They can be produced from renewable substrates and organic residues (Mulligan 2009). A wide range of biosurfactants are potential to apply in various industrial approaches: food, petrochemical, mining, agriculture, cosmetics, pharmaceutical, textile, leather, construction, dyes, and chemicals (Araújo et al. 2016; Ferreira et al. 2017). In addition, these molecules have the ability to decrease surface and interfacial tension. These properties can promote microbial growth, aid microbial enhanced oil recovery (MEOR) procedures

in drilling oil wells, and facilitate bioremediation of pollutants (Zhao et al. 2017). Thus, biosurfactants are a multifunctional material and an important alternative to replace compounds and chemical processes (Silva et al. 2017).

Typically, biosurfactants have hydrophilic structures (amino acids, peptides, mono-/disaccharides, and/or polysaccharides) and hydrophobic structures (saturated and/or unsaturated fatty acids) in their molecules (Smyth et al. 2010; Shao et al. 2015). Biosurfactants are classified by their chemical structure, and this composition depends on the producing microorganisms, the substrate, and the conditions of the fermentation process (Cameotra and Makkar 2004; Nitschke et al. 2005a; Singh et al. 2007; Makkar and Cameotra 1999; Nitschke and Pastore 2006).

Among the various microorganisms able to produce biosurfactants, bacteria belonging to the genus *Pseudomonas* are often the most promising group. They are able to synthesize biosurfactants known as rhamnolipids. These molecules are glycolipids containing fatty acid groups linked to a rhamnose. The lipid portion is composed of β-hydroxydecanoic acid (Benincasa et al. 2004; Mulligan 2009; Abdel-Mawgoud et al. 2010). These different types of rhamnolipids slightly differ on their chemical structures and surface activities. The production of each homolog depends on the nutrient availability, the environmental conditions, and the biosynthesis capabilities of the specific *P. aeruginosa* strain (Oluwaseun et al. 2017; Mondal et al. 2017).

Rhamnolipids are considered as the most promising biosurfactant class in terms of industrial production, due to their physicochemical characteristics, outstandingly high productivity, and deep understanding of the rhamnolipid production (Müller et al. 2012).

2.5 Biological Function of Biosurfactants

Biosurfactants reduce surface tension or emulsify hydrophobic substrates (Diaz de Rienzo et al. 2016). However, the biological function of the biosurfactants into the cell is beyond just solubilizing substrates. From the ecological point of view, biosurfactants provide advantages to surfactant-producing microorganisms in relation to other organisms that do not have such capacity. Biosurfactants also have different chemical structures and are produced by different microorganisms (Mulligan 2009; Abdel-Mawgoud et al. 2010, Banat et al. 2010). These biosurfactants are described in the literature as molecules with antimicrobial and antiviral activities (Plaza et al. 2013, Remichkova et al. 2014). This ability benefits ecological interactions (e.g., competition).

There is a quorum sensing mechanism that controls the genes *rhl* and *pqs* responsible to produce rhamnolipids (Pearson et al. 1997; Dusane et al. 2010). Quorum sensing ("sufficient amount" in Latin) is a mechanism for assessing population density through molecular signals to activate a response that requires a certain population density (Madigan et al. 2015). Rhamnolipids are also important for the formation of water channels in the biofilm. These channels provide the homogenous

flow of nutrients and oxygen in the biofilm, in addition to allowing the release of metabolites. Davey et al. (2003) silenced the rhl gene of *Pseudomonas aeruginosa*, which caused the blockade of rhamnolipid production and prevented the formation of water channels in the biofilm.

Due to the multiple functions of these biosurfactants, their applicability was observed in activities such as the recovery of areas contaminated with hydrophobic compounds (Amani et al. 2013), emulsifiers in cosmetics and the pharmaceutical industry (Ferreira et al. 2017; Bhadoriya et al. 2013), and phytopathogen control (Ongena and Jacques 2008; D'aes et al. 2010, Falardeau et al. 2013). However, the cost of producing these biomolecules today is high, resulting in unfeasible applications. In comparison with synthetic surfactants with an average cost around $ 1 to $ 3 per kg, rhamnolipids cost $ 20 to $ 25 per kg (Chong and Li 2017). To overcome these challenges, many strategies are being adopted to increase productivity and reduce costs. These costs are mainly related to the costly carbon source required for the biosurfactant production and also to extraction and purification processes (Chong and Li 2017).

2.6 Production of Biosurfactants from Alternative Substrates

At the beginning of scientific interest in biosurfactants around 1980 (Syldatk and Wagner 1987), only pure hydrocarbons were used as carbon sources for their production (Fish et al. 1982; Hisatsuka et al. 1971; Itoh and Suzuki 1972; Syldatk et al. 1985). This consequently raised the biosurfactant market value to an unfeasible acceptance scenario. Despite its many advantages over synthetic chemical surfactants, the biosurfactants still have economic obstacles to overcome in the large-scale process, including a drastic reduction in production costs. In fact, there are efforts in the recent decades that focused on minimizing biosurfactant production costs to promote commercial acceptance (Mukherjee et al. 2006; Costa et al. 2008; Nitschke et al. 2011; Heryani and Putra 2017).

Currently, biosurfactants commercialized in the USA are more expensive than synthetic surfactants (Rosenberg and Ron 1997; Maier and Soberon-Chavez 2000; Makkar et al. 2011). In this context, alternative strategies have been adopted to establish a competitive price. Among the strategies are the development of genetically modified microorganisms toward better yields during the fermentation process (Dobler et al. 2016; Du et al. 2017), use of more cost-effective raw materials for biosurfactant production, and the development of economically viable production processes on a large scale (Mukherjee et al. 2006; Hazra et al. 2011; Makkar et al. 2011).

The use of agro-industrial waste becomes an economically interesting strategy since the raw material accounts for about 10–30% of the total cost in this biotechnological process (Makkar and Cameotra 1999; Mukherjee et al. 2006; Makkar

et al. 2011). There are many alternative substrates currently proposed for the production of biosurfactants: residues generated by the vegetable oil manufacturing (peanut, coconut, corn, olive, soybean), cooking oils (sunflower, olive, soy), vegetable processing waste (potato, barley, cashew, cassava, wheat), sugar cane molasses, whey, peat, oily waste from oil refineries, lignocellulosic waste (fruit peels, corncobs), and glycerol from biodiesel production (Desai and Banat 1997; Nitschke et al. 2004; Benincasa 2007; Moldes et al. 2007; Barros et al. 2008; Thavasi et al. 2008; Monteiro et al. 2009; Rocha et al. 2009; Thavasi et al. 2011; Aparna et al. 2012; Cruz et al. 2017; Meneses et al. 2017; Rane et al. 2017). These compounds are known to exhibit high levels of carbohydrates and lipid, both required for the growth of microorganisms and the biosynthesis of biosurfactants (Nitschke et al. 2005b; Nee' Nigam and Pandey 2009; Benincasa 2007; Diaz et al. 2018).

Many microbial genera proved to be able to produce biosurfactants from these residues – *Azotobacter*, *Bacillus*, *Brevibacterium*, *Burkholderia*, *Corynebacterium*, *Flavobacterium*, *Lactobacillus*, *Micrococcus*, *Nocardiopsis*, *Pseudomonas*, *Pseudoxanthomonas*, *Rhodococcus*, *Tsukamurella*, *Candida*, *Pseudozyma*, and *Trichosporon* (Boudour et al. 2004; Thavasi et al. 2008; Monteiro et al. 2009; Thavasi et al. 2011).

Agricultural residues result in lower production costs and a much smaller volume of compounds released into the environment. By successfully developing effective ways of producing surfactants, the environmental impact of surfactant industry may become smaller. Moreover, there is a sustainable gain by recycling waste (Mulligan 2009; Accorsini et al. 2012).

2.7 Conclusion

Microbial ability to use petroleum hydrocarbons as a carbon source enables an alternative treatment based on bioremediation. Moreover, biotechnological strategies are able to improve natural attenuation processes, and they present cost-effectiveness compared to physicochemical treatments. Generally, an accelerated hydrocarbon biodegradation depends on the presence of specific microorganisms and/or bioavailability of pollutant compounds. Thus, environmental contamination by petroleum derivatives presents many hydrophobic molecules, and microbial metabolism can be enhanced by using tensioactive compounds. In this context, biosurfactants are demonstrated to act on water-oil interface thus forming micelles that raise bioavailability of hydrocarbons to biological treatment. This facilitates the metabolism and promotes the development of biomass, hence increasing biodegradation. Therefore, these biocompounds produced by microorganisms increase cellular access to hydrophobic substrates, and they are a natural choice as substitutes for synthetic surfactants. The advantages of biosurfactant application are currently based on technological advancement associated with sustainable development. Biosurfactants still have economic obstacles to overcome in the large-scale process, including a drastic reduction in production costs. In fact, there are efforts in the recent decades

that focused on minimizing biosurfactant production costs to promote commercial acceptance. Hence, the use of agro-industrial waste as a substrate for fermentative processes becomes an economically interesting strategy. There are many alternative substrates currently proposed for the production of biosurfactants, and also many microbial genera proved to be able to produce biosurfactants from these residues.

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Chapter 3
Bioremediation of Petroleum Hydrocarbons in Seawater: Oil Spill Plume Modelling Approaches

Laurencia Debbie Benard and Piakong Mohd Tuah

Abstract Petroleum hydrocarbon and its constituents belong to the family of carcinogens and neurotoxin organic pollutants. The introduction of hydrocarbon contaminants into the environment, particularly in large scale, may cause detrimental effects or impairment to human and other living organisms and also deteriorate the environment. Therefore, it is vital to have efficient corrective measures to tackle petroleum hydrocarbon problem. Most commonly used or conventional marine shoreline cleanup alternatives are of natural, physical and chemical methods. Conventionally, the primary response option is physical containment or recovery of bulk free oil followed by chemical processes and natural attenuation as a last resort. This option of choice has been routinely used worldwide. However, as the first response option, physical removal does not wholly remove oil spills. Chemicals method, on the other hand, has not been extensively used because of the continuous debate on their efficiency and the concerns of their environmental as well as toxicity effects. Ever since the 1989 *Exxon Valdez* spill, the successful application of bioremediation has led this option as a particularly promising choice for elimination of oil from contaminated sites. Bioremediation's ultimate advantage over common technologies is that the operation is comparably low cost and considered to be more environmental-friendly. Since bioremediation establishes on natural processes, it is less interfering and destructive towards the polluted sites. A current biggest challenge in the employment of bioremediation of crude oil is inadequate guidance concerning when and how to use this technology during an occurrence of the oil spill. This chapter aims to present an overview of bioaugmentation of petroleum hydrocarbon using locally isolated beneficial microorganisms (LIBeM) and the potential of incorporating biodegradation data into selected oil spill model to study the fate of oil plume during an occurrence of the oil spill.

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3.1 Introduction

Since the early 1970s until 2016, the International Tanker Owners Pollution Federation (ITOPF) estimated that almost 6 million tons of oil were ceased due to incidents involving tanker accidents (Max Roser 2017). For smaller spills, data was obtained from the tanker owner and their insurers, while for more massive spills, the majority of the data was acquired from the written sources of the shipping press. Current technology by satellite images has enabled oil spills to be detected by computer systems. By nature, oil is toxic to marine life. Marine ecosystem and shoreline serve as public and ecological resources and also provide habitat and protection for biodiversity of wildlife. Marine oil spills will directly affect marine organisms and wildlife including seabirds, marine mammals, and intertidal and subtidal organisms. If oil spill spreads to the shoreline, it may cause erosion to sediments and contamination to vegetation, to natural habitats of wildlife as well as to human. The oil spill is persistent in the sediment and marine environment, causing lethal effects to marine biodiversity.

The abundance of hydrocarbon-degrading microorganisms is not limited to marine, soil and freshwater habitats. Numbers of research have shown that most potential microorganisms, a variety of bacteria, fungi, yeasts and occasionally algae for petroleum hydrocarbon degradation have been screened from areas polluted by oil (Chaerun et al. 2004). Biodegradation by microorganisms is more favourable as these modify crude oils in beneficial ways; the end products are of environmentally safe and eventual removal of petroleum from the contaminated environment. The oil spill stands out due to the destructive and disastrous effect of large-scale incidents. Pollution modelling offers the need to provide actual-time predictions of the oil fate and movement because of the accidental spills being emphasized. It is also an effective tool for providing important data for oil spill readiness and also response activities. Therefore, this chapter reviewed the information on the efficiency of locally isolated beneficial microorganisms (LIBeM) in degrading petroleum hydrocarbon. This chapter also provides information on the importance of oil spill modelling, whether the incorporation of biodegradation data can be used for or as a part of the integrated response to an oil spill at the marine environment.

3.2 Petroleum Hydrocarbon

Petroleum, derived from the Latin word *petra* (means rock) and *oleum* (means oil), are naturally occurring valuable world resources. Petroleum products made up from refining processes of crude oil also called as petroleum. Crude oil is made up of hydrocarbon composition (about 50–98% of total mixture) and non-hydrocarbon composition (e.g. nitrogen, oxygen, sulphur and trace metals) in a broad considerable group or combinations (Clark and Brown 1977). Crude oil and its refined

products are known to be very complex with a wide combination (pertaining to 1000) of singular components which exhibits a broad extent of characteristics and physical and chemical properties. Crude oil also differs very much in appearance depending on its composition. Although it may be yellowish, reddish or even greenish, crude oil is usually black or dark brown. Crude oil may exist in lighter form (gas in the reservoir), heavy form (saline) and semi-solid form or crude bitumen (mixed with sand and water).

Clark and Brown (1977) and the National Academy of Sciences (NAS 1985) have extensively detailed the abundance distribution and properties of petroleum hydrocarbons. Since then, comprehensive oil property databases were established which contain information on over 400 oils, posted by Environment Canada (Jokuty et al. 2000). Crude oil was classified into three, namely, the origin (terrestrial or geological location), API gravity (density) and sulphur content. Further, crude oil with low density is known as light crude oil and with high density is known as heavy crude oil and, if it contains a large amount of sulphur, it is known as sour crude oil. Based on its solubility characteristic in organic solvents, petroleum hydrocarbon components are categorized into four main groups as illustrated in Table 3.1.

There are four important physical characteristics of oil that have adverse effects on its behaviour in the environment as well as spill cleanup responses:

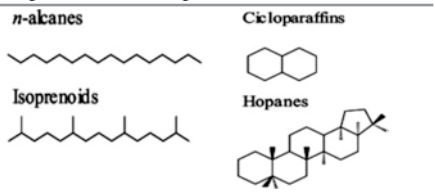
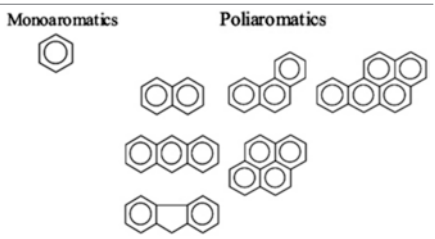
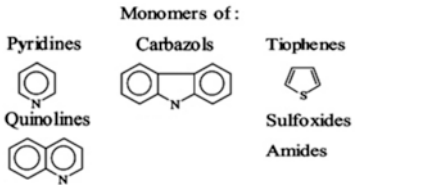
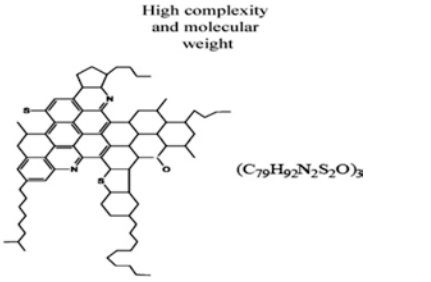
3.2.1 Density

The American Petroleum Institute (API) gravity and specific gravity are two commonly used for the density of oil. Specific gravity is measured as the ratio of the mass of oil to the mass of equivalent water at stated temperature. Meanwhile, the API gravity readily nominates a value of 10° to pure water at 10 °C (60°F). Oils with low densities or low specific gravities will have high API gravities. Clark and Brown (1977) stated that the specific gravities of crude oil are in between 0.79 and 1.00 which equals to API gravities of 10–48. The important index used to predict the oil composition fate in water is the oil density.

3.2.2 Viscosity

Viscosity is the property of a fluid that explains how it withstands a change in shape or motion. As the viscosity of fluid decreases, the ease of fluid flow increases. The viscosity of petroleum is associated with the oil compositions and the surrounding temperature. It also serves as an essential index of spilled oil spreading rate.

Table 3.1 Category of hydrocarbons

Category	The range of carbon atoms	Composition (s)	Representative compound (s)
Saturated	1–45	Normal alkanes	
		Branched alkanes	
Aromatic	4–45	Two or more fused aromatic rings	
Resin	>40	Polar compounds	
Asphaltenes	>40	NSO(s)	
		Poorly characterized hydrocarbons	
		High-molecular-weight compounds	
		Heavy metals	

Source: Adapted from Leahy and Colwell (1990)

3.2.3 Pour Point

The temperature at which oil stops flowing or becomes semi-solid is known as pour point. Temperature (from −57 to 32 °C) is the range of pour point. It is one of the decisive characteristics corresponding to oil fate and cleanup plan of action.

3.2.4 Solubility in Water

The temperature and chemical compositions determine the petroleum hydrocarbon's solubility in water. However, it is usually deficient in the solubility of common crude oil normally around 30 mg/L (NAS 1985). This property is crucial in determining the oil fate, oil toxicity and appropriate bioremediation measures. The most soluble oil components are of low-molecular-weight aromatics (e.g. toluene, benzene and xylene).

3.3 Oil Spill Incident in Marine Environment

An oil spill can be defined as an accidental release of oil into a body of water, as from petroleum-based activities, which posed a threat to marine life and the environment (COED 2002). Oil spills also include the release of a liquid petroleum hydrocarbon or its refined products into the environment, especially marine environment, and were highlighted as a form of pollution. Oil may be released to coastal waters, ocean and also land. However, the term oil spill usually referred to marine oil spills. Even with the latest and advanced technology, accidental spill or discharge of crude oil with its refined products still occurs during upstream (such as extraction, transportation, storage) and downstream processes (refining and distribution) which contributes to environmental pollution.

There are approximately 38 incidents involving super tankers carrying the valuable petroleum hydrocarbons, which unfortunately have affected the coasts of different countries since the past five decades. Extensive damages and great threats to the coastal marine environments are the effects posed by large oil spill accidents in marine. Western and Mediterranean Europe, as well as North Africa, have experienced major oil spill incidents. To date, these countries have experienced 13 out of 20 major spills in the world (ITOPF 2011). Numbers of studies have been done extensively by researchers (Table 3.2), not only to identify the cause of the spills but mostly because the spills have greatly affected not only the ecosystem flora and

Table 3.2 Magnitudes and causes of oil spill events in the world

Ship/spill name	Year	Location	Spill size (Tons)	Cause of oil spill	Reference
Guarapiche oil spill	2012	Maturin, Monagas, Venezuela	41,000	Pipeline ruptured	Reategui-Zirena et al. (2012)
Xingang Port oil spill	2010	Yellow Sea, China	90,000	Pipeline explosion and fire	Garapati (2012)
ExxonMobil	2010	Niger Delta, Nigeria	95,500	Pipeline spill	Garapati (2012)
Deepwater Horizon spill	2010	Gulf of Mexico, US	627,000	Offshore rig blowout	Wang et al. (1998)
Tasman Spirit	2003	Karachi, Pakistan	37,000	Grounding	Aguilera et al. (2010)
Prestige	2002	Galicia, Spain	63,000	Oil tanker sinking	Aguilera et al. (2010)
Amorgos spill	2001	Kenting Peninsula, Taiwan	35,000	Ran aground on a coral reef	Chiau (2005)
Evoikos	1997	Singapore port, Singapore	28,751	Collided with speeding empty tanker	Etkin et al. (2004)
Sea Empress	1996	Milford Haven port, UK	72,361	Grounding due to pilot error in steering	Etkin et al. (2004)
Avila Beach oil spill	1994	San Luis Obispo, California, US	15,646	Cumulative leak	OSPR (1998)
Aegean Sea oil spill	1992	La Coruna port, Spain	74,490	Grounding in bad weather; operator error	Etkin et al. (2004)
Nagasaki spill	1992	Malacca Strait, Malaysia	40,000	Collided with a container vessel	Sandulli et al. (1992)
Exxon Valdez	1989	Prince William Sound, Alaska, US	37,415	Grounding due to negligence	Gilbert (2000)
Lakeview Gusher spill	1910	Kern County, California, US	1,200,000	Wellhead blowout	Wang et al. (1998)

fauna but also human population that is exposed to the noxious properties of the spilled oil. However, it is important, when comparing the seriousness of spills, to factor in the location of the spill, type of oil spilled and also the size of the spill.

The *Exxon Valdez* (1986), *Sea Empress* (1996) and Deepwater Horizon (2010) are among the world's most massive oil spills concerning the worst environmental disaster in history. During the 1989 *Exxon Valdez* spills, a total of 37,000 tons of crude oil has led to fatality and deterioration of more than thousands of marine avi-fauna and mammals. It is estimated that 250,000 seabirds, 2800 sea otters, 300 harbour seals, 250 bald eagles and up to 22 killer whales died along with billions of

salmon and herring eggs. There are a serious decline in intertidal and subtidal communities and also long-term environmental effects (Spies et al. 1996). Edwards and White (1999) and Harris (1997) have recorded the threats caused by Sea Empress that released 72,000 tons of crude oil and 360 tons of heavy fuel, which includes a substantial impact and risk to wildlife, local fisheries and tourism. The amount of oil spilled during the Gulf of Mexico spill in 2010 was estimated as 206 million gallons. The spilled oil affected the Gulf shoreline and more than hundreds of marine avifauna and mammals.

3.4 Bioremediation: A Promising Choice for Oil Removal

Bioremediation is defined as "the act of adding materials to contaminated environments to cause an acceleration of the natural biodegradation processes" (OTA 1991). Principally, crude oils and its refined products consist of hydrocarbons where most of the components are biodegradable (Prince et al. 2003). Since its acknowledged operation during the 1989 *Exxon Valdez* spill, bioremediation has been an option of promising choices for oil removal from contaminated sites and also considered as a secondary treatment option (Bragg et al. 1994; Prince and Clark 2004).

It is highlighted that this technology is established based on the large percentage of oil components which are easily biodegradable (Atlas 1984, 1981; Price 1993). The bioremediation of oil spill successes relies upon the ability to provide and continuously control conditions that favour the rate of biodegradation of oil in the polluted site. Oil spill bioremediation can be classified into two main semblances: (a) bioaugmentation, the addition of known oil-degrading bacteria into contaminated site to supplement the existing microbial population, and (b) biostimulation, the growth of indigenous oil degraders is stimulated by the addition of nutrients or other growth-limiting co-substrates and/or by alterations in environmental conditions (e.g. surf-washing, oxygen addition by plant growth and etc.).

In comparison with conventional technologies (physical and chemical), bioremediation application is comparably low cost and the technology considered to be more environmental-friendly. Typically, physical and chemical approaches will relocate the oil contaminant from one incidental section to another; bioremediation differs greatly as it involves the eventual degradation of oil into harmless biomass (e.g. water and carbon dioxide). The physical method involves the usage of booms, skimmers, manual removing (wiping), mechanical removal, washing, sediment relocation or surf-washing, tiling and in situ burning. Meanwhile, the example technologies for the chemical method are dispersants, demulsifiers, solidifiers and surface film chemicals. Bioremediation is based on natural attenuation. Therefore, it is less interfering and destructive towards the polluted sites. Furthermore, the general public have been able to accept this green technology.

3.5 Bioremediation of Petroleum Hydrocarbon: Environmentally Relevant Microorganisms (ERM)

Biodegradation of crude oil is among the substantial processes which results in the ultimate elimination of hydrocarbon from the contaminated site. Basically, crude oil degradation by microorganisms occurs due to the utilization of hydrocarbons for growth and reproduction, where they are provided with carbon and electron to obtain the required energy. Zobell (1946, 1973), Atlas (1981, 1984), NAS (1985), Foght and Westlake (1987) and Leahy and Colwell (1990) are among the pioneer studies and scientific papers that have encompassed extensive aspects of this environmentally relevant microorganism (ERM) and its processes.

The abundance of hydrocarbon-degrading microorganisms is not limited to marine, soil and freshwater habitats. Back in 1974, the first hydrocarbon-degrading bacterium which was *Pseudomonas putida* was first isolated and registered as a patent for biological remediation agent (Martin 2011). *Pseudomonas* species are known to have a broad affinity for hydrocarbons by being the genus most isolated from hydrocarbon-degrading culture and contaminated sites and also able to degrade selected hydrocarbon components such as aromatics, alicyclic, alkane and thipenes (Dindar et al. 2015; Vankateswaran et al. 1995). However, the potential identification has started way back in the 1960s as there were over 100 species of microorganisms in some 60 genera can oxidize one or more hydrocarbons (Cooney 1974).

Since 1989, studies and researches on potential oil-degrading microorganisms have emerged, following the success application for the *Exxon Valdez* spill (Prince et al. 1994). In 1991, more than 70 oil-degrading microbial genera were being identified (Martin 2011). A recent review listed 79 bacterial genera, 9 cyanobacterial genera and 103 fungal genera that are known to be able to degrade or transform hydrocarbons. However, there are more strains likely to occur in nature and yet unidentified (Hassanshahian and Cappello 2013). Table 3.3 listed for both marine and freshwater some most important hydrocarbon-degrading microorganisms.

There are more than 200 species of bacteria, yeasts and fungi which have proved their ability to degrade hydrocarbons of different ranges (Zobell 1973). From extreme cold Arctic and Antarctic marine environments, bacteria are predominant hydrocarbon degraders (Jordan and Payne 1980). Although algae and protozoa are an important microbial community in the environment, until 2011, there are only 16 algae genera which potentially degrade hydrocarbons and no protozoa identified as potential degraders (Das and Chandran 2011).

Pseudomonas, *Achromobacter*, *Anthrobacter*, *Micrococcus*, *Nocardia*, *Vibrio*, *Acinetobacter*, *Brevibacterium*, *Corynebacterium*, *Flavobacterium*, *Candida*, *Rhodotorula*, and *Sporobolomyces* are categorized as common and significant (based on the frequency of isolation) genera of hydrocarbon utilizers in aquatic environments (Atlas and Bartha 1992). The historical exposure of the environment to hydrocarbons influenced the distribution of hydrocarbon-utilizing microorganisms. Atlas (1981) stated that there would be a higher percentage of hydrocarbon degraders in environments contaminated with oil compared to unpolluted area. A

Table 3.3 List of hydrocarbon-degrading microorganisms in marine and freshwater environment

Genera	Species	References
Bacteria	*Stenotrophomonas maltophilia*	Juhasz et al. (2000)
	Rhodococcus corynebacterioides	Gentili et al. (2006)
	Marinococcus albus	Vyas and Dave (2010)
	Pseudomonas aeruginosa	Agarry et al. (2008), Nurul Huda (2011)
	Pseudomonas flavobacterium	
	Neisseria elongata	Adoki and Orugbani (2007)
	Chromobacterium violaceum	Mukred et al. (2008)
	E. citreus	Udotong et al. (2008)
	Klebsiella pneumoniae	Hii et al. (2009)
	Acinetobacter lwoffii	Alkhatib et al. (2011)
	Bacillus megaterium	Das and Chandran (2011)
	Enterobacter cloacae	Thavasi et al. (2007)
	Brevibacillus parabrevis B-1	Al-Jumaily and Al-Wahab (2012)
	Ochrobactrum anthropi	Bao et al. (2012)
	Vibrio fischeri	Bao et al. (2012)
	Bacillus subtilis	Delille et al. (2005)
	Bacillus cereus	Latha and Kalaivani (2012)
	Bacillus pumilus	Maliji et al. (2013)
	Pseudomonas xanthomarina	Al-Saleh and Obuekwe (2014)
	Bacillus badius D1	El-Sheshtawy and Doheim (2014)
	Aeromonas hydrophila	Sarwade and Gawai (2014)
		Ainon et al. (2010)
Yeast	*Candida maltosa*	Chrzanowski et al. (2006)
	Exophiala xenobiotics	Hoog et al. (2006)
	Candida tropicalis RETL-Cr1	Piakong et al. (2009)
	Trichosporon asahii	Chandran and Das (2010)
	Candida lipolytica	Das and Chandran (2011)
	Pichia ohmen YH-41	Yao et al. (2012)
	Candida tropicalis	Beier et al. (2014)
Fungi	*Aspergillus niger*	Okoro (2008)
	Cochliobolus lutanus	Al-Nasrawi (2012)
	Aspergillus fumigatus	Chaudhry et al. (2012)
	Aspergillus versicolor	Garapati and Mishra (2012)
	Aspergillus saprophyticus	Ekundayo and Osunla (2013)
	Fusarium solani	Al-Jawhari (2014)
Algae	*Prototheca zopfii*	Walker et al. (1975)

total of 100% viable microbial community is estimated in a polluted area while less than 0.1% viable microorganism in pristine or unpolluted ecosystems. To date, there is no single strain of microorganisms with the metabolic capacity to degrade all the components found in crude oil (Zhu et al. 2001).

3.6 Bioremediation: Researches on Potential Hydrocarbon Degrader

In nature, the hydrocarbon-degrading microorganisms are extensive in number and types. Particularly, the removal of non-volatile components of oil from the contaminated site can be accomplished by biodegradation mechanism. Although it requires some time, the microorganisms can degrade a significant amount of spilled oil in marine and freshwater environments as biodegradation is relatively a slow process.

Naturally, crude oil biodegradation usually involves a succession of species within the consortia of microorganisms. The eventual removal of hydrocarbons from the environment may also include the important role played by non-hydrocarbon microorganisms. This is because there is a sequential or progressive reaction to petroleum degradation. The initial attack is carried out by certain organisms on the hydrocarbon components which produces intermediate products which then susceptible to a different group of microorganisms and later results in further degradation (Zhu et al. 2001; Karrick 1977). Most of the previous researches on laboratory studies and field tests have shown that the utilization of microorganisms, locally isolated microorganisms, can enhance biodegradation of hydrocarbon on a contaminated site (Swannell et al. 1996; Price 1993). Table 3.4 shows the previous studies on the performance of *Candida tropicalis* and *Pseudomonas aeruginosa* in degrading hydrocarbons using selected techniques.

Aeromonas hydrophila, *Stenotrophomonas maltophilia*, and *Chromobacterium violaceum* are listed as potential hydrocarbon-degrading microorganisms (Ainon et al. 2010; Udotong et al. 2008) where studies and test were done for their ability to uptake hydrocarbons as energy and main source of carbon. However, most of the studies related to their potential are examined from isolation and identified during characterization, not of hydrocarbon degradation.

Consortia culture which previously exposed to hydrocarbons can become adapted and within times will eventually react to the existence of hydrocarbon pollutant (Atlas and Bartha 1998). Since individual microorganism may only metabolize a limited range of hydrocarbons and crude oil is composed of a mixture of components (Britton 1984; AL-Saleh et al. 2009), crude oil biodegradation requires a mixture of different groups or consortia functionings to degrade a wider range of hydrocarbons (AL-Saleh et al. 2009).

3.7 Bioremediation: Influential Factors

In nature, biodegradation of petroleum hydrocarbons is a highly heterogeneous, complex and relatively slow process. In order for microorganisms to degrade a significant amount of spilled oil, several months to years are required. The key factor for the success of bioremediation of oil depends greatly on the ability to provide and maintain conditions that favour the rate of oil biodegradation in a

Table 3.4 Previous studies on the performance of *Candida tropicalis* and *Pseudomonas aeruginosa* in degrading hydrocarbons

Species	Pollutant	Initial concentration	Technique	Biodegradation performance	Reference
Candida tropicalis RETL-Cr1	Crude oil	5% v/v	Shake-flask culture	40% in 28 days	Benard and Mohd Tuah (2016)
Candida tropicalis NCYC 1503	Phenol	1000 mg/L	Shake-flask culture	100% in 2.5 days	Kuntiya et al. (2013)
Candida tropicalis	Naphthalene and phenol	500–3000 mg/L	Placket-Buman	35–99% in 3 days	Farag and Soliman (2011)
Candida tropicalis NCIM 3556	Phenol	2000 mg/L	Shake-flask culture	95% in 16 h	Varma and Gaikwad (2009)
Candida tropicalis RETL-Cr1	Phenol	282 mg/L	Fed-batch fermentation	100% in 13 h	Piakong (2006)
Candida tropicalis	Phenol	1000 mg/L	Shake-flask culture	100% in 32 h	Yan et al. (2005)
		1800 mg/L		100% in 59 h	
Candida tropicalis PFS-95	Crude oil	NA	Emulsification	68.9% in 16 days	Ijah and Essien (2005)
Pseudomonas aeruginosa BAS-Cr1	Crude oil	5% v/v	Shake-flask culture	30% in 28 days	Benard and Mohd Tuah (2016)
Pseudomonas aeruginosa	Crude oil	0.2% v/v	Shake-flask culture	78.86% in 21 days	Al-Wasify and Hamed (2014)
Pseudomonas aeruginosa	Crude oil	5000 mg/L	Shake-flask culture	85% in 7 days	Latha and Kalaivani (2012)
Pseudomonas aeruginosa	n-hexadecane	2000 mg/L	Shake-flask culture	97.3% in 6 days	Chen et al. (2012)
Pseudomonas aeruginosa NY3	PAHs	25 mg/L	Shake-flask culture	23.1% in 1 day	Nie et al. (2010)
Pseudomonas aeruginosa	Crude oil	4 mL	Fractional recovery	97.2% in 24 days	Ekpo and Udofia (2008)
Pseudomonas aeruginosa	Engine oil	5% v/v	Shake-flask culture	71% in 28 days	Mandri and Lin (2007)
Pseudomonas aeruginosa	Crude oil	700 mg/L	Shake-flask culture	71% in 8 days	Zhang et al. (2005)

Table 3.5 Biodegradation susceptibility of different petroleum products

Biodegradability	Example constituents	Petroleum products
More degradable	n-Butane, n-pentane, n-octane	Gasoline
	Nonane	Diesel fuel
	Methylbutane, dimethylpentanes	Gasoline
	Benzene, toluene, ethylbenzene, xylenes	Gasoline
	Propylbenzenes	Diesel, kerosene
	Decanes	Diesel
	Dodecanes	Kerosene
	Tridecanes	Heating fuels
	Tetradecanes	Lubricating oils
Less degradable	Naphthalenes	Diesel
	Fluoranthenes	Kerosene
	Pyrenes	Heating oil
	Acenaphthenes	Lubricating oil

Source: Adapted from Maila and Cloete (2004)

hydrocarbon-polluted site, with regard to two factors identified as the composition or characteristics of the spilled oil and also environmental conditions (Gavrilescu 2010).

The composition and chemical structure of crude oils strongly affect its biodegradability (Table 3.5). In summary, although not universal, the susceptibility of petroleum hydrocarbons to microbial degradation or attack is as follows: n-alkanes > branched alkanes > low-molecular-weight aromatics > cyclic alkanes (Das and Chandran 2011). Further, their inherent biodegradability is also influenced by the physical state and toxicity of the compounds. For example, n-alkanes are more biodegradable than the $C_5 - C_{10}$ homologues, as they show to be inhibitory to most hydrocarbon degraders due to its ability in disrupting the lipid membrane structures of microorganisms (Okoh 2006).

The concentration of individual oil constituent also influences the rate of biodegradation. The weathering processes influence this factor in different ways. For example, dispersion and dissolution process may dilute the pollutants, while emulsification process may concentrate the pollutants (Venosa and Zhu 2003). Concentrations of hydrocarbons have a negative relationship with the biodegradation rate. Extremely low concentration means unable to stimulate degradation due to unsupported microbial growth, whereas extremely high concentration would inhibit degradation through toxic effects or limitations of nutrient or oxygen (Zhu et al. 2001).

During an occurrence of oil spill, characteristics of contaminated site or environment affected the biodegradation rate of hydrocarbon. The oil biodegradation was affected by major environmental factors: (a) weathering processes, the biodegradation of oil is profoundly affected by the weathering processes; (b) temperature, the populations of microorganisms and spilled oil properties are greatly affected by the ambient or surrounding temperature; (c) concentration and availability of oxygen, oxygenases are required for the process of degradation of oil needed to occur in

Table 3.6 Summary of major environmental factors affecting oil biodegradation

Factor	Microbiological perspective	Reference
Weathering	Weathered oil restricts access of microbe due to limited surface area	Leahy and Colwell (1990)
	Photo-oxidation helps in bioavailability of pollutant	
Temperature	Affects metabolisms of microorganisms and properties of hydrocarbons	Coulon et al. (2005), Zhu et al. (2001)
	Positively correlated with biodegradation rate	
	General optimum temperature is 30–40 °C	
Oxygen	Effective and extensive degradation requires oxygen	Zhu et al. (2001)
	Oxygenases involved in the degradative pathway for saturates and aromatics	
	Limiting factor in subsurface sediments, anoxic zones of water columns and salt marshes	
Nutrients	Deficient nutrient inhibits microbial growth	Martin (2011)
	Excessive nutrient inhibits biodegradation activity	
	The proper ratio of C:N:P is 100:10:1	
pH	Biodegradation rates are highest in the slightly acidic condition	Pawar (2012)
	Fungi are more tolerant of the acidic condition, while others favour pH near neutrality	
	Unfavourable pH reduces microbial activities and causes microbial death	
Salinity	Slight changes affect oil biodegradation via alteration of the microbial population	Zhu et al. (2001)
	Optimum salinity range 2.5–3.5%	

aerobic conditions (Cerniglia 1992; NAS 1985; Atlas 1981); (d) nutrients, 1 g of hydrocarbons is converted to cell materials and requires the utilization of approximately 30 mg of phosphorus and 150 mg of nitrogen (Ron and Rosenberg 2001); (e) pH, a neutral pH is favourable by most heterotrophic bacteria and fungi; and (f) salinity, changes in salinity alter the population of microbe and affected the biodegradation of oil (Zobell 1973). These major environmental factors affecting oil biodegradation are summarized in Table 3.6.

3.8 Oil Spill Plume Modelling: Application of Biodegradation

The answer to numerous questions during a spill response is the oil spill modelling. It is also a very essential tool for providing significant input and data for oil spill response. The modelling outputs can be exploited to predict the oil slick movement trajectory and also future location. Also, any particular habitats or species which

may be at risk due to exposure or contamination of oil can be determined using the output data.

Due to the instantaneous and destructive results of major accidents, oil spills have gained massive attention concerning pollution modelling. There is a need to provide actual-time predictions of the oil trajectory and fate because of the accidental spills being emphasized. A high-resolution model of the area at risk needs to be easily set up as part of a relocatable model system or already set up to ensure effectiveness. Several papers have reviewed the extensive literature on oil spill modelling (Spaulding 1988; Reed et al. 1989). Table 3.7 summarizes the example of existing oil plume model with regard to its limitation.

The important information on controlling factors can be obtained from field-scale experiments and dynamic and static lab tests.

The evaluation or interpretation of a field-scale experiment usually represents a biodegradation process as first-order decay, which may not be valid (Fry et al. 1996; Acton and Barker 1992). A dynamic experiment includes more complex tests. However, the explanation on biological processes is less precise. The biodegradation rates, controlling microbial factors and interactions between organic substrates can be determined, identified and also demonstrated from static microcosm studies (Alvarez and Vogel 1995).

The answer towards either passive (natural attenuation) or active remediation is enough is deemed in actual environment. Passive (natural attenuation) is favoured as it is more promising to (a) permanently remove pollutants via natural processes and (b) to prevent costly treatments (e.g. physical and chemical) and (c) is performed in situ or on-site. Numerical modelling has the ability to fill the gap in oil plume prediction and determine biodegradation's limiting factors and many other questions, only if there is enough availability of data (Essaid et al. 1995).

Still, the predicting capacity for decision-making may particularly obtained from latest and leading pollutant transport models that fully encompass the controlling factors and processes. For feasible and realistic logic, laboratory experiments are conducted in small extent. However, the conversion of observation from laboratory extent to application on field scale most commonly suggests (a) a transport mechanism and mass transfer for field mass; (b) subsistence of multiple phases – pollutants and competing microorganisms; (c) spatial heterogeneities; and (d) limiting factors (e.g. nutrients, pH, redox conditions or temperature) which influence bacterial growth (Sturman et al. 1995). Therefore, great concern is needed in order to deduce the result from laboratory to field scale. Nonetheless, the pollutant loss in most cases of field-scale results is not definitely caused by microbial activity which later brings to factors such as abiotic process and error in measurement (Madsen 1991).

Table 3.7 Examples of existing oil plume model and its limitations

Model	Application	Data needed	Limitation (s)	References
COZOIL	Measures the stranding of oil at the shoreline	"Holding capacity" of the shoreline type, "removal rates" and "half-life" values	Can predict the motion of oil until it reaches the shore but cannot include beach and surf zone processes	Reed et al. (1989)
OILMAP	Predict the transport and fate of the oil from the blowout source on the sea bed until its contact with the shoreline	The transport of oil and hydrated particles released during the blowout	Negligence on biological processes such as biodegradation by microbial activity	Jayko and Howlett (1992), Spaulding et al. (1992)
	Prediction of the oil/gas plume resulting from the blowout, including the effects of hydrate formation and dissociation	The hydrate and oil particle rise velocities are determined by Stokes' law and hence dependent on particle size and density		
ASA's Blowout Model Theory	A simplified integral jet theory employed for the vertical as well as for the horizontal motions of the gas-oil plume	Laws of conservation of water mass, momentum, oil mass and buoyancy	Focusing only on physical parameters	Spaulding (1982)
	Defining the rates of entrainment and spreading of the jet are obtained from laboratory studies		Negligence on biological processes such as biodegradation by microbial activity	
CORMIX	Accounts for both vertical and lateral boundaries through a process called schematization	Uses a collection of jet integral, length scale, integral and passive diffusion approaches to simulate mixing zone	The system only emphasizes the role of boundary interaction and predicts steady-state mixing behaviour and plume geometry	EPA-823-K-07-001, Rev. 5.0, December 2007
	Predicts flow behaviour on shorelines, benthic regions and other biologically important and chemically reactive regions	The system contains a collection of about 30 regional flow modules to simulate the physics of mixing zones	Negligence on biological processes such as biodegradation by microbial activity	

(continued)

Table 3.7 (continued)

Model	Application	Data needed	Limitation (s)	References
USEPA Visual Plume	Simulates single and merging submerged aquatic plumes in arbitrarily stratified ambient flow and buoyant surface discharges	Uses jet integral, length scale and passive diffusion approaches	Does not address the effects of vertical or horizontal boundaries on mixing or on discharge stability	EPA/600/R-94/086
		Features graphics, time-series input files, user-specified units, a conservative tidal background-pollutant build-up capability, a sensitivity analysis capability and pathogen decay model that predicts coliform bacteria mortality	Can only be applied to a stable near field without dynamic attachments	

3.9 Oil Spill Plume Modelling Using MIKE 21/3 OS: A Lab-Scale Experiment on Potential of Locally Isolated Beneficial Microorganisms

A bioaugmentation study was performed by Laurencia and Piakong (2017) to assess the biodegradation of crude oil by five locally isolated beneficial microorganisms (LIBeM) as consortia culture, previously isolated and characterized by Piakong 2006; Piakong et al. (2004). Figure 3.1 shows the colony morphology of LIBeM used in a lab-scale study on bioaugmentation (biodegradation) of 5%, 10% and 15% v/v crude oil by consortia culture. The experiment condition was set up at pH 7 and temperature of 30 °C (incubation). The technique used was shake-flask culture.

MIKE 21/3 OS Oil Spill model was chosen in this study. The MIKE by DHI software was granted by DHI Water and Environment (M) Sdn. Bhd. The selected study area is Kimanis Bay (115.77°N, 5.65°E) where there is a potential for the oil spill to occur during transportation, storage and distribution (routine operations). The oil spill plume modelling is simulated (no calibration) using calculated input parameters from previous field- and laboratory-scale experiments and also from literatures. The simulation was done with the incorporation of biodegradation rate constant obtained from laboratory result of the present study. Transport parameters are as detailed in Table 3.8.

The achieved crude oil biodegradation efficiency by consortia culture was 96%, 96% and 94% for 5%, 10% and 15% v/v crude oil, respectively. The biodegradation rate of consortia culture was 7.84 g/L/d, 9.55 g/L/d and 10.75 g/L/d for 5%, 10% and

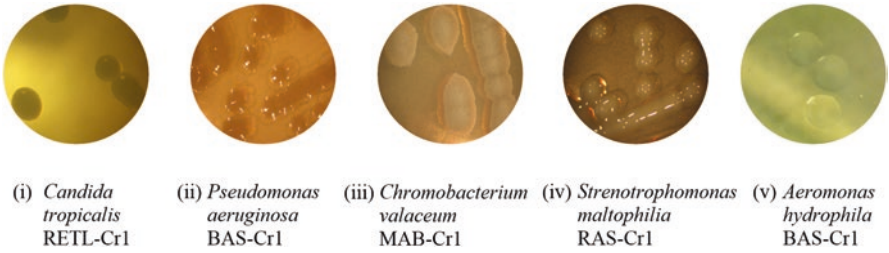


Fig. 3.1 Colony morphology of LIBeM used in the lab-scale study

Table 3.8 Input parameters for MIKE 21/3 OS simulation

Oil type			Heavy fuel oil	Source
Source magnitude	State variables	Unit		
Weight	Volatile oil fractions, heavy oil fractions	wt%	7.5, 77.5	DHI Spill Analysis Data (2014)
	Asphaltene, wax	wt%	8, 7	
Processes	Class constants			
	Schmidt number		2.7	DHI Spill Analysis Data (2014)
	The average molecular weight of volatile fraction	g/mol	123	
	The vapour pressure of a volatile fraction	atm	0.005	
Simple evaporation	Distillation percentage at 180 °C	%	5	
Spreading	Terminal thickness	mm	0.1	
Biodegradation	Decay rate, volatile fraction	Per day	k_{ANBR}, k_1, k_2, k_3	Laurencia and Piakong (2017)
	Decay rate, an involatile fraction	Per day	k_{ANBR}, k_1, k_2, k_3	
Emulsification	Maximum water fraction, Kao constant	m^3/m^3	0.85, 3.3	DHI Spill Analysis Data (2014)
	Law constant, emulsion rate	s/m^2	200, 2.00E-06	
Buoyancy	The density of oil at 20 °C, volatile fraction	kg/m^3	787	
	The density of oil at 20 °C, the heavy fraction	kg/m^3	1011	
Water solubility	Water solubility, volatile fraction	kg/kg	2.00E-5	
	Water solubility, the heavy fraction	kg/kg	2.00E-7	
Volumetric temperature expansion coefficient	Volatile oil fraction	1/°C	0.0007	
	Involatile oil fraction	1/°C	0.0007	

(continued)

Table 3.8 (continued)

Oil type			Heavy fuel oil	Source
Photo-oxidation	Decay rate: volatile fraction, the heavy fraction	Per day	0, 0	
	Light extinction coefficient	1/m	1	
Dissolution	Dissolution rate: light fraction, heavy fraction	Per day	0.4, 0.4	
Vertical dispersion	Wind speed for wave breaking	m/s	5	
	Wave energy dissipation rate	J/ m^3/s	1000	
Vertical limits	Max distance below the surface of the surface amount	m	0.05	
	Max distance above the bed for the bottom amount	m	0.05	
Viscosity	Mooney constant		0.7	
	Dynamic oil viscosity at a reference temperature	cP	209	
	Reference temperature for dynamic oil viscosity	°C	50	
	Coefficient exponential temperature dependency		−0.136	
Oil area	Oil area growth rate constant	Per sec	150	

15% v/v crude oil, respectively. The biodegradation data by consortia culture was then used to calculate the biodegradation rate constant, based on the first-order kinetics model (Abioye et al. 2012) as $C = C_0e^{-kt}$, where C is the hydrocarbon content in water at time t (g/L^{-1}), C_0 the initial hydrocarbon content in water ($g\ L^{-1}$), k the biodegradation rate constant (day^{-1}); and t the time (day). The biodegradation rate constant was 0.005 ($K_{ANBR\ =\ anticipated\ natural\ biodegradation\ rate}$), 0.04639 ($k_{1\ =\ constant\ at\ 5\%\ v/v\ crude\ oil}$), 0.04616 ($k_{2\ =\ constant\ at\ 10\%\ v/v\ crude\ oil}$) and 0.0402 ($k_{3\ =\ constant\ at\ 15\%\ v/v\ crude\ oil}$).

The outputs available are 2D maps, mass budget as a time series and particle tracks and particle properties. 2D maps include oil slick thickness. The mass budget as a time series is for identifying the weathering processes which affect the oil. The particle tracks and particle properties are for illustrating the spreading of the oil. The oil spill simulation is executed using the MIKE ECO Lab engine and a MIKE ECO Lab oil spill template. The execution comprises both Lagrangian particle tracking (including weathering processes) and Eulerian advection-dispersion computations of dissolved oil. To save time, the decoupled mode flow data from a previous MIKE 21/3 simulation are reused. The spreading of oil is calculated by dividing the oil spill into discrete parcels (particles). The movements of the particles are given as a sum of displacement determined by the hydrodynamic flow field (wind) and a dispersive component as a result of random processes. The movement of dissolved oil

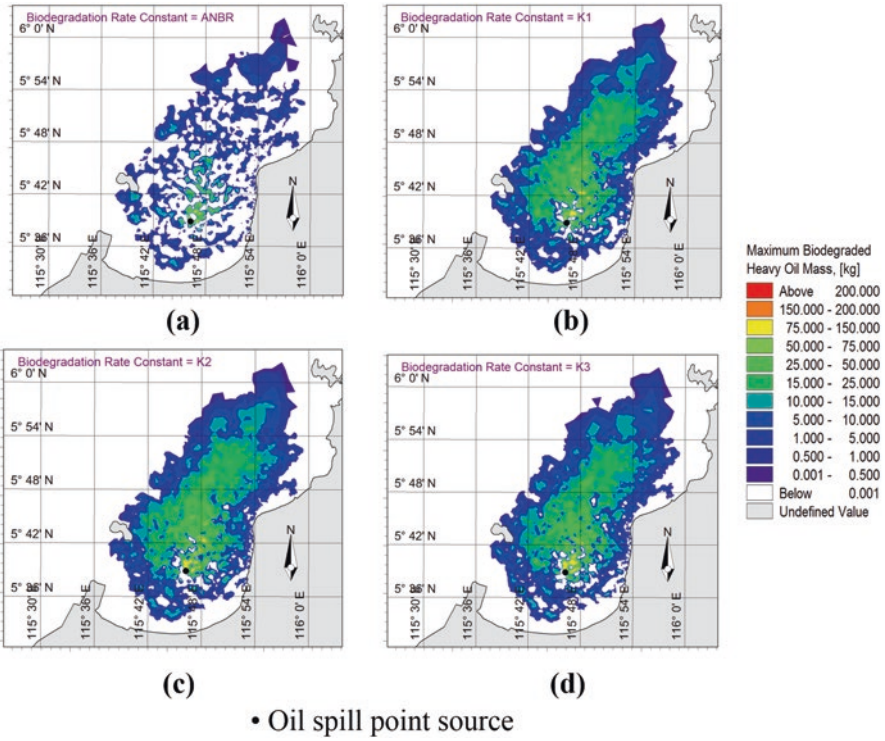


Fig. 3.2 Prediction of the oil spill plume trajectory towards direction NE and total maximum biodegraded heavy oil mass (kg)

is calculated using the advective-dispersion formulation in the transport solver in MIKE 21 and MIKE 3 FM.

Two spill simulations were run to represent the hypothetical spills which occurred at a given study area with a volume of 3000 barrel (bbl), corresponding to tier 1 response. Due to different metocean conditions, one of the spills flows north-eastwardly (NE) (Fig. 3.2), while the other one flows south-westwardly (SW) (Fig. 3.3). For each part, the simulation showed the plot for maximum biodegraded oil (heavy) after 14 days. The simulation results showed the prediction of maximum biodegraded heavy oil with three different values of biodegradation rate constant and anticipated natural biodegradation rate (ANBR) which serve as a control. The spreading of oil is elongated towards NE and SW direction which is manipulated by major environmental factors such as wind, wave, current and tide. The contour (different colours) represents the mass (kg) of oil being degraded.

The result on the incorporation of biodegradation data obtained from laboratory studies showed a promising and a rather significant effect of biodegradation to total oil mass of heavy oils. Heavy oil, due to its complex property and compound, makes it not easily affected by evaporation and photo-oxidation (weathering processes) but

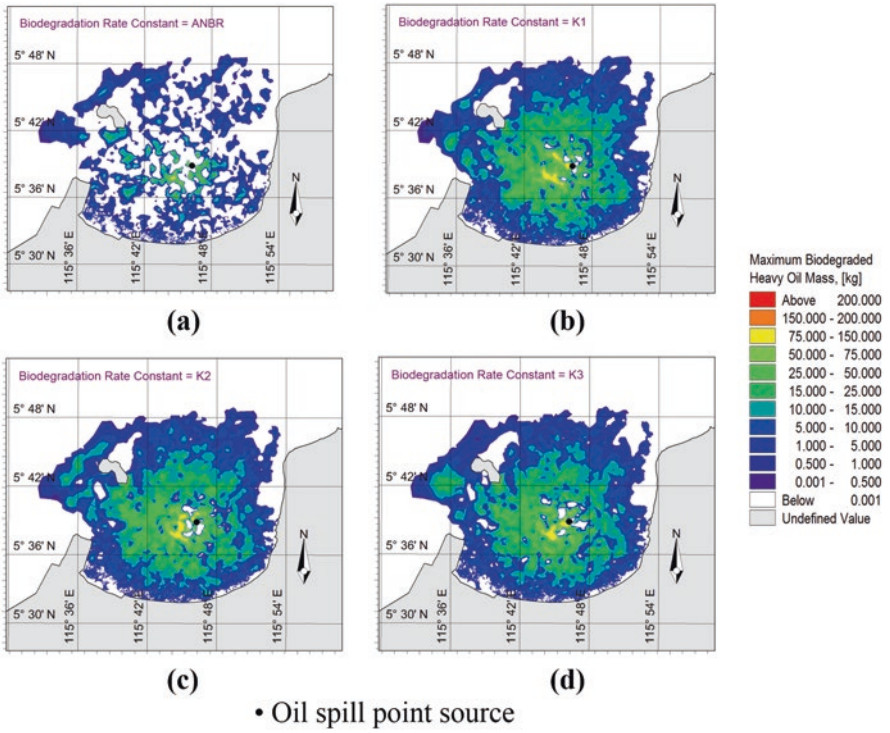


Fig. 3.3 Prediction of the oil spill plume trajectory towards direction SW and total maximum biodegraded heavy oil mass (kg)

is susceptible to biodegradation. In this case, the modelling of biodegradation shows promising results in treating heavy oil spill and could be applied as a response.

Herwig (2013) explained that biodegradation is a complex and dynamic process whose speed can vary dramatically depending on environmental conditions but takes several weeks to several months for most oil compounds. Given that the response phase of most marine oil spills lasts less than a week, biodegradation does not play a major role in most oil spill responses. However, microbial degradation can be of important response when the amount of oil released or the length of time necessary to contain the release extended the response window into the biodegradation timeframe (Atlas and Hazen 2011).

The laboratory-derived biodegradation rate constant based on first-order reaction can be used in MIKE 21/3 OS model to simulate oil spill degradation processes. Based on the conditions of the stated studies and model assumptions, it can be assumed that degradation parameters derived from laboratory experiments adequately describe the degradation of field scale, provided that all the controlling factors are integrated. This work provides an understanding in the application of the biodegradation rate constant into "real-world" extent. Moreover, it gives a better

perception of the input needed for successful modelling of biodegradation at field extent, with regard to the comparison with previous researches.

3.10 Future Aspects

A recent study that focuses more into the processes that influence eventual distribution, effects and recoverability of spilled oil (biodegradation) should be implemented during oil spill modelling to provide a more realistic approach to oil spill plume modelling. Such ability will enable the assessment between actual or potential biological effects of spilled oil and different methods to choose suitable methods of response and mitigation and also when to abolish them. It is crucial to obtain this information (spilled oil and oil samples) from the spill point in order to understand the properties of the oil which aid in the prediction of oil fate and effects.

Oil spill modelling has the answers for many of the inquiries pertaining to spill response. The modelling data can be exploited to forecast the oil slick movement, trajectory and also future location which can be informed to responders, stakeholders and the public. Also, any particular habitats or species may be at risk due to exposure or contamination of oil which can be determined using the output data. Oil weathering processes (e.g. spreading, evaporation, dissolution and entrainment) may also be estimated using the model. The performance of the numbers of common response method (e.g. mechanical and chemical) may also be determined using the response modelling. As it may be carried out at the primary phase of a spill and provide measurable observation of most favourable outcome of the response, the result can be exploited as the physical foundation to bring in supplementary or other response equipment. Other areas to look at in response modelling are user needs, offset of evolving technologies with practical response abilities, data access and incorporating predictions and studies into operations.

The first key question is: Is bioremediation accomplished in the natural environment that includes various uncharacterized organisms? The second key question is: Is oil spill not taking place in an exact environment's condition? For instance, variations occur in the types and amounts of contaminants, climate setting, and hydrogeodynamics. These queries have brought the bioremediation of hydrocarbon to lag behind knowledge-based technologies that are governed by common rationales (Watanabe and Baker 2000). Despite the growing acceptance of bioremediation as a means to treat spilled oil in marine environments, the mechanisms that promote the process under field conditions remain poorly constrained. Bioremediation, therefore, may make a significant contribution towards accelerated habitat recovery by the removal of toxic hydrocarbon components to levels below the toxicity threshold. It is thus an important part of toolkits available for dealing with accidental and deliberate oil releases into the marine environment (Prince 2002). One has to increase the knowledge and information of such microbial interactions and their behaviour before exactly planning to introduce the competent culture in oil spill response action.

3.11 Conclusion

The application of biodegradation data in oil spill plume modelling is a hopeful approach for oil spill response action. Environmentally relevant microorganisms (ERM) are known to have a broad affinity for hydrocarbon degradation. Choosing the best and competent strain culture (single or consortia) is the main focus to achieve high efficiency of hydrocarbon degradation. A key appeal of oil spill modelling is that it may serve as the answer to various questions during a spill; oil spill movement, trajectory, future location and also any particular habitats or species may be at risk due to exposure or contamination of oil. Bioremediation of petroleum hydrocarbon with regard to oil spill modelling approaches provides new insight as well as additional knowledge in the application of a potential locally isolated beneficial microorganism in oil spill plume modelling and whether or not biodegradation can be used as one of the oil spill response actions.

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Chapter 4
Hydrocarbon Degradation Assessment: Biotechnical Approaches Involved

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Abstract The contamination of soil by petroleum hydrocarbons has resulted in an increased attention toward the development of sound and innovative technologies for its remediation. The current chemical and physical treatment approaches are effective for petroleum hydrocarbon degradation, but they stagnate in the desired properties; aside from that, they also commonly generate many harmful compounds that are powerful immunotoxicants and carcinogen to living beings. In contrast to chemical and physical approaches, biotechnical techniques are effectual treatments in terms of cost and safety on long-term use. These methods have displayed a great potentiality and inexpensive privilege because they are environment friendly. The use of biomaterials to accumulate and pre-concentrate hydrocarbon from aqueous solutions or terrestrial ecosystem has been evaluated by many researchers. However, for the predictable future, long-term tolerance studies are looked for. Therefore, this chapter will discuss and provide an overview on biological degradation and fundamental factors for the biodegradation process.

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4.1 Introduction

4.1.1 Critical Importance of Hydrocarbon Remediation

Polycyclic aromatic hydrocarbons (PAHs) are the main source of organic contaminants in an environment. Annually a huge percentage of PAHs are release into the environment through locomotives, boats, ships, and industrial engines. Not only has the oil contamination treated ecological systems and environment but it also caused critical conditions on human health and recreation activities. Indeed, with the increasing demand of fossil fuel energy in recent years, such pollution to soil and water has become the main headline news of mass media. For example, the largest spill in the world history is the Lakeview Gusher spill in California (1,200,000 tons of crude oil) in September 1911 (Harvey 2010). In July 2010, the occurrence of oil spill in the Gulf of Mexico resulted in oil spill along the Gulf Coast with 600000 tons of crude oil. According to the recent report, the total volume of all major spills was about 30 and 37 billion barrels of crude oil in 2006 and 2010, respectively.

On the one hand, all these oil spills have resulted in an immense damage on planet and can remain in an environment for decades or even centuries. Consequently, the impact of this leads to deaths of marine animals and birds and losses of marine ecosystems. On the other hand, cleanup of these contaminated sites and transformation of toxic environment to healthful one require a long time and decades of dedicated work. Moreover, the remediation of these sites is costly; for instance, the USA and UK have spent over 1 and 2 billion dollars to remediate 120,000 contaminated petrol stations in 2001 and 2007, respectively (Collins 2007). This amount is estimated to be raised and reach to 2 trillion dollars in 2040 (Yu and Hill 2006). Crude oil contains a number of toxic and complicated constituents that can affect our dedicated ecological systems. It is also toxic to organisms in an aquatic environment due to release of toxic compounds once exposed to solar ultraviolet radiation. PAHs consist of so many individual constituents; therefore, it is tough to ascertain the effect of each compound within the context of a hydrocarbon mixture. It is cited that aromatic compounds incline to be more toxic compared to aliphatic compounds (Uche and Dadrasnia 2017). For instance, the aromatic compounds have adverse effects on the indigenous microbial flora. It has been found that camphene, isobornyl acetate, limonene, and α-pinene were inhibitors of microorganisms. Derivatives of phenolic and quinonic naphthalene exhibited inhibitory activity against microbial cell growth (Qingren et al. 2011). PAHs have direct effect on the growth and fertility of plants, germination, soil properties (organic matter composition and exchange capacity), and soil chemistry; however, the effects rely on the degree and type of the oil spill. Indeed, there is an irrefutable link between the human health and environment. These poisonous constituents in oil would affect human health through damaging plasma membrane and protein synthesis and act as an inhibition of nerve synapse function (Onwurah et al. 2007; Afuwale and Modi 2012). Light oil compounds contain high proportion of saturated hydrocarbons; so, these can be more harmful compared to heavy oils (Kauppi et al. 2011).

Therefore, it is necessary to take an immediate action to eliminate and remediate these polluted sites after accidental spill. Remediation methods involve applied

chemical, physical, and biological means. This chapter is aimed to illustrate the current understanding of the scientific concepts underlying environmental remediation and to spotlight some of the challenges associated with their effective application.

4.2 Bioremediation of Petroleum Oil-Contaminated Water and Soil

Biodegradation is defined as the breakdown of hydrocarbons via enzymatic activity of the microorganisms (fungi and bacteria), and this depends on the ability of microbes to emulsify the undissolved carbon source in the culture medium (Chrzanowski et al. 2006; Mancera-López et al. 2008). The interaction between microorganisms, plants, and animals is known as limiting degradation factors. Since most hydrocarbons are poor in mobility due to insoluble in water, therefore, their bioavailability is finite in the degradation process (Philp et al. 2005).

4.2.1 Bioaugmentation

Bioaugmentation is one of the main types of bioremediation technologies (Simarro et al. 2013). In fact, this process amounts to the inoculation of nonindigenous microorganisms with a high degradation potential to the soil for promotion of the bioremediation process (Wu et al. 2013). Microorganisms used in this method must be effective in degrading the target pollutant, they should have the ability to adapt to the environment, and they should not include pathogenic agents attacking regional organisms (Szulc et al. 2014).

Recent studies have shown that bioaugmentation increases the biodegradation of hydrocarbon compounds in soils contaminated with petroleum compounds (Taccari et al. 2012; Suja et al. 2014). Nevertheless, the effects of this method are case-specific and vary depending on the type of inoculum and nutritional elements (Abed et al. 2014; Suja et al. 2014). Since the biodegradation process is conducted by several microbes in the soil, understanding how hydrocarbon-degrading microbes are affected by bioaugmentation, the diverse form of microbial populations and their activities, as well as the potential of the inoculum to adjust to new environmental setup and conditions is important in order to ensure the effective bioremediation of contaminated soils (Taccari et al. 2012). However, limited research has been carried out to examine the relationship between the efficiency of petroleum and dynamic hydrocarbon degradation and microbial activity (Abed et al. 2015). A study with the aim of evaluating the effect of bioaugmentation with *Acinetobacter* SZ-1 isolated from KF453955 on the efficiency of petroleum hydrocarbon degradation demonstrated that bioaugmentation had increased the degradation of petroleum hydrocarbons (TPH) up to 34% after 6 weeks of incubation (Wu et al. 2016). In general, the authors reported a positive correlation between the population of TPH

Table 4.1 Some recent studies on bioaugmentation

Microorganisms added	Results	Researchers
Serratia sp. *BF40*	The used strain showed high utilized potential in degradation of crude oil- contaminated saline soils (>60%) because of its high surface activity and salt tolerance	Wu et al. (2012)
Candida tropicalis SK 21	The inoculated yeast resulted in 83% TPH degradation as against 61% inoculating indigenous microorganisms	Fan et al. (2014)
Pseudomonas fluorescens	Augmentation with *P. fluorescens* significantly improved the degradation (>60%) as compared to samples relying on autochthonous microbes	Pavlorkov et al. (2014)
Pseudomonas aeruginosa, *Arthrobacter xylosoxidans*, *and Ochrobactrum intermedium*	Positive effects on biodegradation with substantial reduction in TPH levels were observed due to the bioaugmentation (32.2%)	Colla et al. (2014)

degrading agents in the soil and the efficiency of TPH degradation during the bioremediation process of contaminated soils.

Some studies have shown that biostimulation (increased biodegradation by indigenous bacteria through an increase in the bacterial population as a result of increased nutrients as discussed in Sect. 1.5.3) is more effective than bioaugmentation in enhancing the bioremediation of soils contaminated with petroleum compounds (Sayara et al. 2011; Abed et al. 2014). However, many reports have also demonstrated that the best way to degrade hydrocarbons in soils contaminated with petroleum compounds is through the simultaneous application of biostimulation and bioaugmentation (Taccari et al. 2012; Suja et al. 2014). In a field study intended to assess the potential of bioaugmentation for the remediation of soils polluted with petroleum compounds, it is reported that an increase in fluorescence in *Aeromonas hydrophila*, *Alcaligenes xylosoxidans*, *Gordonia* sp., *Pseudomonas*, *Pseudomonas putida*, *Rhodococcus equi*, *Stenotrophomonas maltophilia*, and *Xanthomonas* sp. has significantly improved the remediation of contaminated soils and was more effective than biosurfactant treatment (Szulc et al. 2014). These researchers reported that an increase in the microbial respiration of the soil and the enzymatic activity of dehydrogenase and catalase is associated with an increase in the rate and amount of hydrocarbon degradation in the contaminated soil. Results of some recent studies on the microbial strain application in order to remediate the crude petroleum-contaminated soils are shown in Table 4.1.

4.2.2 Microbial Strains in the Biodegradation of Hydrocarbons

The most important factor in bioremediation is the presence of microorganisms, especially bacteria and fungi, in the contaminated area (Gong et al. 2007). Hydrocarbons are first degraded by bacteria and fungi in the environment. Different sources have reported different degradation rates for these materials by fungi (about

6–82%), bacteria (about 0.13–50%), and marine bacteria (about 0.003–100%) (Leahy and Colwell 1990). Bacterial and fungal populations are present in soil in abundance and members from both the groups are involved in the biodegradation of hydrocarbons. For example, mineralization up to 82% of n-Hexadecane in a sandy loam soil is associated with bacteria, and only 13% of it is due to fungi (Leahy and Colwell 1990).

Gram-positive bacteria, especially *Bacillus* sp., play an important role in biotechnology and bioremediation processes (Ganesh and Lin 2009). A wide range of bacteria, such as *Pseudomonas*, *Acinetobacter*, *Alcaligenes*, *Nocardia*, and *Rhodococcus*, have the potentiality in aerobic biodegradation of aromatic compounds. For example, numerous studies have been conducted on the degradation of a vast range of aromatic organic compounds, ranging from benzene to benzopyrene, by the *Pseudomonas* genus.

Research conducted on anaerobic biodegradation of aromatic compounds has determined that large groups of bacteria degrade aromatic compounds under anaerobic conditions. There are many bacteria in the environment that can effectively degrade more contamination, and because the rate of degradation is slow, many toxic contaminants accumulate in the environment (Cao et al. 2009). Oxygen is an essential element for many organisms. While oxygen is toxic for some bacteria, most bacteria tend to live where oxygen exists. Aerobic bacteria depend on oxygen to grow. These bacteria obtain their energy through respiration. Anaerobic bacteria can survive in the absence of oxygen. Some of these bacteria breathe through fermentation. The degradation of aliphatic, aromatic, and petroleum hydrocarbon compounds by the oxidase enzyme requires oxygen molecules to function properly.

Therefore, it is necessary to create aerobic conditions in contaminated soils. Morgan and Watkinson reported that between 80% and 100% of BTEX is degraded in the presence of oxygen (Morgan and Watkinson 1992). In general, conversion of toluene and numerous other hydrocarbons into carbon dioxide and water, required about 3 mg/L of oxygen per mg/L of hydrocarbon. The mineralization of naphthalene occurs slowly under anaerobic conditions (about 0.4%), but in aerobic conditions, it is faster to mineralize (about 22.6%). Aeration is essential for gasoline degradation because it provides oxygen for the biodegradation of hydrocarbons in groundwater. The rate at which petroleum hydrocarbons are degraded anaerobically by microorganisms has been made known to be slow and is considered ecologically insignificant (Leahy and Colwell 1990). Evidence suggests that the microbial breakdown of oxidative aromatic compounds like benzoate and halogenated compounds such as benzonatate, chlorophenol, and polycarbophil occurs in anaerobic conditions. There was an evidence also indicating that the metabolic degradation of saturated aromatics including nitrogen, toluene, xylene, and methylbenzene-naphthalene is dependent on oxygen (Ladino-Orjuela et al. 2016). Bioremediation in the presence of oxygen, also called aerobic bioremediation, usually refers to a process that oxidizes toxic substances into nontoxic compounds such as carbon dioxide and water. In anaerobic respiration, as in fermentation, organic pollutants can be mineralized and converted into methane, carbon dioxide, and hydrogen. In another type of anaerobic bioremediation, which reduces dehalogenation, they can be converted into nontoxic materials or chloronitrile.

Numerous organisms were capable of anaerobic hydrocarbon degradation such as ethylbenzene or xylenes (particularly the *Azoarcus* and *Thauera* species) (Dolfing et al. 1990; Fries et al. 1994). Although anaerobic para-xylene degradation has been shown by an undefined nitrate reducing enrichment culture (Häner et al. 1995), there is no available organism reported in pure culture that can anaerobically decompose this compound (Chakraborty and Coates 2004). Anaerobic degradation of ethylbenzene by four organisms capable has been described (Rabus and Widdel 1995; Ball et al. 1996; Kniemeyer et al. 2003) in which strains of EbN1, PbN1 (Rabus and Widdel 1995), and EB1 (Ball et al. 1996) were facultative anaerobic denitrifiers, but the strain of EbS7 was an obligate anaerobic marine sulfate reducer (Kniemeyer et al. 2003). None of these organisms oxidized hydrocarbons aerobically, but all of them were limited in their ability to oxidize alternative aromatic hydrocarbons anaerobically. Other researchers reported the Dechloromonas strain RCB as being capable of anaerobic benzene oxidation (Coates et al. 2001).

Hydrocarbon-degrading bacteria in aquatic and terrestrial environments are as follows: *Arthrobacter*, *Alcaligenes*, *Acinetobacter*, *Achromobacter*, *Pseudomonas* spp., *Nocardia*, *Flavobacterium*, *Escherichia coli*, *Corynebacterium*, and *Bacillus* (Kosaric 2001; Onifade and Abubakar 2007; Abbassi and Shquirat 2008; Cao et al. 2009). Bacterial population capable of degrading hydrocarbons with high molecular weight (such as phenanthrene), includes the genus *Aeromonas*, *Alcaligenes faecalis*, *Alcaligenes denitrificans*, *Arthrobacter polychromogenes*, *Beijerinckia*, *Micrococcus*, *Mycobacterium*, *P. putida*, *P. Paucimobilis*, *Rhodococcus*, *Vibrio*, *Nocardia*, *Flavobacterium*, *Streptomyces*, and *Acinetobacter* (Watanabe 2001; Cao et al. 2009). BTEX degradation starts with the degradation of toluene and is followed by the degradation of benzene, *p*-xylene, *m*-xylene, ethylbenzene, and *o*-xylene. Species capable of degrading toluene are from *Geobacter metallireducens*, *Azoarcus* spp., and *Thauera* spp. In general, the aerobic degradation of simple aromatic compounds is carried out through the metabolic pathway and the presence of an enzyme system in microorganisms (Cao et al. 2009). Research has shown that the genus and species of *Alcaligenes denitrificans* have the potential for fluoranthene biodegradation at about 0.3 mg per ml per day. They can also degrade other polycyclic aromatic hydrocarbons (PAHs), such as piran and benzanthracene. Fluoranthene degradation by *Mycobacterium* species has also been reported (Kanaly and Harayama 2000). The genus and species of *Pseudomonas fluorescens* use chrysene and benz[a]anthracene as sources of carbon and energy (Caldini et al. 1995). *Sphingomonas* has been identified through the 16S rRNA test as a genus that can degrade phenanthrene and use phenanthrene as a source of carbon and energy. This species can degrade other hydrocarbons, including naphthalene, acenaphthylene, acenaphthene, dibenzofuran, fluorene, and anthracene. It can also degrade high molecular weight PAHs such as fluoranthene and piran in a liquid medium containing at least phenanthrene as a source of carbon and energy. Other petroleum products can be degraded by particular bacteria. These include the psychrophilic *Rhodococcus* bacteria isolated from underground waters and capable of growing at temperatures ranging from 4 °C to 30 °C and able to degrade hydrocarbons such as crude oil, diesel oil, and gasoline (Wook et al. 2006).

In PAH-contaminated soils and sediments, the degradation of high molecular weight compounds is very slow compared to that of low molecular weight PAHs (Aitken et al. 1998). It is difficult to explain the mechanism of hydrocarbon biodegradation in a heterogeneous environment where a mixture of different substrates and bacteria are present. Therefore, there is a need for mechanisms to transform, convert, and degrade each of the compounds separately and individually with specific bacteria (Aitken et al. 1998). Thus, the process microbiology has been used as a process for the remediation and elimination of environmental contamination (Dadrasnia et al. 2018). Generally, based on the studies, biodegradation of heavy oil constituents has been carried out in the following order (Plohl et al. 2002) of: Asph altene < Polars < Aromatics > Aliphatics

4.2.3 Biosurfactant Production

One of the prominent features of hydrocarbon-degrading bacteria is their emulsifying capability in a solution due to production of surface-active agents such as biosurfactants. Biosurfactants are directly taking part in the process of eliminating hydrocarbons from the environment by increasing bioavailability and biodegradation because surfactants increase the solubility of hydrocarbons (Ganesh and Lin 2009). Surfactants are produced by microorganisms and have an either positive or negative effect on hydrocarbon degradation. The presence of surfactants increases the solubility and bioavailability of hydrocarbons. Most biosurfactants are high molecular weight fatty acids that are naturally produced under aerobic conditions. Biosurfactants include glycolipids, hydroxylates, mycotic acids, lipoproteins, lipoproteins, fatty acids, and phospholipids (Kosaric 2001).

They are effective in increasing the rate of hydrocarbon biodegradation. Biosurfactants are biotic compounds formed by microorganisms. The production of biosurfactants relies on a variety of factors, including microorganisms and food sources. Biosurfactants are widely used by the industry in the production of food, hair oil, medicine, as well as in oil recycling and environmental remediation. Biosurfactants are toxic to microorganisms to a lesser degree compared to the synthetic surfactants. The major disadvantage associated with biosurfactants is the cost of recycling and producing them on a large scale. By increasing the solubility of petroleum compounds, surfactants increase their bioavailability for biodegradation. Chemical surfactants added to soils contaminated by petroleum products can cause environmental pollution by themselves and can be highly toxic to microorganisms, while biosurfactants increase the degradation of hydrocarbons by microorganisms (Giedraityte et al. 2001). Biosurfactants are effective in surface area changes such as reducing surface tension, wetting and penetrating, spreading or covering, and hydrophobic and hydrophilic interactions. Advantages of biosurfactants include biodegradability, low toxicity, biocompatibility, digestibility, acceptable economic products, and use in environmental control. The surfactant molecule comprises of a group with a hydrophilic end and one or two hydrophobic portions. The hydrophobic end always consists of a long hydrocarbon chain, while its polar tip has a positive

impact on solubility in water. At concentrations higher than the critical micelle concentration (CMC) of surfactants, the solubility of hydrocarbons increases linearly with increases in surfactant levels. By reducing surface tension, surfactants cause hydrophobic contaminants move from a solid phase to an aquatic phase (Jung 2014). Thus, surfactants play an imperative role in the hydrocarbon biodegradation. However, the effect of the same surfactant is not identical when the medium is changed. The effect may also vary when different surfactants are used in the same medium. Toxic surfactants decrease PAH degradation (Maneerat and Phetrong 2007; Jung 2014).The toxicity of a surfactant also depends on its molecular structure.

Surfactants are classified into ionic and nonionic categories depending on the polar ionization of the molecule. Ionic surfactants are divided into cationic, anionic, and Zwitterionic surfactants. In principle, nonionic surfactants have lower toxicity for microorganisms compared to ionic surfactants. The toxicity of surfactants increases in the following order:

Cationic surfactants > anionic surfactants > nonionic surfactants

Research has shown that nonionic surfactants are the best surfactants for enhancing PAH biodegradation due to their lower toxicity. The toxicity of the surfactants varies, and the toxicity of sorbitan polyoxyethylene (Tween series) has been determined to be lower. Surfactant toxicity decreases as their chain length increases. Table 4.2 shows the effect of various surfactants on hydrocarbon degradation in the presence of various bacteria. The selected surfactants should be nontoxic to microorganisms, not hazardous to the environment, and should have sufficient solubility to eliminate contamination (Jung 2014).

Liu et al. (1995), Kim et al. (2001) and Makkar and Rockne (2003)

4.2.4 Bacteria Consortium

A consortium can be defined as a mixture of various microorganisms capable of using hydrocarbons (Sihag et al. 2014). Hydrocarbons vary from those with one carbon molecule to those with numerous carbon molecules. Microorganisms attack hydrocarbon molecules by producing a series of enzymes. These enzymes are a group of proteins that degrade hydrocarbons to simpler compounds. While some microorganisms produce enzymes affecting any type and size of hydrocarbon, others only produce enzymes affecting a specific type of hydrocarbon with a specific molecular size. Once hydrocarbons are degraded, other enzymes may be required to degrade the residual hydrocarbon molecule further. The shortage of specific enzymes for attacking the residual hydrocarbon molecule can be an important barrier to the completion of the degradation process and stop it until the enzyme reenters the system. These complex sequences of degradation processes are called the metabolic pathway (Sihag et al. 2014). No microorganism alone can degrade various hydrocarbon compounds in petroleum products. Therefore, many protein enzymes and metabolic pathways are essential for degradation of a substantial number of compounds in petroleum and other associated products. Once a petroleum

Table 4.2 The effect of various surfactants on hydrocarbon degradation in the presence of various bacteria

Hydrocarbons	Surfactants	Microorganism	Effects	Explanation
Naphthalene	Brij30, triton X-100	RET-PA-101 mixed cultures isolated from soil and contaminated wastes	Positive	Surfactants increase solubility
Naphthalene	Triton X-100, PLE10	*Pseudomonas* 8909N	Positive	Surfactant improves degradation
Naphthalene	Triton X-100	*Pseudomonas* 9816/11	Positive	Surfactants have different effects on different bacteria
Phenanthrene		*Sphingomonas* B8/36	Negative	
Phenanthrene	TritonX-100, SDS, Tween80	*Pseudomonas* Zjf08	Positive	Toin had 20 negative impacts during the degradation
	Tween 20		Positive	
			Negative	

pollutant leaks into the environment, certain microorganisms grow rapidly due to the significant amount of hydrocarbon compounds that can be easily degraded. These fast-growing species can prevent the growth and spread of other species by exhausting the oxygen and nutrients sources of the system. Once readily degradable compounds are consumed, microorganisms die off due to a lack of enzymes required to degrade the remaining hydrocarbons. Then, microorganisms capable of using the remaining hydrocarbons grow and dominate the system. This cycle continues until predominant microbial species degrade all the existing hydrocarbon compounds (Sihag et al. 2014). In Table 4.3 some important enzymes taking part in petroleum hydrocarbon biodegradation are presented. Microbes that readily degrade hydrocarbons present in petroleum products are usually found nearby the soil and water surfaces, which is mainly due to the presence of oxygen, moisture, and food sources (hydrocarbon) near the surface. Although some microorganisms are anaerobes, most microorganisms capable of degrading hydrocarbons are aerobic.

4.2.5 Micro Fungi and Yeast Degradation

Although algae and protozoa are important members constituting the microbial community in both aquatic and terrestrial ecosystems, the extent of their participation in hydrocarbon biodegradation remains unknown. Studies have shown that a number of molds act as decomposers for petroleum products and perform degradation through extracellular enzymes. In general, fungi can grow under extreme environmental conditions. For example, they can grow under conditions with low pH, low nutrients, as well as low moisture. *Trichoderma* and *Mortierella* are among the molds capable of degrading petroleum products (Leahy and Colwell 1990). Molds can produce enzymes capable of degradation due to their rapid growth, production of more biomass, and their large hyphae in the soil. Hydrocarbon-degrading bacteria cause diol formation, followed by ring fission and the formation of dicarboxylic

Table 4.3 Some enzymes involved in biodegradation of petroleum hydrocarbons

Microorganism	Enzyme	Researchers
Acinetobacter sp.	Dioxygenases	Maeng et al. (1996)
Acinetobacter, *Caulobacter*, *Mycobacterium*	Bacterial P450, oxygenase system	van Beilen et al. (2006)
Yarrowia lipolytica, *Candida tropicalis*, *Candida maltosa*	Eukaryotic P450	Iida et al. (2000)
Mycobacterium, *Rhodococcus*, *Burkholderia*, *Pseudomonas*	AlkB related, alkane and hydroxylases	van Beilen et al. (2002)
Methylocystis, *Methylococcus*, *Methylobacter*	Particulate methane, monooxygenases	McDonald et al. (2006)
Methylococcus, *Methylosinus*, *Methylocystis*, *Methylomonas*, *Methylocella*	Soluble methane, monooxygenases	McDonald et al. (2006)

acid. Fungi and other eukaryotes typically oxidize aromatic compounds using monooxygenase and form a trans-diol (Saratale et al. 2007).

In the aerobic degradation of PAHs by prokaryotic microorganisms (such as bacteria), dioxygenase transfers two oxygen atoms to the pollutant and produces compounds with low toxicity, such as acids, alcohol, carbon dioxide, and water. In contrast, degradation by eukaryotic fungi involves the transfer of only one oxygen atom to PAHs, which is similar to the decomposition mechanism in mammals. Although most compounds produced by fungi are less toxic than PAHs, some smaller metabolites produced during the fungal degradation of PAHs are actually more toxic than the original compounds (Frick et al. 1999).

Studies have also shown that yeasts isolate obtained from petroleum-polluted sites are able to utilize these compounds (Spencer et al. 2002; Okerentugba and Ezeronye 2003). Yeasts capable of hydrocarbon degradation include the genus *Candida albicans*, *Candida tropicalis*, *Debaryomyces hansenii*, and *Yarrowia lipolytica*. *Y. lipolytica*, a yeast feeding on alkanes, strongly degrades hydrocarbon substances such as fatty acids, triglycerides (fats and oil), and alkanes (Gargouri et al. 2015). It has been shown that *Y. lipolytica* is the most dominant form of yeast in the degradation of alkanes and emulsifier production (Hassanshahian et al. 2012). The actual participation of yeasts in the hydrocarbon biodegradation in an environment can be more significant than earlier thought due to their metabolic diversity (Sood and Lal 2009). However, the bioavailability of hydrocarbonic organic compounds to microorganisms is generally a limiting step during biodegradation (Wang et al. 2011). In particular, the hydrocarbonic nature of the bacterial surface is considered a crucial factor in cell growth on substrates such as hydrocarbons (Chakraborty et al. 2010). Hydrocarbon substrates should react with the cell surface in order to enter a cell. Two hypotheses can be proposed to describe this process: (1) these compounds can be solved completely or partially in the presence of surface- active compounds (surfactant transfer) or (2) these compounds can be directly attached to the cell wall (Zinjarde et al. 2014). The yeast species mentioned above are capable of producing surfactants during growth on hydrocarbon substrates.

4.3 Biostimulation

Biostimulation is the increase in biodegradation by indigenous bacteria as a result of increased bacterial population due to increased nutrients (Kosaric 2001). If the amount of nutrients and electron acceptors is sufficient, microorganisms will be able to grow and degrade compounds. Nitrogen (NO_3 or NH^{4+}), which is usually an electron acceptor, is the main nutrient element required for the bioremediation process. Urea as ammonium chloride, ammonium salts, and ammonium nitrate is a typical source of nitrogen. However, high amounts of added nitrogen can kill microorganisms. High concentrations of nitrogen can have toxic effects on bacteria. Phosphate, which plays a physiological role in microorganisms and releases energy during metabolism, is the second main nutrient for plants and microorganisms. Due to absorption, chemical stabilization, or both, phosphate is immobilized or less solubilized in soil. Phosphorus is also commonly used as a nutrient in bioremediation. Potassium or sodium phosphate and phosphoric or polyphosphate salts are commonly used as sources of phosphorus (Ha 2007).

Since there are limited quantities of nitrogen and phosphorous in most soils and aquatic habitats, the biodegradation of crude oil and gasoline can be enhanced by adding urea phosphate, NPK fertilizers, and ammonium and phosphate salts (Leahy and colwell 1990). A wide range of optimal C: N and C: P ratios have been reported. Some species require nutrients rich in amino acids, lipids, vitamins, and sugars. Bacteria will grow better if a moderate amount of nutrients, including amino acids and vitamins, are available. However, if excessive nutrients are present, the absorption metabolism of bacteria will be disrupted, and the mortality of bacteria will increase as a result (Iranzo et al. 2001). There are limited amounts of nutrients in aquatic environments and phosphorus: nitrogen: carbon ratios are often not suitable for microbial growth. The limitations in nitrogen and phosphorus availability slow the biodegradation of hydrocarbons in aquatic and soil environments. To improve the biodegradation of crude oil and hydrocarbons, we can improve the carbon/nitrogen/phosphorus ratio by adding fertilizers such as paraffin, urea, octophosphate, ammonium, and phosphate salts (Leahy and Colwell 1990). According to Atlas and Bartha, the concentration of 1 mg of nitrogen and 0.07 mg of phosphorus raised the maximum crude oil degradation per liter (Atlas and Bartha 1973). Upon addition of nitrogen and phosphorus, the rate of degradation of hydrocarbons increased. Another study recommended a 200 mg concentration of nitrogen fertilizers per kilogram of soil for optimum hydrocarbon biodegradation (Morgan and Watkinson 1992). In order to maintain this condition, soil nitrogen levels should be balanced and adjusted as well. The release of hydrocarbons into aquatic environments does not occur if nutrient concentrations are low. Most studies have shown that C/P or C/N ratios are not suitable for bacterial growth and that the in situ bioremediation of contaminated soils increases because of the added nutrients.

Organic nutrients as well as the mineral nutrients have positive effects in stimulation of polyaromatic hydrocarbons (PAHs) and total polyaromatic hydrocarbons (TPHs) degradation (Table 4.4). Based on the presented data in the Table 4.4, organic nutrients are potentially useful sources for stimulating the hydrocarbon biodegradation.

Table 4.4 Organic nutrient application for bio-stimulating

Organic nutrient	Findings	References
Compost (from sewage sludge and wood chips)	100% removal over a 570-day period with removal rate associated with the population of native microbes and enhanced growth in the system	Atagana (2008)
Poultry dropping	Appreciable degradation of PAH and TPH in a bioreactor using 20 g poultry litter and 1 litter seawater	Chikere (2012)
Cow dung	62.96% increase in degradation compared to the control treatment	Orji et al. (2012)
Tea leaf, potato skin, and soy cake	Considerable biodegradation of TPH in the soy cake treatment	Dadrasnia and Agamuthu (2013)
Sugarcane bargasses and oil palm empty fruit bunch	Significant biodegradation	Hamzah et al. (2014)

4.4 Impact of Various Conditions on Biodegradation: Biological and Environmental Factors

The successful application of bioremediation requires information and parameters that contribute to the biodegradation of pollutants. The degradation of hydrocarbons in contaminated soils depends on four basic actions: the presence of hydrocarbon-degrading bacteria, certain environmental conditions promoting degradation activities, the type of hydrocarbons in the soil, and contaminant bioavailability for degrading bacteria.

4.4.1 The Role of Biological Factors

The potential of soil microbial communities for hydrocarbon degradation depends on the size of the microbial population and its catabolic activities. Soil microflora includes a variety of microorganisms such as bacteria, algae, fungi, protozoa, and actinomycetes, which have different capabilities for degrading hydrocarbons. The key factors affecting the rate at which microbes degrade hydrocarbons include the obtainability of pollutants for microorganisms with the catabolic capacity to degrade them, the number of degrading microbes present in the soil, the activity of degradative microorganisms, and the molecular structure of the pollutant (Semple et al. 2003). The number of microorganisms present in the soil is usually in the range of 104 to 107 CFU, to achieve a successful biodegradation process; this figure should not be below 103 per gram of soil. When this figure of microorganisms per gram of soil is less than 103 CFU, it is an indication that the concentrations of organic or inorganic contaminants at toxic level are present (Margesin et al. 2003; Pawar 2012). As will be discussed in a section of environmental factors, the microflora activity of the soil can be measured by these factors. For successful biodegradation, microorganisms must conduct catabolic activities through the following mechanisms: the production of

specific enzymes, the development and creation of new capabilities in metabolism due to genetic changes, and the careful enhancement of organisms capable of transforming target contaminants (Margesin et al. 2003; Pawar 2012). Microorganisms derived carbon and energy from hydrocarbons and depend on them for growth. For availability of carbon, larger hydrocarbon molecules must be broken into simpler ones suitable to be utilized by microorganisms. Other researchers have shown that the use of degrading indigenous bacteria along with available nutrients can be effective in refining soils contaminated with crude oil on a larger scale (Stickney et al. 2010).

Plants capable of accessing absorbed ions by changing pH in the rhizosphere through pumping protons and phenolic compounds outside of the root cells play an imperative role in the bioavailability of pollutants. Exchangeable compounds and fractions absorbed by the plant represent a concentration of elements that are readily available for uptake by plants. There are processes in the rhizosphere that support the transport and bioavailability of hydrocarbon compounds. These processes are more complex in cultivated soils than in unplanted soils. Since root secretions vary in plant species, the microbial population and their demographic structure (their ability to degrade pollutants) largely depend on the choice of plant type. In addition to their effect on nutrient solubility, secretions contribute to the bioavailability of pollutants in the soil. As a result, pollutants would be more readily available to plant roots and microorganisms. Rhizosphere microorganisms use these secretions, which are usually specific to a particular plant, as a source of energy (Wang et al. 2011). Since plant species have different secretion compounds, their effect on the rhizosphere and the microbial community can vary. Therefore, the amount and degree of remediation are depended on the plant species.

In general, the activity of microorganisms in the soil depends on climatic conditions (temperature and humidity), soil pH, the availability of nutrients, etc. (Table 1.5). In temperate and tropical climates, the availability of nutrients, moisture, and oxygen is usually the main factor limiting the degradation of artificial compounds. However, low temperature is the most important limiting factor in the northern soils and polar regions.

4.4.2 Temperature

The amount of decaying and degradation of organic matters is a direct function of the temperature. Temperature affects the range of biochemical activities. Most bacteria grow at best conditions when the temperature is optimized for the species. The temperature varies in many natural environments with the season, while the best temperature for bacterial growth is the temperature ranges of 25–40 °C. The near-freezing temperatures slow down or delay the growth of microbes, and the microbes are stagnated. Because enzymes cannot penetrate from the cells into the cold environments, therefore, the bioremediation is slowly occurred. The largest activity of microbes occurs in the temperature ranges of 20–33 °C, and most microbes can decompose hydrocarbons in this range of temperatures (Iranzo et al. 2001). The

high temperatures (140 °C) may also kill the microbes and the bioremediation process may stop. Bacteria growing at a temperature of 27–32 °C are called mesophiles. Most of the microorganisms in the soil are mesophiles. Bacteria growing at the temperatures ranging from 43 °C to 71 °C are called thermophiles, and bacteria that grow at low temperatures (10–15 °C) are called psychrophiles which grow slowly. Bacteria that grow at very high temperatures are called extrophils. These bacteria live in the bottom of the ocean (Iranzo et al. 2001). Many of the bacteria degrading the oil are phrophil which grow at the temperatures ranging from 20 °C to 40 °C. At first, increasing the temperature allows bacteria to adapt themselves quickly to the surrounding environment. Secondly, the growth rate of the bacteria increases. Each bacterium grows best in the best way that the temperature is optimized for the species. The temperature affects the biodegradation activity. But it does not affect biochemical responses. However, it also affects the soil moisture and redaction potential (Iranzo et al. 2001).

An optimum temperature is required for chemical reactions by enzymes. The faster catalytic activity of bacterial enzymes increases by each 10 °C increase in the temperature. The optimum temperature for biodegradation of the oil is 30–40 °C; most of the oil-degrading bacteria live in this temperature range. The temperature affects the biological degradation of oil by effecting the combination of physical and chemical properties of the oil, the rate of metabolism of hydrocarbons by microorganisms, and the composition of microbial communities. The low temperatures may delay the oil viscosity, as well as the sublimation and evaporation of the oil hydrocarbons, and degradation rates usually decrease with decreasing the temperature. This may be due to a reduction in activity of enzymes and/or by Q10 factor (Leahy and Colwell, 1990). In another study it was stated that the temperature affects the biological degradation and the rate at which hydrocarbons are metabolized by microorganisms, the combination of physical and chemical characteristics of oil, and the composition/structure of the microbial community (Atlas, 1995). He also pointed out that at low temperatures, the viscosity and purification (evaporation) of toxic alkaline chains and their solubility decrease. As a result, the biodegradation of the hydrocarbons is delayed. He said that this reason may be due to reduction in activity of enzymes and/or by Q10 factor. With increasing the temperature, the rate of hydrocarbon metabolism reaches the maximum, usually occurring at temperatures ranging from 30 °C to 40 °C. At higher temperatures, the number of toxic hydrocarbons increases. Climatic conditions and different seasons also affect the population of compatible microorganisms (Bartha and Bossert 1984). The thermophilic conditions may increase the degradation of the hydrocarbons and can easily be carried out in bioreactors by heating the soil or by applying organic matters. Therefore, high soil temperatures may increase the rate of degradation of the soil or can cause purification (evaporation) of thousands of hydrocarbons.

It is reported that low temperatures may delay the evaporation of molecules with a low molecular weight (Atlas and Cerniglia 1995). They also observed the toxic compounds of hydrocarbons at low temperatures and stated that the light oil hydrocarbons were easily decomposed by microorganisms at 10 °C. The lag phase time for bacteria is lower at low temperatures and is longer for light oil compounds. Hydrocarbon degradation occurs in cold ecosystems for a longer period of time.

Table 4.5 Optimum temperature for hydrocarbon degradation in different environments

Environment	Optimum temperature (°C)
Soil environment	30–40
Freshwater environment	20–30
Marine environment	15–20

Other researchers observed the degradation of crude oil with a mixture of microbes on the sea bed soils at the temperatures of 3 °C and 22 °C (Leahy and Colwell 1990). They concluded that by application of 0.1 % oil, only 21% of the oil could be degraded at 3 °C. It was stated that the temperature is the main factor for degradation in marine ecosystems. The temperature also affects many factors, such as, the quality and characteristics of the hydrocarbon composition, as well as the microbial community. The hydrocarbon degradation is slow at temperatures less than 5 °C. The temperature, by affecting the physical and chemical composition of the oil, may affect the biodegradation of the oil, the rate of hydrocarbon metabolism by microorganisms, and the composition of the microbial community. At low temperatures, the oil viscosity increases, and the purification and evaporation of the toxic short chains of alkaline decrease. As a result, the biodegradation of hydrocarbons is delayed, and degradation rates usually decrease with decreasing the temperature. This may be due to a reduction in activity of the enzymes or the effect of the Q10 factor. High velocities may increase the rate of hydrocarbon metabolism, which typically occurs at the temperatures ranging from 30 to 40 °C. At higher temperatures, there are thermophilic bacteria and the presence of toxic hydrocarbons (Leahy and Colwell 1990). The mineralization of oil hydrocarbons in pollutant soils of the South Pole has been very limited at the temperature of 10 °C, because of the low effect of temperature and nutrients (Ferguson et al. 2003). In environments containing the essential nutrients, they have been incubated in a temperature range of 4–42 °C. Octacoan with marked carbon is added bearing a 14 °C, and a direct relationship was found between the temperature and mineralization. In this case, most of mineralization occurred in samples with the highest temperature. The mineralization is stopped at temperatures less than or equal to the freezing point of the water.

Generally, in Table 4.5, optimum temperature for degradation of hydrocarbon in soil, fresh water, and marine environments is presented (Bartha and Bossert 1984; Cooney 1984).

4.4.3 Nutrients

Nutrients are classified into three groups of macro, micro, and trace elements based on the quality and the essential requirements of microorganisms. Macronutrients contain carbon, nitrogen, and phosphorus, which compose up to 50, 14, and 3% of the dry weight of the microbial cells. Sulfur, calcium, and magnesium are also microelements that contain 0.5 and 5% of the dry weight of a cell. Rare elements are not needed by all organisms. Most of the rare elements are iron,

manganese, cobalt, copper, and zinc. Sufficient soil nutrients are needed for plant growth and their dependent microorganisms. This amount of availability can be changed when the plants and microorganisms are affected by the stresses of the pollutants. In a study it was reported that the oil hydrocarbons can greatly reduce the availability of the plant access to nutrients to the soil (Graj et al. 2013). The results of low nutrient availability showed that in fact, the oil hydrocarbons contain a lot of carbon, but they are very poor in terms of nitrogen and phosphorus. Microorganisms that degrade the soil hydrocarbons, either consume the available nutrients or they fix the nitrogen and phosphorus. This may reduce the nutrients in the polluted soil. The oil hydrocarbons may also reduce the availability of plants and microorganisms to the nutrients, by reducing their solubility in the water. In general, the deficiency of nutrients in the soil caused by oil hydrocarbons can be offset by fertilizer applications or plants to soil such as clover, which may strengthen the soil.

4.4.4 pH

The pH is one of the environmental factors that affects the growth of bacteria. The optimum value of the pH for biodegradation of the hydrocarbons in the soil is in the range of 5–7.8 (Dibble and Bartha 1979). Each organism has an optimum pH for living in its habitat. Microbes and bacteria can grow at their proper pH levels and degrade the hydrocarbons. Fungi and algae prefer acidic pHs, while most bacteria prefer alkaline pHs instead of acidic pH levels. These bacteria have mechanisms for maintaining normal physiological concentration of H+ ions into the cell. Acidic solutions prevent the growth of bacteria (Iranzo et al. 2001). Most bacteria grow at the pH ranges between 6 and 8, but the bacterial growth potential occurs at pH values around 7. Most of the environmental factors and conditions for bioremediation action are pH and temperature. Bioremediation tends to occur more frequently at pH levels near 7, and it is successful at pH values ranging from 6 to 8 (Iranzo et al. 2001; Mammitzsch et al. 2014). The pH affects the growth of bacteria and microbial species, and when pH reaches less than the optimum level for the growth of microbial species, the growth of the cells is stopped and their ability is reduced. The pH also affects such factors as the availability of carbon and nutrients and the solubility of heavy metals (Mousavi et al. 2017). Therefore, it is one of the important factors of microbial community (Wang et al. 2017). The rate of bioremediation of the oil compounds usually increases with increasing pH and decreases with decreasing pH. At pH values less than 5.5, the rate of mineralization of the hydrocarbons is significantly reduced. Because in these values, growth rates of the majority of the bacteria are reduced. Biodegradation of the oil compounds depends on the presence of certain enzymes that are largely dependent on pH value (Wang et al. 2017). The pH is effective on the ability of organisms to perform cellular functions, cell membrane transfer, and so on. Most bacteria grow in neutral to slightly alkaline pHs, and in most cases, the growth of bacteria is

weak at pH = 5 or less. The degradation of oil hydrocarbons at pH = 7 is faster than that of for pH = 5, but it should be noted that among these organisms, fungi tend to pH = 7 (Das and Chandran 2010).

4.4.5 Oxygen

Bioremediation can take place in both aerobic and anaerobic conditions. In aerobic respiration, all microbes use oxygen as an electron receptor in their metabolic reactions and during oxidation (reduction) of their processes. In anaerobic respiration, microbes use nitrate-iron sulfate and CO_2 are used as electron receptor (Ladino-Orjuela et al. 2016). In these conditions, the availability of nutrients such as moisture, nitrogen, phosphorus, and other elements is essential (Collins et al. 2003). When the energy required to break the chemical bonds and the transmission of electrons is supplied from the pollutants, this calls reductive oxidation. Aerobic bioremediation occurred at the presence of oxygen or air, and it is the process in which the toxic matters are oxidized to nontoxic compounds such as carbon dioxide. Under anaerobic conditions, mineralized organic pollutants are converted to CO_2 and water (Rockne and Reddy 2003). In bioremediation process, an electron receptor is often added to the microorganisms for stimulation, and the electron receptor is usually oxygen, and research has shown that there is a strong correlation between the number of bacteria and the amount of oxygen. The increase in the population of bacteria is consistent with the quality of degrading pollutants (Atlas and Cerniglia 1995; Atlas 1995; Collins et al. 2003). Aliphatic and aromatic catabolism are oxygenized by bacteria and fungi including oxidation steps by enzyme. Therefore, the presence of an oxygen molecule is essential. Oil hydrocarbon degradation occurs under aerobic conditions faster than anaerobic conditions, and then hydrocarbons are converted to H_2O and CO_2. Therefore, the rate of bioremediation can be increased upon addition of oxygen. Oxygen can be injected into the surface of the soil or contaminated water in the bioreactor system. This is done in order to grow the microorganism activities. This technique is called biorenting. Biosparing is also the injection of air or oxygen into groundwater at low concentrations to increase the rate of degradation. (Atlas 1995) stated that about 3 kg of oxygen is needed to disperse 1 kg of oil hydrocarbon.

4.4.6 Moisture

Bacteria need moisture to grow. As a result of drying, the growth of bacteria is also stopped. The need for bacteria to water is higher than other organisms, including yeast and fungi. Soil water not only affects the available moisture content of microorganisms but also affects aeration and the nature and amount of atmospheric pressure and pH. Soil microorganisms can easily degrade oil hydrocarbons with a limited and appropriate range of soil moisture conditions. With very dry soil, the growth and metabolic

process of bacteria will be lowered or prevented. On the contrary, with very wet soil, aeration in soil will be difficult. Optimum humidity is occurred to motivate the degradation of hydrocarbons in the range of 50–80% of moisture content for the crop capacity of contaminated soils (Bartha and Bossert 1984). Thus, the content of crop capacity varies with different soils. Therefore, crop capacity is important for studies and experiments, and it decreases greatly for oily soils. Due to the reduction in the crop capacity in the oily soils, the addition of organic matters to the soil is important (such as straw and wood particles, fertilizer, sludge, etc.) (Dibble and Bartha 1979). Moisture is a very important and relevant biodegradation variable. Soil moisture content has an impact on the availability of pollutants, the stages of the movement, growth of organisms, the distribution of species, the transfer of gases, and the effective level of toxicity of pollutants. Low moisture levels may reduce the microbial activity through limiting cellular movement and metabolic activities. Moisture content of about 80% of the field capacity has been reported as an optimum value for most self-remediation processes.

4.4.7 Salinity

Entering the salinity into the environment affects plants and microorganisms. However, some organisms adapt themselves to live in aquatic and soil environments. Typically, increasing the salinity may decrease the growth of the small microorganisms in the water and soil environment. Some animal groups are more susceptible to salinity. Typically, the community of fish and vegetables, as well as algae and plants, seems to be more susceptible to the salinity. Fungal cells have been adapted to high salinity conditions. Most species of Halotolerant fungi include *Aspergillus* and *Penicillium* (Al Tamie 2014). Typically, excessive salt concentrations prevent the growth of bacteria, and the reason is that high salt concentrations lead to a lack of water in the cells of the bacteria, leading to cell death, and this has a negative effect on the hydrocarbon degradation of soil microorganisms (Iranzo et al. 2001). Many studies have been carried out to investigate the effect of salinity on hydrocarbon degradation, and all of them have showed that there is a positive relationship between salinity and mineralization of the hydrocarbons, in which the mineralization process of the hydrocarbons increases in lower salinities (Qin et al. 2012) and the rate of hydrocarbon metabolism decreases with increasing the salinity levels. This reduction is related to the reduction in oxygen levels or the availability of nutrients for enrichment using microorganisms in saline waters that were unable to use oil as a source of carbon and energy, but at an extremely low salinity, the biodegradation was significantly reduced. Microorganisms require a slightly different salt to grow and for metabolism. In general, they do not have a unique need for NaCl, because many halophiles need the lower levels of K^+, potassium dioxide, Mg^{2+}, and other cations. Therefore, the obvious need for NaCl for bacteria is not specific, and other salts can be substituted (Abdulkarim et al. 2009). In Table 4.6 optimum conditions for microbial degradation of hydrocarbons are presented.

Table 4.6 Optimum conditions for microbial activity in hydrocarbons (HC) degradation (Sihag et al. 2014)

Environmental factor	Microbial activity	HC degradation
Capacity of water holding	25–28	40–80
Soil pH	5.5–8.8	6.5–8.0
Oxygen	10%	10–40%
Heavy metals	Less than 2000 ppm	Less than 700 ppm
Contaminants	Not too toxic	HC 5–10% of dry weight of soil
Temperature (°C)	10–45	20–30
C: N: P	100:10:1(0.5)	100:10:1(0.5)

4.5 Bioavailability Reduction Mechanisms

As we know, bioremediation is a general term for the elimination of environmental pollution by biological processes and by microorganisms in soils and contaminated waters (Asha and Sandeep 2013). Bioremediation is the use of living organisms, such as microorganisms, bacteria, protozoa, and fungi, to degrade pollutants into nontoxic or low toxic compounds (Asha and Sandeep 2013). Due to the differences in the physical, chemical, and biological properties of soils, the interaction of organic hydrocarbons and soils also undergoes major changes. The bioavailability of polycyclic aromatic hydrocarbons is reduced after entering the soil due to the reaction with the soil components. Important processes involved in reducing the bioavailability of pollutants are their adsorption by clays, adsorption by soil organic matter, and the release into micro- and nanoparticles (Schreck et al. 2011). In stabilizing the pollutants within soil organic matters, pollutants are rapidly absorbed into the outer surfaces of the soil and then slowly enter into the solid organic matter. The absorption of polycyclic hydrocarbons by soil involves the following two processes: rapid initial absorption, which is attributed to the adsorption of hydrophobic compounds to hydrophilic surfaces, and a slow absorption of pollutant migration into places within the soil matrix, in which the availability of degrading microorganisms is limited to them. Although the mechanisms for stabilizing the organic pollutants are still not clear, most of the assumptions are based on fixation through adsorption, containment in nanoparticles, separation in soil organic matter, or a combination of these mechanisms.

4.6 Effect of Hydrocarbon Contaminations on Crustacean

Hydrocarbons are compounds which are primarily composed of hydrogen and carbon. These substances are major product of pesticides, gas, and oil. These components contribute to the green house effects and cause global warming and ozone layer deletion, increase manifestation of cancer, reduce plants photosynthetic ability and overlay, and do untold damage to ecosystems.

The most suspected areas for hydrocarbon pollution are coastal waters which are the habitat of many crustaceans. In these areas hydrocarbon pollutions appear as sink sediments. Temperature and salinity have effect on hydrocarbon uptake and delegation by affecting the physiological ability of animals to respond to the absorbed hydrocarbons. The effects of temperature are complex, but higher temperature increases the hydrocarbon contaminants absorbance and accumulation in tissues. It also affects the metabolic rate in tissue which confirms the higher activity of immune systems.

Hydrocarbons, in the notorious form of oil spills in the ocean, treat aquatic animal's life by accumulating in their tissues and affecting their metabolite and immune system (Kan-atireklap et al. 2005). The molecular weight and methylation of hydrocarbons affect the accumulation of these chemicals in animal tissues. Methylnaphthalenes accumulate slowly, but aromatic hydrocarbons accumulate in tissues faster than the aliphatic hydrocarbons. Its concentration in the tissues of blue muscle and yellowfin sole (*Limanda aspera*) was monitored from 1990 up to 1997 (Karinen and Rice 1974; Karinen 1977). They reported the high-level accumulation of hydrocarbons but low concentration of polyaromatic hydrocarbons. Hydrocarbons have several different effects ranging from behavioral to physiological effects (Rice 1973; Rice et al. 1979).

Up to now, few studies have been done on the tolerance of crustaceans to contaminants. In a study model, the genotoxic effect of hydrocarbon contaminants has been studied in crustacean (Kvenvolden and Cooper 2003). In this study effects of polyhalogenated hydrocarbons and aromatic hydrocarbons on aryl hydrocarbon receptors showed that these receptors mediate many toxic responses. Also it was confirmed that different dimmers of AhR nuclear translocators from heat shock protein family make a complex with aromatic hydrocarbons and aryl hydrocarbon receptors. This complex modifies the transcription which is involved in the immune system. It is assume that decreasing in the reproductive quality of the crustacean in oil exploitation zone could be associated with the effects of hydrocarbons on immune system regulation through effects on gene transcription (Alcaraz et al. 1999).

The amount of dissolved oxygen decreases due to the increasing water temperature by both global warming and presence of the contaminations. And also hydrocarbon contamination effects on crustacean immune system cause mortality in coastal areas and coastal located aquaculture farms (Gullaya Wattayakorn and Rungsupa 2011). So, hydrocarbon contaminations have direct and indirect effects on crustacean health and productivity (Robin et al. 2017).

4.6.1 Effect of Hydrocarbons on Aquatic Animal's Organs and Immune Pathways

Hemolymph and interstitial fluid which also named extracellular fluid are circulating in a crustacean. This circulating fluid will control the crustacean cell functions (Monahan-earley et al. 2013). Factors affecting its immune functions consist of

body temperature, pH value of the serum, the osmotic pressure of the serum, and the concentration of the oxygen and carbon dioxide in the serum. This organ maintaining a suitable environment for all the enzyme functions efficiently and hence enables all biochemical reactions that were carried out at their maximum rate. Hydrocarbon contamination effects on the enzyme functions of this fluid by penetrating through lymphatic membrane create trigger complex with heat shock protein families which will further the effect of immune gene transcription (Alcaraz et al. 1999).

Over 70% of aquatic animal's anatomy consist of muscle, which has the main role of animal's movement and a specific role in animal's immune system activity. The muscle is known to accumulate contaminations such as heavy metal and hydrocarbons over time. The importance of this aquatic animal muscle contamination is when human consume them as seafood. The baseline distribution assessment of the total hydrocarbons in the water and benthic sediments in a contaminated costal area showed the muscle of crustacean is accumulated by hydrocarbons (Abu-Hilal and Khordagui 1994).

The accumulated hydrocarbon can diffuse in to circulatory system from the stomach by food. These contaminants will affect the gills which have the largest exposed surface to water environment and will cause respiratory deficiency which will further cause hypoxia. Hypoxia and non-efficient transcription will cause deficiency in defense mechanisms in crustacean which are prophenoloxidase (proPo) system, phagocytosis, encapsulation, antimicrobial proteins, encapsulation, lectins, and cuttable proteins. Each of these mechanisms would be activated by the main domain protein which is expressed by immune-related genes immediately after receiving invader attack signals (Ai et al. 2009). This alarm signal may be received by hydrocarbon contaminant or different pathogens and environmental stresses such as temperature shock and salinity. But, defective transcription due to the effect of the hydrocarbons causes disorders in immune system functions and makes the animals sensitive to invader attack.

4.6.2 Importance of Aquatic Animals

Attention on the existence of aquatic animals around the world has increased in recent years. Aquacultures are an enormous constituent of human nutritional supplies, especially for their protein content. So, aquaculture farming has grown rapidly over the last four decades. Also as the population around the world grows, the demand on seafood increased. Thus the improvement of aquaculture industry by improving husbandry, management, and environment factors and decreasing environmental pollutions is essential. Also, sensitivity of aquatic animals such as fish, invertebrate, and crustacean to the environmental pollution made them indicators of environmental issues in research.

4.7 Phytoremediation

Phytoremediation is generally termed as the application of plants and associates microbes to contain, deactivate, or degrade environmental contaminants that are harmful on the living body. It restore contaminated site, which is gaining more attention (Vangronsveld et al. 2009). In phytoremediation, grasses and trees are commonly used, with grasses more commonly applied in remediation of total petroleum hydrocarbons and polycyclic aromatic hydrocarbons (PAHs), whereas trees are usually being considered for remediation of benzene, toluene, ethylbenzene, and xylene (BTEX) which are volatile organic compounds (Cook and Hesterberg 2013). PAHs are recognized chemicals with mutagenic and carcinogenic effects on living system (Bisht et al. 2015). At concentrations levels found in urban environment, BTEX are also identified with toxic, mutagenic, and carcinogenic effects (Masih et al. 2016).

Hence, the need of effective technologies toward minimizing or eliminating the harmful threat is posed by these compounds. Once plant-based remediation is established, it works with minimal maintenance, and it is also considered less devastating to the environment, with high possibility of public acceptance (Singh et al. 2003). However conventionally, in an effort to remove harmful organic pollutants in situ, different physical and chemical techniques have been used including aeration, soil washing, pumping, extraction, and incorporation of oxidants like potassium permanganate ($KMnO_4$) and hydrogen peroxide (H_2O_2), or sometimes incineration is applied. Still, these approaches could lead to secondary contamination and environmental damage. In addition, these conventional techniques are high-cost (Kang 2014). Moreover, most of these conventional techniques do not provide permanent solution to the remediation and often considered completely ineffective (Kong and Glick 2017). In phytoremediation, many approaches and applications are involved. The techniques vary in process where plants can get out, immobilize, or deteriorate contaminant (Bolan et al. 2011). Mechanisms involved in phytoremediation are described below and illustrated in Fig. 4.1.

4.7.1 Rhizoremediation

Primarily, remediation of hydrocarbons using plant depends on rhizoremediation, whereby contaminants are broken down as a result of microbial activity taking place at the roots (Rohrbacher and St-Arnaud 2016). This phytoremediation process relies on the interactions among plants, microorganisms, and soils (Cook and Hesterberg 2013). A zone of soil where microorganisms are immensely influenced by the root system is termed as rhizosphere which serves as home to unnumbered microorganisms (Meena et al. 2017). The concept of rhizosphere was elaborated by Lorenz Hiltner in 1994. The roots of plants provide a large surface area where microorganisms can proliferate, it also supplies nutrients, and oxygen exchange is facilitated through root penetration, thereby permitting aerobic microorganism

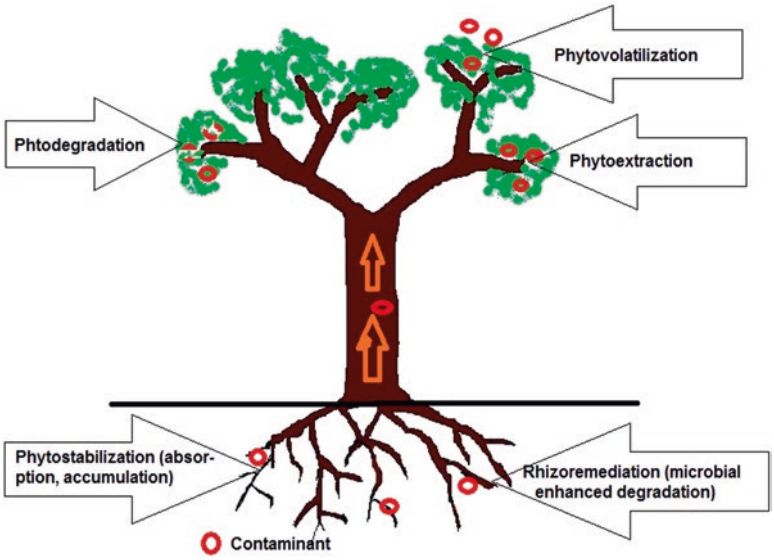

Fig. 4.1 Mechanism involved in phytoremediation

proliferation. In addition, different phenolic compounds present in root exudates serve as inducers in catabolic pathways of various contaminants (Segura et al. 2009). The creation of a potent environment in which microorganisms can develop and relate can be termed as rhizosphere effect (Nihorimbere et al. 2011). Rhizosphere effects facilitate hydrocarbon degradation; plants give out organic compounds via their roots, affecting the abundance and diversity or influencing the activity of microorganisms having the capability to degrade hydrocarbons in the zone surrounding the roots (Phillips et al. 2012). The carbon dioxide (CO_2) captured by plant during photosynthesis is translocated into the roots. To so extend, under genetic, hormonal, and nutritional control, roots can increase which as a result put down carbon as root biomass. This could be linked to the fact that the root exudes all sorts of carbon-containing constituents into the rhizosphere (Kell 2012). Plant root exudates are composed of several compounds including monomeric and polymeric carbohydrates, amino acids, and organic acids. It also consists of protein enzymes and plant mucigel or mucilages (Rugova et al. 2017). Microorganisms are chemoattracted by the plant root exudates, resulting in their multiplication and colonization (Allard-Massicotte et al. 2016). Despite the role of root exudate as a carbon source and energy for microbes, it stimulates hydrocarbon degrader, thereby enhancing degradation of hydrocarbon in the rhizosphere (Rohrbacher and St-Arnaud 2016). Organic contaminant can also be degraded effectively through rhizosphere microbe-assisted phytoremediation. Teng et al. in 2011 conducted a pot experiment to study the phytoremediation by alfalfa grown for 90 days when inoculated with *Rhizobium meliloti*. The experiment was carried out on polycyclic aromatic hydrocarbon (PAH)-contaminated agricultural soil. Their results revealed that planting with non-inoculated alfalfa and alfalfa inoculated with *Rhizobium*

meliloti significantly decreases initial soil concentrations of PAH by 37.2% and 51.4%, respectively, in comparison to unplanted control soil (Teng et al. 2011).

4.7.2 Phytostabilization

Phytostabilization is an approach aimed at containing contaminants with an unsaturated zone (vadose zone) via accumulation using roots or by means of precipitation within the rhizosphere. It therefore serves to avert offside contamination by the way of their movement through wind and water erosion. It can also be referred to as an establishment of plant cover on the surface of polluted soils, thereby reducing the exposure of contaminants to water and wind as well as minimizing direct contact with animals/human (Bolan et al. 2011). The process of phytostabilization of organic contaminants including petroleum hydrocarbons could include incorporation of the pollutants into humic matters within the rhizosphere. In this case, plants could supply an enzyme capable of binding the contaminant into soil organic material or may elevate the amount of organic matter of the soil, thereby leading to an increased humification (Germida et al. 2002).

4.7.3 Phytoextraction

In phytoextraction, otherwise termed as phytoaccumulation, contaminants from water or soils are removed by plants or algae into harvestable biomass. Plants with the ability to take larger concentration of contaminants above normal are termed as hyperaccumulators (Vaziri et al. 2013). Denise et al. studied phytoextraction of total petroleum hydrocarbon at laboratory scale using a sea weed, *Heteranthera callifolia.* The plant was grown for 4 weeks at concentrations of 0%, 2%, 4%, 6%, and 8% water-saturated fraction of hexane. The results indicated bioaccumulation of total petroleum hydrocarbon in roots, petioles, and leaves with values 0.096 ± 0.080 mg/L, 0.434 ± 0.170 mg/L, and $(0.202\ l \pm 0.116)$ mg/L, respectively. The highest concentration was recorded in leaves (Denise et al. 2013).

4.7.4 Phytovolatilization

In phytovolitization, some of the contaminants of lower molecular weight are often removed from the soils and discharged via leaves by means of evapotranspiration processes (Gerhardt et al. 2009). In phytovolatilization approach, volatile contaminants are transformed due to metabolic activity taking place in plants in association with the microbes and then discharged them into the atmosphere (Qixing et al. 2011).

4.7.5 Phytotransformation (Phytodegradation)

In phytotransformation, environmental contaminants are chemically modified as results of metabolic processes in plants, often leading to their deactivation, immobilization, or degradation (Vaziri et al. 2013). It is also termed as contaminant destructive process whereby plant produced protein enzymes that catalyze the metabolism of contaminants and still remain active in rhizosphere. Plants synthesized enzymes such as dehalogenase, laccase, nitrilase, nitroreductase, and laccase which have been found in both soil and plant sediments (Ansari and Sharma 2017).

4.8 Nanoremediation

Recently, application of nanotechnology in the field of bioremediation by using nano materials has been an interested area of research at pilot scale (Sharma et al. 2017). Nanoremediation techniques involve the application of reactive nanomaterials to transform and detoxify pollutants. These nanomaterials are characterized by their ability to reduce or weaken the effects of the pollutants through both chemical reduction and catalytic process (Ingle et al. 2014).

4.8.1 Nanoparticles in Biodegradation of Hydrocarbons

Nanotechnology involved the use of very tiny particles manufactured; the particles are characterized with dimension <100 nm, termed as nanoparticles (atomic or molecular aggregates). Their dimension varies ranging between 1 and 100 nm which can seriously alter their physicochemical characteristics when compared to the bulk material (Yadav et al. 2017). Synthetic petroleum plastics such as polyethylene are inevitable daily requisite, but as a result of its poor waste disposal, it caused high degree of environmental pollution, and hence better means of its degradation is urgently needed (Bhatia et al. 2013). Microorganisms were reported as mediators of synthetic plastic degradation (Bhatia et al. 2014). Pathak and Kumar demonstrated the use of silicon oxide (SiO_2) nanoparticles on low-density polyethylene biodegradation. In their studies, SiO_2 at concentration of 0.01% (w/v) was used as a supplement to low-density polyethylene containing minimum salt medium. *Pseudomonas* sp. C 25 and *Bacillus* sp. V8 strains were reported to exhibit most potentiality for polymer degradation. Their results further revealed that SiO_2 nanomaterial enhanced growth profiling, and it also effectively increased the biodegradation capability of the strains. This study indicates a significant influence of bacterial-nanomaterials interactions toward polyethylene biodegradation (Pathak and Kumar 2017). A study was also carried in an attempt to demonstrate the influence of

bacteria-nanoparticle interactions in biodegradation of low-density polyethylene using superparamagnetic iron oxide nanoparticles (SPION, of size ranging between 10.6 nm and 37.8 nm). In this study, microbial consortium including Bacterium Te68R, *Microbacterium* sp., and *Pseudomonas putida* on minimal broth Davis medium deficient of dextrose and iron was used. The results of this work revealed an influence of SPION nanoparticle in accelerating the rate of bacterial growth; it also enhanced the durability of exponential phase of growth by 36 h. The study signifies the important influence of bacteria-nanoparticle in biodegradative process (Kapri et al. 2010).

4.9 Conclusions

The quality of life on earth has directly link to the overall quality of our environment. It was a common belief that aquatic and terrestrial systems were sufficient to break down the pollution from farming and industry; however, today these sources are not enough to absorb the pollutants due to the greater or careless usage of human beings. The currently accepted technique to degrade hydrocarbon pollution is through microbial activities which are very important for the renewal of our environment and maintenance of the global carbon cycle. Amid at breakdown the substances which can be transformed or degraded by microorganisms. This process is known as bioremediation which is relatively low cost and can be carried out on-site and has a high public acceptance.

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Chapter 5
Microbes and Petroleum Bioremediation

Bruna Martins Dellagnezze, Milene Barbosa Gomes, and Valéria Maia de Oliveira

Abstract Petroleum pollution is an environmental issue often reported, including oil spills that occur accidentally worldwide. The release of large quantities of oil causes directly or indirectly huge environmental and economic impacts and may persist for decades. Bioremediation processes, such as biostimulation and bioaugmentation, among others, represent an eco-friendly and effective way to treat impacted areas based on the use of biological agents, associated or not to other compounds like biosurfactants in order to mineralize or complex organic and inorganic pollutant compounds. Therefore, this book chapter will review some topics related to bioremediation, including several in situ and ex situ techniques employed to treat polluted areas and the use of biosurfactants produced by several microorganisms. Moreover, oil spills and how they can affect marine and terrestrial environments are also mentioned, based on recent reports available in literature and according to organizations responsible for environmental impact monitoring. Hydrocarbonoclastic microorganisms have been described in both environments as well as the community dynamics of specific groups as a function of oil compounds input. In marine environments, a high abundance increase of a specific group called "obligate hydrocarbonoclastic bacteria (OHCB)" has been reported after an event involving petroleum contamination. Similar observation has been reported for mangroves, showing that oil or its derivatives allow the selection of microorganisms capable to degrade hydrocarbons. Petroleum contamination in cold environments, as Arctic and Antarctic regions, represents a huge challenge since management of contaminated sites and bioremediation effectiveness in these regions depend on several factors influencing oil degradation under cold conditions facing intrinsic limiting factors. In conclusion, bioremediation is not only a scientific concept described in literature but a concrete and applied efficient tool to treat polluted environments. The increasing number of bioremediation companies and patents also corroborates the tendency in search for new technologies and approaches focusing on sustainable management of polluted areas.

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5.1 Introduction

Crude petroleum and its derivatives are still the main energy source for several countries and are among the most widely used substances in industrial processes. The world production of crude oil in 2015 was 41.4 million barrels per day (OPEC 2016). Activities related to petroleum industry as transport, extraction, refining, and distribution can affect directly or indirectly the surrounding ecosystems or even those in the transportation route. Due to the extensive application in several industrial sectors, petroleum hydrocarbons (PHCs) have been reported as a great pollutant source in the environment. Accidental oil spills, whether offshore or onshore, have been often reported and imply in major impact, affecting all the food web involved as well as human resources, mainly local communities economically dependent on natural resources for living.

Remediation techniques are already described and applied for contaminated environments, based mainly on physical treatment for the removal of contamination source. Instead, bioremediation is a technique employing microorganisms or their products to degrade or inactivate toxic compounds. Nevertheless, the success of this process is dependent on many biotic and abiotic factors.

Several microorganisms, as bacteria, fungi, and yeasts, whether from marine or terrestrial environments, are reported in literature as being capable to degrade diverse hydrocarbon compounds and/or used in bioremediation processes. In addition, some of them can produce biosurfactants, complex molecules which increase hydrocarbon degradation.

This book chapter will review some of the topics concerning bioremediation research. Contamination and fate of spilled petroleum in environment, as well as some of the microorganisms capable to degrade hydrocarbons, are described. In addition, some commercialized bioremediation products and how different bioremediation approaches have been investigated and applied for petroleum contamination as an eco-friendly and efficient tool for polluted environments are discussed.

5.2 Bioremediation

Anthropogenic activities have resulted in the dump of hazardous waste into the environment. These wastes represent pollutant sources of diverse types, such as pesticides, heavy metals, polyaromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCB), antibiotics, dyes, and cleaning products (disinfectants and detergents), among others. Most of them are toxic to humans and animals, offering risks of diseases or even death (Verma and Jaiswal 2016). They can contaminate soil, surface water, groundwater, and air causing unpredictable environmental catastrophes. For this reason, remediation of contaminated environments has been an arduous task with countless researches aiming low cost-efficient solution.

Table 5.1 Advantages and disadvantages of bioremediation techniques compared to conventional technology

Advantages	Disadvantages
Lower cost	May be difficult to control
Contaminants usually converted to innocuous products	Amendments introduced into the environment to enhance bioremediation may cause other contamination problems
Contaminants are destroyed, not simply transferred to different environments	May not reduce the concentration of contaminants to required levels
Persistent use	More time-consuming

In order to remove pollutants from contaminated sites, several physicochemical methods have been developed, such as solidification and stabilization, soil vapor extraction, soil washing, air sparging, thermal desorption, and incineration (Dadrasnia et al. 2013).

Such methods might be highly expensive and laborious, and, moreover, there is an inherent risk of worsening the situation by spreading pollutants (Salleh et al. 2003). Other common technologies used are evaporation, burying, dispersion, and washing (Das and Chandran 2011), but as a disadvantage, they often lead to incomplete decomposition. Then currently a simple and cost-effective method for hydrocarbon removal is necessary.

Bioremediation, although not a recent term, describes a natural process that uses biological agents (microorganisms or their products) in order to promote pollutant mineralization and recovery of the contaminated site. This approach presents lower costs and other advantages when compared to physicochemical processes (Table 5.1). According to the American Environmental Protection Agency (EPA), the definition of bioremediation consists in a "treatment that uses naturally occurring organisms to break down hazardous substances into less toxic or nontoxic substances."

According to literature, romans may have been the first ones to discover bioremediation during the development and establishment of biological treatment of wastewater and sewer. However, bioremediation process using microorganisms was invented by the American scientist George M. Robinson. He worked as an assistant petroleum engineer at Santa Maria & Company in California in the 1960s and devoted himself to experiments with a series of microbes in contaminated flasks (Sonawdekar 2012). The concept of commercial utilization gained acceptance throughout the 1960, but only in 1970 Dr. Chakrabarty described a crude oil-degrading bacterial strain of *Pseudomonas putida*, and 2 years later the first bioremediation commercial product was launched (Kundu et al. 2017). As consequence of Exxon Valdez oil spill event in 1989 in Alaska, the remediation using dispersants started to gain more visibility.

There are many different bioremediation techniques reported in literature using biological compounds (bacteria, fungi, plants) or enzymes. Although the techniques described can be applied to a diverse range of contaminants, hydrocarbons are the most reported class of pollutants due to their wide contamination in water and soils (Firmino et al. 2015). The main goal of this technique is to reduce or eliminate toxic substances from contaminated sites through different pathways such as degradation, assimilation, or transpiration in the atmosphere, yielding nontoxic final products such as inorganic molecules, water, carbon dioxide, and microbial biomass (Van Hamme et al. 2003).

Bioremediation techniques can be managed *on-site* (or in situ*)*, treating the contaminated material at the impacted site and *off-site* (or ex situ*)*, which removes the contaminated matrix to be treated in other location (Azubuike et al. 2016). Several factors as costs, site characteristics, and type and concentration of pollutants can determine if bioremediation should be carried out ex situ or in situ (Fig. 5.1). There are many types of bioremediation treatments such as application of specific fungi species (mycoremediation), plants (phytoremediation, rhizofiltration), and microorganisms in general and their subproducts or nutrients (bioaugmentation, biostimulation, biopiles, natural attenuation, and biosurfactants), among others (Banerjee et al. 2016; Marykensa 2011).

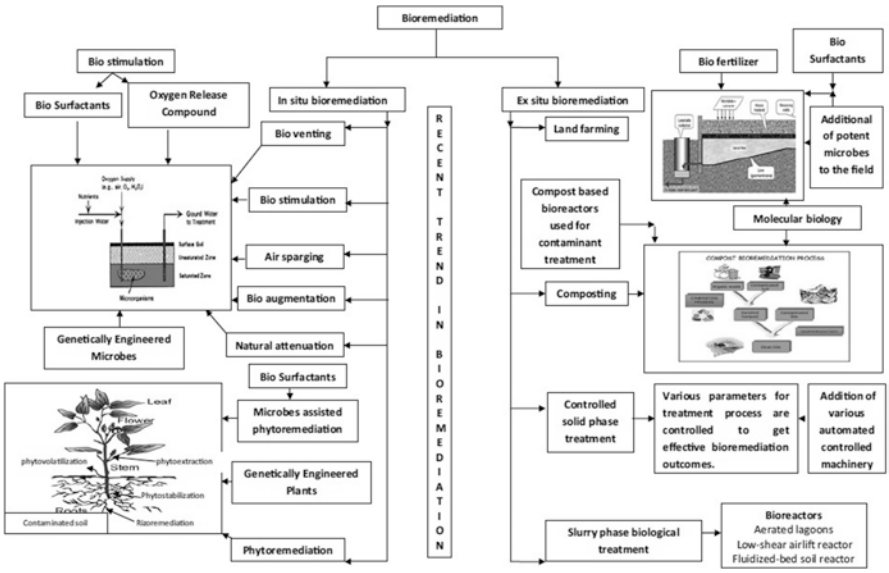

Fig. 5.1 Bioremediation techniques (in situ and ex situ). (Source: Juwarkar et al. 2014)

5.2.1 Methods of In Situ Bioremediation

5.2.1.1 Bioventing

It is an in situ technique used to degrade pollutants in subsurface soil through the action of autochthonous microorganisms. In this case, air/nutrients are injected into wells dug at the site of contamination above water level. The injection of air leads to oxygenation of the contaminated soil, stimulating autochthonous microorganisms and accelerating biodegradation of pollutants. This technique has been proved to be very effective in remediating petroleum-contaminated soil, also for aromatic compounds, known as recalcitrant in the environment. The use of this approach has been reported for soil contaminated with phenanthrene, which was almost totally removed from soil matrix after 7 months (Hohener and Ponsin 2014; Frutos et al. 2010).

5.2.1.2 Bioaugmentation

Bioaugmentation consists of adding specific indigenous or genetically engineered microorganisms to the contaminated site (soil or water). This technique can be employed when pollutants are very complex and native soil microorganisms are not capable of degrading them. The addition of microorganisms enhances the metabolic capability of indigenous microbial populations, thus increasing the extent of degradation. Moreover, a "consortium" (a pool of metabolically diverse microorganisms) can be used at the polluted site and act in synergism with indigenous microbiota to improve bioremediation. Also, genetically engineered microorganisms (GEM) have been strongly considered to be applied in bioremediation process. Joutey and coworkers (2013) described the degradative capacity of GEM and demonstrated the degradation of various pollutants, as hydrocarbons and some heavy metals, under controlled conditions. According to Dellagnezze et al. (2016), the application of bioaugmentation strategy using a consortium composed by metagenomic clones and a strain of *Bacillus subtilis* (CBMAI 707) contributed to increased aromatic compounds degradation of crude oil in mesocosm assay. However, the major obstacles for GEM application are still ecological and environmental concerns and regulatory constraints (Juwarkar et al. 2014; Menn et al. 2008).

5.2.1.3 Biosparging

Similarly to bioventing, biosparging also uses indigenous microorganisms to degrade the pollutants, however in a saturated zone, where the pores and rock fractures are filled with water. Using the same purpose in order to stimulate and enhance the microbial metabolic activity, nutrients and/or oxygen are injected into the saturated zone (Garima and Singh 2014). It is used to reduce the petroleum products often dissolved in groundwater or adsorbed to the soil below water level. However,

this technique is more efficiently used for the removal of medium-weight petroleum products, like diesel, kerosene, etc. This process can also be a mix between anaerobic and aerobic metabolic processes. Kao et al. (2008) have described a (BTEX)-contaminated aquifer plume treated with biosparging and observed this metabolic variation, proven by some alterations in several parameters such as dissolved oxygen, redox potentials, nitrate, sulfate, total culturable heterotrophs, total anaerobes, and methanogenic microorganisms.

5.2.1.4 Biostimulation

In some cases, effective remediation is not achieved based solely on indigenous microbial populations grown under environmental conditions; thus some additional nutrient source is needed in order to stimulate microbial activity by optimizing the surrounding environment of the contaminated site. By addition of oxygen, or other electron acceptors, autochthonous microbiota is stimulated and may improve degradation rates. Stimulants are added into the subsurface through injection wells (Adams et al. 2015).

5.2.2 Methods of Ex Situ Bioremediation

5.2.2.1 Landfarming

Landfarming is a remediation technology implemented above ground for contaminated soils through biodegradation where the contaminated soil is hollow out, added by microorganisms and nutrients, and spread out on the ground surface or liner. The soil is regularly stirred for aeration and mixing of microorganisms, nutrients, and pollutants. Biodegradation efficacy can be enhanced by optimizing temperature and nutrients in the contaminated soil. Addition of co-substrates and anaerobic pretreatment of soil also enhances the degradation process. This technique has been successfully used for bioremediation of benzene, toluene, and xylene (BTX pollutants) (Sonawdekar 2012).

5.2.2.2 Composting

Composting is a controlled biological process, where aerobic and thermophilic conditions prevail for the microbial degradation of contaminated materials resulting in stable end products that can be safely disposed into environment. In general, composting is accomplished by autochthonous microorganisms and wastes are transformed into less complex materials with mass decrease. The contaminated soil is excavated and blended with organic substances, like wood, animal and vegetal wastes, etc. Aeration, temperature, and moisture are closely monitored to achieve

higher degradative efficiency. The thermophilic composting has been used to reduce the concentration of toxic compounds and to treat sewage sludge, diesel-contaminated soil, brewing wastes, antibiotic fermentation waste, and waste from processing units (Khan and Anjaneyulu 2006).

5.2.2.3 Biopiles

Biopile is a technique that combines the use of two other techniques, landfarming and composting, where compost piles remain under well-aerated condition. It is more refined in comparison to the landfarming method and controls the spread of contamination by volatilization and leaching. This technique is used for treatment of surface contamination of spilled hydrocarbon pollutants, mainly petroleum products, allowing the growth of autochthonous microbiota, whether aerobic or anaerobic (Onweremadu 2014).

5.2.2.4 Bioreactors

Biodegradation of contaminants can be carried out in large bioreactors to treat both solid and liquid waste. The solids or liquids contaminants are subjected to bioremediation under controlled conditions in specifically designed bioreactors. Many parameters such as nutrient supply, temperature, aeration, moisture, and the contact between microorganisms and pollutants are maintained at optimal conditions. Hence the degradation is very rapid and efficient. However, running costs of bioreactors are very high (Azubuike et al. 2016).

5.2.3 Effect of Biosurfactants on Bioremediation

Biological alternative for the use of chemical surfactants are biosurfactants. Martins et al. (2009) define the term "biosurfactant" as a compound obtained from an organism that acts in interfaces and significantly reduces the surface tension. Since biosurfactants are surfactants, they are also amphiphilic compounds containing one hydrophobic and one hydrophilic moiety (Darvishi et al. 2011).

Biosurfactants production is the action response of microorganisms to the limited bioavailability of hydrophobic organic and hydrophilic compounds. Bacteria, yeast, and fungi have already been described as biosurfactant producers. Due to their biological origin, they present some advantages compared to chemical surfactants, like better biocompatibility and biodegradability, reduced toxicity, activity and stability at extreme conditions of temperature, pH, and salinity (Abdel-Mawgoud et al. 2010; Calvo et al. 2009).

Biosurfactants can be characterized based on their physicochemical properties, microbial origin, and chemical composition (Pacwa-Plociniczak et al. 2011).

According to their molecular weight, two groups can be defined. The first group consists of low-molecular-weight compounds such as lipopeptides, glycolipids, and phospholipids. These molecules can act in surface and interfacial tension, increasing the surface area of insoluble organic compounds. Also, they can encapsulate hydrophobic compounds in the surfactant micelle core, resulting in an increase of the availability of hydrophobic compounds for microorganisms capable to degrade such compounds (Banat et al. 2010). The second group consists of high-molecular-weight compounds such as polysaccharides, proteins, lipopolysaccharides, lipoproteins, and biopolymers. High-molecular-weight bioemulsifiers promote the stabilization of emulsion formed by hydrocarbons and water, increasing the surface area for biodegradation (Banat et al. 2010; Darvishi et al. 2011).

However, glycolipids are the only microbial surfactants fully commercialized as a mixture for bioremediation purposes, and rhamnolipids, trehalolipids, and sophorolipids are among the most well-known and intensively studied low-molecular-weight biosurfactants (Shekhar et al. 2015; Franzetti et al. 2010).

5.2.4 Recent Strategies for Bioremediation

Adams et al. (2015), in a recent review, described techniques that are gaining visibility using GEM capable to degrade specific contaminants. These techniques were firstly reported in the late 1980s and early 1990s, but due to the widespread use and rapid development of molecular tools, they have gained prominence. Engineering microorganisms aiming degradative properties is based on several possibilities to explore and discover new metabolic and genetic diversity of microorganisms (Fulekar 2009).

Microbial electrochemical technologies (MET), a recent bioremediation strategy, consist in anaerobic systems where microorganisms can act through electrodes that can be placed in the contaminated area (Palma et al. 2017). In addition, nanoparticles have been used for bioremediation purposes. They can act increasing the bioavailability of hydrophobic components (Rizwan et al. 2014). Further, nanoparticles can also be used to immobilize bacterial cells that are capable of degrading specific toxic compounds or to biorecover certain compounds (Kumar et al. 2016; Shan et al. 2005).

5.2.5 Microbial Bioremediation of Hydrocarbons

Degradation of vast hydrophobic compounds by microorganisms has contributed to the application of bioremediation as a biotechnological process. The use of microorganisms has been considered an important tool, since it can promote the complete removal of pollutants from different contaminated environments (Demnerova et al. 2005). The capability for organic pollutant degradation is reported occurring in

many species whether through aerobic or anaerobic process, leading to the modification of complex and recalcitrant lipophilic organic molecules into simple water-soluble products. However, the degradation of a broad range of compounds cannot be achieved by only one single bacterium. In order to potentialize the degradation of complex compounds, the use of a microbial association or consortium is more suitable in which the combination of diverse genetic background can provide a more efficient process (Joutey et al. 2013). They attack the organic chemicals by their enzymatic apparatus after getting into contact with specific or structurally related compounds which induce or depress the microbial enzymatic activity. This process occurs by a successive chain of reactions that usually involves a microbial consortium and their ecological interactions (synergism and co-metabolism).

According to Chikere et al. (2011), there are 81 genera of microorganisms described as being able to carry out biodegradation of petroleum or its derivatives in different environments. These microorganisms include members of the phyla Proteobacteria (*Achromobacter*, *Acinetobacter*, *Alcaligenes*, *Sphingomonas*, *Pseudomonas*), Actinobacteria (*Arthrobacter*, *Corynebacterium*, *Dietzia*), Firmicutes (*Bacillus*), and Bacteroidetes (*Flavobacterium*), unculturable bacterial clones, and members of the fungal phyla Ascomycota (*Aspergillus*, *Penicillium*), Zygomycota (*Cunninghamella*), subphylum Mucoromycotina (*Mucor*), and Basidiomycota (*Phanerochaete*, *Sporobolomyces*), among others.

The degradation ability of natural microbial communities from different habitats makes their catabolic potential even more versatile to transform organic compounds into inert and nontoxic molecules (Banerjee et al. 2016).

5.3 Oil Spills and Contamination

Petroleum is composed by a blend of hydrocarbons and can contain dozens of thousands of organic compounds chemically diverse, including some heavy metals. Petroleum chemical and physical properties may vary according to some features from reservoirs as age, location, and depth (Van Hamme et al. 2003; Head et al. 2003). The general classification of crude oil is resumed to light, medium, or heavy oil. This specification is based on their composition (proportions of the high-molecular-weight compounds) and products generated from their respective distillation process such as paraffins, naphthenes, or aromatic compounds. Light oils contain more saturated and aromatic hydrocarbons and a small proportion of resins and asphaltenes. On the other hand, the largest fraction of heavy oils consists of polar compounds, whereas saturated and aromatic hydrocarbons are in lower proportion (Head et al. 2003).

In accordance with Van Hamme and collaborators (2003), the biodegradability level of the oil components is dependent on their structure, meaning the more structurally complex the molecule, the more complex is its degradation process. Generally, biodegradation starts from structurally simpler molecules as n-alkanes and moves forward to compounds of higher structural complexity such as branched-

chain alkanes and low molecular-weight n-alkyl aromatics, until more complex molecular structures as monoaromatics, cyclic alkanes, polycyclic aromatic hydrocarbons (PAHs), and asphaltenes.

Biodegradation of crude oil leads to changes in its composition. However, in general, biodegraded oils present high quantity of polar compounds which are more complex molecules and, in turn, more resilient to degradation than saturated and aromatic hydrocarbons. Moreover, heavier fractions of petroleum are more toxic and persistent and when released in the environment can cause long-term impact (Hassanshahian and Cappello 2013).

5.3.1 Oil Spills in Marine Environments

Energy use from oil sources has increased after World War II, in which the demand for economic development is directed toward oil exploration and exploitation which, inevitably, were accompanied by oil spills. Marine oil spills have become a category of anthropogenic disasters that seriously affect humans in several ways as ecologically and economically, besides the long-term damages to marine ecosystem (Mei and Yin 2009).

The most frequently described accidental oil spills occur offshore, where the marine contamination is broadly reported (Vieites et al. 2004; Doval et al. 2006; Wieczorek et al. 2007; Outdot and Chaillan 2010; Hazen et al. 2010). Hassanshahian and Cappello (2013) reported an estimate on the general causes of oil spills in which almost 50% come from natural seeps and less than 9% from catastrophic releases. However, 40% of marine oil input is related to consumption and urban discharge.

The release of large quantities of oil impacts directly or indirectly marine environments, affecting all food web, from phytoplankton to large mammals (Perelo 2010; Peterson et al. 2003). These damages can occur immediately and persist for decades. Besides affecting the environment, oil spills have major impacts on economy and human resources, mainly for communities that depend on marine resources, as fishermen. In 2012, a comparative study involving two cases of massive oils spills (Exxon Valdez and Deepwater horizon) assessed the mental health of affected people. Researchers observed high stress levels and also depression associated to economic losses from affected natural resources (gill et al. 2012).

The last update report developed by ITOPF (the International Tanker Owners Pollution Federation) published in February 2017 describes the major spillages from tankers since 1970 (Table 5.2). Main reasons of oils spills are related to structural damages, collision, grounding, fires, and explosions. Spills are characterized by the type of oil spill and the cause and location of accident. Moreover, spills are categorized by size (amount): (1) higher than 7 tonnes, (2) between 7 and 700 tonnes, and (3) higher than 700 tonnes.

The case of petroleum tanker Exxon Valdez at Alaska coast, in 1989, had a large and long-term impact, releasing about 40 million liters that spread more than 1300 km in the shoreline of the Gulf of Alaska. The application of commercial fer-

Table 5.2 Major accidents involving oil spills from tankers since 1967. Source: Oil Tanker Spill Statistics, 2017 (ITOPF)

Position	Ship name	Year	Location	Spill size (tonnes)
1	Atlantic Empress	1979	Off Tobago, West Indies	287.000
2	ABT Summer	1991	700 nautical miles from Angola	260,000
3	Castillo de Bellver	1983	Off Saldanha Bay, South Africa	252,000
4	Amoco Cadiz	1978	Off Britany, France	223,000
5	Haven	1991	Genoa, Italy	144,000
6	Odyssey	1988	700 nautical miles of Nova Scotia, Canada	132,000
7	Torrey Canyon	1987	Scilly Isles	119,000
8	Sea Star	1972	Gulf of Oman	115,000
9	Irene Serenade	1980	Navarino Bay, Greece	100,000
10	Urquiola	1976	La Coruna, Spain	100,000
11	Hawaiian Patriot	1977	300 nautical miles off Honolulu	95,000
12	Independenta	1979	Bosphorus, Turkey	95,000
13	Jakob Maersk	1978	Oporto, Portugal	88,000
14	Braer	1993	Shetland Island, UK	85,000
15	Aegean Sea	1992	La Coruna, Spain	74,000
16	Sea Empress	1996	Milford Haven, UK	72,000
17	Mark V	1989	120 nautical miles off Morocco	70,000
18	Nova	1985	Off Kharg Island, Gulf of Iran	70,000
19	Katina P	1992	Off Maputo, Mozambique	67,000
20	Prestige	2002	Off Galicia, Spain	63,000
21	Exxon Valdez	1989	Prince William Sound, Alaska, USA	37,000
22	Hebei Spirit	2007	South Korea	11,000

tilizers as biostimulation approach was done throughout the coast, with 2237 fertilizer applications along the shoreline. After 3 years of the accident, most part of oil had been removed and the cleanup process was concluded (atlas and Hazen 2011). However, years after the accident, in a toxicological research, authors still observed abnormalities in algae populations, invertebrates, some species of birds, and mammals that were exposed to chronic pollution, even in significantly less (sublethal) quantity (Peterson et al. 2003).

A more recent case involving the oil company British Petroleum (BP) occurred in 2010, in Mississippi, when oil and gas with high pressure escaped from BP's Deepwater Horizon well with subsequent explosion causing 11 deaths. The oil flowed out from the well for a period of 84 days, releasing about five million barrels spread along 690 miles of US coastline. Several measures were taken to retain the oil, as follows: 3% of oil were skimmed (skimmers are collector vessels containing devices where the superficial layer of the oil is drawn), 5% were burned (burning of oil is a strategy used in a controlled area in order to clear areas in a short time), 8% were chemically dispersed (approximately 1.4 million gallons of dispersants were

used), 16% underwent natural dispersion, 17% were captured, 25% underwent evaporation or were dissolved, and 26% were remnant (Chen and Denison 2011; Atlas and Hazen 2011). In this case, biostimulation was not a feasible strategy, since the large amount of oil added of a great amount of nutrients or oxygen could lead to eutrophication. Nonetheless, several works have mentioned a shift in marine microbial communities, with significant increase in microbial groups capable of degrading hydrocarbon molecules, observed during or after petroleum leakage (Hazen et al. 2010; Kostka et al. 2011).

5.3.2 *Fate of Oil in the Sea*

When an oil spill occurs in the marine environment, petroleum derivatives can undergo several physicochemical and biological processes, called weathering. Physicochemical processes include evaporation, dissolution, dispersion, emulsification, photooxidation, adsorption sinking, and sedimentation. Biological weathering consists in biodegradation through microorganisms and ingestion by other organisms (Hassanshahian and Cappello 2013).

Moreover, in case of oil spill, many factors, as temperature, wind, and sea conditions, can determine the fate of oil in the sea and the contamination of ecosystems (Fig. 5.2).

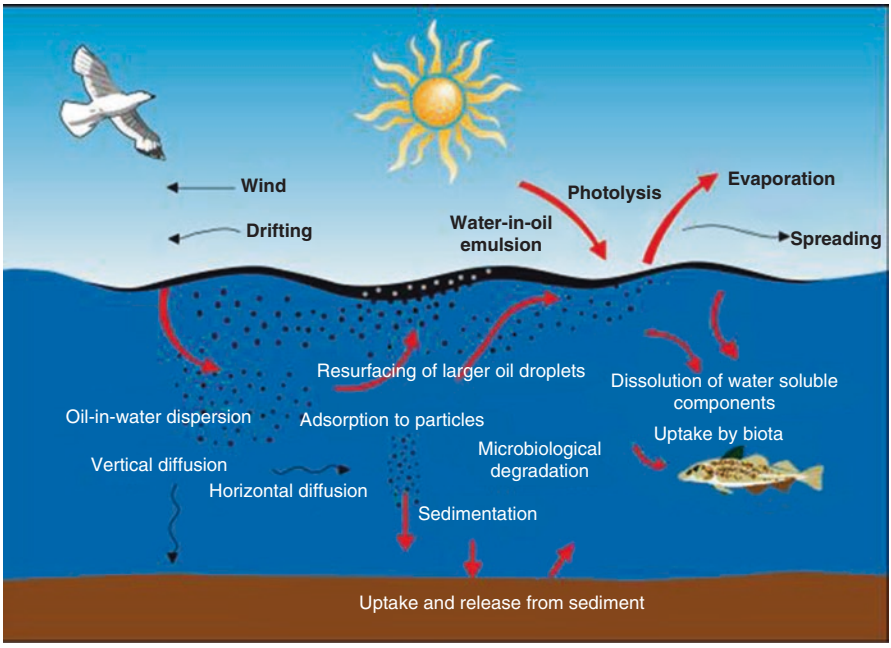

Fig. 5.2 Fate of oil in seawater by different pathways. (Source: Chen and Denison (2011))

As consequence of spill, oil spreads on the surface forming a slick layer and interfering in nutrient and light penetration in water column, with negative impact on the photosynthesis process from phytoplankton (Carrera-Martínez et al. 2010; González et al. 2009). Volatile compounds and petroleum light fraction evaporate more rapidly, also depending on the type of oil and environmental parameters. Other fraction can adhere to suspended solids in the water column and contaminate deep sediment layers.

Sunlight-dependent photooxidation reactions (photolysis) also can occur, being restricted by the privation of sunlight. However, products from photooxidation process, such as toxic acids and phenolic compounds, are normally diluted into the vast volumes of seawater and thus do not cause harmful toxic effects (Kingston 2002). Hydrocarbon dissolution can occur mainly for low-molecular-weight molecules (less than 1%), which in turn can be easily degraded. Dispersion is possibly the main process responsible for natural removal of the majority of petroleum pollution on surfaces. As consequence, oil is "partitioned" in droplets and mixed to the water column, thus becoming more easily accessed for bacterial degradation (Chen and Denilson 2011).

Under specific conditions, oil-water emulsion can be formed. This process happens when water droplets are mixed to the oil layer on the surface, forming a viscous substance called "mousse"; its stability and formation are also dependent on the type of spilled oil. Mousse formation can determine the persistence of oil layer on the ocean surface (Kingston 2002).

Biological recovery of an ecosystem affected by oil spill is related to toxicity level as well as other elements that cause risks to normal biological functions. Thus, the recovery of an impacted environment can start from the decline of toxic compounds until a tolerable level for most of organisms and will depend on several abiotic and biotic factors, such as time of year, availability of colonizing microorganisms, and biological and climate interactions, among others (Kingston 2002; Chen and Denison 2011).

5.3.3 Marine Hydrocarbon Degrading Bacteria

Marine bacterial communities have been studied in order to identify possible microbial degraders and their metabolic potential. Thus, studies regarding the discovery and identification of key organisms capable to degrade contaminants are highly relevant, especially for the development of new in situ bioremediation strategies (Hassanshahian and Cappello 2013).

There are some typical microbial patterns after an event involving petroleum contamination, with large increase in abundance of some groups, such as *Alcanivorax* spp., reported as alkane degrader; *Cycloclasticus* spp., which degrade PAHs; *Marinobacter* and *Thalassolituus*, also present in temperate environments; and

Table 5.3 Main bacterial genera belonging to the OHCB (obligate hydrocarbonoclastic bacteria) group

Bacterial genus	Substance	References
Alcanivorax	Crude oil	Yakimov et al. (1998), Kotska et al. (2011), and Santisi et al. (2015)
Cycloclasticus	Crude oil/ PAH	Kasai et al. (2002), Maruyama et al. (2003), and Kimes et al. (2014)
Marinobacter	Crude oil/ phenanthrene	Jurelevicius et al. (2013), Fathepure (2014), and Gomes et al. (2018)
Oleispira	Crude oil	Yakimov et al. (2007) and Brooijmans et al. (2009)
Thalassolituus	Crude oil	Yakimov et al. (2007), McKew et al. (2007), Dong et al. (2014), and Sanni et al. (2015)

Oleispira, an obligate alkane-degrading psychrophile present in cold marine environments. Some marine degraders are highly specialized obligate hydrocarbon utilizers, called marine "obligate hydrocarbonoclastic bacteria (OHCB)" (Table 5.3). Approximately 90% of the microbial community present when an oil spill occurs consist of obligate hydrocarbon-degrading bacteria, which play a significant role in the natural remediation of oil-polluted marine environment (Yakimov et al. 2007; McGenity et al. 2012).

The OHCB are widely distributed; however some species belonging to this group have only been detected in cold waters (e.g., *Oleispira antarctica*). Also, it has been reported that the type of hydrocarbon contamination can select specific genera, such as aliphatics degrading *Alcanivorax* and aromatic compounds degrading *Cycloclasticus* (Berthe-Corti and Nachtkamp 2010).

Despite the bacterial genera comprised in the OHCB group, other genera have been reported as hydrocarbon degraders in seawater.

Microbacterium and *Porphyrobacter* strains were isolated from enrichments containing benzo[a]pyrene. Also, strains belonging to the genera *Vibrio*, *Marinobacter*, *Cycloclasticus*, *Pseudoalteromonas*, *Marinomonas*, and *Halomonas* were reported isolated from sediments and able to grow on phenanthrene or chrysene (McGenity et al. 2012). Recently, PAH degraders were isolated from a Brazilian marine oil terminal (TEBAR), including *Idiomarina* sp. R2A 23.10, able to degrade phenanthrene; *Marinobacter flavimaris* R2A 36.J, able to degrade pyrene; and *Modicisalibacter tunisiensis* MOD 31.J, able to degrade phenol (Gomes et al. 2018). *Dietzia maris*, *Micrococcus* sp., and *Bacillus* sp. were also related with hydrocarbon degradation in marine environments (Dellagnezze et al. 2014; Kleinsteuber et al. 2006; Nakano et al. 2011). In addition, members belonging to some fungal genera, including *Aspergillus*, *Mucor*, *Fusarium*, and *Penicillium*, have been reported as capable to degrade petroleum (Xue et al. 2015).

5.3.4 Contamination in Terrestrial Environments

Oil spill affecting soil is one of the major global concerns today. Petroleum contamination of soil causes serious hazards to human health and pollution of groundwater and surface water bodies, which limits their use and decreases agricultural productivity, resulting in obvious economic losses. Health risks that emerge from direct contact with the contaminated soil and secondary contamination of water supplies are the major concerns (Thapa et al. 2012). Terrestrial spills tend to be from pipelines, railway accidents, and tank storage. The spread and pathway of oil spilled in terrestrial environments depends on the type of terrain, soil, and vegetation. Moreover, oil (or its derivatives as volatile monoaromatic compounds) can seep through soil and migrate to groundwater or even attach to soil particles. Subsurface contamination is thus affected by soil/sediment characteristics as grain size and by oil features as viscosity and weathering state (Pearson and Fleece 2014).

In addition, volatile organic compounds (VOCs), mainly BTEX compounds (benzene, toluene, ethylbenzene, and xylene), have been considered as major contributors to the deterioration of water and air quality. BTEX are prevalent in the environment as consequence of combustion processes as well as vehicle exhausts. They are also used as industrial solvents for the synthesis of several organic compounds (e.g., plastics, synthetic fibers, and pesticides) and are present in many petroleum derivatives (El-Naas et al. 2014).

Another ecosystem constantly threatened by petroleum contamination is intertidal wetlands. Environments such as salt marshes and tropical mangroves play a crucial role in life cycle of many species. Besides high primary productivity, these environments function as protection barrier from wave and storm damage, provide food and shelter for fish and other marine species, and help dissipate greenhouse gases. Nevertheless, these environments are extremely susceptible to oil input, not only by receiving oil-contaminated seawater but also due to their proximity to oil refineries and industries (McGenity et al. 2014).

Mangrove sediments can retain pollutants increasing their toxicity and impacting the ecosystem integrity. Petroleum compounds are the most damaging, and in addition to the type, concentration, and weathering of oil, climatic and tide conditions may worsen the mortality of the ecosystem. In case of severely harmful oil spill, causing even the death of vegetal species and trees, oil degradation in sediments can be diminished and remediation alternatives are needed (Santos et al. 2010).

Recent mangrove studies have focused on microbial indicators of hydrocarbon pollution on mangrove ecosystems. The interaction dynamics among key groups represents an important parameter to evaluate pollution and its mitigation. Santos and collaborators (2011) carried out a study at Marambaia mangrove (Rio de Janeiro, Brazil) based on 454 pyrosequencing of 16S rRNA amplicons to identify candidate indicator groups in contaminated and pristine sediment samples. The authors showed that some groups as *Cycloclasticus, Marinobacter*, and

Marinobacterium can thrive in the oil presence, whereas others like the members belonging to the order *Chromatiales* and the genus *Haliea* are much more sensitive to it. This work enlarged the understanding about microbial indicators of oil pollution in mangroves. In another study, the presence of specific bacterial groups was correlated with the distribution of petroleum pollutants, corroborating that bioindicators of oil pollution can be used as a suitable tool to better analyze contamination (Ghizelini et al. 2012).

5.3.5 Petroleum Contamination in Cold Environments

Areas contaminated with petroleum hydrocarbon in polar environments as Antarctic and Arctic represent a serious environmental problem. The management of contaminated sites in these regions faces challenges naturally associated with intrinsic environmental conditions. Several technologies have been improved and adapted for in situ application in these regions, as bioremediation (bioaugmentation and biostimulation), landfarming, biopiles, and others. However, the choice of the most suitable treatment strategy takes into consideration several aspects, as climate and soil characteristics, costs, environmental regulations, logistics, and infrastructure (Camenzuli and Freidman 2015).

Oil degradation and bioremediation efficiency under cold conditions are mainly dependent on temperature, which influence several parameters as chemical composition of oil; rate of hydrocarbon degradation; bioavailability of compounds, nutrients, oxygen rate (aerobic conditions), or other electron acceptors (anaerobic conditions); and also composition and abundance of microbial communities (Yang et al. 2009).

Parameters that influence the biodegradation process in Arctic soils are similar to those in marine and terrestrial environments, such as climate conditions, features of the soil and oil, costs, infrastructure, and environmental regulations (Naseri et al. 2014, Yang et al. 2009).

Based on the quoted authors, such parameters are described in detail below:

- *Type of hydrocarbons*: Molecular structure of compounds influences their biodegradation rates.
- *Bioavailability*: Temperature, oil viscosity, water solubility, amount of spilt oil, and soil characteristics influence hydrocarbon bioavailability. For example, during winter, when the soil pore water is frozen avoiding the transfer of nutrients, oxygen, and hydrocarbon molecules, bioavailability is critical.
- *Cold-adapted, oil-degrading microorganisms*: To achieve an effective biodegradation process, microbial degraders must be suitable and resist environmental changes.
- *Soil temperature, nutrient levels, and humidity*: Low temperature affects oil weathering processes and influences the metabolic activity of hydrocarbon degraders, inhibiting biodegradation process or reducing it at extremely low rates

for most of the year in polar soils. Also, in cold conditions, rate of nutrient recycling decreases in the ecosystem, resulting in a scarcity of nitrogen and phosphorous. In the same way, soil humidity can influence the biodegradation rate due to its effects on hydrocarbon bioavailability and also the transfer and diffusion process of other materials, such as gases and nutrients.

Biostimulation and bioaugmentation have been studied and applied in cold environments (Kasanke and Leigh 2017; Wang et al. 2015; Margesin and Schinner 2001), as well as other bioremediation approaches, like biopiles and landfarming (Camenzuli and Freidman 2015). Moreover, soil warming or heat injection can be carried out through engineered biopiles, in order to achieve optimal temperature. However, excessive heat leads to the evaporation of soil pore water, which decreases hydrocarbon bioavailability. To circumvent this limitation, the use of humidified air is recommended, as well as water that must be provided during the process to equalize the dry portions (Naseri et al. 2014).

5.4 Bioremediation Studies and Practical Aspects

As previously described, bioremediation involves different approaches aiming at the mineralization of organic compounds by using microorganisms or their products.

A recent example of natural attenuation (in situ bioremediation) was observed after the Deepwater Horizon (British Petroleum) platform explosion accident, quoted previously. Hazen et al. (2010) evaluated deep water samples from across the Gulf of Mexico aiming the comprehension of the impact of the deep hydrocarbon plume on the marine microbes and the rates of hydrocarbon biodegradation. They reported a shift in the marine bacterial community exposed to the oil, with an increase in the abundance of microorganisms from the order *Oceanospirillales* (class ϒ-Proteobacteria), suggesting a faster acclimation and ability of such bacteria to thrive in the oil.

To be an effective process, biostimulation is dependent on availability and capability of intrinsic microorganisms to perform the complete degradation of a target pollutant. Still, the amount of specific degrading groups (in colony forming units – CFU/mL or gram of soil) must be abundant (Luqueño et al. 2011). Liu and coworkers (2010) reported the use of biostimulation in polycyclic aromatic hydrocarbons (PAH) contaminated soil by the addition of organic fertilizer. After 360 days, there was a reduction of 58% PAH in the amended treatment when compared to the control without any fertilizer. Moreover, the authors observed a smaller number of degrading bacteria in the control samples than in the treated ones and concluded that at concentrations below 10^5 UFC g^{-1}, bioremediation process may not occur significantly. Thus, the higher the abundance of degrading microorganisms within an area under remediation treatment, the faster and more efficient the process is.

Other works have reported the use of biostimulation approach whether in both soil and aquatic environments, resulting in an effective removal of pesticides (Kanissery and Sims 2011), PAH and petroleum (Nikolopoulou and Kalogerakis 2008; Nikolopoulou and Kalogerakis, 2009; Delille et al. 2009; Yu et al. 2011), and organohalogenates (Major et al. 2002).

In some cases, bioaugmentation is considered as an alternative remediation technique when biostimulation or natural attenuation fails. This may happen when (1) there is low abundance of degrading microorganisms in the treated area and (2) the native microbiota do not present physiological capability to degrade pollutants (Fantroussi and Agathos 2005; Tyagi et al. 2011).

There are different strategies of bioaugmentation, as follows: (1) reinoculation of potential oil degraders from autochthonous microbial community, (2) selection of suitable target microorganisms from contaminated environments similar to the target area to be treated, and (3) use of genetically modified microorganisms (GMO) aiming to potentiate the degradation process (Mrozik and Piotrowska-Seget 2010, Hosokawa et al. 2009). Hosokawa et al. (2009) reported the bioaugmentation approach using ABA (autochthonous bioaugmentation) in Hokkaido Island in petroleum-contaminated sediments, comparing different consortia. The consortium previously isolated from contaminated sediment showed higher efficiency to degrade petroleum compounds.

Several bacterial and fungal strains have been used by different authors in bioaugmentation strategies for petroleum and derivative compounds (Table 5.4).

Despite several advantages in bioaugmentation, as low cost and high efficiency, there are some limitations involved in its application. Strain selection, microbial ecology aspects, and inoculation procedure may influence directly in the process effectivity. Parameters such as availability and amount of water, oxygen, nitrogen, and phosphorus and, on top of that, the ability of microorganisms to degrade the target contaminants

Table 5.4 Microorganisms used in bioaugmentation approach for degradation of diverse pollutants from petroleum and derivatives

Microorganism	Compound	References
Alcanivorax	Petroleum hydrocarbons	McKew et al. (2007), and Gertler et al. (2009)
Bacillus	Diesel oil; quinolone	Bento et al. (2005) and Tuo et al. (2012)
Rhodococcus	Diesel oil; PHA	Kuyukina and Ivshina (2010) and Lee et al. (2011)
Pseudomonas	Petroleum; simazine	Stallwood et al. (2005), Morgante et al. (2010), and Mei-Zhen et al. (2012)
Burkholderia	Carbofuran; ethylenediaminetetraacetic acid (EDTA)	Chen et al. (2005) and Plangklang and Reungsang (2011)
Aspergillus	Anthracene, naphthalene (PAH)	Ye et al. (2011) and Ali et al. (2012)
Penicillium	Petroleum/crude oil	Ojeda- Morales et al. (2013), and Crisafi et al. (2016)

and compete/act synergically with autochthonous microbiota are essential factors to achieve a complete degradation of the pollutant. Remediation process under natural conditions may be inefficient in absence of any of the abovementioned parameters (Boopathy 2000; Tyagi et al. 2011). Gentry (2004) reported some methods that may enhance the activity of exogenous microorganisms or genes in the environment: (1) development of methods to enhance the tolerance and resistance of exogenous microorganisms into contaminated areas, (2) research for genetically engineered microorganisms with remediation potential, (3) monitoring of the activity and/or presence of introduced microorganisms through reporter genes, and (4) control of the released genetically engineered microorganisms through suicide genes.

The combined use of biostimulation and bioaugmentation for hydrocarbon removal is mentioned in previous literature and may be a useful approach for accelerating bioremediation. Exogenous and indigenous microorganisms can be supported from biostimulation due to nutrient addition and electron acceptors (El Fantroussie and Agathos 2005).

Yu and collaborators (2005) reported the degradation of a mixture of three types of polyaromatic hydrocarbons (PAH) in mangrove sediments, fluorene (Fl), phenanthrene (Phe), and pyrene (Pyr), using three approaches individually: natural attenuation, biostimulation, and bioaugmentation during 4 weeks. At the end of the last week (week 4), natural attenuation (only autochthonous microorganisms) removed more than 99% of fluorene and phenanthrene but only about 30% pyrene. Biostimulation, adding mineral salt medium allowed more than 97% degradation of all three PAHs, showing that nutrient amendment could enhance pyrene degradation. However, bioaugmentation, using a PAH-degrading bacterial consortium enriched from mangrove sediments, was not able to stimulate PAHs degradation, and biodegradation percentages were similar to those obtained by natural attenuation.

Crisafi et al. (2016) reported the treatment of oil-contaminated seawater after an oil spill event occurred in the Gulf of Taranto (Italy), using different bioremediation approaches. The authors concluded that biostimulation based on inorganic nutrients allowed 73% hydrocarbon biodegradation; bioaugmentation using selected hydrocarbonoclastic consortium composed by *Alcanivorax borkumensis*, *Alcanivorax dieselolei*, *Marinobacter hydrocarbonoclasticus*, *Cycloclasticus* sp. 78-ME, and *Thalassolituus oleivorans* allowed approximately 79% degradation, while the addition of nutrients and a washing agent allowed 69% degradation. Nevertheless, the authors also could observe harmful effects of the washing agent on the microbial community.

5.5 Bioaugmentation Using Genetically Engineered Microorganisms (GEM)

Almost 40 years ago (in the decade of 1980), the search for bacterial genes encoding catabolic enzymes for degradation of recalcitrant compounds gained attention, along with their cloning and genetic characterization, increasing global interest

toward the metabolic potential of microorganisms for biodegradation processes. The first genetic study on microbial degradation was performed with a *Pseudomonas* strain developed to degrade several compounds such as camphor, octane, salicylate, and naphthalene (Chakrabarty 1972; Chakrabarty et al. 1973). This work resulted in a patent [US Patent #425944] (Cases e De Lorenzo 2005).

GEM might be a useful biological tool to treat polluted environment. Biodegradation rates of several contaminants could be enhanced by genetic engineering through cloning of genes involved in degradation pathway(s) with wider substrate specificities. However, the critical point before the release into the environment is to check the stability of any GEM. In addition, the competence to thrive in natural environments can determine the fate of released GEM (Samanta et al. 2002).

Nevertheless, the use of GEM for bioremediation faces restrictive legislation that forbids in situ application. However, using GEM for bioremediation purposes might be a viable and effective alternative, including the performance and degradation time, and it could be considered in containment conditions (de Lorenzo 2010).

5.6 Marketable Bioremediation Agents

The United States Environmental Protection Agency (US EPA) considered as bioremediation agents microbiological cultures, enzymes, or nutrient additives, which can boost biodegradation processes and mitigate contaminated areas. In the year 2001, the same agency compiled a list of 15 bioremediation agents in the scope of National Oil and Hazardous Substances Pollution Contingency Plan (NCP) Product Schedule, and in the next year, this list was modified, totalizing only 9 bioremediation agents. Several new companies have strengthened commercial products in order to clean and treat contaminated environments whether using lyophilized microbial consortia or their enzymes or metabolites, like biosurfactants or other polymers, which currently tend to be a growing market (Randhawa and Rahman 2014).

However, studies have reported that the efficiency of bioremediation products may vary between laboratory and field conditions. Due to the limitation to simulate environmental conditions in laboratory tests (biological interactions, influence of abiotic effects, such as climate and nutrient mass transport), the biological product may fail in the field application. For this reason, field studies are required as the final step to testify the effectiveness of bioremediation products (Das and Chandran 2011).

A recent review provides a list of bioremediation companies all over the world, such as the German “AB enzymes” and the American “EOS Remediation” among others, which are also involved in development of biosensors for detecting pollutants (Mahmutoglu et al. 2010).

Along with emerging bioremediation companies, researchers have developed several methods in order to restore contaminated sites. Based on Thomson Innovation patent database, the work carried out by Kapoor and co-workers (2013) analyzed a total of 125 patent applications and their approaches involving oil-

degrading microorganisms to achieve bioremediation. Still, in a rank for bioremediation technology, the United States of America is the leading country followed by China, Korea, Japan, and Russia. Several companies worldwide known for developing innovative approaches currently work toward eco-friendly products and sustainable solutions, including DuPont, Biosaint, and others.

5.7 Conclusion

In conclusion, given the increasing number of patents and companies all over the world, bioremediation is not a mere theoretical scientific concept but a concrete and applied efficient tool to treat polluted environments, ensuring a minimum impact on the ecosystem. Moreover, this scenario opens the possibility to uncover new technologies and approaches that may be used individually or in combination, as well as the improvement of known practices, aiming at eco-friendly treatments for environment decontamination and waste management in general.

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Chapter 6
Microbial Degradation of Petroleum Hydrocarbons: Technology and Mechanism

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Abstract The petrochemical industry has received considerable attention from many sectors in our society. Petroleum spill has been frequently reported due to the lack of appropriate protocols during exploration, refining, transportation, and storage. An in-depth knowledge of petroleum compounds before planning the best strategies of pollutant bioremediation. The petroleum composition is a mixture of different hydrocarbons. Typically, the most found molecules are alkanes, cycloalkanes, and hydrocarbon mono-aromatics, known as BTEX (benzene, toluene, ethylbenzene, and xylene isomers, ortho-, meta-, and para-xylene). Besides the environmental contamination, BTEX compounds deserve attention regarding their high toxicity and a potential threat to human health. Among the available technologies for remediating areas that were impacted by petroleum-derived fuels, microbial biodegradation has emerged as a very effective technique. These technologies can be used as a complementary action to other conventional treatment technologies. Many microorganisms can use BTEX as their only carbon source. An optimized BTEX biodegradation requires an abundant presence of electron acceptors, a high enzymatic expression and an enhanced microbial access to mono-aromatics hydrocarbons. The metabolic pathways related to hydrocarbon degradation will always depend on the microorganism and the growth conditions. Also, compounds will undergo biodegradation only if there are enzymes capable of catalyzing them. The microorganism *P. putida* has an outstanding metabolic versatility that allows its growth in many different carbon sources. There are many natural plasmids found in *P. putida*, including the TOL plasmid that provides the genes for degrading toxic mono-aromatic hydrocarbons. However, the strongest motivation behind biodegradation studies is to seek microorganisms with a wide range of metabolic pathways

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to degrade various pollutants with cost-effective procedures. Therefore, the purpose of this chapter is to expand the discussion about the BTEX bioremediation and microbial metabolism of hydrocarbons.

6.1 Introduction

Environmental contamination by organic compounds is one of the main concerns of the twenty-first century (Coledam et al. 2017). Petroleum contamination from the petrochemical industry has received considerable attention from many sectors in our society. Fossil fuels are currently classified as priority pollutants due to many potential environmental problems they may cause (Noel et al. 2016; Dong et al. 2017; Mnif et al. 2017; Varjani 2017).

Accidents linked to petroleum hydrocarbons have been reported quite frequently, mostly due to the lack of appropriate protocols during exploration, refining, transportation, and storage. These activities result in considerable environmental degradation, especially when the limited capacity of these ecosystems to absorb impacts is considered (Cesarino et al. 2013; Turner and Renegar 2017). An in-depth knowledge of petroleum compounds before planning the best strategies of pollutant bioremediation. Thorough analyses of the affected areas, as well as the ultimate destination of oily effluents, the crude oil biodegradation mechanisms, and the controlling factors of microbial consumption rates, are imperative for proper environmental management (Varjani 2017).

Some petroleum-based products, such as diesel and gasoline, have a very low solubility in water. Besides, some organic compounds from the composition of these fuels, such as volatile mono-aromatic hydrocarbons known as BTEX (benzene, toluene, ethylbenzene, and three isomers of xylene, ortho -, meta-, and para-xylene), are listed as priority pollutants by the USEPA – United States Environmental Protection Agency (Qu et al. 2015; Lueders 2017). The BTEX compounds are also included in the Hazardous Air Pollutants List, as one of the 275 substances identified as a potential threat to human health (Rahul and Balomajumder 2013; Akmirza et al. 2017).

These volatile compounds are the most soluble fraction of refined petroleum and are abundant in gasoline, which causes high toxicity, mobility, and consequent bioavailability (Mitra and Roy 2011). In other words, BTEX can migrate from the ground to the groundwater, thus reaching water supplies far from their contamination sources. The main characteristics of BTEX are the presence of the benzene rings among its molecular structures. Benzene has the potential for high pollution due to its scientifically proven neurotoxic, carcinogenic, and teratogenic properties. Such properties pose a serious risk to the environment, local fauna, and humans (ATSDR 1999; Fuchs et al. 2011; Almeda et al. 2013).

Among the available technologies for remediating areas impacted by petroleum-derived fuels, microbial biodegradation has emerged as a very effective technique that can be used as a complementary action to other conventional treatment technologies. Biodegradation is a relatively low-cost and noninvasive alternative to

physical and chemical processes (Otenio et al. 2005; Das and Chandran 2011; El-Naas et al. 2014; Varjani 2017).

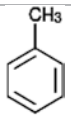
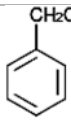
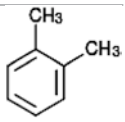
The purpose of this chapter is to expand the discussion about the bioremediation of petroleum hydrocarbons by providing an up-to-date review on the degradation of such pollutants by microorganisms, especially BTEX compounds, to further expand our comprehension of the challenges related to bioremediation.

6.2 BTEX Compounds

BTEX mono-aromatic hydrocarbons are among the contaminants that require major attention due to the issues associated with their environmental release (Qu et al. 2015; Xie et al. 2016). BTEX are often found in surface and groundwater as a result of accidental spills, leaking of storage tank and pipelines. They are frequently found due to the intentional disposal of oils or oily residues in water, especially nonregulated industrial effluents (Mitra and Roy 2011; El-Naas et al. 2014; Fathepure 2014). These compounds are relatively stable, have low molecular weight, and are found in many refined petroleum products. The chemical structure and properties of BTEX are shown in Table 6.1.

These compounds are widely used by chemical and pharmaceutical industries as intermediates during the synthesis of thousands of organic compounds including adhesives, coatings, degreasers, detergents, dyes, explosives, fuels, varnishes, paints, pesticides, enamels, resins, and solvents (Weelink et al. 2010; Bolden et al. 2015).

Table 6.1 Physicochemical properties of BTEX compounds

	Benzene	Toluene	Ethylbenzene	o-Xylene
CAS number	71-43-2	108-88-3	100-41-4	95-47-6
Chemical structure				
Molecular formula	C_6H_6	C_7H_8	C_8H_{10}	C_8H_{10}
Molecular weight g Mol^{-1}	78.11	92.14	106.17	106.17
Boiling point (°C)	80.1	110.6	136.2	144.5
Solubility in water (20 °C) (mg L^{-1})	1770	520	150	170
Density (20 °C) (mg L^{-1})	0.877	0.867	0.867	0.880
Vapor pressure (25 °C) (mmHg)	74.6	22.0	7.0	7.0
Viscosity (20 °C) (cP)	0.647	0.580	0.678	0.802
Autoignition temperature (°C)	498	480	432	463
Characteristics	Carcinogenic	Inflammable	Inflammable	Inflammable
	Inflammable	Harmful	Harmful	Harmful
	Toxic			Irritant

Source: Adapted from Weelink et al. (2010) and El-Naas et al. (2014)

In gasoline, the BTEX compounds are the most abundant hydrocarbons. They represent 18% to 25% (m/m) of gasoline formulation (ANP 2016). The individual percentages of BTEX compounds in gasoline are presented, according to Mitra and Roy (2011), as 11% benzene, 26% toluene, 11% ethylbenzene, 12% o-xylene, 31% m-xylene, and 9% p-xylene. These compounds are very specific indicators of gasoline contamination in polluted areas.

The presence of ethanol in gasoline, which is added to Brazilian fuels to improve performance and reduce carbon dioxide emissions, makes the dissolved aromatics amount even greater because of a cosolvency effect. This effect is defined by the ability of a given solvent to increase the solubility of a solute in another solvent. Once dissolved, BTEX is easily transported by groundwater movement. Mobility may be impaired by sorption in soil grains, but the main transport limiting mechanisms are biodegradation and, to a lesser extent, volatilization (Nascimento Filho et al. 2013).

Besides the environmental contamination, BTEX compounds deserve attention in regard to their high toxicity. They are harmful to human health in many ways: carcinogenesis, hematopoietic syndromes, and central nervous system depression. BTEX release is an important public health issue (Otenio et al. 2005; Xie et al. 2016). Benzene acts in the immune system, as well as hematopoietic and reproductive functions, causing deformities in the fetus, infertility, and abortion. Therefore, benzene rings are the most toxic compounds of BTEX mixtures (Kinder et al. 2017).

Moreover, toluene, which is another component of BTEX, is associated with hepatic and gastrointestinal dysfunctions. It is also teratogenic and a strong depressant of the central nervous system (Bowen and Hannigan 2006). According to the International Agency for Research on Cancer (IARC 2000), ethylbenzene also has carcinogenic potential, as well as damaging to the respiratory system. Ethylbenzene is often associated with eye irritation and fainting. The most common disturbances caused by chronic exposure to xylene vapors are fatigue, headache, irritability, weakness, memory loss, drowsiness, mood and balance disorder, tinnitus, nausea, and loss of appetite (El-Naas et al. 2014). At high concentrations, xylene can lead to unconsciousness and death, due to central nervous system depression (ATSDR 2004).

6.3 Environmental Impacts

Oil spills in maritime and river areas have received attention from many researchers worldwide. When in contact with water, a thin layer of the hydrophobic compounds appears on the liquid surface, which prevents the gaseous exchanges between water and air. This layer is thick enough to decrease sunlight incidence through the water, thus inhibiting photosynthesis and respiration. The main petroleum waste impact is the collapse of the food chain (Freitas et al. 2016).

During large-scale accidental leaks and spills, action should be taken immediately to remove contaminants, such the immediate recovery of the pollutant. On the other hand, chronic releases (e.g., barely detectable leaks in underground gas sta-

tions tanks) apparently cause smaller contaminations, but their continuous and prolonged effects have profound consequences to the environment (Mapelli et al. 2017).

Both spill and leakage of gasoline in soil lead to vertical migration of pollutants through the unsaturated zone via interconnected pores within soil particles. Under the influence of gravitational force, BTEX penetrates lower layers with a velocity greater than water and expel interstitial water and air. Thus, the formation of several phases occurs, where the contaminants can transit from one phase to another. Their permanence in each one of those layers is determined by physicochemical properties of both contaminant and environment. BTEX percolation can be retarded by volatilization and sorption in soil grains, especially if it is rich in organic matter. However, when the leak reaches the aeration zone, the compounds come into contact with the groundwater and then dissolve, facilitating permeability (Xu et al. 2015). Once the contamination is established, it can act in three different levels: soil, groundwater, and atmosphere. Thus, the task of assessing the extent, risks, and dynamics of BTEX is a very demanding task. There is a high degree of complexity in determining the concentration of such contaminations, including the best remediation strategies (El-Naas et al. 2014).

6.4 Biotechnological Process and the Biodegradation of Hydrocarbons

The susceptibility of petroleum constituents to biodegradation changes according to the hydrocarbon molecule size and its concentration (Souza et al. 2016). According to Kanaly et al. (2000) and Yu et al. (2005), an increase in the number of aromatic rings leads to the higher chemical stability and hydrophobicity of the molecule, making them less available to biodegradation processes.

Low biodegradability and high persistence of hydrocarbons yield significant environmental impacts. The development of innovative, cost-effective, and ecological technologies is required to minimize these impacts (Sandu et al. 2017). Many conventional methods of ex situ physical-chemical treatments include the chemical inactivation, soil washing, and incineration. Most of these procedures only move the contaminants from the affected area to another place. All the physical-chemical treatments share many limitations, especially their high costs (Trellu et al. 2016; Varjani 2017). When hydrocarbons percolate the soil, a large amount remains sorbed to the soil matrix (approximately 50%), reducing the removal efficiency. The unfeasible resource expenditure and limited effectiveness of these conventional treatments encouraged new technologies for in situ bioremediation (Varjani 2017).

In this context, microorganisms with biodegradable potential have arisen as a promising technology to modify or degrade certain pollutants in contaminated areas and thus reduce overall toxicity. The process of bioremediation emerged as a much better alternative to the legacy remediation technologies available remove organic pollutants (Das and Chandran 2011; Montagnolli and Bidoia 2012; Das and Dash 2014).

Bento et al. (2005) demonstrated that microbial populations can simultaneously degrade aliphatic and aromatic hydrocarbon fractions, although certain compounds and the composition of some mixtures themselves may interfere with the biodegradation.

Biodegradation is based on three basic principles: the presence microorganisms with metabolic capacity, either autochthonous or inoculated (bioaugmentation), the availability of the contaminant as an energy and carbon source, and the appropriate conditions for microbial growth and activity (Ortiz-Hernández et al. 2014). Biotransformation consists of altering the molecular structure of organic compounds to a molecule with characteristics different from the original, which may or may not be less toxic than its precursor (Oberoi and Philip 2017). Mineralization, in turn, represents the complete degradation of organic molecules into new inert inorganic substances, such as carbon dioxide, water, and minor waste compounds (Das and Chandran 2011; El-Naas et al. 2014).

It is generally observed that any improved hydrocarbon biodegradation depends on the presence of specific microorganisms. The local microbial population is directly affected by the environmental conditions and hydrocarbon structure (El-Naas et al. 2014). According to Colla et al. (2008), contaminated sites containing target pollutants are actually a very good source of microorganisms for bioremediation studies, since the contaminated site acts as a selective medium for these microorganisms. Therefore, the remaining microbes in these sites become adapted to specific pollutants.

Almost three decades ago, Atlas (1991) found that microorganisms that have the capacity to degrade hydrocarbons in nonpolluted ecosystems are lower than 0.1% of the total microbial community, but this amount increased from 1 to 10% of the total microbiota biomass when the medium was contaminated by petroleum hydrocarbons. However, it is well established that in polluted environments, general microbial diversity decreases, resulting in the appearance of dominant populations within these disturbed communities, which may endure the toxic contaminants (Kumar and Khanna 2010; Chikere et al. 2011; Varjani 2017).

Microbiological degradation follows a preferred sequence of compounds to be degraded. Aliphatic hydrocarbons (alkanes and alkenes), for example, are the fastest and most easily degraded compounds, followed by aromatic hydrocarbons and finally cycloalkanes (Varjani 2017). In general, branched and polynucleated compounds are more difficult to degrade than mono-aromatic or single-chain molecules. Also, as the degree of halogenation of the molecule increases, biodegradability decreases.

6.5 Microbial Bioremediation

It is a well-documented that some microorganisms can degrade toxic substances (Prince et al. 2016; Padhi and Gokhale 2017; Oberoi and Philip 2017; Varjani 2017). Their ability to consume BTEX hydrocarbons as a sole source of carbon was

observed in 1908 when the bacterium *Bacillus hexavarbovorum* was obtained. It has been confirmed that this species can grow aerobically by metabolizing toluene and xylene (Gibson and Subramanian 1984; Alvarez and Hunt 2002).

Countless other microorganisms have been reported to grow aerobically in BTEX as their only carbon source (Table 6.2). Most of them are bacteria, but they may also include actinomycetes, fungi, and yeasts (Varjani 2017). The members of the genus *Pseudomonas*, for example, have a notable ability to degrade hundreds of organic compounds, which includes the consumption of BTEX (Otenio et al. 2005; Varjani and Upasani 2016).

Gusmão et al. (2006) verified the formation of biofilms in a bacterial consortium using a fixed bed horizontal anaerobic reactor containing BTEX. They found that degrading microorganisms were members of the genus *Bacteroides* and *Pseudomonas*.

Khodaei et al. (2017) experiments with *P. zhaodongensis* strains from petroleum-contaminated groundwater identified their ability to degrade all the BTEX compounds, either individually or mixed. However, when grown in a non-contaminated solution, the bacterial strains were not able to degrade m-xylene. Other experimental setups also proved that specific bacterial species can degrade all the compounds in a BTEX mixture, thus indicating a co-metabolic process. However, in natural environments, many other microorganisms such as filamentous fungi and protozoa may also contribute to molecular breakdown (Ortiz-Hernández et al. 2014; Nwankwegu and Owosi 2017).

According to Das and Chandran (2011), some yeast genera isolated from contaminated water (*Candida*, *Rhodotorula*, *Geotrichum* sp., and *Trichosporon*) are also able to degrade hydrocarbons.

Table 6.2 The potential for degradation of BTEX compounds by different microorganisms

Microorganism (strain)	Substrate	References
Bacillus firmus	m-Xylene	Irshaid and Jacob (2015)
Bacillus subtilis and *Pseudomonas aeruginosa*	BTEX	Mukherjee and Bordoloi (2012)
Candida tropicalis	Toluene	Ahmed and Song (2011)
Cladophialophora sp.	BTEX	Prenafeta-Boldú et al. (2002)
Cladophialophora immunda	Toluene	Blasi et al. (2017)
Desulfotomaculum	o- and m-Xylene	Morasch et al. (2004)
Enterobacter cloacae SG208	Benzene	Padhi and Gokhale (2017)
Marinobacter spp.	Benzene	Nicholson and Fathepure (2004)
Pseudomonas putida and *Pseudomonas fluorescens*	BTEX	Shim et al. (2005) and Mazzeo et al. (2010)
Pseudomonas putida CCMI 852	Toluene and xylene	Otenio et al. (2005)
Pseudomonas putida F1	Benzene	Reardon et al. (2002)
Pseudomonas thivervalensis MAH1	BTEX	Qu et al. (2015)

6.6 Biological Aspects of BTEX Degradation

Changes in pollutant concentration are linked to biodegradation processes. The analysis of microbial activity is extremely important to develop proper bioremediation frameworks. An optimized BTEX biodegradation requires an abundant presence of electron acceptors, a high enzymatic expression, and an enhanced microbial access to mono-aromatics (Varjani and Upasani 2017).

The biodegradation process is based on biological oxy-reduction reactions. In petroleum biodegradation, the hydrocarbons are oxidized in the presence of electron acceptors, which are reduced. There are different compounds that can act as electron acceptors: oxygen (O_2), nitrate (NO_{3-}), iron oxides ($Fe(OH)_3$), sulfate (SO_4^{-2}), and water (H_2O). In addition to electron acceptors, the environmental conditions (i.e., temperature, pH, and redox potential) are related to microbial dynamics (Das and Chandran 2011; El-Naas et al. 2014; Corseuil et al. 2015).

Electron transfer is essential for cellular respiration as it produces the energy (mostly stored as ATP) required for basal functions. Aerobic bacteria use oxygen as an electron acceptor, producing carbon dioxide and water, whereas anaerobic bacteria use other compounds, such as nitrate (NO^{3-}), Fe^{3+} ion, and sulfate (SO_4^{2-}), producing methane and water (El-Naas et al. 2014).

Most petroleum hydrocarbons are biodegradable under aerobic conditions, in which oxygen acts in two roles: as a co-substrate for the enzymes that initiate hydrocarbon metabolism and as the final electron acceptor (Wolicka et al. 2009; Irshaid and Jacob 2015). The most common limitation to aerobic biodegradation in the subsurface is due to the low oxygen solubility in water.

The biodegradation process is limited by many other processes. The high concentration of pollutants, for example, may decrease microbial growth and negatively impact biodegradation rates (Mnif et al. 2017). Slower rates during in situ biodegradation are likely due to a restricted contact with the pollutants as well. Thus, degradation mostly depends on substrate transfer. On the other hand, carbon sources that are easily metabolized, such as the ethanol added to gasoline, can competitively decrease the biodegradation rate of target hydrocarbons (Deeb et al. 2002).

The low concentrations of nutrients, such as phosphorus and nitrogen, can limit the extent and viability of biodegradation (Hollender et al. 2003; Benincasa 2007). Micronutrients and macronutrients are necessary to the synthesis of cellular components. Nitrogen is needed to form amino acids and proteins, phosphorus is essential to ATP and DNA, sulfur is part of many proteins and coenzymes, calcium is important to keep the cell wall, and magnesium stabilizes ribosomes. Microorganisms need large amounts of nitrogen and phosphorus to increase their biomass, so the availability of these nutrients in a contaminated area is critical for biodegradation effectiveness (Abouseoud et al. 2008).

An optimal pH range for most microorganisms is close to neutral, yet many microorganisms adapt quite well to pH values between 5 and 9 (Madigan et al. 2016). However, pH usually decreases during biodegradation due to the production of acid metabolites and CO_2, which may decrease microbial activity.

Temperature is also another important factor toward microbial growth and activity. The thermal energy available influences both the physicochemical nature of the pollutants and bioavailability. The solubility of hydrophobic compounds is temperature-dependent, since the higher the temperature, the less viscous they are. Temperature can affect the degrees of dispersion, volatilization, and diffusion rates of organic compounds. Enzymatic kinetics are also affected by the temperature and broadly varies across microorganism species (Martínez-Alonso and Gaju 2005). Temperatures above the optimal value can lead to denaturation and inactivation of proteins, enzymes, and nucleic acids. Most bacteria present in the subsurface operate more efficiently in a 20–40 °C range. This range is also the standard temperature of many other natural environments (Varjani and Upasani 2017).

Finally, biodegradation can also be limited by the lack of initial biomass. For Corseuil and Weber (1994), low populations of microorganisms may result in extremely lengthy lag phases, yielding an insignificant biodegradation output, even under favorable oxygen and nutrient conditions.

6.7 Enzyme Expression: Plasmid TOL

The fate of degraded hydrocarbons depends on which microorganisms have been able to develop strategies to increase their bioavailability. These microorganisms should be able to trigger specific enzymes and their respective degradation pathways that allow hydrocarbons to be used as energy and carbon sources (Mapelli et al. 2017). However, the complexity of the metabolic processes required for biodegradation often leads to complex microbial consortia, consisted by bacteria of different genera and species. In a consortium, each microbe is specialized to degrade one or several hydrocarbon fractions in a multi-organism process (Littlejohns and Daugulis 2008).

The simultaneous consumption of different substrates is a clever survival strategy of microorganisms to metabolize dozens of organic compounds at the same time while equipping the cell with a kinetic advantage and metabolic flexibility. Besides, this allows fast growth even if the concentration of each nutrient is minimum. This strategy may indicate how microorganisms cope with the low availability of substrates (Semple et al. 2007).

In recent years, many papers have described the mechanisms and the metabolic pathways related to hydrocarbon degradation (Meckenstock et al. 2016; Wilkes et al. 2016; Varjani and Upasani 2017).

The metabolic pathways will always depend on the microorganism and the growth conditions (Joutey et al. 2013). Compounds will undergo biodegradation only if there are enzymes capable of catalyzing them (Diez 2010).

P. putida has an outstanding metabolic versatility that allows its growth in many different carbon sources. This microorganism has an enormous importance for the natural degradation of contaminated environments (Otenio et al. 2005; D'Alvise et al. 2010). The wild-type *P. putida* has plasmids (i.e., genetic information besides

chromosomal DNA) that do not encode essential functions for the bacterium; however, they carry genes that influence the cellular physiology of the bacterium (Madigan et al. 2016), including enzymes related to petroleum biodegradation.

There are many natural plasmids found in *P. putida*, including the TOL plasmid that provides the genes for degrading toxic mono-aromatic hydrocarbons. The plasmid TOL (pWW0), originally isolated from the *P. putida* mt-2 strain by Williams and Murray (1974), is one of the most studied catabolic plasmids, due to encoding enzymes for the biodegradation pathway of toluene, xylene, and ethylbenzene (Burlage et al. 1989; Greated et al. 2002; Otenio et al. 2005; D'Alvise et al. 2010; Domínguez-Cuevas and Marqués 2017).

The plasmid TOL (pWW0) is 117 kb long and belongs to the Incp-9 incompatibility class. It is stable in several microorganisms, such as *P. aeruginosa*, *P. putida*, *P. corrugata*, and *Escherichia coli* (Greated et al. 2002). Otenio et al. (2005) searched the *P. putida* CCMI 852 strain for plasmids and successfully found the TOL plasmid (pWW0) in it. This microorganism was isolated from a domestic sewage treatment plant and further analyzed using gel electrophoresis.

There must be an induction of appropriate degrading enzymes to initiate the degradation process. The concentration of an inducer (which may be the substrate/contaminant to be degraded) must be in higher than the minimum induction limit so that sequences within the bacterial genome are activated (Rüegg et al. 2007; Cozzareli et al. 2010). In general, such limit is low, and enzyme induction hardly compromises BTEX bioremediation. A target substrate (e.g., toluene) starts some cascade biochemical reactions that lead to the transcription of specific genes (from the plasmids) to encode an enzymatic set that degrades the target compound. Any confirmation of plasmids in a hydrocarbon-degrading bacterium is used as a biological tool.

Plasmids carrying the essential genetic information to initiate the degradation of hydrocarbons can certainly be found in certain places. Underground contaminations by BTEX, for example, are often found several years after leakage began. Therefore, microbiological adaptations and enzyme induction have been occurring for a long time (Carmona et al. 2009) in these places.

The main aerobic pathways of degradation that lead to the mineralization of BTEX compounds are bound to two enzymatic groups: dioxygenase and monooxygenases. The dioxygenase route, also called the TOD route, breaks the aromatic ring, whereas monooxygenases, also called TOL route, breaks methyl substituents (El-Naas et al. 2014).

Aromatic hydrocarbons are generally hydroxylated to catechols. The rings are subsequently degraded to intermediates, which can be readily converted to the Krebs cycle intermediates, such as acetic acid, acetaldehyde, or pyruvic acid, as shown in Fig. 6.1 (Fuchs et al. 2011; Peixoto et al. 2011).

Otenio et al. (2005) studied the action of *P. putida* CCMI 852 with the plasmid TOL on benzene, toluene, and xylene individually and in mixtures. The authors concluded that *P. putida* CCMI 852 metabolized toluene and xylene, but no benzene uptake was detected. In fact, they noticed that in the presence of benzene, bacterial performance on the toluene and xylene substrates occurred at a 50% lower rate

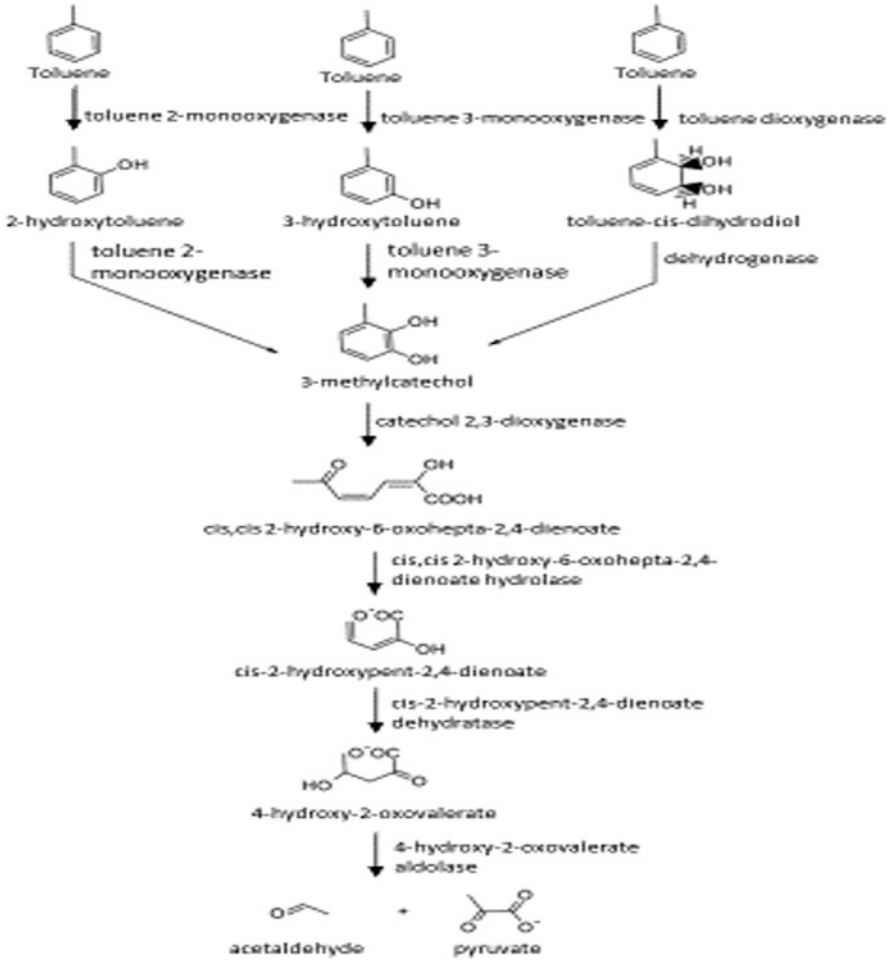


Fig. 6.1 Aerobic degradation pathways of toluene (through the action of the enzymes toluene 2- and 3-monooxygenases and toluene dioxygenase)

compared to solutions containing only toluene and xylene. According to Lee et al. (1994) and Martino et al. (2012), benzene can be metabolized via TOD only, whereas toluene and xylenes may be subject to oxidation by both routes.

Cavalca et al. (2000) selected bacteria to degrade various aromatic compounds, including BTEX. They found genes encoding xylene monooxygenase, toluene dioxygenase, and catechol 2,3-dioxygenase enzymes on their isolates. Fifteen out of 50 strains were identified as *P. putida*, *P. aureofaciens*, *P. aeruginosa*, and *Alcaligenes xilosoxidans*. These species grew in media composed of various aromatic hydrocarbons, including BTEX. Benzene, m-xylene, and p-xylene uptake occurred in 53.3% of the 15 species, as well as ethylbenzene (66.7%). All 15 species consumed toluene as a substrate, but none of them grew in o-xylene. The TOL pathway was found in

P. putida and *A. xilosoxidans*, whereas the TOD pathway was found in *P. aureofaciens*, *A. xylosoxidans* and *P. putida*. Therefore, the growth of *P. putida* CM23 in media containing benzene, toluene, and m-p-xylene suggested both metabolic routes (TOD and TOL).

BTEX compounds in mixtures and individually were also studied by Zhang et al. (2013). He applied a *Mycobacterium cosmeticum* byf-4 strain to metabolize all aromatic the compounds. Their experiments proved that TOD plasmid was necessary to synthesize of all the enzymes related to the degradation of such mono-aromatics.

The strongest motivation behind biodegradation studies is to seek microorganisms with a wide range of metabolic pathways to degrade various pollutants with cost-effective procedures. The biotechnological efforts related to the genetic modification of bacteria have been developed to find fully optimized microorganisms that incorporate metabolic pathways and ultimately mineralize all BTEX compounds. Therefore, a complete mineralization of the mixture of BTEX compounds by *P. putida* is achievable when genetic modifications combine the TOD and TOL pathways (Lee et al. 1995).

6.8 Conclusion

The petroleum biodegradation into the environment is a concern worldwide. An expanded knowledge about biodegradation processes is highly significant for environmental studies, in which autochthonous microorganisms can transform or mineralize organic contaminants using different biochemical pathways of biodegradation. The microbial degradation process helps to eliminate oil spills from polluted areas. The contaminant removal occurs due to a combination of many physical and chemical methods. The enzymatic systems that target different hydrocarbons allow microorganisms to biodegrade them as their source of energy and carbon.

Therefore, based on the current review, microbial-driven degradation is a key element in any bioremediation strategy of petroleum hydrocarbons.

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Chapter 7
Biosurfactant-Enhanced Petroleum Oil Bioremediation

Agus Jatnika Effendi, Edwan Kardena, and Qomarudin Helmy

Abstract Hydrocarbon compounds, ranging from crude oil to its derivative products, have the potential to cause environmental problems on a global scale. The emergence of these hydrocarbon compounds in the ecosystem makes it a concern to general health of public and environmental aspects, owing to their toxic nature, low to less biodegradability, and potentially accumulated in the food chain. Various technologies have been employed for cleaning contaminated sites with hydrocarbon compounds, including physical and chemical approaches such as thermal desorption, soil washing, soil flushing, and solvent extraction technique. Increasing public awareness of more environmentally friendly methods encourages the use of biodegradation concept in its effort to decontaminate sites. This chapter will discuss the hazard of petroleum oil contamination to the environment, its cleanup methods including physical-chemical-biological process, and biosurfactants role in enhancing the remediation process. It is well known that some bacteria are capable to produce surface-active agent of what is so-called biosurfactants. Biosurfactants have some advantages compare to its synthetic counterparts, i.e., biodegradable, low level of toxicity, biocompatible, high availability of production of raw materials, and very useful for environmental management, i.e., in oil spills handling and bioremediation of industrial wastes contaminated soils. However, the most important biosurfactant weaknesses are in the case of large-scale production. High production costs are not suitable for environmental remediation field applications that require surfactants at low cost and large volumes. Some strategies to overcome these issues and also a case study of field-scale application are presented in this chapter.

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7.1 Introduction

Environmental pollution occurs every second during the life of mankind meeting its needs. Pollution occurs when liquids, solids, and/or gases of hazardous materials are mixed with the naturally occurring water, soil, and/or air. The increase growth of the chemical and petrochemical industries of the twentieth century produces a wide range of chemical products needed in everyday life. It is estimated that approximately 8–16 million chemical compounds either natural or man-made organic compounds can be found in the earth where approximately 40,000 chemical compounds are contained in daily human needs products (Singh et al. 2009). Soil and waters are the most commonly used places for disposal of various contaminants that may affect the biodiversity and function of the soil or water. Such contaminants may be alkanes, aromatic compounds, PAHs, chlorinated hydrocarbons, nitroaromatics, and heterocyclic nitrogen. Contaminants entering the environment are often complex mixtures of various chemical compounds commonly found in the petrochemical industry, oil refining, petroleum exploration and production, and other industries. In most cases, pollutants attach to the soil both physically and chemically bonded. Oil and gas industry activities that often cause pollution problems in the environment include:

- Crude oil spills during its distribution and/or transportation.
- Residual originally from wastewater treatment facilities, e.g., oil catcher, API separator, and dissolved air flotation.
- By-product/waste deposits originally from petroleum and gas industry activities in the form of oil slurry, drilling wastes in the form of drill mud, and drilling powders containing oil residues.
- Storage tank or separation tank, e.g., tanker, floating storage, and ground storage tank).
- Residue from cleaning process of tools in oil and gas industry activities.
- Offshore oil drilling potentially contributes to continuous pollution from drilling wells leaking in the seabed and spilled oil distribution from wells to tankers and from ships to land (Bishop 1991).

The discussion of environmental pollution by petroleum oil will be focused on soil contamination by oily sludge, oil-contaminated soil, and its remediation technology which will be described in separate subchapters.

7.1.1 Petroleum Oil Contamination

Hydrocarbon compounds including petroleum oil derivation products and petrochemical products as well as oil sludge as a by-product have the potential to form contaminants on a large scale. The existence of these compounds in the environment is considered both environmentally, and public health is very dangerous,

Fig. 7.1 Typically abandoned oily sludge pit in petroleum and gas industry activities

because of the ability of these compounds to accumulate, persist, and have high toxicity value (Prasetya et al. 2006; Taha et al. 2010). Oil sludge is formed from two main factors that control its formation: First is inorganic residues such as sediments, sand, scale, and other impurities. Second is the precipitation process of paraffin wax due to temperature changes, where wax precipitates tend to dissolve in crude oil. In addition, the oxidation process of organic compounds in the raw oil owing to climatic changes and microbial activity and the presence of disturbances in mass equilibrium due to the loss of its volatile compounds and also the tendency of asphaltenes, resins, and also other polymeric substance to precipitate is the initial formation of oil sludge (El-Hamied and Ahmed 2004). In general, the oil sludge deposited in the crude oil storage tanks will be removed and stored in the typically sludge pit as shown in Fig. 7.1.

Oil sludge is considered harmful to the environment due to its characteristics as a representation of the stability of the three components of the system in the cycle/fate of contaminants, i.e., solid/oil/water stabilized by gas phase and intermediate products in the biological degradation process of organic compounds (Faye and Sinyavskiy 2008). Due to the evaporation of moisture content in the oil sludge stored in the pit/pond/storage tank, the liquid-liquid phase of the oil sludge will be lost. The loss of the liquid forms a thick, sticky solid matrix with different plasticity levels. Under these conditions oil sludge is not dusty, has thixotropic properties (viscous gels or viscous fluids under normal conditions but can be strongly viscous

Table 7.1 Chemical and physical descriptions of typical oil sludge and oil-polluted soils

No	Parameter	Type of waste	
		Oil sludge	Oil-contaminated soil
1.	Density at 20 °C (kg/m^3)	989.0	996.0
2.	Coking capacity (mass %)	12.5	32.1
3.	Acidity (mg KOH/100 ml)	21,0	–
4.	Element composition (mass %)		
	Carbon	82.3	84.2
	Hydrogen	11.3	9.5
	Nitrogen	0.4	0.3
	Sulfur	1.0	2.1
5.	Component in the organics phase (mass %)		
	Oils		
	Paraffin-naphthalene	40.8	6.5
	Aromatic	26.1	28.1
	Total oil	66.9	34.6
	Tars (mass %)		
	Petroleum ether-benzene	11.5	35.8
	Benzene	4.3	3.1
	Ethanol-benzene	4.5	14.6
	Total tars	20.3	53.5
	Asphaltenes	12.8	11.9

Adapted from Ongarbayev and Mansurov (2008)

when stirred/agitated), and gives only a maximum of 1–3% (mass) moisture in the topsoil where it is stored. The oil sludge characteristic as shown in Table 7.1 is favored by industrial managers as it makes easier in terms of transportation as well as in the case of stockpiling in storage pit/bunker (Ongarbayev and Mansurov 2008).

The chemical and physical composition and characteristics of organic compounds extracted from oil sludge and oil-contaminated soil are shown in Table 7.1. In general, oil sludge and oil-contaminated soil have a density of 989 and 996 kg/m^3 and contain high sulfur (1–2% mass), coking capacity (carbonated solids derived from oil refining or other cracking processes), and high acidity levels. Although the main content of oil sludge is paraffin-naphthalene hydrocarbon, with high acidity, it indicates intermolecular interactions between oil sludge components that make it sticky/plasticity (Faye and Sinyavskiy 2008). The composition of chemical components removed from oil sludge is naphthalene-paraffin hydrocarbons (40.8% mass) and aromatic petro-hydrocarbons (26.1% mass). The composition of tars (sticky substance) in oil sludge reaches 20.3% mass, while the asphaltenes (aromatic hydrocarbon fraction with high molecular weight with soluble properties in aromatic solvents but not in aliphatic solvents) reach 12.8% mass. Chemical compositions with high paraffin hydrocarbon and asphalt-resinous characteristics cause complications in further processing of oil sludge. The result of spectral analysis on the organic phase separated from the oil sludge indicates that the carbon-based phase of oil sludge contains copper, chromium, and manganese with high

concentration while the organic phase extracted from oil-contaminated soil contains vanadium, iron, magnesium, silicon, and aluminum with high concentrations. Heavy metals contained in both oil sludge and oil-contaminated soil are affected by the origin of the crude oil extracted from its reservoir. From the oil sludge characteristic data, it can be deduced that the carbonic stage of the oil sludge tends to be weighty petroleum deposit. These properties are characterized by high tars and asphaltenes, while high densities of waste are affected by climatic factors as they are stored in open storage ponds for a long time.

7.1.2 Effect to the Environment

The toxicity of oil sludge is determined by the composition and content of the compounds contained therein such as heavy metals and PAHs. The effects of toxicity depend on the target exposed. The effects of toxicity are divided into two groups: human toxicity and ecotoxicity effects (Krishnamurthi et al. 2003). The degree of petroleum oil toxicity to some living classes can be seen in Table 7.2.

Morelli et al. (1999) confirmed that the toxicity rate of oil sludge is mutagenic after passing through various bioassays and toxicological analyses, e.g., *Bacillus cereus* spot test, resazurine reduction test, and bioluminescence test with *Photobacterium*. Characteristics of oil sludge are also reported to be the same or unchanged after being stored for 6 months. The genotoxicity effect of oil sludge is reported by Krishnamurthi et al. (2003) in a study of Chinese hamster ovary cell cultures. The results showed that oil sludge can cause DNA damage, chromosomal abnormalities, and cell death due to exposure to oil sludge extract. PAHs is one component of compounds contained in oil sludge which is suspected by Krishnamurthi et al. (2007) plays a role in the oil sludge toxicity properties in the environment. Exposure to various doses of oil sludge (25, 50, 100 μL) can cause DNA damage and chromosomal abnormalities with a confidence level of $P < 0.001$ compared with controls. The genotoxicity of oil sludge is indicated by the presence of identified PAHs from GC-MS analysis.

In addition to containing PAHs, oil sludge also contains several types of metals that are toxic to living things. Some metals commonly contained in petroleum include arsenic, chromium, cadmium, lead, vanadium, nickel, copper, iron, and mercury (Faye and Sinyavskiy 2008; Taha et al. 2010). The degree of toxicity and the effects of various heavy metals contained in the oil sludge are shown in Table 7.3.

Assuming the national crude oil production target of 1,000,000 BOPD (barrel oil per day), the potential for oil sludge production in Indonesia is estimated to reach 60,000 tons per year. The amount of oil sludge is based on Pertamina Ltd. Business Unit Tanjung, South Kalimantan, that producing 5000 BOPD will produce oil sludge of 300 m^3 per year (Cahyono et al. 2009). This large generation does not include oil sludge which has been stockpiled by other oil and gas companies operating in Indonesia. Santa Fe Ltd. in Papua and Vico Ltd. in Kalimantan are known to store about 20,000 m^3 and 15,000 m^3 of oil sludge (Suryatmana et al. 2005), while

Table 7.2 Levels of petroleum hydrocarbon toxicity to living organisms

Hydrocarbons	Organisms	Endpoint	Conc. (μg/l)
1, 1′-biphenyl	Water flea	LC50 48 h	3660 μg/l
1, 1′-biphenyl	Brine shrimp	LC50 24 h	4009 μg/l
1, 1′-biphenyl	Rainbow trout	LC50 96 h	1500 μg/l
1, 1′-biphenyl	Bluegill	LC50 96 h	4700 μg/l
1,2-Dimethylbenzene	Water flea	LC50 48 h	3168 μg/l
1,2-Dimethylbenzene	Rainbow trout	LC50 96 h	8050 μg/l
1,2-Dimethylbenzene	Bluegill	LC50 96 h	16,100 μg/l
1-methylnaphthalene	Brine shrimp	LC50 24 h	2560 μg/l
1-methylnaphthalene	Dungeness/crab	LC50 48 h	8200 μg/l
1-methylnaphthalene	Fathead minnow	LC50 96 h	9000 μg/l
Anthracene	Opossum shrimp	LC50 48 h	3.6 μg/l
Anthracene	Scud	LC50 240 h	5.6 μg/l
Anthracene	Bluegill	LC50 48 h	12.02 μg/l
Benzene	Water flea	LC50 24 h	18,400 μg/l
Benzene	Fathead minnow	LC50 96 h	24,600 μg/l
Benzene	Brine shrimp	LC50 48 h	97,800 μg/l
Benzene	Bluegill	LC50 24 h	400,000 μg/l
Benzene	Rainbow trout	LC50 96 h	21,637 μg/l
Benzene	Laboratory animal	LD50 oral	4 ml/kg[a]
Benzo(a)pyrene	Water flea	LC50 96 h	5 μg/l
Benzo(a)pyrene	Fathead minnow	LT50 40 h	6 μg/l
Ethylbenzene	Fathead minnow	LC50 96 h	9090 μg/l
Ethylbenzene	Brine shrimp	LC50 24 h	11,326 μg/l
Ethylbenzene	Water flea	LC50 48 h	75,000 μg/l
Ethylbenzene	Bluegill	LC50 24 h	90,000 μg/l
Ethylbenzene	Rainbow trout	LC50 24 h	14,000 μg/l
Fluoranthene	Opossum shrimp	LC50 96 h	87,600 μg/l
Fluoranthene	Water flea	LC50 48 h	45 μg/l
Fluoranthene	Scud	LC50 96 h	85 μg/l
Fluoranthene	Bluegill	LC50 96 h	12.3 μg/l
Phenanthrene	Water flea	EC50 48 h	212 μg/l
Phenanthrene	Brine shrimp	LC50 24 h	677 μg/l
Phenanthrene	Rainbow trout	LC50 552 h	40 μg/l
Phyrene	Opossum shrimp	LC50 48 h	24.8 μg/l
Phyrene	Clam	LC50 96 h	1.68 μg/l
Toluene	Fathead minnow	LC50 96 h	25,000 μg/l
Toluene	Rainbow trout	LC50 96 h	6780 μg/l
Toluene	Bluegill	LC50 96 h	13,000 μg/l
Toluene	Laboratory animal	LD50 oral	6–8 ml/kg[a]
Xylene	Brine shrimp	LC50 24 h	5.7 μg/l
Xylene	Bluegill	LC50 24 h	25,600 μg/l

(continued)

Table 7.2 (continued)

Hydrocarbons	Organisms	Endpoint	Conc. (μg/l)
Xylene	Rainbow trout	LC50 24 h	8300 μg/l
Xylene	Laboratory animal	LD50 oral	4 ml/kg[a]
Hexane	Brine shrimp	LC50 24 h	3533 μg/l
Octane	Brine shrimp	LC50 24 h	400 μg/l
Pentane	Brine shrimp	LC50 24 h	11,905 μg/l
Total petroleum hydrocarbon	Earthworm	EC50 14d	644 ppm[b]
Total petroleum hydrocarbon	Wheat	EC50 germination	30,400 ppm[c]
Total petroleum hydrocarbon	Maize	EC50 germination	28,600 ppm[c]
Total petroleum hydrocarbon	Luminescent bacteria	EC50	4700 ppm[c]

Summarized from Battelle (2007)
[a]Raisbeck (2013), [b]Hentati et al. (2013), [c]Tang et al. (2011)

Table 7.3 Impact and toxic amount of heavy metals in oil sludge (ATSDR 2001)

Metals	Acute	Chronic	Toxic concentration
As/arsenic	Diarrhea, dizziness, vomiting, pain, encephalopathy	Diabetes, hyperkeratosis, cancer in the lung, bladder, skin, encephalopathy	24-h urine:
			≥50 μg/l urine or 100 μg/g of creatinine
Cd/ cadmium	Pneumonia	Protein in urine, osteomalacia, lung cancer	Protein in urine and/ or ≥ 15 μg/g of creatinine
Cr/ chromium	Bleeding gastrointestinal organs, hemolysis, acute renal failure	Lung cancer (inhalation), pulmonary fibrosis	Not determined standard
Fe/iron	Vomiting, bleeding gastrointestinal organs, cardiac depression, metabolic acidosis	Liver cirrhosis	Nontoxic: <300 μg/dl
			Severe: >500 μg/dl
Pb/lead	Dizziness, vomiting, encephalopathy (headache, seizures, decreased consciousness)	Encephalopathy, anemia, abdominal pain, nephropathy/renal abnormality, foot drop/ wrist-drop	Children, symptoms occur in [Pb] ≥45 μg/dl (blood); adult, [Pb] ≥70 μg/dl
Hg/ mercury	Inhaled: fever, vomiting, diarrhea; ingestion: sore throat	Dizziness, metal taste, tremor, headache, kidney disorder, hypersensitivity (pink disease)	Normal limit,
			10 μg/l (blood); toxic, 20 μg/l (24 h urine)
Ni/nickel	Dermatitis, myocarditis, encephalopathy	Occupational (inhalation): pulmonary fibrosis, nasopharyngeal tumor, reduced sperm count	Serious poisoning:≥500 μg/l (8 h urine)
			Excessive exposure: ≥8 μg/l (blood)

Pertamina Ltd. stockpiles 15,000 m^3 of oil sludge in Cilacap and Balikpapan, 500 m^3 in Plaju, and 16,000 m^3 in Indramayu oil processing facility. To date, the number of sludge generation will vary due to stringent standards regulated by Indonesian government that they must comply. The magnitude of oil sludge is potentially high in

environmental pollution, either pollution to soil and groundwater or air pollution (Kostecki and Behbehani 1999). Soil and groundwater contamination occurs due to leaking of stockpile facility accompanied by infiltration of leachate into soil and groundwater, while air pollution occurs due to volatile compounds that evaporate to the environment around the stockpile facility (Singh et al. 2009). The compounds contained in oil sludge as described previously contain a variety of toxic compounds, namely, PAHs, as well as heavy metals that are harmful to environmental health (Faye and Sinyavskiy 2008).

7.2 Cleanup Methods

Various technologies are applied to overcome contaminated sites such as remediation techniques with physical processes such as centrifugation (Adams 1982), soil washing (Bhandari et al. 2000; Urum and Pekdemir 2004), soil flushing (Shin and Kim 2004), soil vapor extraction, electrokinetic remediation, wet classification, encapsulation (Singh et al. 2009), and photocatalysis (da Rocha et al. 2010) and remediation techniques with chemical processes such as chemical extraction (Taiwo and Otolorin 2009) and solidification/stabilization. Remediation techniques with thermal processes include incineration, thermal desorption, wet oxidation, vitrification, and supercritical oxidation (Shie et al. 2003; Shie et al. 2004), while remediation techniques with biological processes include ex situ bioremediation (land farming, windrow, biopile, composting), in situ bioremediation (biosparging, air sparging, bioleaching), and phytoremediation (Cortes et al. 2009; Liu et al. 2009).

7.2.1 Physical Remediation Technique

Naturally, the pollution removal mechanism can occur through natural processes such as advection of pollutants both horizontally and vertically, evaporation of volatile pollutants, emulsification with water to form emulsions, and the oxidation of pollutants by sunlight/photooxidation (Bishop 1991).

(a) Centrifugation

The sludge present at the bottom of the crude oil storage tank is generally a mixture of inorganic compounds, sand, wax, and heavy crude oil. The conventional method of oil sludge management is by heating the oil sludge to facilitate the pumping and then flowed on a three-phase centrifuge as shown in Fig. 7.2, to separate the water phase, oil phase, and solid phase. This process normally leaves residual solid waste containing 6–15% hydrocarbon and able to recover for almost 90% of crude oil (Adams 1982).

(b) Soil Washing and Soil Flushing

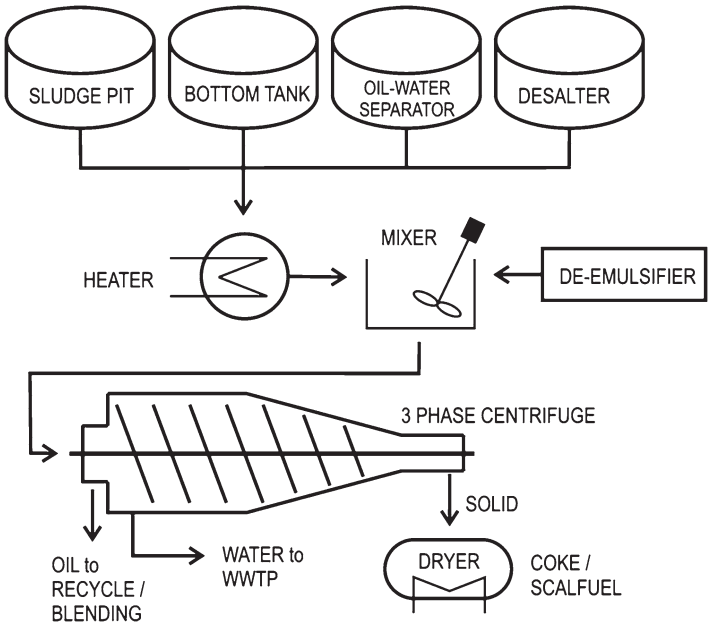

Fig. 7.2 Three-phase centrifuges in oil sludge handling

In soil washing, water is used with additives in the form of surfactants and mechanical processes (stirring, spraying, and suctioning) in the effort to wash the contaminated soil of petroleum hydrocarbons (Chu 2003; Urum et al. 2005). While in soil flushing, extractor fluid is used to rinse (flushing) the polluted soil (Shin and Kim 2004). This process is usually accompanied by a process of biodegradation to treat the fluid from the washing/flushing of the contaminated soil (Joseph and Joseph 2009).

(c) Soil Vapor Extraction

This technique is capable of treating gases and volatile and semi-volatile organic compounds from contaminated soil petroleum hydrocarbons by a vacuum pump process as shown in Fig. 7.3 (USACE 2002). The working principle of soil vapor extraction is similar to that of water sparging wherein the construction of a well in contaminated soil and air is pumped out of the well. Airflow created due to the vacuum situation around the well will accelerate the process of volatilization of polluted constituents. One of the things that can interfere with this process is when there is a high concentration of iron compounds in the pollution site. The disturbance occurs because iron/Fe^{2+} will oxidize and will be precipitated in contact with oxygen. The iron precipitation will cause the soil permeability to decrease so that the oxygen flow in the soil is disturbed. The process criteria in soil vapor extraction include intrinsic soil permeability, $k > 10^{-9}$ cm^2, concentration of iron in groundwater <10 mg/l, volatility of the hydrocarbon petroleum fraction to be treated <250 °C, and Henry's law constant >100 atm (Kucharski 1999).

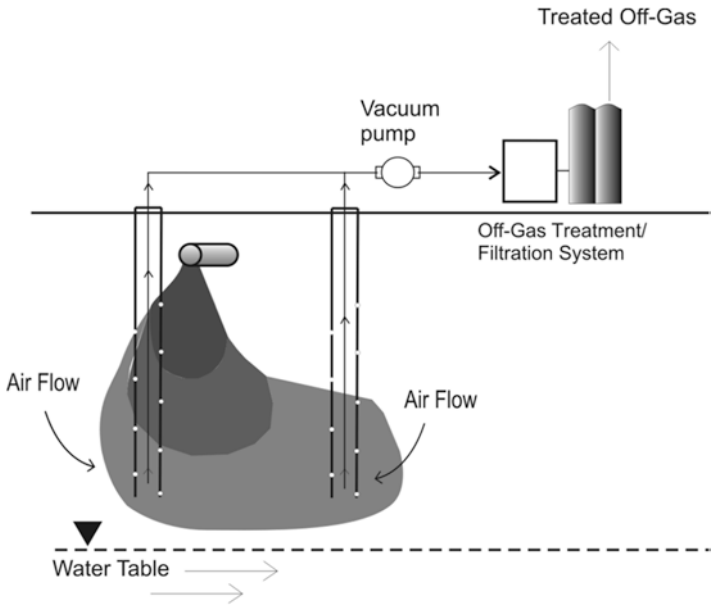

Fig. 7.3 Illustration of soil vapor extraction process in processing contaminants (USACE 2002)

7.2.2 Chemical Remediation Technique

The remediation technique of petroleum hydrocarbons contaminated soil with chemical processes is generally done by extraction. The principle of this technique is to separate and collect contaminants into smaller residues (separation). Water with or without additives is used as an extraction agent (Liu et al. 2009).

(a) Chemical Extraction

The most common method applied to solve the oily sludge problem is the extraction using chemical solvent (Taiwo and Otolorin 2009; Zubaidy and Abouelnasr 2010). This method is very effective in treating oil sludge, but there are constraints related to the application of this method that is expensive solvent prices and related to the compatibility with the environment (Martia et al. 2009). This is because the toxicity of the solvent is as high as the oil sludge itself (Morelli et al. 1995; Krishnamurthi et al. 2007). The chemical extraction scheme is displayed in Fig. 7.4.

(b) Solidification/Stabilization

Solidification/stabilization technique is a method that uses chemicals and or physical processes to minimize the release of contaminants contained in the waste to the surrounding environment. Stabilization refers to efforts to minimize the potential spread of contaminants chemically so that the contaminants become insoluble, immobile, or nontoxic, while solidification refers to the effort of compacting con-

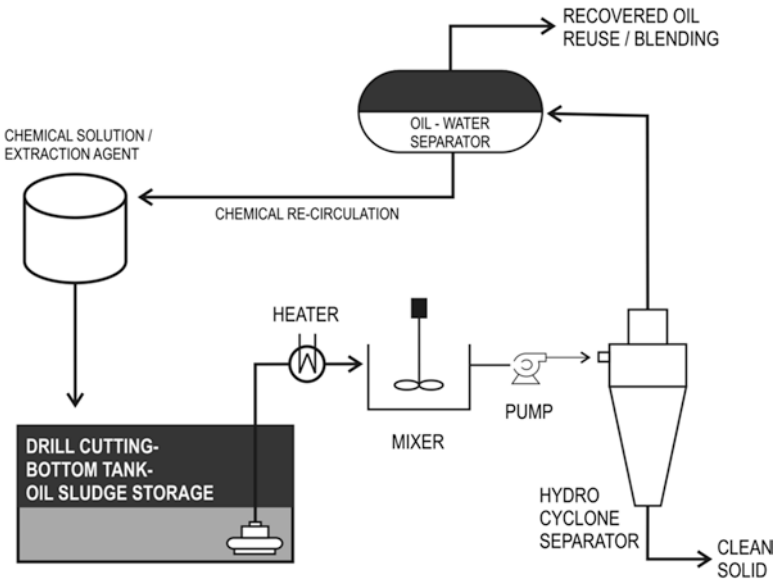

Fig. 7.4 Schematic of oil sludge chemical extraction process

taminants into a solid material. The advantages of this technique are simple and easy to apply; the additive/mixer is easy to obtain and relatively cheap, while the weakness is the volume of contaminants that will increase due to the addition of additive (Taha et al. 2010). Construction materials such as bricks and paving blocks can usually be produced from contaminated soil, while volatile constituents will disappear during cooking. The process criteria in solidification/stabilization include soil particle size>200 mesh, constituents in semi-volatile petroleum hydrocarbons <10,000 ppm, and cyanide concentrations <3000 ppm (Kucharski 1999). In addition to these criteria, the process of solidification/stabilization must pass through several tests of permeability test, leachability test/TCLP, and compressive strength/UCS (Taha et al. 2010).

(c) Asphalt Incorporation

Handling of oil sludge with asphalt incorporation process or as an asphalt mixer material is a productive method in utilizing waste. Asphalt brewing machine/mixer will remove volatile components from oil sludge in combustion chamber and mix nonvolatile components with other asphalt materials (Kucharski 1999). Oil sludge characteristic of heavy petroleum residue with high tars and asphaltenes content makes it suitable as mixed materials in asphalt manufacture (Ongarbayev and Mansurov 2008). The process criteria in oil sludge handling with asphalt incorporation process are particle size <200 mesh (less than 10%), minimum organic compound content, and minimum volatile constituents of petroleum hydrocarbon (Kucharski 1999).

Fig. 7.5 Cleanup process of abandoned oily sludge pit (upper left to lower right): site investigation ➔ excavation ➔ containment ➔ labeling ➔ transportation ➔ coprocessing in cement kiln

7.2.3 Thermal Soil Remediation Process

The thermal remediation process of the soil is the easiest way to be implemented because oil sludge has an energy/heat value and is a fuel source. Burning oil sludge directly with incinerator (Shie et al. 2004) and pyrolysis (Shie et al. 2003) can be applied with various technical considerations including destroying harmful compounds contained in oil sludge and minimization effort of oil sludge used in coprocessing as auxiliary fuel in the cement-burning furnace (Liu et al. 2009).

(a) Incineration and Coprocessing

Oil sludge handling by burning is a very simple method and the easiest to do. However, the application of this method is not recommended with regard to the excess of air pollution due to incinerator emissions. The scheme of the oil sludge incineration/coprocessing process is shown in Fig. 7.5. In principle, coprocessing is also an incineration process, i.e., utilizing heat to burn contaminant/waste. The basic difference between the two is in incineration; combustion only reaches an 800 °C furnace temperature which still potentially produce waste in the form of bottommost ash and should be handled beyond as per the applicable rules and regulations. As in coprocessing the working temperature can reach as high as 1500 °C and almost without producing bottom ash. The remaining bottom ash in the co-handling process is extremely less amount and can be used in making products from cements. The amount of harmful chemicals in the co-handling is very less or is present as trace elements that result in making safer products of cement. Other dissimilarities, burning at high temperature, are classified as another disposal form, while co-handling is classified as efforts for recovery. Toxic waste utilization with

the concept of 3R (recycle, recovery, and reuse) is governed by the Government of Indonesia Regulation Number 101/2014 regarding hazardous waste management (Cahyono et al. 2009; Helmy and Kardena 2015).

(b) Thermal Desorption

The thermal desorption process uses internal heat source, e.g., an oil heated screw feeder or with an external heat source, e.g., hot air flow to treat the contaminated soil. The main difference of thermal desorption process with incineration is that in thermal desorption, volatile material is separated/treated/condensed from contaminated soil. The amount of contaminated soil and water is directly correlated with the amount of energy that must be added to the thermal desorber system to evaporate the hydrocarbon compounds in the contaminated soil. Thermal desorbers are operated at high temperatures reaching 650 °C, whereas other thermal systems only use temperatures between 200 °C and 350 °C. To know the optimum operating temperature of the thermal desorbers unit, it is necessary to analyze the boiling point value of the petroleum hydrocarbon mixture which will be processed (Kucharski 1999). Gasoline evaporates at a temperature of between 40 and 225 °C, diesel at 200 and 340 °C, kerosene at 180 and 300 °C, and heavy crude oil and oil residue and lubricating oil having a volatile constituent at >400 °C. These volatile gases are released into the environment without being processed first or can be reburned, condensed, or adsorbed using activated carbon. The process criteria in the desorption thermal process include soil particle size <4.4 mm, moisture content of 10–25%, the ratio of soil heating value per petroleum fraction <2000 BTU/lb, and the boiling point value of the petroleum compound to be evaporated <650 °C (Kucharski 1999).

Of the various remediation techniques of petroleum oil remediation, physical, chemical, and thermal remediation as described previously has not been able to demonstrate a satisfactory performance in waste management solution. That's because the use of the techniques described above still poses a new problems. Remediation by thermal process may cause new contamination in the air, whereas remediation by the physicals process only removes the source of the pollutants elsewhere, and the remediation by chemical processes is constrained by high toxicity of the chemicals used. Therefore, an alternative technique is needed that effectively eliminates pollutants thoroughly and more friendly to the environment.

7.3 Biological Remediation Technique

Biological degradation is an interesting method of remediating contaminated sites of harmful compounds. In this method, hydrocarbons containing high organic matter are used as food sources for microorganisms. Microorganisms in biological processes are effectual in oxidizing soluble carbon-based compounds of pollutants comprising emulsified oils. Limitation in the biodegradation process is that most

hydrocarbons are less soluble making it difficult for microorganisms to degrade them. One of the steps applied to increase the biodegradation rate of organic compound with low solubility is to add biosurfactants. Biosurfactants are produced by microbes extracellularly containing both hydrophilic and hydrophobic moiety. Biosurfactant affects the rate of biodegradation of hydrocarbon compounds and enhances the degradation process in two ways, i.e., changing the affinity amid the microorganisms and hydrocarbon compounds by surface tension reduction between the two phases thereby reducing the hydrophobicity of the cell surface of the microorganism and by enhancing the dispersion and solubility of hydrocarbons. The role of biosurfactant in biodegradation of fuel oil will be described in separate subchapters which will discuss, i.e., the definition and classification of biosurfactants, biosurfactants production, and its application in petroleum oil degradation.

Biological waste treatment is still considered relatively more efficient and environmentally friendly compared to physical-chemical waste treatment (Atlas and Cerniglia 1995). This is because the process is not too complicated, does not require complex tools and facilities, is relatively cheaper when compared with other techniques, and does not cause toxic side effects that are more secure to the environment. But in addition to these advantages, the biological degradation of petroleum oil has several shortcomings, among others, limited application. According to Schindler and Buhler (1984), this is because microorganisms have limited specificity and capacity in the processing of complex waste and require a relatively long time. Some of the techniques in treating petroleum waste biologically that are often applied are the following.

7.3.1 Bioremediation

Frequent bioremediation processes include several systems, including:

(a) Solid- and slurry-phase bioremediation: Aims to treat waste in both solid and slurry form. These include land farming, composting (soil-pile; windrow; in-vessel), and slurry treatment (Anderson 1995).
(b) Liquid-phase bioremediation: Aims to treat waste in liquid form. This process uses a common type of reactor, i.e., activated sludge, SBR, trickling filter, RBC, and fluidized bed reactor (Cookson 1995).
(c) Vapor-phase biological treatment: Aims to treat off gases from ground remediation and groundwater remediation (bioventing, air sparging) operations (Eweis et al. 1998).

7.3.2 Phytoremediation

Phytoremediation is an advanced development of bioremediation techniques that utilize the ability of various plants as biological agents to restore contaminated soil or water bodies through the principle of optimization of plant root system

conditions that can stimulate indigenous microorganism activity to degrade target compounds. It is cost-effective in addition to environmental friendly, but it might take additional time compared to established bioremediation approaches since phytoremediation is a native procedure. Some plants species have also been demonstrated to have the potential to flourish in polluted soil and truly uptake or extract the pollutants from growth media moved to its tissue. Such plants function in numerous dissimilar manners, e.g., certain plants will be able to cause hyperaccumulation of lethal heavy metals in their cells; some plants converting pollutants into less toxic compounds and vaporizing them also can sieve water pollutants (Ndimele 2010; Brooks 1998; Brooks and Robinson 1998). There are six mechanisms identified where plants are able to influence the level of contaminants in water, soil, and sediment. Phytoextraction denotes capability of plants to eliminate pollutants, heavy metals, and other toxic components from beneath the soil surface and transport these components to plant leaf and other parts. In that case, the plants might be reaped and physically removed from that particular location. The application of phyto-extraction is generally restricted to heavy metals and other inorganic soil components of sediment and soil part (Pajević et al. 2016; Mahmood 2010). Phytodegradation signifies the capability of plants to degrade contaminants after they are absorbed by the roots. In phyto-extraction, plant absorption occurs when contaminants hydrophobicity and solubility fall into a particular acceptable scale (Park et al. 2011). Rhizodegradation signifies the potential of plants actions to degrade harmful compounds within plant root zones by the action of bacteria or other microorganisms that grow and develop in the rhizosphere (Tangahu et al. 2011). Phytovolatilization implicates the absorption process of certain pollutants from root to the plant body and then release the volatile compound of the contaminant or the volatile degradation products that occur with water vapor from the leaves (Sakakibara et al. 2010). Phytostabilization is the attachment of certain pollutants to the plant roots that cannot be absorbed into the plant stem. These substances are attached to the roots so that they will not get carried away by the flow of water in the media. Rhizofiltration refers to absorption/biofiltration of the contaminants into its biomass and separated from the contaminated location after harvesting the plants. Rhizofiltration is typically exploited in surface water, groundwater, or wastewater for removing heavy metals, toxic chemicals, or other mineral contaminant. Each of these six mechanisms will cast an influence on the metal mobility, concentration, or metal toxicity of unwanted pollutants (USEPA 2000; Marques et al. 2009).

7.3.3 Mycoremediation

In the bioremediation system, microorganism (in this case bacteria) utilizes organics compound by uptake of the compound into its cells (e.g., by a cell wall diffusion process) and utilizes an intracellular enzyme (an enzyme within the cell) in the degradation process. With this mechanism, the diffusion of pollutant compounds into the cell wall is limited by the molecular size of the pollutant compound, the size of the cell wall, and the toxicity of the compound that potentially can disrupt or even

kill bacteria. Fungi have different degradation mechanisms with bacteria. In the fungi system, the degradative enzyme is secreted by the fungus from its mycelia or is called an extracellular enzyme. Thus, the biodegradation process takes place outside the fungal cell or its miscellaneous. With this mechanisms can overcome the problem of molecular size of pollutant compounds and toxicity of pollutant compounds against degrading microorganisms. Widely studied fungi in bioremediation application are white-rot fungi. The study of white-rot fungal species and its application has been done by many researchers in the world. Some oil-degrading fungi that have been isolated from the contaminated sites show excellent performance in degrading petroleum oil compare to their fellow bacteria. Other white-rot fungi group also shows their superior capability in degrading persistent hydrocarbons compounds, e.g., *Pleurotus pulmonarius*, *Pleurotus ostreatus*, *Pleurotus tuber-regium*, *Trametes versicolor*, *Bjerkandera adusta*, *Agaricus bisporus*, *Lentinula edodes*, and *Irpex lacteus* (Singh 2006; Adenipekun and Lawal 2012).

7.4 Biosurfactant-Enhanced Bioremediation

The term biosurfactant (biological surface-active agents) refers to the various types of compounds produced by microorganisms that are as surface-active agents or emulsifying agent (Noordman 1999). Biosurfactants can be produced continuously in a constitutive manner even though the microorganisms are not grown on hydrophobic substrates. This indicates that the function of biosurfactants is not only to enhance the hydrophobic substrates surface area but also employed to augment the bioavailability of hydrophobic substrates through the process of desorption or solubilization. Biosurfactant also govern the removal and attachment of microbes from various surface. The biosurfactants can act as a regulator of cell movement by controlling cell adhesion to the surface as well as cell detachment from the surface. In terms of its chemical structure, biosurfactants possess both hydrophobic and hydrophilic moiety making them to collect at boundaries amid fluids having dissimilar polarities, for instance, oil and water, making the interfacial surface tension between both phases decreasing (Vijayakumar and Saravanan 2015). Figure 7.6 shows a scheme of a chemical structure of biosurfactants comprising hydrophobic and hydrophilic groups and its micelle formation.

Unlike synthetic surfactants, biosurfactants are generally classified by their molecular weight, chemical composition, or microbes that produce them. Ron and Rosenberg (2001) stated that biosurfactants or bioemulsifiers generated by numerous microbes based on their molecular weight are basically split into two major class, i.e., low molecular weight biosurfactants and high molecular weight biosurfactants. The biosurfactants having low molecular weight are glycolipids such as rhamnolipid and sophorolipid or lipopeptides such as polymyxin and surfactin, while high molecular weight biosurfactants include lipoprotein, lipopolysaccharide, and amphipathic polysaccharides. Biosurfactants having low molecular weight can serve to decrease surface tension, while biosurfactants having higher molecular

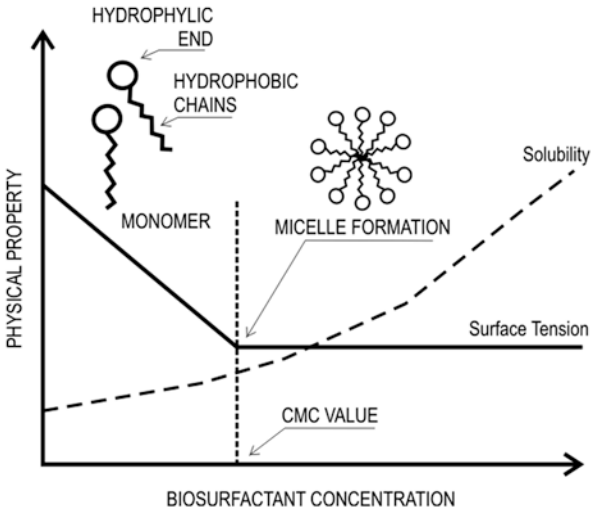

Fig. 7.6 Surface tension value as a function of biosurfactant concentration, CMC represent critical micelle concentration

weight are highly efficient for oil emulsion stabilization in aquatic conditions. Desai and Banat (1997) classify biosurfactants based on the composition of their chemical structures. Biosurfactants comprise a varied group of surface-active compounds and occur in nature with diversity of biochemical structure, such as lipopeptides, glycolipid, phospholipids, neutral lipids, fatty acids, particulate, and polymeric structures (Huszcza and Burczyk 2003). The naturally produced biosurfactants cause no harm to environment, as compared to synthetically manufactured surfactants. Moreover the natural biosurfactants are cost-effective compared to man-made surfactants (Rashedi et al. 2006; Helmy et al. 2011). The most common type of biosurfactant is glycolipid. Glycolipids are a combination of carbohydrates having long chain of aliphatic acids or hydroxy aliphatic acid. Glycolipids are divided into rhamnolipid, trehalolipid, and sophorolipid. The numerous findings and studies by several scientists with regard to biosurfactant which improved biological degradation of contaminants are recapitulated in Table 7.4.

Pollutant degrader microbe concentration in an open system, such as a hydrocarbon-contaminated soil, oil spill in the water usually contain not high enough value to effectively degrade the pollutant compounds. Because indigenous microorganisms have not been well adapted to the pollutants, the concept of "bioaugmentation" is introduced. A well-adapted and well-acclimatized microorganism of a particular type of pollutant is introduced into the system. With this method, microorganisms can immediately degrade the pollutant into a simpler or less harmful material to the environment. One factor influencing the biological degradation process is the availability of substrates for microorganisms to attack. There are two possibilities for the bioavailability of the substrate to the microorganism, i.e., high substrate solubility and low substrate solubility. The soluble

Table 7.4 Various study related to biosurfactants-mediated remediation process

Pollutants	Biodegrader microbial agent	Biosurfactants	References
Petroleum hydrocarbon			
Biodiesel and diesel oil	Mixed culture microflora	Rhamnolipids	Chrzanowski et al. (2012)
Diesel oil and biodiesel	Mixed culture microflora	Rhamnolipids	Owsianiak et al. (2009)
Diesel oil	Indigenous soil microflora	Surfactin	Whang et al. (2007)
Diesel oil	Indigenous soil microflora	Rhamnolipids	Whang et al. (2007)
Crude oil	*Pseudomonas* sp. *BP10 and Rhodococcus* sp. *NJ2*	Glycolipids	Kumari et al. (2014)
Crude oil	*Lactobacillus delbrueckii*	Glycolipid	Thavasi et al. (2011)
Crude oil	Indigenous marine microflora	Rhamnolipids	McKew et al. (2007)
Crude oil	Mixed culture microflora	Rhamnolipids	Abalos et al. (2004)
Crude oil, naphthalene, hexadecane, pristane	Indigenous soil microflora	Sophorolipid	Kang et al. (2010)
Crude oil spill	Indigenous marine microflora	Biosurfactants from strain JE-1058 of *Gordonia* sp.	Saeki et al. (2009)
Oil mud or sludge	*P. aeruginosa and Rhodococcus* sp.	Raw biosurfactant	Cameotra and Singh (2008)
Petroleum hydrocarbon	Indigenous soil microflora	Rhamnolipids	Benincasa (2007)
Hexadecane	*C. tropicalis*	Rhamnolipid	Zeng et al. (2011)
Drill cuttings (oil-based)	Mixed culture microflora	Rhamnolipids	Yan et al. (2011)
Kerosene	Not specified	Mannosylerythritol lipids (MELs)	Hua et al. (2004)
Benzene, toluene, xylene	*R. pickettii* and *A. piechaudii*	3-hydroxy fatty acid	Plaza et al. (2007)
Phenanthrene	*G. etunicatum*	Lipopeptide (*Bacillus subtilis* BS1)	Xiao et al. (2012)
Phenanthrene	Consortium microflora	Biosurfactant from *A. calcoaceticus*	Zhao et al. (2011)
Phenanthrene	*Sphingomonas* sp.	Rhamnolipid	Pei et al. (2010)
Phenanthrene	*B. parabrevis* strain PDM-3	Glycolipid	Reddy et al. (2010)
PAHs	Activated sludge microflora	Rhamnolipid	Sponza and Gok (2011)

(continued)

Table 7.4 (continued)

Pollutants	Biodegrader microbial agent	Biosurfactants	References
PAHs	AMF + alfalfa + bacterial consortium of PAHs biodegraders	Rhamnolipid	Zhang et al. (2010)
Phenanthrene and benzo (a) pyrene	*B. subtilis* strain B-UM.	Biosurfactant of *A. calcoaceticus*	Wong et al. (2010)
Pyrene	*Pseudomonas fluorescens*	Rhamnolipid	Husain (2008)
Anthracene	*Sphingomonas* sp. and *Pseudomonas* sp.	Rhamnolipid	Cui et al. (2008)
Anthracene	*Pseudomonas* sp.	Rhamnolipid	Santos et al. (2007)
Fluoranthene	*Pseudomonas alcaligenes* PA-10	Rhamnolipid	Hickey et al. (2007)
Chlorinated PAHs, nitroaromatic compounds, pesticides			
Polychlorinated biphenyls	Consortium microflora	Rhamnolipid	Viisimaa et al. (2013)
4-chlorophenol	Activated sludge microflora	Biosurfactant	Uysal and Turkman (2007)
PCBs (2, 3, and 4 Cl)	Mixed culture microflora	Rhamnolipids	Fiebig et al. (1997)
Carbazole	*Pseudomonas* sp. GBS.5	Rhamnolipid	Singh et al. (2013)
Beta-cypermethrin	*P. aeruginosa* strain CH7	Rhamnolipid	Zhang et al. (2011)
Copper, zinc	*Bacillus subtilis*, *Pseudomonas aeruginosa*, *Torulopsis bombicola*	Surfactin, rhamnolipid, sophorolipid	Mulligan et al. (2001)
Cadmium, lead	–	Rhamnolipids	Juwarkar et al. (2007)
Copper, zinc, and nickel	–	Rhamnolipid	Dahrazma and mulligan (2007)
Lead, cadmium	*Pseudomonas* sp. strain LKS06	Rhamnolipid	Huang and Liu (2013)
Lead, zinc, copper	*Acinetobacter* sp. *Pseudomonas putida* T1(8)	Rhamnolipids	Hidayati et al. (2014)
Zinc, lead, chromium	Isolates of KDM3, KDM 4, KDM 6	Biosurfactant (not specified)	Vijayanand and Divyashree (2015)

substrates will be more easily degraded by microorganisms because microorganisms are only able to metabolize a compound when available in a soluble form. As with the less to nonsoluble substrate, microorganisms can only metabolize the solute compound alone, while the nonsoluble compounds will remain unchanged. Biosurfactant at cell membrane level demonstrates higher bioactivity. These alterations might consequence in improved hydrophobicity, which is deliberated to be positive in case of bioremediation process (Lawniczak et al. 2013).

7.5 Biosurfactant-Producing Microorganisms

Microorganisms producing biosurfactants as reported by Desai and Desai (1993) and Desai and Banat (1997) consist of various types of bacteria and fungi. Some of these include *Pseudomonas* sp., *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Arthrobacter* sp., *Azotobacter chroococcum*, *Azotobacter vinelandii*, *Bacillus licheniformis*, and *Bacillus subtilis*. Several types of biosurfactants can also be produced from fungi such as sophorolipid produced by *Torulopsis bombicola*, *Torulopsis apicola*, *Candida lipolytica*, *Candida tropicalis*, *Candida antarctica*, and *Candida glabrata*. Biosurfactants based on their constituent structures can be glycolipids, phospholipids, polysaccharides and lipids complex, lipoprotein, and cross-linked and hydroxylated fatty acid and may be a whole cellular structures. The biosurfactants classification based on molecular weight, chemical composition, and its producing microorganisms can be seen in Table 7.5.

7.6 Enhanced Bioremediation Mechanisms

A specific problem that often becomes an obstacle in the process of hydrocarbon biodegradation is the readiness of the target compound as a substrate for immediate degradation by degrading (petrophilic) microorganisms. Mass transfer factor in the use of substrates by bacteria is one of the regulating factors in the process of substrate uptake. Uptake process is strongly influenced by the structure and size of the substrate to be absorbed, while the role of biosurfactant is to break large droplet oil into micelle oil that can be absorbed by bacteria. With the increasing process of micelle oil formation, the bacteria will more quickly obtain the substrate necessary for its growth. The formation of micelle oil occurs by a mechanism that refers to the characteristics of biosurfactants having two structures at once, i.e., hydrophobic and hydrophilic groups. When the surfactant dissolves in a polar solvent due to its hydrophilic nature, distortion occurs in the liquid structure of the solvent so that the surfactant will be more easily transported to the surface of the intercellular layer and lowers the tension on the surface area, while the hydrophobic structure binds to the surface of large droplet oil causing the surfactant to be partially retained in the solution to form micelle structure of micron size, resulting in oil dispersion process in solution (Mahro 2000).

Some of the steps applied to enhance the biodegradation rate of carbon-based compounds with low solubility is by adding biosurfactants. Biosurfactants are produced by microbial cells extracellularly that contain hydrophilic and hydrophobic charges. Biosurfactant affects the biodegradation rate of hydrocarbon compounds and increases the degradation process in two modes by enhancing the dispersion and solubility of hydrocarbon compounds and by regulating the interaction amid microorganism cells and hydrocarbon compounds by diminishing the surface tension between two stages, thus increasing the hydrophobicity of the cell surface of

Table 7.5 Biosurfactants classification, type, and its microbial producers

Classification	Microbial producers	Biosurfactants	Application	References
Glycolipids	*Pseudomonas aeruginosa*, *Serratia rubidaea*, *S. rubidaea* SNAU02	Rhamnolipids	Hydrocarbon removal/ degradation, enhanced oil recovery, removal of heavy metals antifungal	Bai et al. 1997, Nalini and Parthasarathi. 2017, Rahman et al. 2002, Santa Anna et al. 2007 and Whang et al. 2007
	S. marcescens, *Rhodococcus erythropolis*, *Tsukamurella* sp.	Glycolipids	Biological degradation of raw oil	Mulligan et al. 2001
	Candida lipolytica, *C. apicola*, *C. bogoriensis*, *Rhodotorula babjevae* YS3, *Candida bombicola*	Sophorose lipids	Biodegradation of crude oil, heavy metals removal, antifungal	Mulligan et al. (2001) and Sen et al. (2017)
	Corynebacterium spp., *Rhodococcus erythropolis, Nocardia* sp., *Mycobacterium* spp., *Arthrobacter paraffineus*	Trehalose lipids	Antimicrobial, antiviral, antifungal, cosmetics, bioremediation	Singh et al. (2007)
Peptides, lipopeptides	*Bacillus pumilus*, *Bacillus subtilis*, *Bacillus* sp.	Surfactin	Antimicrobial, antiviral, antifungal, bioremediation	Mulligan et al. (2001)
	Azotobacter chroococcum, *Azotobacter vinelandii*	Exopolysaccharide (EPS)	Hydrocarbon removal/ degradation	Suryatmana et al. (2005)
			Soil washing	Levisauskas et al. (2004)
			Emulsifier	Thavasi et al. (2009)
	Pseudomonas sp., *Rhodococcus* sp.	Lipopolysaccharides	Surface-active agent	Van Hamme and Ward (2001)
	Pseudomonas syringae	Syringafactin	Swarming motility	Berti and Thomas (2009)
	Pseudomonas sp.	Arthrofactin	Emulsifications	Elazzazy et al. (2014)

(continued)

Table 7.5 (continued)

Classification	Microbial producers	Biosurfactants	Application	References
	B. licheniformis	Lichenysin	Heavy metal removal	Zouboulis et al. (2003)
	Bacillus amyloliquefaciens	Bamylocin	Emulsification	Lee et al. (2007)
	Streptococcus mutans, *S. salivarius*	Lantibiotics	Antibiotic, food industry, agriculture	Gomes et al. (2017)
	Paenibacillus polymyxa	Polymyxins	Antibiotics	Deng et al. (2011)
	Streptomyces sp.	Streptofactin	Surface-active agents	Straight et al. (2006)
	Pseudomonas fluorescens	Viscosin	Surface-active agents	Neu et al. (1990)
Lipids, fatty acids	*Penicillium spiculisporum*, *Capnocytophaga* sp., *Nocardia erythropolis*	Fatty acids	EOR	Makkar and Cameotra (1999)
	Acinetobacter sp.	Phospholipids	Emulsion breaker	Singh et al. (2007)
	Clostridium sp.	Neutral lipids	Surface-active compounds	Christofi and Ivshina (2002)
Polymeric biosurfactants	*Acinetobacter* spp.	Emulsan	Emulsifier	Christofi and Ivshina (2002)
	Candida lipolytica	Liposan	Emulsifier	Cirigliano and Carman (1984)
	Acinetobacter radioresistens	Alasan	PAH biodegradation, emulsification	Mulligan (2005)
	Acinetobacter calcoaceticus	Biodispersan	Emulsifier	Rosenberg et al. (1988)
	Acinetobacter calcoaceticus	Particulate surfactant	Emulsifier	Rosenberg (1993)

the microorganism. The chief motives for enhanced perseverance of higher molecular weight hydrophobic compound are their poor solubility in water, thus making it unfavorable for microorganisms to utilize as carbon source. Biodegradation is inhibited when carbon-based compounds are irreversibly bound to the surfaces. The biosurfactant with its amphipathic structure can augment growth of microbes certain substrates by increasing substrates solubility in water or by desorbing them from the surfaces (Ron and Rosenberg 2002). On the other hand, when the biosurfactant is dispersed in water with very less concentration, the biosurfactant will form a monomer. Under these circumstances, hydrophobic tail does not form the bonds of hydrogen and disrupts the surrounding structure of water, causing an increase in the system's free energy. At higher concentrations of biosurfactants, the free energy can be decreased by aggregating biosurfactant molecules into micellar

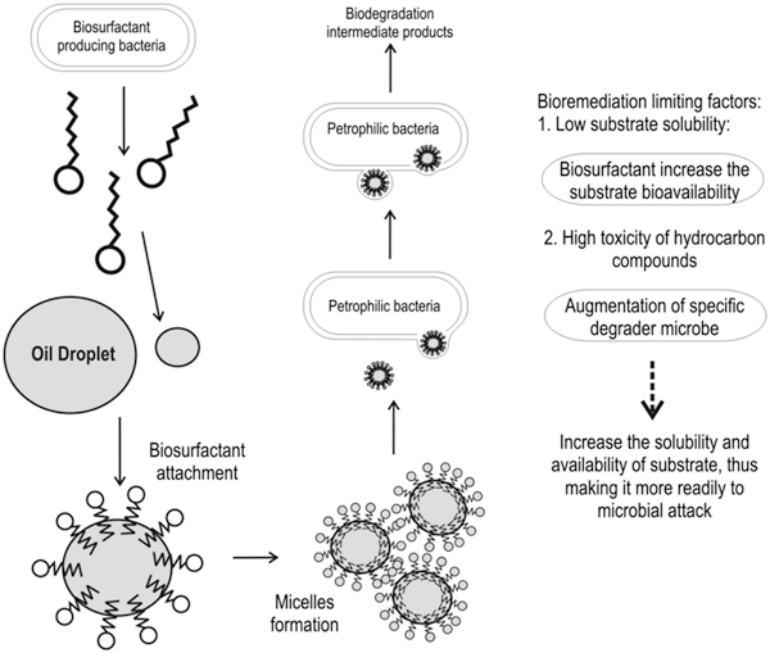

Fig. 7.7 Mechanisms of microbial degradation of hydrophobic compound with the aid of biosurfactants

structures, where the hydrophilic heads are uncovered to the aqueous water stage, while hydrophobic tails are attached within the micelle structure (Banat et al. 2010). Mechanisms of microbial degradation of hydrophobic compound with the aid of biosurfactants are shown in Fig. 7.7.

An assuring technique that can enhance biodegradation efficiency of hydrophobic compounds is the employment of natural biosurfactants. Biosurfactants can improve a low solubility compounds biodegradation by two mechanisms.

1. Enhancement of the solubility by means of emulsification of the hydrophobic compounds, making it more available for microorganisms attack
2. Facilitated transport and mobilization of hydrophobic compounds, allowing it to connect more with microbial cells

By decreasing the interfacial and surface tension with biosurfactant addition, the surface area of hydrophobic compound will increase, which leads to enhanced bioavailability and mobility. The emulsification mechanisms are promoted by high molecular mass of biosurfactants, whereas the mobilization mechanisms are promoted by low molecular type of biosurfactants (Bustamantee et al. 2012). The bioremediation success is managed by three significant parameters, i.e., microbial availability, pollutants availability, and favorable environmental factors. Bioremediation efficacy depends on the ability of microbes to biodegrade difficult combinations and their rate-limiting kinetics (Cameotra and Makkar 2010). Owing

to the high water solids distribution and hydrophobicity ratio, these pollutants try to interrelate with the aqueous phase and soil carbonaceous substance and develop unavailability for microbial biodegradation because bacterial cells are well known to biodegrade contaminants which are soluble in water. To achieve a well-organized, effectual, and comprehensive biological degradation process, hydrocarbon solubilization with biosurfactant prior to the bioaugmentation is recommended. In addition, augmentation of biosurfactants producing microorganisms along with hydrocarbon degrading microorganisms in the bioremediation process offers the benefit of a sustainable allocation of nonnoxious and cost-effective biosurfactants (Moran et al. 2000; Rahman et al. 2003; Kardena et al. 2015).

7.7 Challenges

Despite the many advantages possessed by biosurfactants when compared to synthetic surfactants, the most important biosurfactant weaknesses are in the case of large-scale production. A large quantity of surfactant is required by the petrochemical industry used in its environmental applications. In addition, the problem of getting pure compounds from biosurfactant becomes one of its weaknesses. The pure compounds of surfactants are essential in the pharmaceuticals, food, and cosmetics industries (Kosaric 1992). With regard to the real field-scale biosurfactant application for enhanced biological remediation process, the biological mediators (biosurfactant-producing microbes and/or pollutant degrading microbes) can be externally added. It can be done by spraying of the microorganisms or their secondary metabolites on to the polluted sites, or these can be produced on location which could demonstrate very encouraging results for remediation.

Consecutive steps that should be considered in designing biosurfactant-enhanced bioremediation process include:

(a) Suitable biosurfactants selection and/or we may go for those microbes which produce biosurfactants and preferentially are from native microflora, e.g., rhizobacteria or endogenous soil microbes isolated from contaminated site. Suitable biosurfactant applications refer to its potential toxicity when launched to the surrounding ecosystem. The noxiousness of biosurfactant may occur in the entire ecosystems or toward surrounding degrading microorganisms. Many researchers reporting the adverse capability of biosurfactant such as the toxicity potential.
(b) Evaluation of probable biosurfactants produced toxicity, if any. The surface-active substances themselves may lead to pollution when launched in the ecosystem. The noxiousness of biosurfactants could be toward the microbes or toward environmental system; thus it might inhibit the biological degradation of contaminants. Ivshina et al. (1998) reported the toxicity potential of glycolipid biosurfactant produced from *Rhodococcus ruber* AC 235 with effective concentration (EC50) of 650 mg/l. Earlier study by Munstermann et al. (1992) found that rhamnolipid biosurfactant from *P. aeruginosa* with EC50 of 50 mg/l, treha-

lose dicorynomycolate, and trehalose tetraester produced from *Rhodococcus erythropolis* has the EC50 of 49 mg/l and 286 mg/l, correspondingly. The major mechanisms of biosurfactant toxicity are the cell membrane disruption by interaction with the lipid component and also the reaction of biosurfactant molecule with protein that are indispensable for cell function. This assessment may be a consideration because of the antimicrobial and antifungal attributes of several biosurfactants to other microbial diversities in its surrounding environment although the toxicity of biosurfactants is very low compared to its synthetic counterparts (Klosowska-Chomiczewska et al. 2011). Surfactin biosurfactants produced by *Bacillus* sp. are known to have adverse properties to other microorganisms. Many studies have revealed that some biosurfactants facilitate the mechanisms of organisms biocontrol such as opposition and parasitism activities (Mujumdar et al. 2016).

(c) Selection of methods for field-scale applications. Direct spraying of cell-free cultivated broths is more advantageous since purification process of biosurfactants is very expensive (Kardena et al. 2013). The downstream process for biosurfactants purification can reach about 50% to 60% of the total production cost. High production costs as well as refining processes are incompatible with environmental remediation applications that require low-cost and high-volume biosurfactants (Lotfabad et al. 2016).

(d) The use of low-cost substrates to produce biosurfactant. Production costs are a major constraint in biosurfactant production, estimated in most biotechnology processes that raw material accounts for 20–30% of total cost of production (Chong and li 2017). Strategies to reduce these costs are to use cost-effective raw substances such as waste materials, plant-derived oils, waste oils, starches, lactate water, and refinery waste for biosurfactant production (Makkar and Cameotra 1999). On-site biosurfactants production is suggested to overcome the production economy for its application in environmental remediation.

7.8 Case Study: From Lab Scale to Field Scale

Bioremediation is an activity undertaken to remediate contaminated site that requires an understanding of many chemical, physical, and biological phenomena. Treatability studies conducted on a lab or bench scale may require adjustment if applied on a pilot and full-scale applications (Whyte et al. 2001; Kim et al. 2005; Lamichhane et al. 2012). The purpose of the treatment study is to confirm that prearranged remedial actions have a high degree of achievement. The type and number of treatment findings depend on site qualities and types of pollutants. The effort levels that is anticipated in the treatment study is proportional to ambiguity about location characteristics and contaminants, whereas less ambiguity signifies less attempt can guarantee the accomplishment, while higher ambiguity may require widespread efforts to safeguard successfulness of applied treatment (Rittmann and McCarty 2001). The methods of treatment including laboratory evaluation, pilot studies, and field demonstrations are shown in Fig. 7.8.

Fig. 7.8 Treatment study of petroleum oil-polluted soil: from flask to laboratory microcosm (upper) and large-scale presentation (lower)

Treatability assessment is useful for determining the potential for successful bioremediation project: whether the biodegradation rate can be accelerated, how to conduct the best way to do process engineering in the field, and how to verify the bioremediation process has occurred. These issues cannot be properly addressed by observations made at a single scale alone (Zhang et al. 2012). A number of laboratory-scale studies (see Table 7.5) have reported the role of biosurfactants in pollutant removal/degradation, washing/leaching, and enhanced solubilization, but some information are found in research papers on comprehensive field implementation of this method.

In a study reported by Kardena et al. (2017), a bench scale of crude oil biodegradation where biosurfactants produced from *Pseudomonas aeruginosa* PAU01 (BS-1) and *Burkholderia* sp. PAU02 (BS-2) also Tergitol NP-10 surfactant were tested in its performance in enhancing the total petroleum hydrocarbon (TPH) removal process by petrofilic (PF) consortia. Results shows that both biosurfactants have superior performance in enhancing the biodegradation process compare with its synthetic counterpart (Fig. 7.9). Control reactor contains petrofilic bacteria alone able to decrease the TPH content from initial 5% down to 2.62% with 47.4% removal efficiency after 10 days of incubation time. Biosurfactant produced from *Pseudomonas aeruginosa* PAU01 and *Burkholderia* sp. PAU02 could enhance the efficiency of the petrofilic culture in degrading crude oil. Reactor with addition of PF + BS-1 and PF + BS-2 able to decrease the TPH content from initial 5% down to 1.72% and 1.68% with its removal efficiency of 65.4% and 66%, respectively. Contrary results is shown in the reactor with addition of Tergitol NP-10, where the addition of synthetic surfactant tends to inhibit the efficiency of the petrofilic culture in degrading crude oil. PF + Tergitol NP-10 able to decrease the TPH content from

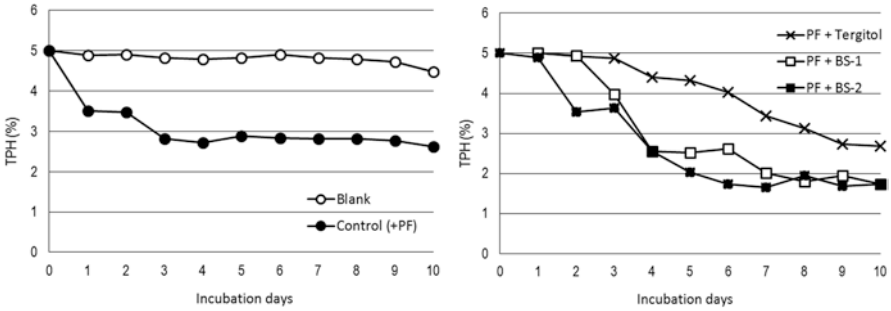

Fig. 7.9 Biodegradation assay of crude oil in a bench scale (left) with addition of petrofilic bacteria/PF in control (-●-) and blank (-○-), (right) performance of Tergitol (-x-), BS-1/*Pseudomonas aeruginosa* PAU01 (-□-), and BS-2/*Burkholderia* sp. PAU02 (-■-) in enhancing the biodegradation process

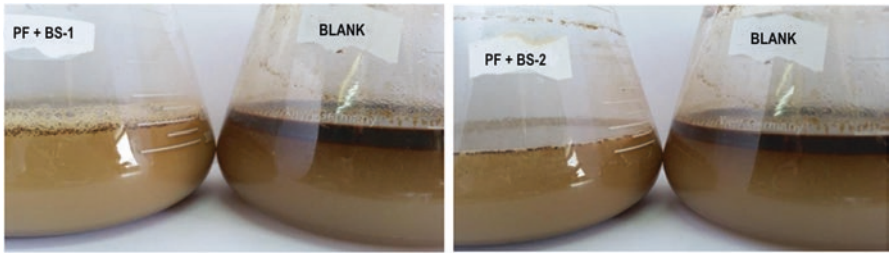

Fig. 7.10 Visual image of bench-scale reactor PF + BS-1 (left); PF + BS-2 (right) compared with blank reactor. There is no significance oil layer was found in the PF + BS-1 and PF + BS-2 reactors compare with blank reactor, indicating that biosurfactants able to emulsifying oil in the water phase

initial 5% down to 2.84% with 39% removal efficiency observed (lower removal efficiency compared with control reactor) (Fig. 7.10).

Contaminant removal rate observed at the bench or laboratory scale may not necessarily apply at the pilot-scale demonstration. Observed contaminant removal rates in the pilot-/field-scale, for example, in general likely to be 4–10 times prolovnged than laboratory-determined values. Bioremediation project engineer must consider all relevant phenomena to determine which will limit contaminant biodegradation rate for a particular site, because in the full-scale project, the site are typically heterogeneous which can cause different phenomena to limit biodegradation rates. Kildisas et al. (2003) reported the pilot-scale treatment of oil-contaminated soil by washing. The applied technology comprises washing/leaching of the migration fraction with the biosurfactants application, separation of oil from water and soil, and biological degradation of remaining oil fraction which was not migrated. Kosaric (2001) reported an application field of biological remediation at numerous contaminated locations in Middle East region and Canada with addition of biosurfactants to the microbial growth medium. These locations represent sand and soil polluted by petro-hydrocarbons, mainly originated from industries. Biological

remediation was enhanced when nutrients and glycolipids biosurfactant was supplemented at a concentration of 0.5 kg of biosurfactant/ton of soil was applied. Christofi and Ivshina (2002), in Russia (Perm region), demonstrated a field scale of biopile biological remediation of raw oil-polluted soil in oil fields. The introduction of biosurfactant from *Rhodococcus erythropolis* and *Rhodococcus ruber* into petrohydrocarbon polluted soil and enhanced biological remediation process by 20–25%. Instantaneous employement of *R. ruber* and *R. erythropolis* demonstrated as an effective and efficient microbes. This treatment within a period of 3 months caused a decrease of 75.5% oil amount. Martienssen et al. (2003) also showed the efficacy of Bio Versal FW surfactant for in situ bioremediation of extremely polluted locations at Halle-Saale, Germany. The treatment might attain elimination of 50 g petrohydrocarbons/kg of soil in 15 months duration under field studies. Kardena et al. (2015) reported the field-scale bioremediation project designed with addition of both biosurfactant-producing microbe and petrophilic degrader microbe that were able to remove total 46 g petro-hydrocarbon/kg soils from 4883 cubic meter of polluted oil mud during a treatment period of 16 months (Fig. 7.11). Both petrofilic

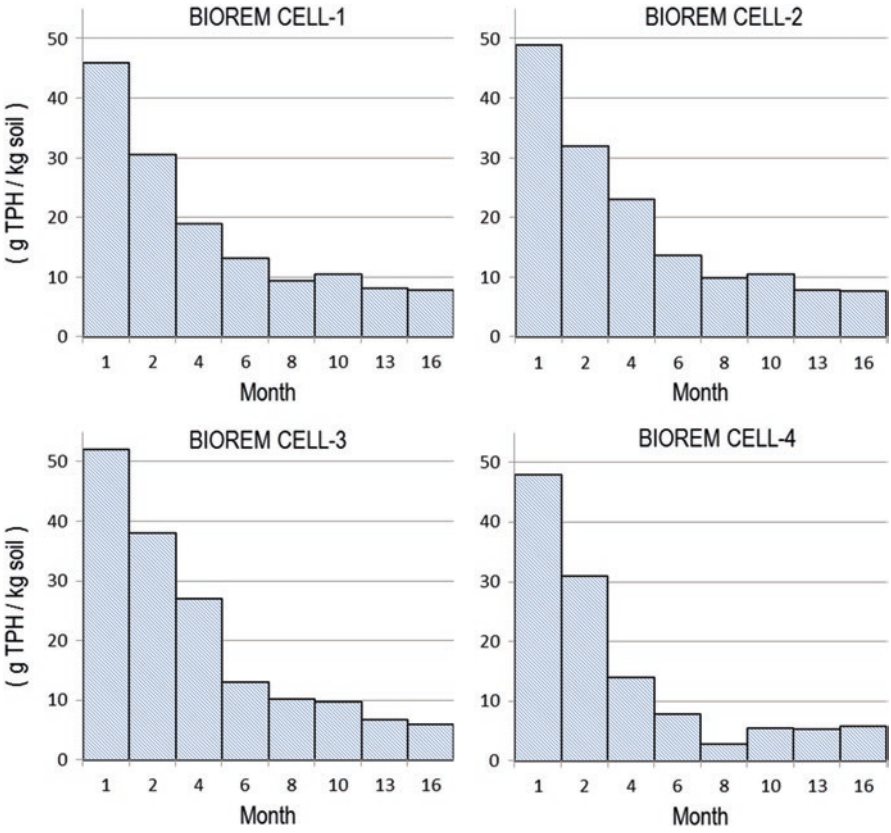

Fig. 7.11 Field-scale application performance of 4883 cubic meter of oil mud-polluted soil biological remediation procedure with an average original oil content of 48.7 g TPH/kg soil

Fig. 7.12 On location production of petrofilic consortia and biosurfactants producing microbe on a 56 m^3 tank to enhanced the bioremediation process performance, (upper image) bioreactor installation, (lower image) nutrient and carbon source addition

consortia and biosurfactant-producing bacteria were produced on-site on a large-scale bioreactor as shown in Fig. 7.12.

7.9 Conclusions

One promising method to enhance the bioremediation performance of petroleum oil-polluted ecosystems is the application of effective biosurfactant agents. The biosurfactants may augment petroleum oil biological remediation by means of increasing the bioavailability of substrates for various microorganisms' attack. On the other hand, the biosurfactants also lead in interacting with microbial cell surface. This helps in lowering of the surface interfacial tension and allows the hydrophobic substances to combine easily with microorganisms. In any case, biosurfactants might encourage petroleum biodegrading bacterial strains by providing co-substrates and improve their capability to consume hydrophobic substances as their

energy source. The efficiency of biosurfactants for enhancing biodegradation of contaminants necessitates a consideration of the biological availability of target pollutant. The proliferation, survival, and activities of the augmented microbial cultures (both biosurfactant-producing and contaminant biodegrading microbes) along with indigenous communities will affect the degradation process. Another factor will be in understanding the concept of interaction amid biosurfactants and its producing bacterial strains, and the climatic conditions also determine the rate and efficiency of biological remediation of petro-hydrocarbons. Addition of biosurfactant as reported by many researchers can stimulate some organisms, but some case have adverse effect to the growth of some microorganisms. Suggested strategy suitable for effective bioremediation would be to stimulate biosurfactants produced by indigenous degrading population that found to be already present and well adapted at the contaminated site. The effective microbiological approach in biological remediation of petroleum oil-contaminated soil is the application of biosurfactants product without essentially characterizing or purifying the surface-active components chemical structures. Biosurfactants contained in broth media (without cells) could be utilized straightforward or it may be diluted (if required) suitably to the polluted locations to enhanced the biodegradation process.

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Chapter 8
Modeling Applications in Bioremediation of Hydrocarbon Pollutants

Mahmoud Nasr

Abstract This chapter addressed various statistical and modeling techniques that have been recently employed for studying the bioremediation of hydrocarbon pollutants. Isotherm adsorption models such as Temkin, Freundlich, Langmuir, and Dubinin-Radushkevich were used for describing the removal of hydrocarbon contaminants from aqueous phases. Statistical techniques, viz., regression analysis, quadratic model, and response surface methodology, were performed to demonstrate the effects of operational conditions on the remediation of water contaminated with hydrocarbon. Artificial intelligence including artificial neural network (ANN) and fuzzy inference system (FIS) was also presented as a black-box model for the prediction of hydrocarbon removal efficiencies. In addition, this chapter included literature studies that have implemented advanced modeling techniques within the field of hydrocarbon bioremediation.

8.1 Introduction

The term "bioremediation" is used to define the reduction, degradation, detoxification, and mineralization of pollutants via biological mechanisms (Olawoyin 2016). The objective of bioremediation is to transform contaminants into less harmful substances using microorganism and biomasses. Bioremediation technologies are classified into in situ and ex situ, depending on several factors such as source and concentration of pollutants, site characteristics and type, and cost saving (Sanusi et al. 2016). The bioremediation process undergoes a high degree of nonlinearity regarding physical, chemical, and biological reactions. In addition, bioremediation is influenced by several factors such as medium pH, temperature, aeration rate, agitation speed, and substrate to inoculum ratio (San-Valero et al. 2015). Hence, a significant effort should be exerted for developing adequate modeling techniques that can address the performance of bioremediation.

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The term "modeling" is used to describe a particular system using mathematical language that comprises a set of factors, variables, and equations (Bas and Boyacı 2007). The designed models should be able to simulate, predict, and control the behavior of the system under study with a reasonable accuracy. Modeling of the bioremediation process is an essential procedure for reactor design and performance prediction (Nasr et al. 2017). The bioremediation process can be addressed by either a white-box model (also known as deterministic models, physically based models, or knowledge-driven models) or a black-box model due to missing process information (Pakravan et al. 2015). In white-box systems, the process variables are transformed into a number of mathematical equations. However, this type of model requires all necessary information and a lot of assumption to improve the prediction accuracy (Zadeh 1997). Black-box models are used to provide an adequate description of a system when the process knowledge is not enough. Artificial intelligence, which is defined as a black-box model, can be used for the prediction of nonlinear and complex systems. Multivariate analysis is another reliable black-box modeling technique that can be employed as a statistical tool for isolation, monitoring, and assessment (Alalm et al. 2016). Other modeling methods such as hybrid and stochastic gray-box systems have been employed in bioremediation studies for the determination of microorganisms' activities.

Sequential steps should be conducted to develop a reliable model. The procedures include (a) problem identification, (b) model selection, (c) data collection and preparation, (d) model calibration and parameters estimation, (e) model validation, and (f) testing and scenario evaluations (Nasr et al. 2014). Model calibration is an important step, which is used to find a reliable explanation of a particular set of data. During calibration, the model parameters are adjusted to improve fitting accuracy (Fawzy et al. 2017). The parameters used as initial conditions can be obtained from the literature. In the validation procedure, the readings not used for calibration are compared with the model outputs to obtain a reliable model. The model inadequacy can result from different sources such as input and output data, physical properties and configuration of the system, operational conditions, and model structure (Panja et al. 2017).

Several hazardous pollutants can result from sewage, hydrocarbons, dyes, agrochemicals, chlorinated compounds, and heavy metals. Hydrocarbons are considered as an essential cause of environmental damage and several health risk problems (Nwadiogbu et al. 2016). Most studies on bioremediation have focused on hydrocarbons due to their toxic impact on soil and groundwater. In addition, aquatic systems receive significant variations in wastewater discharge and composition, which may contain multiple hydrocarbon contaminants (Srinivasan and Viraraghavan 2010a, b).

This chapter attempted to cover different modeling and statistical techniques that have been recently employed for describing the bioremediation of hydrocarbons. Different statistical and artificial intelligence methods were used to represent the highly complex models that undergo the bioremediation process. The application of adsorption isotherm models such as Temkin, Freundlich, Langmuir, and Dubinin-Radushkevich was also demonstrated. In addition, this work covered literature studies that have employed reliable techniques within the field of hydrocarbon bioremediation.

8.2 Stoichiometry and Kinetics of Bacterial Activity

Several microorganisms have been found to have important applications in the bioremediation of hydrocarbon-contaminated water (Nasr and Ismail 2015). The biological activities of these organisms are influenced by various physicochemical and environmental parameters. Some mathematical models are based on the theory that microorganisms can utilize hydrocarbons from the aqueous medium. Other models are used to couple mass transfer with Monod or first-order kinetics for hydrocarbon biodegradation (Boparai et al. 2011).

Monod equation, as expressed by Eq. (8.1), is a kinetic model employed to determine the microbial growth via the correlation between substrate concentration and specific growth rate (Ateia et al. 2015). Monod-type model is also used to predict the substrate removal efficiencies in bioremediation processes at a large-scale application.

$$\mu = \mu_{\max}\left(\frac{S}{S + K_S}\right) \tag{8.1}$$

where μ is specific growth rate constant (1/day), $\mu_{\max}$ is maximum specific growth rate (1/day), S is limiting substrate concentration (mg/L), and K_S is half-saturation constant (mg/L), provided at $\mu = 0.5\mu_{\max}$. The correlation between μ and S is used to estimate the bio-kinetic growth constants (i.e., $\mu_{\max}$ and K_S) by either statistical or graphical technique.

The Monod equation can be employed to calculate the bacterial growth rate, as given by Eq. (8.2).

$$\frac{dX}{dt} = \mu X \tag{8.2}$$

where dX/dt is biomass growth rate (mg/L/d) and X is biomass concentration (mg/L).

The stoichiometric correlation between the consumed substrate and produced biomass can be presented by Eq. (8.3).

$$\frac{dX}{dt} = Y\frac{dS}{dt} - k_d X \tag{8.3}$$

where Y is the stoichiometry of biomass yield coefficient (dimensionless) and k_d is the kinetic rate of cell decay (1/day).

The specific substrate utilization rate is calculated by Eq. (8.4).

$$U = \frac{dS}{X \cdot dt} \tag{8.4}$$

where U is the specific substrate utilization rate (1/day) and dS/dt is substrate utilization rate (mg/L/d).

As presented by Eq. (8.5), a plot of μ versus U results in a linear line having a slope of Y and an intercept of k_d.

$$\mu = Y \times U - k_d \tag{8.5}$$

8.3 Bacterial Behavior in a Controlled Batch System

Figure 8.1 displays a batch reactor that contains an initial substrate concentration (S_o) and a biomass concentration (X). The reactor is operated under an aerobic and completely mixed condition; thus, the concentration of dissolved oxygen (DO) is not a limiting factor for bacterial growth. During batch experiments, microorganisms utilize substrate for synthesis of new cells, energy generation, and formation of by-products (Eq. 8.6). Hence, as time proceeds, the substrate concentration decreases (negative dS/dt) along with an increase in the microorganisms concentration (positive dX/dt).

$$\text{Substrate} + \text{Biomass} \rightarrow \text{New cells} + \text{Energy} + \text{Byproducts} \tag{8.6}$$

Figure 8.2 shows a plot of biomass concentration versus time, resulting in a growth curve that contains five distinct phases. The phases can be illustrated as follows (Gupta et al. 2017):

(a) Lag phase, which diminishes when the cells are acclimated (adapted) to the environmental condition. This phase occurs directly after bacterial inoculation.
(b) Exponential phase, where the biomass concentration increases steadily due to the utilization of substrate for growth.
(c) Stationary phase that occurs when essential substrates (e.g., carbon and nutrient species) and/or DO reach a threshold level. Under this condition, the bacterial population is neither growing nor decreasing.
(d) Death phase, in which some bacterial cells are damaged due to death and lysis. Under this environment, the net biomass growth becomes negative.

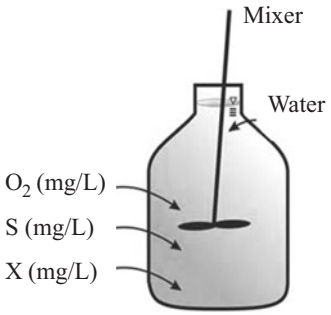

Fig. 8.1 Mixed batch reactor supplied with substrate, inoculum, and dissolved oxygen

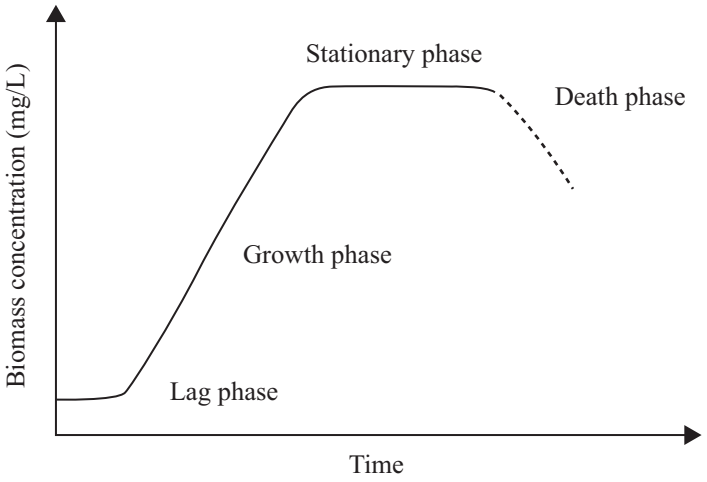

Fig. 8.2 Typical biomass growth curve in a batch system

8.4 Mathematical Modeling of Trickling Filter for Bioremediation Application

San-Valero et al. (2015) developed a mathematical model used for estimating the removal of the hydrophilic volatile organic compound by biotrickling filters. Their study reported that the mass balance of pollutant (or DO) in the gas phase could be represented by Eq. (8.7).

$$\theta_{\mathrm{G}} \frac{\partial C_{\mathrm{G}}}{\partial t} = -v_{\mathrm{G}} \frac{\partial C_{\mathrm{G}}}{\partial z} - K_{\mathrm{L}} a \left(\frac{C_{\mathrm{G}}}{H} - C_{\mathrm{L}} \right) \tag{8.7}$$

where C_{G} and C_{L} are concentrations of gas and liquid phases, respectively (mg/L), $K_{\mathrm{L}}a$ is mass transfer coefficient (1/s), H is Henry's law constant (dimensionless), t is time (s), z is vertical distance from the bottom of the reactor (m), v_{G} is superficial air velocity (m/s), and θ_{G} is porosity of the bed (dimensionless).

The mass balance of the mobile liquid phase is given by Eq. (8.8).

$$\theta_{\mathrm{L}} \frac{\partial C_{\mathrm{L}}}{\partial t} = -v_{L} \frac{\partial C_{\mathrm{L}}}{\partial z} - K_{\mathrm{L}} a \left(\frac{C_{G}}{H} - C_{\mathrm{L}} \right) - \frac{D \cdot a}{\beta} \left(C_{\mathrm{L}} - S \right) \tag{8.8}$$

where D is diffusion coefficient of contaminant (or DO) in water (m^2/s), a is specific surface area of the packing medium (m^2/m^3), β is the thickness of liquid-biofilm interface (m), S is the concentration of pollutant (or DO) in biofilm interface (mg/L), and v_{L} is superficial liquid velocity (m/s).

The mass balance of the biofilm is represented by Eq. (8.9).

$$\frac{\partial S}{\partial t} = f\left(X_v\right) D \frac{\partial^2 S}{\partial x^2} - \frac{\mu_{max} X_v}{Y} \frac{S_O}{S_O + K_O} \frac{S_P}{S_P + K_P} \tag{8.9}$$

where S is concentration inside biofilm (mg/L); $f(X_v)$ is correction factor of diffusivity in solution due to biomass (dimensionless); X_v is the concentration of biomass (mg/L); μ_{max} is maximum specific growth rate (1/s); K_O and K_P are the half-saturation constants of oxygen and pollutant, respectively (mg/L); and Y is yield coefficient (dimensionless).

8.5 Adsorption Models

Adsorption isotherms and kinetics are appropriate models that can be used to investigate the removal of hydrocarbons from water bodies (Fawzy et al. 2016a). The most common isotherm models are Langmuir, Freundlich, Dubinin-Radushkevich (D-R), and Temkin. In addition, pseudo-first-order and pseudo-second-order are performed for examining the kinetic studies of adsorption.

8.5.1 Langmuir Adsorption Isotherm

Langmuir isotherm is applied to quantitatively describe the transfer of pollutants from the aqueous solution to the solid surface at equilibrium. Langmuir model has been developed according to the following assumptions (Langmuir and Waugh 1940):

(a) Monolayer coverage; i.e., the outer surface of adsorbent is covered by a single layer of adsorbate.
(b) The solid surface contains a finite number of vacant pores, where each site occupies one molecule, and no interaction occurs among adsorbate species.
(c) The solid surface is homogeneous; i.e., adsorption sites are identical (equal size and shape), and the heat of adsorption is uniform for each site.

The Langmuir model in Eq. (8.10) demonstrates that a linear plot of C_e/q_e against C_e gives a slope of $1/Q_m$ and an intercept of $1/(K_L \cdot Q_m)$.

$$\frac{C_e}{q_e} = \left(\frac{1}{Q_m}\right) C_e + \frac{1}{K_L \cdot Q_m} \tag{8.10}$$

where C_e is the concentration of adsorbate at equilibrium (mg/L), q_e is the milligram of adsorbate per gram of adsorbent at equilibrium (mg/g), Q_m is the maximum monolayer capacity (mg/g), and K_L is the Langmuir isotherm constant (L/mg).

The Langmuir-type adsorption is then used to determine the isotherm shape in terms of a separation factor (Li et al. 2010); see Eq. (8.11).

$$r = \frac{1}{1 + K_{\mathrm{L}} \cdot C_{\mathrm{o}}} \tag{8.11}$$

where r is a separation factor (dimensionless), K_{L} is Langmuir constant (L/mg), and C_{o} is initial adsorbate concentration (mg/L).

The factor "r" is used to evaluate the favorability of Langmuir adsorption based on the following classifications: "unfavorable" at $r > 1$, "linear" at $r = 1$, "favorable" at $0 < r < 1$, and "irreversible" at $r = 0$.

8.5.2 Freundlich Adsorption Isotherm

Freundlich isotherm model is developed to describe the adsorption of a single solute onto heterogeneous surfaces (Freundlich 1906). The model describes the distribution of adsorbate between the solid and liquid phases, assuming an exponential distribution of adsorption energies. The Freundlich formula in Eq. (8.12) implies that a plot of $\log(q_{\mathrm{e}})$ against $\log(C_{\mathrm{e}})$ results in a linear form with a slope of $(1/n)$ and an intercept of $\log(K_{\mathrm{F}})$.

$$\log\left(q_{\mathrm{e}}\right) = \left(\frac{1}{n}\right)\log\left(C_{\mathrm{e}}\right) + \log\left(K_{\mathrm{F}}\right) \tag{8.12}$$

where K_{F} is Freundlich constant indicating the adsorption capacity $((\mathrm{mg/g})\cdot(\mathrm{L/mg})^{1/n})$, and 1/n represents surface heterogeneity or adsorption intensity, in which the sorbent surface is more heterogeneous at 1/n close to zero.

The Freundlich exponent "1/n" reveals the type of isotherm, which is "unfavorable" at $1/n > 1$, "favorable" at $0 < 1/n < 1$, and "irreversible" at $1/n = 0$ (Saruchi and Kumar 2016). The value of 1/n lower than 1 implies a chemisorption process, whereas $1/n > 1$ indicates a cooperative process.

8.5.3 Dubinin-Radushkevich (D-R) Isotherm

Dubinin-Radushkevich (D-R) isotherm model assumes that the sorption mechanism undergoes pore-filling rather than layer-by-layer surface coverage. The model is applied to structurally homogeneous systems, i.e., micropores having similar dimensions (Hutson and Yang 1997). In addition, this isotherm is temperature dependent and valid for physical adsorption processes involving van der Waals forces (Boparai et al. 2011).

The linearized form of D-R isotherm equation is expressed by Eq. (8.13). A plot of $\ln(q_e)$ versus ε^2 results in a straight line having a slope = $-K_{DR}$ and an intercept = $\ln(q_s)$.

$$\ln\left(q_e\right) = -K_{DR}\left(\varepsilon^2\right) + \ln\left(q_s\right) \tag{8.13}$$

where q_e is the milligram of adsorbate per gram of adsorbent at equilibrium (mg/g), q_s is the theoretical isotherm saturation capacity (mg/g), K_{DR} is D-R isotherm constant that describes adsorption energy (mol^2/kJ^2), and ε is Polanyi potential or the mean free energy (kJ/mol).

The Polanyi potential can be calculated by Eq. (8.14).

$$\varepsilon = RT\ln\left[1 + \frac{1}{C_e}\right] \tag{8.14}$$

where R is the gas constant (8.314 J/mol/K) and T is the temperature (K).

As seen in Eq. (8.15), the value of K_{DR} is used to determine the mean sorption energy.

$$E = \frac{1}{\sqrt{2K_{DR}}} \tag{8.15}$$

where E is the mean sorption energy (kJ/mol).

8.5.4 Temkin Isotherm

The Temkin isotherm model describes the interaction effect of adsorbent/adsorbate in terms of the binding heterogeneity (Temkin 1941). The model assumes that the heat of adsorption of the molecules in a particular layer decreases linearly rather than logarithmically while neglecting deficient and high concentrations (Aljeboree et al. 2014). In this isotherm, adsorption is characterized by a uniform distribution of binding energies up to a certain extent. The Temkin isotherm is presented by Eq. (8.16), which shows that a linear plot of q_e vs. $\ln(C_e)$ gives a straight line with a slope = B and an intercept = $B{\cdot}\ln(A_T)$ (Boparai et al. 2011).

$$q_e = B\cdot\ln\left(C_e\right) + B\cdot\ln\left(A_T\right) \tag{8.16}$$

where A_T is the equilibrium binding constant equivalent to maximum binding energy (L/mol) and B is a constant related to the heat of sorption (J/mol), and it equals $R{\cdot}T/b_T$, in which R is the universal gas constant (kJ/mol/K), T is the adsorption temperature (K), and b_T is Temkin isotherm constant.

8.5.5 Pseudo-First-Order Kinetic

Pseudo-first-order model assumes that the interaction between sorbate and sorbent occurs due to hydrogen bonds and/or van der Waals forces, suggesting that the reaction is possibly physisorption (Saruchi and Kumar 2016). The formula of Eq. (8.17) presents the linear equation of the pseudo-first-order model. A plot of $\ln(q_e - q_t)$ versus t obtains a straight line with a slope of k_1 and an intercept of $\ln(q_e)$.

$$\ln\left(q_e - q_t\right) = -k_1 \times t + \ln\left(q_e\right) \tag{8.17}$$

where q_e and q_t are the milligram of adsorbate per gram of adsorbent at equilibrium and time t, respectively (mg/g), and k_1 is the pseudo-first-order rate constant (1/min).

8.5.6 Pseudo-Second-Order Kinetic

Pseudo-second-order model assumes that electrons are covalently exchanged between adsorbate and adsorbent via chemical interaction, also known as chemisorption (Fawzy et al. 2016b). According to Eq. (8.18), a plot of t/q_t against t results in a linear relationship with slope and intercept of $1/q_e$ and $1/(k_2 \times q_e^2)$, respectively.

$$\frac{t}{q_t} = \left(\frac{1}{q_e}\right)t + \frac{1}{k_2 \times q_e^2} \tag{8.18}$$

where k_2 is the rate constant of second-order adsorption (g/mg/min).

8.5.7 Application of Adsorption for Hydrocarbon Remediation

Okiel et al. (2011) investigated the adsorption of oil from oil-contaminated effluents using deposited carbon (DC), bentonite, and powdered activated carbon (PAC). Their study found that at initial oil concentration of 1000 mg/L and for 30 min, the adsorption capacities were 250, 244, and 150 mg/g for DC, bentonite, and PAC, respectively. In addition, Freundlich isotherm provided a better description of the adsorption data rather than Langmuir model.

Rasheed et al. (2016) investigated the removal of polycyclic aromatic hydrocarbons, namely, anthracene and pyrene, from wastewater using PAC. Their study found that the removal efficiency of hydrocarbons was above 99% after an adsorption time of 4 h. The experimental data fitted well to Elovich model, suggesting that chemisorption was dominant during the adsorption process.

Nwadiogbu et al. (2016) investigated the treatment of oil spill through adsorption onto an agricultural waste of corncobs. Their study indicated that the adsorption process was described by surface reaction and intraparticle diffusion mechanisms. In addition, Langmuir isotherm provided a better fit to the adsorption data than the Freundlich model, and the maximum monolayer sorption capacities ranged between 0.0043 mg/g and 0.0768 mg/g.

Li et al. (2010) studied the application of coal for remediation of oily wastewater. The experimental factors were medium pH, oil concentration, coal type, particle size distribution, and contact time. Their results indicated that the equilibrium time was 1.5 h and the adsorption process followed the Freundlich isotherm. The adsorption capacities were 23.8 and 840.0 mg/g at initial oil concentrations of 160.5 and 1023.6 mg/L, respectively. The absorption mechanism comprised physical and chemical processes.

Angelova et al. (2011) revealed that rice husks could be used as a promising environmental material for the remediation of water contaminated with oil and oil products. Their study created a correlation between morphology and surface functional groups of the sorbent and adsorption mechanisms of the material.

Srinivasan and Viraraghavan (2010a, b) investigated the application of different types of biomaterials, i.e., *Mucor rouxii* and *Absidia coerulea* cultured in chitosan and walnut shell media, for the removal of oil from aqueous solutions. The selected oil types were cutting oil, standard mineral oil, and vegetable oil, achieving adsorption capacities of 84.0, 77.2, and 92.5 mg/g, respectively. The treatment efficiencies of oil-contaminated water by the fungal biomass of *Mucor rouxii* ranged between 77% and 93% at pH of 5.0.

Srinivasan and Viraraghavan (2008) investigated the adsorption of oil from aqueous solutions by walnut shell media. The findings depicted that the sorption capacities were 580 mg/g for Bright-Edge oil, 300 mg/g for standard mineral oil, and 510 mg/g for vegetable oil.

Ibrahim et al. (2010) examined the remediation of wastewater contaminated with emulsified oil using agricultural waste barley straw. The experimental factors were solution pH, temperature, loading of adsorbent, and particle size. Results revealed that the adsorption capability was favorable at a neutral pH environment. Langmuir model described well the experimental data, and the monolayer adsorption capacity was 576.0 ± 0.3 mg/g at 25 °C.

8.6 Design of Experiments

Design of experiments is a statistical approach employed to estimate the influences of multiple independent factors on a single variable. The optimization of experiments can be considered using different techniques such as one-factor-at-a-time and factorial design (Elhalil et al. 2016). The results of experimental design can be graphically displayed using a response surface methodology (RSM). RSM undergoes different mathematical and statistical techniques for optimizing, predicting, and improving a

study of interest (Bas and Boyacı 2007). It can be applied to define the effects of multiple independent variables on chemical and biochemical processes. In addition, RSM can be employed for the determination of enzyme stability and kinetic constants.

8.7 One-Factor-at-a-Time Statistical Method

In a one-factor-at-a-time method, only one variable (or factor) differs with the experimental time, whereas other inputs are maintained constant. The optimum value of the first variable is used for the subsequent experimental runs, in which this step is repeated for other variables. However, this method fails to consider the interaction effects between factors.

8.8 Factorial Design Statistical Method

Factorial design is used to simultaneously determine the effects of two or multiple factors on output. In addition, factorial design can be developed to describe the interaction effects between the independent variables (Nasr et al. 2017). This technique can predict accurate outputs with a minimum number of experiments and reduced time. Factorial design is classified into full-factorial and fractional-factorial.

A full-factorial design, which contains *n*-factors and each factor has *m*-levels, is termed as a m^n factorial experiment. For instance, a full-factorial design noted as 2^3 describes three factors (e.g., pH, time, and temperature) with two levels for each factor (e.g., minimum and maximum); i.e., hence the number of experiments is $2^3 = 8$. Similarly, a 3^2 factorial design has two factors, each with three levels (e.g., minimum, average, and maximum), and $3^2 = 9$ experimental runs. Based on the aforementioned, full-factorial design represents all possible combinations/interactions among factors, which can then be displayed in a single interface.

However, when the number of input variables is large (e.g., more than four factors), the full-factorial design becomes time-consuming. Under this condition, fractional-factorial design, which investigates the most important correlations between factors using a minimum number of experiments, becomes preferable (Srinivasan and Viraraghavan 2010a, b). Fractional-factorial design can be performed using central composite and Box-Behnken methods.

8.9 Application of Design of Experiments for Hydrocarbon Remediation

Srinivasan and Viraraghavan (2010a, b) employed a factorial design analysis to describe the removal of oil from aqueous solution by a fungal biomass, namely, *Mucor rouxii*. The selected oil types were cutting oil, standard mineral oil, and

canola oil. The experimental factors were solution pH (3–9), temperature (5–30 °C), sorbent mass (0.05–0.5 g), initial oil concentration (50–350 mg/L), and mixing speed (100–200 rpm). The results of their study revealed that the medium pH was the most influential factor, in which the removal efficiencies ranged between 80% and 99% at pH of 3.0.

Tansel and Regula (2008) conducted a 2 × 2 × 3 factorial design experiment to determine the impacts of operational factors on the remediation of water contaminated with petroleum hydrocarbons (PHC). The input attributes were oil concentration (150 ppm "low" and 3000 ppm "high"), coagulant type (Cat floc K-10, Cat floc T-2, and no coagulant), and the source of water (pond and brackish). The model outputs were (a) turbidity removal and (b) petroleum hydrocarbon removal. Results indicated that the highest turbidity removal of 93.53% was obtained at "low" oil concentration, "pond" water source, and "Cat floc K-10" coagulant. In addition, a PHC removal of 92.53% was developed at "low" oil concentration, "brackish" water source, and "Cat floc K-10" coagulant.

Sivagurunathan et al. (2003) investigated the effects of several factors of medium pH (5, 7, and 9), temperature (18, 22, and 26 °C), and agitation speed (50, 150, and 250 rpm) on the bioremediation of water containing hydrocarbon residues using *Pseudomonas fluorescens*. A face-centered cube design having three factors and three levels for each factor (i.e., 3^3) was employed. A RSM was applied to plot the results of a quadratic equation having linear, second-order, and interaction terms. Results indicated that the optimum condition was temperature 22.48 °C, pH 7.31, and agitation speed 206 rpm, achieving a total biodegradation of toluene (R^2-value 0.98).

8.10 Artificial Intelligence Modeling

Artificial intelligence (AI) is the development of computer-based systems able to achieve tasks that involve human intelligence, including decision-making, speech recognition, translation between languages, and visual perception (Fawzy et al. 2017). AI can be employed for organization and classification of large datasets, as well as for capturing complex relationships. It is a black-box model that uses machine learning such as artificial neural networks (ANN) and fuzzy logic concepts.

8.11 Artificial Neural Network

Artificial neural network (ANN) is a computer-based approach that implements learning procedures similar to the nervous system of the human brain (Nasr et al. 2017). ANN is composed of a large number of interconnected nodes, also known as neurons, which are organized in layers. The input layer receives experimental

data and transfers results to the last layer through successive hidden layers. The neurons in a particular layer are fully interrelated to those in the subsequent layer through weights and biases (Panja et al. 2017). In addition, activation functions are used to transfer results between successive layers. During training, the weights and biases are adjusted until the mean squared error (MSE) between the simulated outputs and the actual results is minimized. A back-propagation method with a Levenberg-Marquardt (trainlm) algorithm has been widely used for network training (Olawoyin 2016). In this method, the training process undertakes two phases: i.e., in the forward phase, the external signals are propagated from the input layer to the output layer, whereas in the backward phase, the error between the predicted and observed values at the output layer is propagated backward to modify weights and biases (Pakravan et al. 2015). These iterations are successively repeated until achieving the minimum MSE. After network training, a portion of data is used for validation and testing processes to prevent data overfitting and examine the stability level of the trained network. These procedures result in attaining a high degree of prediction accuracy even when ANN receives noisy and/or erroneous input data (Sanusi et al. 2016).

8.12 Fuzzy Inference System

A fuzzy inference system (FIS) is employed to describe nonlinear and complex relations between a number of input factors and one (or more) output (Zadeh 1997). As shown in Fig. 8.3, a FIS is achieved by conducting three major steps (a) fuzzification, (b) "if-then" rules, and (c) defuzzification (Gupta et al. 2017). During fuzzification, crisp (numeric) values are converted into fuzzy inputs using fuzzy linguistic variables, fuzzy linguistic terms, and membership functions. For example, an input factor such as hydrocarbon concentration can be converted according to linguistic concepts into "low," "medium," and "high." Each linguistic expression can be graphically represented as a membership function, e.g., linear, trapezoidal, and Gaussian fuzzy sets (Nasr et al. 2014). After that, an inference engine is performed using a set of "if-then" rules, in which a single fuzzy rule has the form "if x is A, then y is B."

Assume x and y are the variables "bioremediation" and "residual hydrocarbon," respectively, whereas A and B are linguistic variables "high" and "low," respectively. The "if-then" rule will have the form "if bioremediation is high, then residual hydrocarbon is low."

The aggregation of rules is undertaken when the rule-based system comprises several numbers of "if-then" rules. Finally, defuzzification step is carried out to convert the fuzzy result into a crisp output (Zadeh 1997). The widely used defuzzification methods in the literature are center of gravity, mean-max, max-membership, weighted average, and center of sums. Mamdani, Sugeno, and Tsukamoto are different types of fuzzy inferences that have been widely used to implement the fuzzy logic procedures (Nasr et al. 2014).

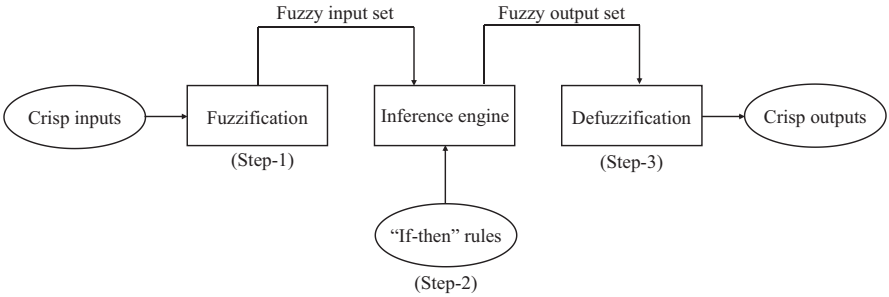

Fig. 8.3 A fuzzy inference system

8.13 Application of Artificial Intelligence for Hydrocarbon Remediation

Sanusi et al. (2016) applied an ANN model for optimizing the degradation performance of total petroleum hydrocarbon by *Paspalum scrobiculatum* L. Hack with R^2-value over 0.95. Their study found that the optimum condition achieving a removal efficiency of 85.5% was an aeration rate of 1.02 L/min, diesel concentration of 3%, and 72 sampling days.

Olawoyin (2016) proposed an ANN model with the Levenberg-Marquardt backpropagation training algorithm for the prediction of potential toxicity of polycyclic aromatic hydrocarbons in soils. The input parameters were treated soil (I and IV), pH (5.02–7.25), electrical conductivity (54–195 mS/cm), and dissolved organic carbon (31.18–62.96 mg/L). The model achieved a high accuracy with R^2-value above 0.99.

Panja et al. (2017) developed an ANN with a structure of 8–14–3 to predict the production of hydrocarbon from shales. The eight input factors were bottom hole pressure (500, 1000, and 1500 psi), gas relative permeability (1, 2, and 3 ng), hydraulic fracture spacing (60, 180, and 300 ft), initial dissolved gas-oil ratio (800, 1900, and 3000), initial reservoir pressure (4000, 5250, and 6500 psi), reservoir permeability (10, 225, and 5000 nD), rock compressibility (4×10^{-6}, 4×10^{-5}, and 4×10^{-4} 1/psi), and slope of gas-oil ratio (0.50, 0.65, and 0.80). The input factors were distributed according to Box-Behnken design of experiment. The model outputs were oil recovery, gas recovery, and gas-oil ratio. The model showed a high predictive accuracy in terms of coefficient of determination (R^2-value) and normalized root mean square error.

Vaferi et al. (2014) applied an ANN model to predict the treatment efficiency of wastewater contaminated with aromatic hydrocarbons. The ANN structure was multilayer perceptron with one hidden layer containing 15 neurons. The input attributes were contact time (0–1440 min), initial concentration of H_2O_2 (0–1942 mg/L), pollutant concentration (200–840 mg/L), pH (3.1–11.6), temperature (25–86 °C), and UV intensity (225–304 nm). The output variable was final pollutant concentration (4–840 mg/L). It was found that the optimum experimental factors were three UV lights illumination and acidic pH of 3.1. The proposed model predicted the

degradation of aromatics hydrocarbon with a mean square error of 5×10^4 (i.e., high accuracy).

Pakravan et al. (2015) investigated the effects of pH (1.5–10.5), initial COD (200–800 mg/L), concentration of H_2O_2 (2.2–15.4 mM), and contact time (45–135 min) on the treatment of petroleum refinery wastewater. The data were obtained from 30 experimental runs, i.e., a central composite factorial design of $2^4 + 6$ center points + 8 star points. It was found that at an initial COD concentration of 300 mg/L, the optimum condition was pH, 5; H_2O_2, 8.8 mM; and time, 120 min. A RSM, along with a quadratic regression model, was employed to describe the relationship between the input factors and the output variable (i.e., COD removal efficiency). The findings of the statistical modeling technique were compared to those obtained from ANN (as typical artificial intelligence method). For this purpose, a feed-forward back-propagation ANN model with a structure of 4–5–1 was applied for the prediction of COD removal efficiency. Results indicated that ANN (R^2, 0.96; adj-R^2, 0.96) provided a higher predictive capability than RSM (R^2, 0.94; adj-R^2, 0.91). A sensitivity analysis using the network weights was employed, which indicated that the initial COD concentration was the most dominating factor.

8.14 Conclusion

This study presented recent applications of white-box and black-box models that have been used for the prediction of bioremediation performances of hydrocarbon pollutants. In addition, this chapter described various physicochemical and environmental parameters that affect the biological activities of hydrocarbon degradation. Isotherm and kinetic studies that have been employed for the adsorption of hydrocarbon contaminants from aqueous solution were investigated. The sorbent materials used in the literature were deposited carbon, bentonite, powdered activated carbon, coal, microorganisms, and agricultural waste (e.g., corncobs, rice husks, and barley straw). Factorial design experiments were demonstrated to determine the effects of several factors, viz., culture pH, temperature, pollutant concentration, mixing speed, and reaction time, on hydrocarbon removal efficiencies. Artificial neural network and fuzzy inference systems were also applied for modeling, developing, controlling, and simulating hydrocarbon remediation processes.

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Chapter 9
Biodegradation of Polycyclic Aromatic Hydrocarbons (PAHs) by Microbes Isolated from the Marine Sponge *Biemna fortis* (Topsent 1897)

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Abstract Industries rely on oil-based products as a significant source of energy. Spillages and accidental leakages are frequent during the extraction, refinement, transportation, and hoarding of oil and their products. The living beings on earth are foremostly contaminated by hazardous polycyclic aromatic hydrocarbons (PAHs); therefore, their degradation is essential. The inadequate use of chemical and mechanical techniques to expel hydrocarbons from the sullied marine ecosystem is not cost-effective. The conversion of complex natural contaminants to other simple natural substances by biodegraders such as microorganisms may allude to absolute mineralization into carbon dioxide, water, and inorganic substances through the mechanism of bioremediation. Previous research works on PAH-degrading bacteria are mainly focused on the utilization of terrestrial microbes; however, the potential use of marine microbes is unexplored. There is an enduring international interest in exploring the application of microbes isolated from marine sponge *Biemna fortis* having high PAH-degrading potential. This book chapter represents an updated overview of the potential application of microbes isolated from marine sponge *B. fortis* for PAH degradation.

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9.1 Introduction

The remediation of surroundings defiled with perilous materials has got a lot of consideration about the potential unfriendly impacts of toxicants on civic well-being. The genotoxic, mutagenic, and carcinogenic properties of polycyclic aromatic hydrocarbons (PAHs) involved in a congregation of natural contaminations are of great concern (WHO 1983; Cerniglia 1992; Mastrangelo et al. 1996; Schützendübel et al. 1999). PAHs have fused aromatic rings in linear, angular, or cluster arrangements which are electrochemically stable, resistant to biodegradation. The carcinogenic index generally tends to increase with an increment in the number of aromatic rings, structural angularity, and hydrophobicity, and an increase in the molecular weight decreases its volatility (Mackay and Callcott 1998; Marston et al. 2001).

Small PAHs include up to six aromatic rings, and large PAHs include more than six aromatic rings. However, PAHs may also contain four (chrysene, naphthacene, pyrene), five (benzo(a)pyrene, pentacene), six (coronene), seven, or more rings (ovalene with ten rings). The igniting of remnant fuels, gas produced by the combustion of engine oil, production of bituminous coal and gas, and the burning of waste generate PAHs which contaminates soil (Harvey 1991; Cai et al. 2007; Das et al. 2008). PAHs are the most extensive organic pollutants of soil, and water bodies (Puglisi et al. 2007), if inappropriately managed and/or fortuitously released to the ecosystem, may endure in soil for a longer duration causing serious damage (Chaineau et al. 2000, 2005).

The US Environmental Protection Agency (USEPA 2002) has documented 16 PAHs as the major environmental pollutants on the basis of profusion and toxicity (Liu et al. 2001; Samanta et al. 2002; Bamforth and Singleton 2005; Puglisi et al. 2007) as depicted in Fig. 9.1. Some of them are carcinogenic, mutagenic, and teratogenic (Adonis et al. 2003; Cai et al. 2007).

PAHs are omnipresent, nonpolar, and extremely hydrophobic, have affinity for fatty tissues (Van der Oost et al. 2003), and are therefore being considered as substances of prospective human health hazards and marine life (Mastrangelo et al. 1996; Hughes et al. 1997; Binkova et al. 2000; Marston et al. 2001; Xue and Warshawsky 2005; Okafor and Opuene 2007; Fagbote and Olaufekum 2010; Lee and Byeon 2010).

The bioaccumulation of PAHs in diverse food chains in the environment is quite frightening (Morehead et al. 1986; Xue and Warshawsky 2005). Awareness of the ecological fate and biodegradation mechanisms of PAHs is incited by their indestructive impacts on human well-being. The coastal and oceanic sediments are eventual sinks for the readily adsorbed particulate matter by hydrophobic PAHs (Hughes et al. 1997; Yu et al. 2005; Osuji and Ezeburio 2006). PAHs cause significant hazards attributable to their cancer-causing nature in marine life forms, for example, benthic, demersal and pelagic fishes, crustaceans, and shellfish (Peruguni et al. 2007). The destinies of PAHs in the surroundings are linked with both abiotic and biotic events including volatilization, photooxidation, concoction oxidation,

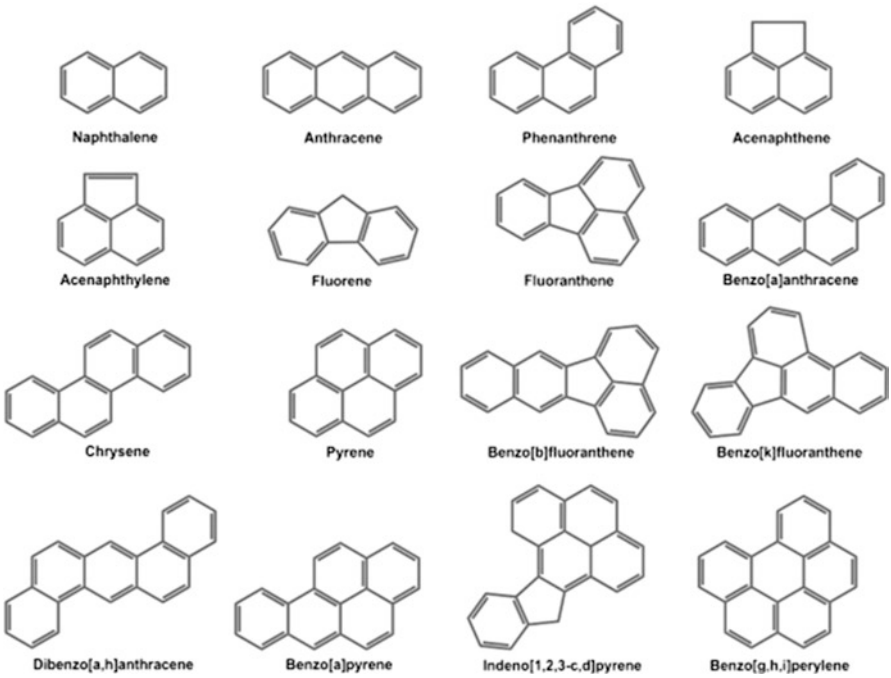

Fig. 9.1 Structure of the16 PAHs enlisted as priority pollutants. (Bamforth and Singleton 2005)

bioaccumulation, and microbial change. The microbial association with other organisms has been viewed as the most powerful and noteworthy reason for PAH degradation (Cerniglia 1993; Nwuche and Ugoji 2008; Haghighat et al. 2008; Agbozu and Opuene 2009; Atlas and Bragg 2009).

Research on the organic disparagement of PAHs has revealed that microorganisms, fungi, and algae have catabolic capacities that might be used for the remediation of PAH-sullied soil and water (Albert et al. 2005). There has been an increasing concern in the bioremediation of terrestrial and marine surroundings contaminated with PAH and have lethal effects on human well-being. The utilization of microorganisms for the bioremediation of PAH-contaminated environments accounts to be an appealing innovation for reclamation of polluted sites (Mrozik et al. 2003).

The man-made aromatic substances which are incorporated into pesticides, cleansers, oils, solvents, paints, and explosives can be naturally biodegraded enzymatically by the microorganisms (Dagley 1975). The presence of chlorine substituent in these compounds increases their stubbornness to enzymatic degradation leading to their decreased solvency and chemical reactivity (Reineke and Knackmuss 1988). Therefore, as compared to the parent hydrocarbons, chlorinated aromatic compounds are harder to degrade.

The degradation of numerous ecological pollutants like oil and other hydrocarbons through bioremediation process which utilize microorganisms to detoxify or expel pollutants inferable from their diverse metabolic capacities are an advancing

strategy assumed to be noninvasive and relatively less expensive (Nilanjana and Preethy 2011). It is intricate to investigate the biodegradation of PAHs in indigenous habitats. The rate of biodegradation and the degree of bacterial digestion can be attributed to various environmental components, viz., temperature, pH, oxygen concentration, saltiness, light intensity, co-substrates, and season. Besides, in the presence of other nutrients, the level of degradation of polycyclic aromatic hydrocarbons essentially increases (Mrozik et al. 2003).

The sea has been considered as a rich wellspring of compounds having novel structures and biological activities (Archana et al. 2005). The bioremediation of marine environment is significant since seas and estuaries have been the major site of the oil spillage, which happens amid routine tasks of raw petroleum extraction, refining, and circulation and due to the intense mishaps (Anupama and Padma 2009).

Mrozik et al. (2003) demonstrated that the pure and mixed cultures of microorganisms extracted from water bodies can metabolize anthracene and phenanthrene as the sole carbon source. Consequently, another population of marine hydrocarbon-degrading microorganisms has been characterized with a significant role in the biodegradation of hydrocarbons and other related compounds. These microorganisms are isolated from different aquatic sources, for example, sponges, ocean weeds, and so on.

9.2 Marine Sponges

Marine sponges are sedentary organisms which are inhabitants of ocean bed and represent a significant component of the marine benthic environment. Sponges are composed of layers of cells with no clear tissues or organs and appropriate nervous system. Sponges, being ciliates, are filter feeders and feed on planktons and small marine organisms through minute body pores by pumping the circulating water using specialized paddles and tails. They have been living in aquatic habitat for more than 600 million years. More than 10,000 species of sponges have been identified around the world. Numerous sponges harbor microorganisms with bioactive properties, of which a few can be potentially used as pharmaceutical leads (Hill 2004).

Sponges encompass a diverse group of green algae, red algae, cryptophytes, dinoflagellates, diatoms, and a rich variety of microorganisms in their cells (Simone et al. 2005). *Biemna fortis* is one such form of sponge that harbors a large number of bacterial groups, which have the capacity to degrade the high concentrations of polycyclic aromatic hydrocarbons (PAHs) (Farhan and Mahesh 2015). The investigation of biodegradation of aromatic hydrocarbon by microorganism relies on techniques, viz., denaturing gel gradient electrophoresis (DGGE), microbial community fingerprinting by T-RFLP and ARISA, DNA hybridization assay, thin layer chromatography (TLC), and 16S rRNA gene sequencing including fluorescent in

situ hybridization (FISH). Sequencing the DNA of biodegrading microorganisms has opened our scientific outlook experiences into the systems, the event, and the character of dynamic microbes that impact biodegradation of natural ecological contaminations.

9.3 Classification of *Biemna fortis*

Biemna fortis is a marine sponge belonging to the lineage: cellular organisms, Eukaryota, Opisthokonta, Metazoa, Porifera, Demospongiae, Heteroscleromorpha, Biemnida, and Biemnidae. There are several other species belonging to the genus *Biemna* which is diverse in nature (Table 9.1).

Table 9.1 List of species listed under the genera *Biemna*, class Demospongiae

Biemna species	Described by the scientists
B. anisotoxa	Lévi, 1963
B.bihamigera	Dendy, 1922
B.caribea	Pulitzer-Finali, 1986
B.chilensis	Thiele, 1905
B.chujaensis	Sim & Shim, 2006
B. ciocalyptoides	Dendy, 1897
B. cribaria	Alcolado & Gotera, 1986
B. dautzenbergi	Topsent, 1890
B. ehrenbergi	Keller, 1889
B. fistulosa	Topsent, 1897
B. flabellata	Bergquist, 1970
B. fortis	Topsent, 1897
B. fragilis	Kieschnick, 1900
B.gellioides	Lévi & Lévi, 1989
B.granulosigmata	Lévi, 1993
B.hongdoensis	Jeon & Sim, 2009
B.humilis	Thiele, 1903
B. jeolmyongensis	Sim & Shim, 2006
B.laboutei	Hooper, 1996
B. liposigma	Burton, 1928
B.liposphaera	Hentschel, 1912
B.macrorhaphis	Hentschel, 1914
B.megalosigma	Hentschel, 1912
B.megastyla	Burton, 1959
B.microacanthosigma	Mothes, Hajdu, Lerner & van Soest, 2004
B.microstrongyla	Hentschel, 1912
B.microstyla	de Laubenfels, 1950
B.microxa	Hentschel, 1911

(continued)

Table 9.1 (continued)

Biemna species	Described by the scientists
B.mnioeis	de Laubenfels, 1954
B.novaezealandiae	Dendy, 1924
B.omanensis	van Soest & Beglinger, 2002
B.parthenopea	Pulitzer-Finali, 1978
B.pedunculata	Lévi, 1963
B.peracuta	Topsent, 1927
B.philippensis	Dendy, 1896
B.plicata	Whitelegge, 1907
B.polyphylla	Lévi, 1963
B.rhabderemioides	Bergquist, 1961
B.rhabdostyla	Uriz, 1988
B.rhadia	de Laubenfels, 1930
B.rufescens	Bergquist & Fromont, 1988
B.saucia	Hooper, Capon & Hodder, 1991
B.seychellensis	Thomas, 1973
B.spinomicroxea	Mothes, Campos, Lerner, Carraro & van Soest, 2005
B.strongylota	Rios & Cristobo, 2006
B.tenuisigma	Pulitzer-Finali, 1978
B.tetraphis	Tanita & Hoshino, 1989
B.thielei	Burton, 1930
B.trirhaphis	Topsent, 1897
B.trisigmata	Mothes & Campos, 2004
B.truncata	Hentschel, 1912
B.tubulata	Dendy, 1905
B.variantia	Bowerbank, 1858
B.victoriana	Hallmann, 1916

9.4 Distribution of *Biemna*

The marine sponges *Biemna* are widely distributed and are also known to live in the western and central Indian Ocean Regions.

- *Biemna anisotoxa* (LEVI), from South Africa (Levi 1963).
- *Biemna ciocalyptoides* sensu (BURTON), from the Red Sea (Burton 1959) and Seychelles (Van Soest 1994) (homonym *of B.ciocalyptoides* (Dendy 1897)) from southern Australia.
- *Biemna seychellensis* (THOMAS), from the Seychelles Is (Thomas 1973), originally described as a variety of the N Atlantic *B. variantia* (BOWERBANK).
- *Biemna fords* (TOPSENT), from Ambon, Indonesia (Topsent 1897; Desqueyroux-Faundez 1981), Arafura Sea (Hentschel 1912), Straits of Malacca (Sollas 1902), Bay of Bengal (Burton 1930; Burton and Rao 1932), Red Sea (Topsent 1897;

Burton 1959), Mombasa (Pulitzer-Finali 1993) and Sulawesi, Indonesia, Gulf of Thailand, and Truk Atoll, Micronesia.

- *Biemna humilis* (THIELE) from Indonesia (Thiele 1903), Zanzibar and Shimoni (Pulitzer-Finali 1993).
- *Biemna microstrongyla* (HENTSCHEL) from Indonesia (Hentschel 1912) and Mombasa (Pulitzer-Finali 1993).
- *Biemna pedonculata* (LEVI), from South Africa (Levi 1963).
- *Biemna polyphylla* (LEVI), from South Africa (Levi 1963).
- *Biemna sigmodragma* (LEVI), from South Africa (Levi 1963) (originally described as a subspecies of *B. megalosigma* HENTSCHEL from SE Indonesia).
- *Biemna trirhaphis* (TOPSENT), from Ambon, Indonesia (Topsent 1897; Desqueyroux-Faundez 1981), Red Sea (Burton 1959; Levi 1961), and Mombasa and Zanzibar (Pulitzer-Finali 1993).
- *Biemna tubulata* (DENDY) from Sri Lanka (Dendy 1905), NW India (Dendy 1916), the Mergui Archipelago and Andaman Sea region (Burton and Rao 1932), Providence Reef (Dendy 1922), and Seychelles Is (Thomas 1973).
- *Biemna truncata* (recorded from Aru I., Indonesia (Hentschel 1912), Sri Lanka (Burton 1930), and the Seychelles (Thomas 1973).
- *Biemna bihamigera* (from Providence Reef (Dendy 1922), Aldabra (Levi 1961) and Shimoni, East Africa (Pulitzer-Finall 1993).
- *Biemna democratica* (from the Straits of Malacca; Sollas 1902).
- *Biemna saucia* is a toxic sponge from the NE Indian Ocean (Hooper et al. 1991).

9.5 Description of *Biemna fortis*

Biemna fortis is a massive sponge (15 cm long and 9 cm in diameter), erect with chimney-like projections, often compressed with large fistulose surface processes and terminal oscules; peduncle is masked beneath the sediment with tubular projections noticeable at the surface; the sponge is tough and hispidous; ostia are not obvious, while the oscula (3–8 mm) are terminally situated; surface is woody and cork-like (Fig. 9.2).

The surface is rough, fibrous, compressible, and harsh to touch and has a firm consistency. The ectosomal skeleton has protruding choanosomal megascleres but without any special spicules or structures. The choanosomal skeleton is cavernous, disorganized halichondrid reticulate. Megascleres are exceptionally long and thickest in the basal third of the spicule. Megascleres are styles, smooth, and marginally bended upward (extend: 929–1283 × 16.2–36.5 μm; mean: 1121 × 28.6 μm); microscleres are sigmas, abundant with pointed tips (run: 71–93 × 3.1–5.3 μm; mean: 85 μm × 4.3 μm). Microscleres include sigmas of two sizes, raphides, and microxeas. The ectosomal skeleton is a mass of extraneously arranged spicules; choanosome rarely contains fiber tracts and is made of bounteous felted spicules scattered with numerous sigmas. They flourish well in territories of saline conditions, found in coral reef and the sandy substrate with coral patches (Belinda et al. 2005).

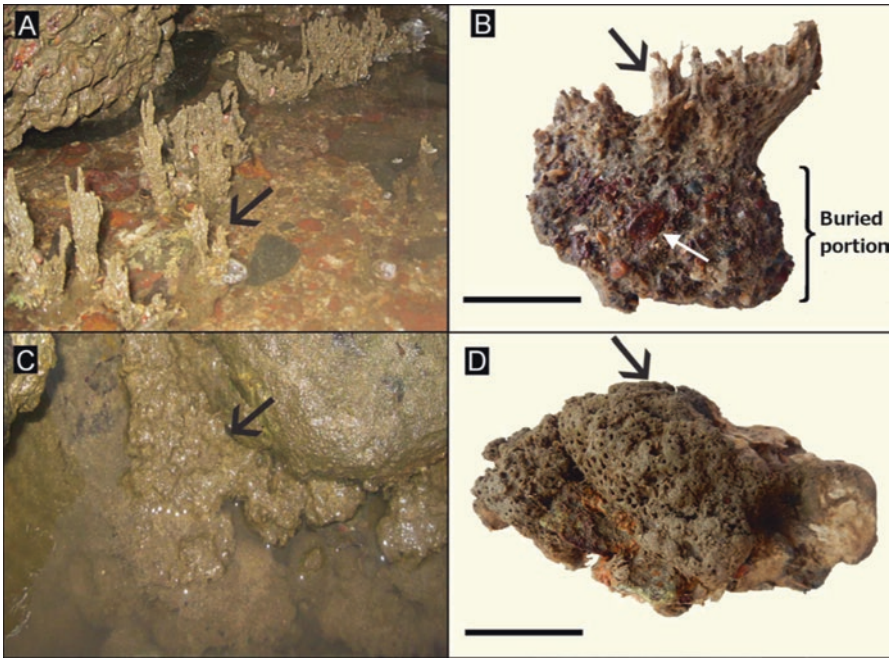

Fig. 9.2 *Biemna fortis* growth forms (Dahihande and Thakur 2017). (**a**) *Biemna fortis* growing (**b**) Partially buried growth form, with the underground, buried body mass (**c**) *Biemna fortis* growing (**d**) Massive growth form, most of the growth is above surface. Black arrows indicate the portion above ground surface, white arrow indicates the sediment inclusion in the buried body mass

The color of the sponge varies in live and in a preserved sample: yellowish-green to darkish-yellow color can be observed in the part buried in the ground; the tip of the projection is dull green to dim, and core is sandy gray. Differences in color are because of aggregated debris.

9.6 Sponge-Associated Microorganisms

Marine sponges inhabit diverse microbial communities with remarkable biological and biotechnological implications. Sponges are filter feeders and devour microorganisms from the surrounding seawater. Any microbes that endure the digestive and immune responses of sponges are symbiotically associated. In marine sponges, ten characterized bacterial phyla have been identified (Taylor et al. 2007; Hentschel et al. 2012; Webster and Taylor 2012). Majority of sponges are widely associated with α, β, γ, δ, and ε *Proteobacteria* and *Chloroflexi* bacteria (Hentschel et al. 2002; Taylor et al. 2007).

Sponges form harmonic relations with diverse bacteria and form the most vital portion of sponge's diet. In some cases, the bacterial cells make up to 38% of sponge

wet weight (Vacelet and Donadey 1977; Taylor et al. 2007; Hentschel et al. 2012). Albeit sponge-specific archaea, eubacteria subsist exclusively within sponge hosts but do not inhabit the surrounding marine environment (Hentschel et al. 2002). The microbiomes of marine sponge reliably constitute archaea and fungi exhibiting species-specific host-related functions (Taylor et al. 2007; Thomas et al. 2010).

Prabha et al. (2010) isolated and cultured several bacteria from the sponge *Halichondria* sp., collected from the Gujarat coast of the Indo-Pacific region, and the antibiotic activity was assayed against 16 strains of clinical pathogens. Further investigation was carried out on the most potent *Bacillus* sp. (SAB1) and the fungus *Aspergillus fumigatus* which was antagonistic to several clinically pathogenic Gram-positive and Gram-negative bacteria.

Four species of marine sponges, viz., *Echinodictyum* sp., *Spongia* sp., *Sigmadocia fibulatus*, and *Mycale mannarensis*, were found to harbor 75 bacterial strains from the Tuticorin coast, Gulf of Mannar region (Anand et al. 2006). Four bacteria, viz., *Bacillus subtilis*, *Escherichia coli*, *Vibrio parahaemolyticus*, and *Vibrio harveyi*, and one fungal pathogen, viz., *Candida albicans*, were used to screen for antibiotic production by these strains by the agar overlay method.

Ocky Karna Radjasa (2007) isolated 90 bacterial isolates found associated with sponges collected from the marine regions of Indonesia.

Saravanakumar et al. (2011) reported antibiotic-producing bacteria, isolated from 14 species of sponges from Indian waters. One hundred and nine bacterial strains were screened for antibiotic production against five fish pathogens, namely, *Vibrio fischeri*, *Vibrio vulnificus*, *Vibrio harveyi*, *Aeromonas hydrophila*, and *Aeromonas sobria*.

Santos et al. (2010) isolated and characterized bacteria with antimicrobial activities against pathogenic bacteria from Brazilian sponges. The sponge-associated bacterial strains were subdivided into three different clusters based on the comparative sequence analysis of 16S rRNA genes, among which, three with alpha-Proteobacteria (*Pseudovibrio* sp.), four with gamma-Proteobacteria (genera *Pseudomonas* and *Stenotrophomonas*), and five strains were affiliated with Firmicutes (genera *Bacillus* and *Virgibacillus*).

9.7 Antimicrobial Activity of Sponge-Associated Bacteria

The microorganisms associated with numerous sedentary marine sponges serve as food particles and chemical defenses against potential predators (Albrecht et al. 2007), inhibiting cancerous growths (Belinda et al. 2005). Sponge-associated bacterial strains represent a rich source of bioactive metabolites (Kalirajan et al. 2013) and are regarded as gold quarry due to their vast applications in pharmaceuticals, nutritional supplements, cosmetics, agrochemicals, molecular probes, enzymes, and fine chemicals (Isaac et al. 2012). Only a few of the bioactive compounds discovered in sponges have been commercialized (Archana et al. 2005, Kalirajan et al. 2013).

The quantitative and qualitative condition of the surrounding water varies according to the bacterial colonies (Albrecht et al. 2007). The bacteria developing on the surface of sponges reside in a highly competitive environment and have limited access to space and nutrients (Burgess et al. 1999). Sponge-associated bacteria produce secondary metabolites which exceed planktonic bacterial metabolite production (Lemos et al. 1986; Jensen and Fenical 1994). A number of impending therapeutic substances of the sponges have remarkable similarities to metabolites derived from their associated microorganisms and are a rich source for the manufacture of antibiotics (Proksch et al. 2002).

The microbes associated with sponges have the novel genes such as polyketide synthases (PKS) and nonribosomal peptide synthetases (NRPS) for synthesizing a broad range of structurally diverse natural compounds (Isaac et al. 2012). These bioactive substances have significant medical and industrial applications and are important for the epibiotic defense of the marine invertebrates (Archana et al. 2005). *Pseudomonas* sp. 1531-E7 was isolated from the marine sponge *Homophymia* sp. leading to the discovery of antiviral compound 2-undecyl-4-quinolone (1) (Bultel-Poncé et al. 1999).

Fourteen isoprenylated cyclohexanols and truncateols A-N isolated from the sponge-associated fungus *Truncatella angustata* were tested in vitro against the influenza A (H1N1) virus reported by Zhao et al. (2015). Reimer et al. (2015) isolated *Streptomyces* sp. that was associated with the marine sponge *Dysidea tupha*.

Hundred heterotrophic, halophilic bacterial bionts isolated from one bivalve, five, and nine corals sponges were investigated for the antagonistic activities (Sheryanne and Irene 2012). Among these 46 bionts were active against human pathogenic bacteria, namely, *E. coli*, *A. aerogenes*, *S. marcescens*, *S. citreus*, *P. vulgaris*, and *S. typhi*. Due to the immense activity, biochemical accessibility, and stability than the terrestrial counterparts, marine sponge-related microorganisms have drawn tremendous consideration as a reserve for new secondary metabolites (Skariyachan et al. 2014; Kiran et al. 2014).

9.8 Biodegradation of PAHs Using Sponge *Biemna fortis*-Associated Bacteria

Virtually, all aquatic sponges contain a numerous microorganisms in their tissues. From the marine sponge *Callyspongia diffusa*, a total of 101 microbial isolates were obtained, and the biosurfactant producers were *B. subtilis* MB-7, *B. amyloliquefaciens* MB-101, *Halomonas* sp. MB-30, and *Alcaligenes* sp. (Asha et al. 2015). The sponge-associated microorganisms in aquatic environments can be effectively used in the bioremediation of PAH.

In oil spilled environment, microorganisms are the most active primary oil degraders (Rahman et al. 2003; Brooijmans et al. 2009) and feed exclusively on hydrocarbons (Yakimov et al. 2007). Twenty five genera of hydrocarbon-degrading bacteria and fungi were enlisted by Floodgate, 1984, which were isolated from

marine environment. Kiran et al. (2010) reported that cultivable microorganisms from marine sponge may act as potent sources of glycolipid and lipopeptide biosurfactant.

The *Biemna fortis*-associated bacterial strains, namely, *E. coli*, *P. aeruginosa*, *S. aureus*, *S. typhi*, *S. flexneri*, *K. pneumoniae*, *V. cholera*, *A. baumannii*, methicillin-resistant *S. aureus*, *P. macquariensis*, *K. varians*, *M. luteus*, *C. xerosis*, and *M. varians* were successfully isolated using the enrichment process. The bacteria, viz., *C. xerosis*, *K. varians*, *P. macquariensis*, *M. luteus*, and *M. varians*, were the most influential and significantly biodegraded-specific PAHs like phenanthrene, fluoranthene, naphthalene, pyrene, and anthracene (Farhan and Mahesh 2015). These four bacterial isolates were utilizing fluoranthene, pyrene, and naphthalene as a sole source of carbon and energy for growth. Naphthalene being the simplest PAH has a fused pair benzene rings and has increased water solubility at 25 °C resulting in greater accessibility of the substrate to the microorganisms.

The majority of the isolates from marine sponge *Biemna* were *P. macquariensis*, *K. varians*, *M. luteus*, *C. xerosis*, and *M. varians* grows on or mineralizes pyrene as reported by Farhan and Mahesh (2015). Since pyrene is structurally similar to several carcinogenic PAHs, it has been used as a model compound for biodegradation of high molecular weight PAH.

Paenibacillus macquariensis was an effective degrader to metabolize three PAHs with more complex structure, i.e., naphthalene, fluoranthene, and pyrene, and consequently may be employed in metabolizing different recalcitrant PAHs having lesser solubility. Hence, it can play an efficient role in cleaning up of numerous PAHs in the contaminated sites (Farhan and Mahesh 2015). Xuezhu Zhu et al. (2016) recommended that phenanthrene, fluorine, and naphthalene contributed as co-substrates, and the degradation of these compounds proceeded at a moderately faster rate when compared to the biodegradation of substrates alone. Thavamani et al. (2012) reported that *Paenibacillus* sp. PHE-3 could biodegrade PAHs through co-metabolism and degrade benzo[a]pyrene utilizing phenanthrene as a co-substrate. Daane et al. (2002) investigated the degradation of naphthalene from petroleum hydrocarbon-contaminated sediment and salt marsh rhizosphere from the isolated *Paenibacillus* sp. which were able to use aromatic substrates.

Bacillus gordonae sp. (*P. validus* by Heyndrickx et al. 1995) described by Pichinoty et al. (1986) utilized phthalate, protocatechuate, p-hydroxybenzoate, isophthalate, phenol, trimellitate, p-cresol, quinate, and naphthalene as a sole source of carbon. *Paenibacillus* sp. was isolated as a PAH-degrading microorganism from tar oil-contaminated soil (Meyer et al. 1999). Daane et al. (2001) revealed that *Paenibacillus* sp. (strain PR-P1) facilitated pyrene degradation in sediment slurry microbes utilizing naphthalene or phenanthrene as a sole source of carbon.

Kocuria varians was found to degrade naphthalene (Farhan and Mahesh 2015). Tumaikina et al. (2008) recognized the ability of other *Kocurial* species to grow on oil and other hydrocarbons as a sole carbon and energy sources. For example, naphthalene, phenanthrene, fluoranthene, and crude oil were degraded by *K. flava* and *K. rosea*.

Micrococcus sp. was reported to have high interaction on naphthalene and was apparent that the antagonistic incident may result in blocking appropriate degradation pathways for other PAHs (Farhan and Mahesh 2015). The rates of degradation for these compounds delayed considerably when more than one compound was present in the same sample showing an antagonistic effect on the degrading abilities of isolated strains which could co-metabolize other PAHs. *M. luteus* degraded the compounds at a faster rate than *K. rosea*, and the utmost degradation was observed for naphthalene followed by phenanthrene, fluoranthene, and pyrene (Haritash and Kaushik 2016). However, the mechanism of biodegradation of benz-α anthracene, benz-α pyrene, and pyrene is still unclear. Othman et al. (2010) revealed that under optimum conditions, only *M. diversus* had a high tendency for degradation of two ring naphthalenes. Additionally, similar results were obtained by Narasimhulu and Setty (2011) who isolated and characterized the naphthalene-degrading bacteria in soil.

Corynebacterium sp., a GC-rich Gram-positive bacterium, employed in the development of bio-production of diverse compounds such as amino acids, alcohols, and organic acids can utilize naphthalene as the main source of carbon and energy (Farhan and Mahesh 2015). *Pseudomonas*, *Sphingomonas*, *Nocardia*, *Beijerinckia*, *Rhodococcus*, and *Mycobacterium* can completely mineralize anthracene forming the dihydriol as an initial oxygenated intermediate (Sudip et al. 2002).

Mycobacterium sp. has been reported to degrade >95% fluoranthene efficiently in a mineral medium supplemented with organic nutrients. *Mycobacterium* sp., *Rhodococcus* sp., and *Gardona* sp. isolated from numerous actinomycetes bacteria utilize fluoranthene from varying hydrocarbon polluted soils (Sudip et al. 2002).

9.9 Microbial Biodegradation Mechanism of PAH

Bacteria have developed several approaches for imbibing energy from nearly all compounds and have been considered as nature's vital scavengers due to their rapid flexibility to degrade or remediate ecological hazards.

Numerous bacteria can biodegrade PAHs, and few can consume low-MW PAHs as their carbon source. To investigate PAH degradation, culture-based approaches have been extensively employed, and several bacterial species have been capable of doing it.

The biochemical pathways for the bacterial biodegradation of PAHs have been thoroughly studied for anthracene and acenaphthene (Dean-Ross et al. 2001; Pinyakong et al. 2004), naphthalene (Resnick et al. 1996; Annweiler et al. 2000), and phenanthrene (Menn et al. 1993; Kiyohara et al. 1994; Pinyakong et al. 2003a, b). The most representative genera responsible for PAH degradation are *Acidovorax*, *Acinetobacter*, *Aeromonas*, *Alcaligenes*, *Arthrobacter*, *Bacillus*, *Brevibacterium*, *Comamonas*, *Corynebacterium*, *Flavobacterium*, *Micrococcus*, *Mycobacterium*, *Nocardia*, *Pseudomonas*, *Rhodococcus*, *Sphingomonas*, *Stenotrophomonas*, *Streptomyces*, and *Xanthomonas* (Doyle et al. 2008). These monoculture studies

have been extremely valuable since the microorganisms metabolize different compounds in pure cultures.

Based on the presence or absence of oxygen, two main approaches (aerobic and anaerobic degradation) are being followed to degrade PAHs. Oxygen is not only the final electron acceptor in the aerobic catabolism of aromatics but also acts as a co-substrate for the hydroxylation and oxygenolytic aromatic ring cleavage. On the contrary, based on reductive reactions, the anaerobic catabolism of aromatic substances employs a completely different strategy to cleave the aromatic ring (Carmona et al. 2009). The anaerobic catabolism of aromatic compounds is unclear with respect to microbial potentiality.

Under aerobic conditions, the degradation of the majority of organic pollutants by microorganisms is more rapid. The foremost step in aromatic hydrocarbon degradation is the accumulation of one or two oxygen atoms which are then converted into phenol (aliphatic) or alkanol (aromatic). The first intermediate is an epoxide in some species activating the hydrocarbon to make it more soluble in water and tags and commence a reactive site for the next reaction. The energy required for the reaction is generated by the oxidation of a reduced bio-intermediary, for instance, NADH is re-oxidized by an electron acceptor. HMW PAHs (two and three rings) are readily degradable substrates which are less specifically metabolized by the catabolic enzymes due to their low solubility when compared to LMW PAHs (Cerniglia and Heitkamp 1989; Molina et al. 1999). These aromatic hydrocarbons get transformed into products like alkanes, alkenes, and cycloalkanes. The degradation pathway for alkanes and cycloalkanes includes subsequent formation of alcohol, aldehyde, and fatty acids. Different enzyme systems accomplish the primary attack on alkanes for the degradation.

Depending on the chain length, specific enzyme system attacks PAH rings and relies on molecular oxygen for the biodegradation (Table 9.2). The chief mechanism for aerobic bacterial metabolism of PAHs relies on the preliminary oxidation of the benzene ring by the action of multicomponent enzyme systems (oxygenases, peroxidases, and dioxygenase) to form cis-dihydrodiols as a preliminary by-product. *Mycobacterium* sp. is competent of oxidizing PAHs by the enzyme cytochrome P-450 monoxygenase to form *trans*-dihydrodiols (Kelley et al. 1990). Dioxygenases cleave these intermediates resulting in the formation of intradiol or extradiol ring (Cerniglia 1992; Eaton and Chapman 1992; Gibson and Parales 2000), which is then metabolized to carbon dioxide and water via catechols (Kelly et al. 1990). The enzymatic reactions and metabolic pathways implicated in the microbial degradation of naphthalene have been illustrated in Fig. 9.3.

The microbial degradation of oil, chlorinated hydrocarbons, fuel additives, and many other compounds are efficiently degraded by cytochrome P450 alkane hydroxylases belonging to the superfamily of heme-thiolate monooxygenases (Van Beilen and Funhoff 2007).

Pseudomonas along with diversified bacteria is capable of oxidizing naphthalene utilizing dioxygenase enzymes. Ensign (2001) isolated and characterized three proteins of omega-hydroxylase system (the rubredoxin reductase, a rubredoxin, and an omega-hydroxylase) from *Pseudomonas*. Fatty acids are the intermittent prod-

Table 9.2 Enzymes implicated in biodegradation of aromatic hydrocarbons

Enzymes	Substrates	Microorganisms
Soluble methane Monooxygenases	C_1 to C_8 alkanes, alkenes, and cycloalkanes	*M. cella* *M. coccus* *M. cystitis* *M. monas* *M. sinus*
Particulate Methane Monooxygenases	C_1 to C_5 alkanes and cycloalkanes	*M. bacter* *M. coccus* *M. cystitis*
AlkB-related Alkane Hydroxylases	C_5 to C_{16} alkanes, fatty acids, alkyl benzenes, cycloalkanes, etc.	*Burkholderia* *Mycobacterium* *Pseudomonas* *Rhodococcus*
Eukaryotic P450	C_{15} to C_{16} alkanes, fatty acids	*C. maltosa* *C. tropicalis* *Y. lipolytica*
Bacterial P450 Oxygenase system	C_5-C_{16} alkanes, cycloalkanes	*Acinetobacter* *Caulobacter* *Mycobacterium*
Dioxygenases	C_{10}-C_{30} alkanes	*Acinetobacter* sp.

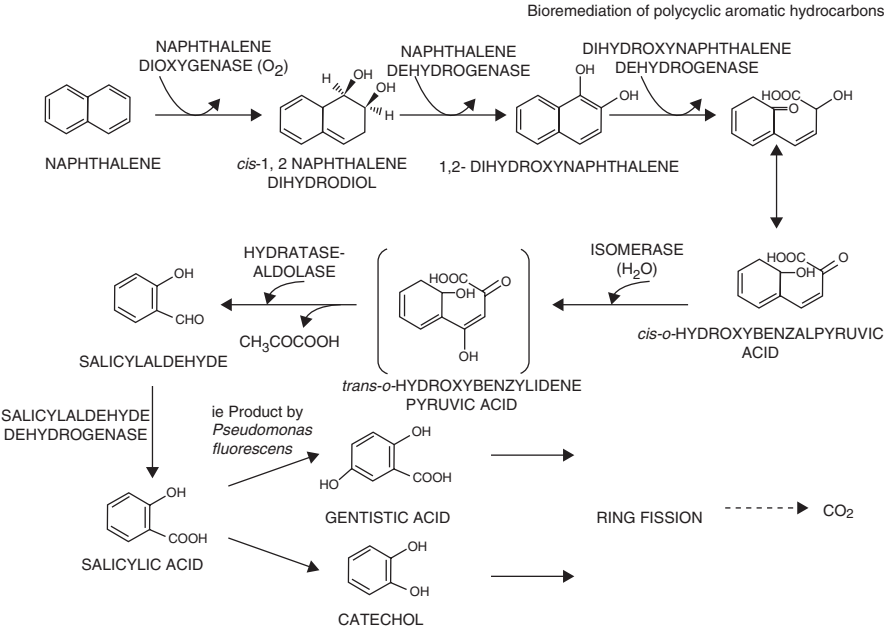

Fig. 9.3 Mechanism of polycyclic aromatic hydrocarbon degradation by microbes. (Bamforth and Singleton 2005)

ucts of the alkane degradation produced from the alkanols via aldehydes and further decomposed by carboxylic acid degradation pathway. These acids are excreted by the cells and accumulate in the environment and thus, serve as a carbon source for microbial community.

9.10 Application of Bioremediation Agents

The bioremediation agents defined by the United States Environmental Protection Agency (USEPA 2002) include microbial cultures, enzyme/nutrient additives enlisted in Table 9.3. considerably enhance the rate of biodegradation to alleviate the effects of the discharge (Nichols 2001).

The bioremediation product may be efficient in the laboratory but less efficient in the field (Venosa et al. 1996; Lee et al. 1997; Mearns 1997). Since laboratory studies always cannot imitate change in the macro environmental conditions, field studies are the most convincing expression of the efficacy of these products.

Inipol EAP22 is a nutrient additive consisting of urea as a nitrogen source (microemulsion), phosphorus source (sodium laureth phosphate), 2-butoxy-1-ethanol (surfactant), oleic acid (hydrophobic agent), and oil spill cleanup agent famous for bioremediation (Table 9.3). The merits of Inipol EAP22 include (1) prevention of water-in-oil emulsification by minimizing the interfacial tension and oil viscosity, (2) controlling the release of phosphorus and nitrogen for oil biodegradation, and (3) nontoxicity to living organisms and superior biodegradability (Ladousse and Tramier 1991).

Table 9.3 Commercially available bioremediation agents

Sl. No.	Bioremediation agents/products
1	BET BIOPETRO
2	BIOCATALYSTIOS-500
3	BIO-D NUTRIENTS
4	BIOREN 1 AND 2
5	ENVIROZYME BR
6	HYDROCARBON D-GRADER
7	INIPOL EAP22
8	IOS-500
9	LAND AND SEA
10	MEDINA MICROBIAL ACTIVATOR
11	MICRO-BLAZE
12	OIL SPILL EATER –II
13	PETRO-CLEAN
14	WAPED
15	WMI-2000

Oil Spill Eater II (OSEII) is an enzyme/nutrient stabilizer consisting of "nitrogen, phosphorus (ready carbon availability), and vitamins (quick bacterial colonization)" (Table 9.3). A field investigation was carried in a fuel-contaminated area of Marine Corps Air Ground Combat Center (MCAGCC) in California to test the efficacy of OSEII for enhancing hydrocarbon biodegradation (Zwick et al. 1997).

BIOREN 1 and 2 are the derivatives of fish meal with urea and superphosphate (in a granular form as nitrogen source) and phosphorus sources and proteinaceous material (carbon source). BIOREN 1 contains a biosurfactant leading to enhanced oil degradation, while BIOREN 2 without biosurfactant attributes to greater bioavailability of hydrocarbons to microbial attack (Le Floch et al. 1997, 1999).

9.11 Conclusion and Future Prospective

The main threat to the aquatic environment is through oil leakage and by lethal polycyclic aromatic hydrocarbons into the food chain which is due to the toxic, mutagenic, and carcinogenic properties (Sei and Fathepure 2009).

The quick elimination and cleanup of PAHs by the physicochemical methods such as volatilization, photochemical oxidation, and bioaccumulation are seldom successful when compared to microbial bioremediation (Prince 1997; Zhao et al. 2008).

Bacteria are omnipresent and predominantly found in the marine environment and are considered as a potent hydrocarbon-degrading agents (Dasgupta et al. 2013). The degradation of oil-rich and potentially toxic environments solely depends on the novel microorganisms associated with the sponge *Biemna fortis*. The native and exogenous microbes used as inoculants can be applied to hydrocarbon-polluted environments depending on their biodegrading capabilities (Venosa and Zhu 2003; Díaz-Ramírez et al. 2008).

The major requirement for the bioremediation of oil spill depends on the microorganisms with suitable metabolic competence (Venosa et al. 2001). An array of microorganisms has been isolated from the marine sponge *Biemna fortis* for PAH degradation yielding beneficiary result (Farhan and Mahesh 2015). The degradation of aromatic hydrocarbons by PAH-degrading bacteria may detoxify or even contribute to the nutrition of the sponge remains uncertain.

Therefore, several PAH-contaminated sites require the cleansing, impending remediation methods that have to be explored and applied. Further investigations required to evaluate the functions of other invertebrates for the associations of PAH-degrading bacteria in oil-seep environs should yield remarkable outcome. The associations of a multifaceted bacterial community in sponge *Biemna fortis* capable of scavenging PAHs have been translated. Thus, we could suggest that these microbial populations may pave to the success of the sponge *Biemna fortis* living in such unique ecosystems by degrading PAHs.

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Chapter 10
A Comprehensive Review on the Bioremediation of Oil Spills

Mahsa Baniasadi and Seyyed Mohammad Mousavi

Abstract Oil spills are probable accidents occurring mostly during transportation and processing of oil that can contaminate marine, soil, sediments, and other environments. Oil spill is a special challenge to be remediated due to its several environmental, economic, and social threats. Several physical (mechanical), chemical, and biological methods are available as response to the oil spills. Among them bioremediation proved to be a promising technique for treatment of oil spills especially after being applied successfully for Exxon Valdez oil spill. Bioremediation is a greener approach in comparison with physicochemical methods, which is more cost-effective with less disruptive effect on the environments. In this method the natural or genetically manipulated microorganisms are applied to the polluted site and/or the polluted environment is enriched with nutrients, which are called bioaugmentation and biostimulation, respectively. These methods have been examined by researchers for treatments of oil spills mostly in laboratory scale and in less extent in real fields. One novel approach in this area of the research is focused on the novel material addition to the polluted environment for biostimulation of the treatment process. Novel materials include organic sources to provide nitrogen and phosphorus for the medium such as compost, biowastes, biofuel, etc. Biosurfactant addition is another promising method that improves the bioremediation by reducing the surface tension. Some polymeric materials can be added for improving the immobilization of microorganisms and consequently enhancing the degradation rate. Novel bioaugmentation approaches are conducted by manipulating microorganisms with the aim of modification of enzymatic characteristic, metabolic pathway design, expansion of substrate rate, enhancing the genes resistance toward catabolic activities, etc. However, still there are several resistances toward the application of these microorganisms to the real field, due to the environmental concerns. Another novel approach is the integration of electrochemical methods and biological routes. Several achievements were reported by researchers for the remediation of oil spills by using bioelectrochemical systems (BES). Microbial fuel cells are another

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technique to convert chemical energy into electricity concurrent with contaminant degradation. The future research on the oil spill bioremediation must be focused on these new aspects of the process and finally pave the way for application of bioremediation in real field to obtain promising pollutants degradation results.

10.1 Introduction

Oil spills occur when large quantities of petroleum hydrocarbons leak in the environment from storage tanks, pipelines, drilling process, non-suitable waste disposal practices, leaching from landfills, etc. (Pontes et al. 2013; Li et al. 2017). These can be originated from cleaning process of the equipment and unit or residues in containers and outdated chemicals and accidents during transportation (Helmy et al. 2015). Majorly, petroleum hydrocarbon spillage accident occurs during shipping, offshore and onshore exploration and production, and transportation (Atlas 1995).

Oil spills can occur in marine and terrestrial environments and threat the ecosystem and human health (Cheng et al. 2017). This environmental contamination can pollute the drinking water, cause fire and risk of explosion, ruin the water and air quality, destroy the recreational areas, waste the nonrenewable resources, and have huge economic costs. Oil spill's negative impacts have different economic, environmental, and social aspects. The consequence of oil spills on the ecosystem and natural resources are widespread and long term. Therefore, there is a need for a supportive logistic and trained workforce who can take the suitable responses in a short time after the occurrence of oil spills (Marzan et al. 2017).

Studies on oil spills treatment and removal strategies are usually considered in different mediums. Occurrence of oil spills in the sea and shoreline is the most common, since the petroleum is usually transported through marine transportation. The costs of these environmental disasters such as Exxon Valdez and Deepwater Horizon are incalculable and can influence the whole wildelife and human health. The carcinogenic and mutagenic effects of the oil spills in the sea have been proved. The marine oil spills, once occur, prevent light diffusion and oxygen penetration in the bottom layers of the sea (Bovio et al. 2017). Oily layer on the water surface threatens the existing marine flora and fauna (Jain et al. 2011). Destroying widelife, contamination of sea food, and reduction of tourism industry are all consequences of oil spills in the sea (Ng et al. 2015).

Some accidents have polluted the shorelines such as Amoco Cadiz spill which happened in Brittany shoreline (Atlas 1995). Shoreline contamination can occur due to the tidal and wave actions as well and adherence of the oil spillages to the soil (Lim et al. 2016).

The heavy components of the oil may sink to the sediments in the sea and form a tarry layer or get buried (Martin et al. 2015). The buried oil treatment is a totally different process than superficial oil. In the case of superficial spills, the treatment process is applied directly to the layers of the oil. These methods are not effective

for the treatment of buried oil, since the materials used for treatment may not reach the polluted layer in bottom of the sea (Pontes et al. 2013).

When the oil spill occurs in soil, the contaminants are attached to the soil physically or chemically or trapped in the soil matrix. The severity of the problem of oil spills depends on the oil type. Heavy oil spreads slower in the soil and can reach the lower layers of the soil. Therefore, the faster the response to the oil spills is performed, there is more chance for stopping the contamination (Helmy et al. 2015). An indicator to quantify the contamination in the soil is total petroleum hydrocarbon (TPH) concentration, which in the case of oil spills can reach 20–50 g kg^{-1} in the soil. This level of contamination can threat human health and environment (Xu et al. 2017). Soil contamination occurs most of all in the petrol stations and refineries, where the soil is exposed to the small but constant leakage of petroleum (Rhykerd et al. 1995).

Several guidelines, regulations, and directives are available to take care of oil spill prevention, preparedness, management, and compensation. However, accidental oil spill are inevitable, and therefore the governments should be prepared to perform the best response in case of spill. Development of federal government's blueprint (National Contingency Plan (NCP) which is for responding to oil spills and release of hazardous substance), assigning a competent (national) authority, and response capability are among the duties of governments (Walker 2017). Oil spill prevention and preparedness are among the top priorities of United States Environmental Protection Agency (US EPA 2013). Several physical, chemical, and biological methods are available for remediation of oil spills; among them bioremediation is the most promising method, which is the scope of this chapter.

10.2 Oil Spill Accidents in History

The first oil spill happened in the year 1907 and 7400 tons of paraffin oil entered to the sea and coastline of United Kingdom as a consequence. After that, about 140 large spills occurred, and in total seven million tons of oil have entered freely in to the environment. However, more than 90% of the oil pollutions are either natural such as runoff from land-based sources or has anthropogenic sources (not necessarily accidents) such as normal ship operation and deballasting and tank washing (Mapelli et al. 2017).

The largest oil spills in the history occurred in the sea such as Gulf War, Deepwater Horizon, Ixtoc 1 oil well, Amoco Cadiz, and other famous oil spills (Lim et al. 2016).

Amoco Cadiz accident happened in 1978 and released 227,000 tons of crude oil and bunkers in marine and contaminated 320 km of the shoreline length up to as deep as 20 inches (Lim et al. 2016).

Ashland oil spill occurred in 1988 when a four million gallon tank containing diesel oil collapsed and the oil dumped into the Monongahela River (Miklaucic and Saseen 1989). In 1989, Exxon Valdez oil spill occurred when a tanker crashed a reef

Fig. 10.1 Image of Exxon Valdez oil spill 1989 ("The Exxon Valdez Oil Spill: 25 Years Ago Today – The Atlantic" n.d.)

in Alaska, which spilled thousands of tons of oil into the sea (Jain et al. 2011). The sea and shoreline contamination, caused sever localized ecological damage to nearby community (Atlas 1995). It was reported that more than 250 thousand seabirds were killed due to this spill (Mapelli et al. 2017). Figure 10.1 is a photo of Exxon Valdez oil spill, where bioremediation was applied effectively as a strategy.

The largest inland oil spill happened in 1992 in Fergana Valley Uzbekistan, when 88 million gallons of oil released from an oil well into land. The ground absorbed this spill, and no cleaning was possible ("10 Largest Oil Spills in History – Telegraph" n.d.).

Prestige accident occurred in 2002 by the sinking of tanker and affected kilometers of the coastline with loosing up to 66% of the species richness in the region as a consequence (Bovio et al. 2017).

One of the most famous oil spills was the Deepwater Horizon (DWH) accident which occurred in the year 2010 in Gulf of Mexico during the drilling rig explosion. During this disaster, more than 700 thousand tons of crude oil was released into Gulf of Mexico (Mapelli et al. 2017). This accident decreased the biodiversity of the vertebrates and metazoan meiofauna. The cleaning cost of that spill was estimated to be 10 billion USD (Alessandrello et al. 2017). This accident can be called the worst oil accident in the history (Ng et al. 2015).

10.3 Oil Spills Removal Strategies

The faster response to the spill leads to more chance to prevent and stop contamination (Helmy et al. 2015). The aim of oil spill responds is mitigating the adverse impacts of the oil spill rather than monitoring the contaminants and allowing its natural attenuation. The first aim of a respond to oil spill is controlling the source and preventing the oil spread. The respond could be any strategy, method, technology, or equipment to control the spill and remediate its negative consequences. In addition to the fast response, stewardship is necessary to monitor and foresee the movement of oil. Use of mechanical equipment for spill removal such as skimmers, booms, barriers and sorbents, dispersants, and controlled in situ burning are among the response strategies (Walker 2017).

The aim of environmental treatments and remediation methods is to degrade and transform the contaminants into less harmful and even harmless compounds; when not possible, treatments is done by reduction of contaminants mobility and migration to prevent their spreading into uncontaminated areas. With this approach, the contaminant toxicity does not change, but the probability of their further distribution to the environment is reduced. Several treatments and responses to the oil spills are available which include physical, chemical, and biological methods (Jain et al. 2011).

The common mechanical strategy used for marine oil spills is controlling the oil from spreading and reaching shorelines with the application of barriers and then concentrating the oil into thick layer by booms to facilitate the oil removal by different types of skimmers like suction skimmers oleophilic and weir. Natural or synthetic polymers are used as sorbent for small spills (Mapelli et al. 2017). After in situ burning, toxicity assessments must be performed on burned residues. This approach was applied in the case of Deepwater Horizon accident (Mapelli et al. 2017).

Solvents and dispersants can be applied to reduce the size of spill into small droplets (Mapelli et al. 2017). The use of dispersant is a strategy to reduce the size of oil droplets to make it consumable by microorganisms more easily. For this strategy, the consumption of 830,000 gallons of chemical dispersant in both below and above sea surface is needed. The bacterial growth is also enhanced by the use of dispersant (Martin et al. 2015). In order to be effective, the dispersants must be added immediately after spill, before volatilization of light hydrocarbons. The function of dispersants is affected by salinity of water, water temperature, and wave action. Size reduction of oil droplets increases the surface area and reduces the interfacial water-oil tension and accelerates the biodegradation (Mapelli et al. 2017).

Another factor that limits the hydrocarbon degradation rate is the solubility of hydrocarbons. Surfactants are used to modify the hydrophobicity of cell membrane and modulate the bioavailability. In order to make this approach more sustainable, the nontoxic biosurfactants are currently replacing chemical surfactants (Mapelli et al. 2017).

The choice of the best cleanup technique is quite complex and is based on several factors including type of oil, location and size of spill, and local regulations and standards. For selecting the best methods, various criteria including efficiency, time, cost, reliability, effect on oil characteristic, and necessity of the post-remediation treatment of the applied method must be considered (Marzan et al. 2017).

Oil spill removal in the sea is done conventionally by using booms, skimmers, and big sponges as sorbents, skimming and mechanical removal using sorbents, vacuuming, in situ burning, and chemical dispersants (Marzan et al. 2017; Ng et al. 2015). However, all these methods have harmful environmental effects and endanger the ecosystem. The limitation of adsorbents application is the possibility of the erosion by the moving wave and lack of knowledge about its effectiveness (Helmy et al. 2015; Ng et al. 2015). Using dispersant as an oil spill removal method does not degrade the pollutants but just transforms it to another phase, which has still difficulty to be removed (Bovio et al. 2017).

Considering the case of soil, the available methods are solvent extraction, chemical oxidation, electrokinetic movement of contaminants, thermal desorption, flotation, washing with cosolvents or surfactants, using chemical agents for oxidation-reduction, physical removal such as ultrasonication, excavation of soil and sediment or groundwater pumping, and biological methods (Lim et al. 2016; Balba et al. 1998).

For remediation of sediments, different physicochemical methods are available such as ozonation, dredging, and electrochemical degradation. These methods have aggressive nature and are expensive and energy intensive (Li et al. 2017).

The conventional physical and chemical treatment methods are proved to be effective for removal of oil spills, but they produce several hazardous compounds which are still immunotoxicant and carcinogenic (Jain et al. 2011). Necessity of addition of chemicals for better removal makes chemical and physical treatment processes more costly (Marzan et al. 2017). Biologic methods detoxify hazardous substances, while physical methods usually transfer the hazardous substances to another environment. In addition, biological methods are less disruptive than excavation methods to the environment in the case of soil (Helmy et al. 2015).

10.4 Bioremediation of Oil Spills

Among the available methods, bioremediation is the most benign method which aims at enhancing the microbial metabolic activity and consequently stimulates the oxidation-reduction of the contaminants. During bioremediation, microorganisms degrade the organic contaminants (as their carbon and energy source) (Balba et al. 1998). However, the capability of microorganisms in degrading petroleum hydrocarbons is highly dependent on available chemical compounds and the conditions of the environment (Jain et al. 2011). This method has been developed in 1940s and became popular after Exxon Valdez oil spill in 1980s (Lim et al. 2016). Bioremediation is a quite slow process which requires weeks or months for effective cleanup. Although

detailed economic analysis of this process is not performed yet, properly done bioremediation is a cost-effective method (Jafarinejad 2017). Not having significant adverse effects such as production of secondary contaminants (Cheng et al. 2017), minimal physical disruption of the site, effectiveness in removing toxic compounds, simpler mechanical technologies, and less economical cost are other advantages of this process. Necessity of the specific approach for each polluted site and each spill type is a disadvantage of this process. Bioremediation is a less effective treatment strategy in the sea (Jafarinejad 2017), and the available knowledge is still rough and mainly focused on the application of prokaryotic organisms (Bovio et al. 2017).

Microorganisms use enzymes and oxygen and break down the structure of hydrocarbons. They use the petroleum hydrocarbons as substrates to produce biomass and decompose pollutants into water (Martin et al. 2015), carbon dioxides, and other harmless compounds (Atlas and Barsa 1992) such as fatty acids (Marzan et al. 2017). When considering bioremediation as a treatment to the oil spills, the aim is addition of materials to the contaminated environment to accelerate the natural biodegradation process. As an example, the addition of nutrients enhances indigenous organism's growth and activity. Another approach is exposure of the polluted environment to nonindigenous microorganisms with enhanced ability for hydrocarbon degradation. Bioremediation is considered as a complementary treatment after conventional cleanup (Jafarinejad 2017). Auxiliary treatments such as aeration and temperature adjustment can improve the bioremediation process (Lim et al. 2016). During bioremediation, petroleum hydrocarbons are used either as growth medium or as co-metabolism. This means that the contaminants can be considered as carbon and energy sources and be totally degraded and mineralized or be used as extra nutrition source in combination of growth substrate (Lahel et al. 2016). However, long period is needed for an effective bioremediation, and in the case of highly polluted environment, the process is less effective (Soleimani et al. 2013).

When considering bioremediation for treatment of buried oil, one must ensure that the added materials (microorganism and nutrients) can reach the polluted environment (Pontes et al. 2013). In the case of shoreline, such as oil spill in Brittany coastline, bioremediation was reported to be fast and effective method. The reason for that could be the adaptation of the indigenous microorganism of that region to the release from ballast water tanks, constant aeration with wave action, and presence of nitrogen and phosphorus nutrients from the agricultural runoff. However, the formation of emulsion which is resistant to biodegradation can prevent the process, since the microorganisms may colonize on the surface of emulsion but cannot reach within the mass of emulsion (Atlas 1995). Limiting factors for bioremediation in marine environment are usually nonbiologic factors (e.g., oxygen, phosphate, and nitrogen concentration) (Atlas 1995). For soil bioremediation, the limiting factors are aging of the spill, ambiguity of the soil matrix type, and nature of the contaminants (Xu et al. 2017).

Research on the bioremediation of oil spills must consider all different aspects such as effects of environmental parameters, metabolic pathways, basis of hydrocarbon breakdown as substrate (dissimilation) from genetic point of view, and effects of hydrocarbon contaminants on microorganism. The basis of this study

originated from monitoring the fate of hydrocarbon contaminants in the environment and search for the methods to accelerate the natural degrading process by overcoming the rate-limiting factors (Jain et al. 2011). Accelerating methods include addition of microbes with higher oil-degrading capacity or nutrients such as nitrogen and phosphorous (Marzan et al. 2017). For an effective bioremediation process, the presence of microorganism with desirable physiological characteristic and enzymatic capabilities, proper growth and activity conditions, and bioavailability of active microbial consortia play an important role (Lahel et al. 2016).

As already mentioned, several parameters can influence the bioremediation. Physical parameters (temperature, pressure, contaminant surface area) and chemical parameters (nutrient and oxygen availability, acidity, salinity, and contaminant nature and composition) have major effects on bioremediation. Among them most of the factors can be manipulated to accelerate natural biodegradation, while factors such as salinity are not adjustable in real field (Jafarinejad 2017). Among the biological factors metabolic parameters, mass transfer parameters in cell membrane and bioavailability must be considered (Gonzalez and Sanchez 2011).

Temperature can impact viscosity and consequently the toxicity; since at higher viscosity, the toxic light hydrocarbons are less volatile. The solubility of petroleum hydrocarbons changes with temperature as well. At low temperatures, alkanes with shorter chains are more soluble, while higher temperatures are favorable for solubility of several light aromatics. In all ranges of the normal seawater temperature (2–35 °C), biodegradation can take place. However, the rate decreases with decrease of temperature. The optimum temperature for biodegradation is 30–40 °C in soil and 20–30 °C in freshwater. For marine environment, it is reported to be 15–20 °C. Temperature has significant impact also on the microbial growth and activity. Dissolved oxygen is required for degradation and oxidization of the pollutants. Usually there is no oxygen limitation on superficial water in the sea and freshwater. However, oxygen may be limited in some subsurface sediments such as anoxic zones in water columns. Dense marine shorelines, tidal flats, coastal salt marshes, freshwater wetlands, and bottom layer of soil are other examples of the environment with lack of oxygen. The availability of oxygen also depends on water and wave turbulence, oil physical state, and availability of substrate. However, it was reported that anaerobic degradation of certain pollutants can occur in negligible extent as well (Rastegar et al. 2017; Nasirpour et al. 2015). Systems such as upflow anaerobic sludge blanket (UASB) are some bioreactor systems used for ex-situ bioremediation of petroleum hydrocarbons pollutants in wastewater obtained from a refinery. The advantages of anaerobic system over aerobic system is utilization of less space and no energy requirements (Rastegar et al. 2011).

Pressure can impact the rate of bioremediation. At higher pressure such as in the deep ocean, the rate of biodegradation decreases. The surface area of the contaminants can impact the interface of oil and water. Biodegradation rate improves with increase of surface area. In marine, turbulence of the sea surface can affect the process by influencing dispersion. This causes dilution of the available nutrients and spread of the oil (Jafarinejad 2017). Also the degree of spreading can affect the surface area. In aquatic system, oil normally spread and form a thin slick (Atlas 1991).

At higher pH, the rate of hydrocarbon degradation increases. Marine environments usually have alkaline conditions. The pH in salt marshes is lower (around 5), and pH in freshwater and soil is very variable (Jafarinejad 2017).

The presence of nutrients including nitrogen and phosphorous is a more limiting factor than oxygen. The nutrients are consumed not only by pollutant degrading microorganism but also other microorganism such as phytoplankton. Precipitation of phosphorus may also compete with oil-degrading microorganism (Jafarinejad 2017).

The adaptation skills of the microorganisms and their resistance to extreme environmental pollutions are an important factor as well (Bovio et al. 2017). However, even adapted microorganisms are not effective for biodegradation of extremely high amount of pollution. For instance, earthworms could not survive in the soil in which oil content contamination is more than 3%. At oil content of 1%, almost 100% inhibition of bacterial activity was observed (Lim et al. 2016). During oil spills, the concentration of petroleum hydrocarbons is far excess of tolerable limits (Atlas 1991).

Bioremediation in cold environments such as Alaska, northern Russia, and Canada need more studies and considerations. Between 1996 and 1999, 407 spills on average occurred annually in Alaska. Even higher risk of pipeline damage and petroleum hydrocarbon pollution is available there, in comparison with moderate climates. In cold zones, the oil spill impacts the microbial population, freeze-thaw processes, thermal and moisture regimes, as well as oxygen availability and pH of the soil. Environmental impact of the oil spill is harsher in the cold environments, since the cold ecosystems are more sensitive. Furthermore, low temperature results in higher viscosity, lower volatile evaporation rate, and higher water solubility of the oil which can delay the biodegradation process. However, successful bioremediation of oil spill was achieved in several cases such as arctic and subarctic regions (Montagnolli et al. 2015).

Bioremediation of pollutants in highly salinated areas is also particular due to the effect of salinity on microbial population (Si-Zhong et al. 2009).

Different microorganisms including bacteria, fungi, yeast, and microalgae are able to degrade petroleum hydrocarbons. The bioremediation can be performed in situ or ex situ (Lahel et al. 2016). In ex situ process, the contaminated matrix is extracted elsewhere to be treated, while during in situ treatment, the treatment occurs in the place of contamination (Balba et al. 1998). The bioremediation of soil was conducted effectively both with in situ and ex situ approaches (Lim et al. 2016). However, in situ approach is more cost-effective and safer than ex situ with less disruption of the polluted environment (Lahel et al. 2016).

Another common approach is supplying electron donors to stimulate the reduction reactions and degradation of halogenated compounds, or electron acceptors to stimulate the oxidation reactions and degradation of non-halogenated compounds (Balba et al. 1998).

Recently novel approaches have emerged that integrate physicochemical methods with biological approaches (Balba et al. 1998), which will be discussed thoroughly later on this chapter.

10.5 Microorganisms for Bioremediation of Oil Spills

It was reported that more than 200 different species of bacteria, fungi, and yeasts are able to degrade petroleum hydrocarbons. These microorganisms can be found naturally in marine, freshwater, and soil. The biodegradable hydrocarbon compounds range from methane to C_{40} compounds. To classify, almost 79 bacterial, 9 cyanobacterial genera, 103 fungi, 14 algae, and 56 yeasts are able to degrade the hydrocarbon pollutants (Jafarinejad 2017; Gonzalez and Sanchez 2011).

Different groups of indigenous soil bacteria can degrade different compounds of petroleum hydrocarbons. These bacteria include *Pseudomonas* strains isolated from soil and aquifers to degrade polycyclic aromatic hydrocarbons (PAHs) (Atlas 1995). Other microorganisms with the ability to degrade petroleum hydrocarbons are *Yokenella* sp., *Alcaligenes* sp., *Alcanivorax* sp., *Microbulbifer* sp., *Sphingomonas* sp., *Micrococcus* sp., *Cellulomonus* sp., *Dietzia* sp., *Roseomonas* sp., *Stenotrophomonas* sp., *Gordonia* sp., *Acinetobacter* sp., *Corynebacterium* sp., *Flavobacter* sp., *Streptococcus* sp., *Providencia* sp., *Sphingobacterium* sp., *Capnocytophaga* sp., *Bacillus* sp., *Enterobacter* sp., and *Moraxella* sp. (Jain et al. 2011).

Alcanivorax sp. bacteria and *Cycloclasticus* sp. can use aliphatic and aromatic hydrocarbons, as their carbon source, respectively. Some bacteria can help to produce biosurfactants which can enhance the bioremediation by reducing surface tension and increase of crude oil uptake. However, factors such as availability of nutrients and nature of oil contaminants are influential in degrading the petroleum hydrocarbons (Bovio et al. 2017).

Some fungi are also capable of degrading petroleum hydrocarbons. However, they need longer time for effective degradation. Fungus belonging to *Aspergillus* sp., *Amorphoteca* sp., *Penicillium* sp., *Graphium* sp., *Neosartorya* sp., *Fusarium* sp., *Paecilomyces* sp., and *Talaromyces* sp. are among the microorganisms with the ability to degrade petroleum hydrocarbons. White rot fungi are reported to be able to degrade compounds such as polychlorinated biphenyls (PCBs) and PAHs (Baniasadi et al. 2018). Some yeasts including *Candida* sp., *Pichia* sp., and *Yarrowia* sp. also reported to have the potential to degrade the compounds available in oil contaminants (Jain et al. 2011). Some researchers suggests that in some specific circumstances, fungi can degrade petroleum better than bacteria. However, there is not much information available for fungal bioremediation of marine contaminated sites (Bovio et al. 2017).

Marzan et al. isolated bacteria for bioremediation from Shela River which was polluted with an oil spill in the year 2014 for their oil-degrading potential. They have isolated seven distinct bacterial colonies to degrade the furnace oil. Among the isolated bacteria, the top three with the oil-degrading capabilities were assessed to be *Pseudomonas aeruginosa*, *Bacillus* sp., and *Serratia* sp. (Marzan et al. 2017).

Using indigenous microorganisms available in the polluted site is suggested to be a promising method for bioremediation of petroleum hydrocarbon contaminants, since these native microorganisms are adapted to the available conditions. However,

microorganisms with enzymatic ability for pollutant degradation may be absent which leads to very long process. For example, *Bacillus subtilis*, *Pseudomonas aeruginosa*, and some other microorganisms are isolated from soil contaminated with petroleum hydrocarbons (Gonzalez and Sanchez 2011).

In the work of Bovio et al., fungal community capable of degrading oil spills were isolated from Mediterranean marine (67 strains) and sediments (17 strains). The fungal growth was stimulated by crude oil which was the carbon source. Among them *A. terreus*, *T. harzianum*, and *P. citreonigrum* yileded the highest dichlorization percentage, and *A. terreus* reported the highest yield in decreasing hydrocarbon compounds (Bovio et al. 2017).

When microorganism communities are exposed to contaminants (hydrocarbons), they adapt gradually and undergo selective genetic enrichment. After adaptation, the population of the bacteria capable of degrading hydrocarbons and plasmids of the bacterial cells that encode hydrocarbon catabolic genes is increased (Lahel et al. 2016). The increase in population of oil degrader microorganisms has been observed for *Alcanivorax* sp. and *Cycloclasticus pugetii* (Gonzalez and Sanchez 2011).

When the existing microbial population of the environment is not capable or sufficient for degradation of the pollution, the addition of oil-degrading microorganisms to the contaminated area is conducted. This approach is called bioaugmentation and is explained further in the next section (Jafarinejad 2017). Recently, the researchers are searching to manipulate the microorganisms genetically to enhance their oil-degrading ability (Martin et al. 2015).

Bioaugmentation is the method in which microorganisms with high oil-degrading ability are added to a contaminated environment as adjunct for the indigenous microbial population to achieve the effective biodegradation. It was reported that petroleum biodegradation is performed better in the presence of consortium of microorganism in comparison to monospecies activities (Jain et al. 2011). Singh et al. have used consortia of different bacterial strains (mixture of *Micrococcus* sp. GS2-22, *Flavobacterium* sp. DS5-73, *Corynebacterium* sp. GS5-66, *Bacillus* sp. DS6-86, and *Pseudomonas* sp. DS10-129) to perform bioremediation of petroleum hydrocarbon contaminated soil. In their work, oil degradation rate of 78% was achieved after 20 days (Singh et al. 2012).

10.6 Mechanism of Bioremediation of Oil Spills

Petroleum oil spill is complex mixture of different compounds. More than 17,000 chemical components have been identified in crude petroleum that contains large amounts of aliphatic, branched, and aromatic hydrocarbons. The majority of nonpolar fraction is composed of saturated and aromatic hydrocarbons. Petroleum hydrocarbons also contain halogenated hydrocarbons (Jain et al. 2011; Balba et al. 1998). The oil spill composition includes alkanes (both linear and branched), aromatics, and cycloalkanes and some NSO (nitrogen, sulfur, and oxygen)-containing compounds such as thiophene, phenol, and indole (Gonzalez and Sanchez 2011).

More methyl-branched compounds and/or condensed aromatic rings content make oil spill nature more complex and lead to slower degradation rate and possibility of accumulating of partially oxidized intermediary metabolites (Atlas 1995).

The enzymatic capability of microorganism enables them to degrade petroleum hydrocarbons. Some types of microorganisms are alkanes (linear, branched, and cyclic paraffins) degrader, and some are aromatics degrader and some both. The degradation of normal alkanes (C1-C26) is the easiest and fastest one. However, degradation of toxic light aromatics (like benzene, toluene, and xylene) by marine microorganisms is fast and easy as well (Ronald M. Atlas 1995). Low water solubility as well as high sorption capacity makes degradation of PAHs more difficult especially in cold climates (Si-Zhong et al. 2009).

The highest biodegradation rates are for saturates and then light aromatics. The order of petroleum hydrocarbon component degradation is normal alkanes followed by branched alkanes and alkenes, light n-alkyl aromatics, single aromatics, cyclic alkanes, polycyclic aromatic hydrocarbon, asphaltenes, and resins (Jafarinejad 2017; Si-Zhong et al. 2009).

Benzene, toluene, ethyl benzene, and xylene and in general aromatic hydrocarbons can be degraded by microorganisms such as *Pseudomonas*, *Rhodococcus*, and *Ralstonia*. The microorganisms suitable for degradation of polyaromatic hydrocarbons are *Pseudomonas* for naphthalene, *Pseudomonas* and *Haemophilus* for phenanthrene, *Rhodococcus* for anthracene, *Haemophilus* and *Mycobacterium* for pyrene, and *Rhodococcus* and *Mycobacterium* for benzo[a]pyrene (Gonzalez and Sanchez 2011).

It must be noted that the composition of oil spill may be changed by evaporation and dissolve of light aromatics and alkanes which are further metabolized by microorganisms. As a consequence, heavier components may remain (Jafarinejad 2017).

Crude oil never biodegrades completely. It was reported that in some days and weeks, more than half of the heavy oil can be degraded and a black complex residue is always left after biodegradation, mostly containing asphaltic compounds. However, bioremediation is still considered effective, since this residue is not toxic and has low bioavailability, and inasmuch as coating and suffocation of the polluted area do not occur, it can be considered environmentally inert (Helmy et al. 2015).

The bioremediation of petroleum oil can be conducted under aerobic as well as anaerobic conditions. During aerobic metabolism, oxygen-oxidizing enzymes which convert the O_2 to reduced substrate are needed. The lack of contact with water-insoluble hydrocarbons is a problem that bacteria can solve by two general strategies. The contact is enhanced by a particular adhesion mechanism in which emulsifying agents are produced extracellularly (Jain et al. 2011).

The mechanism of oil spill degradation is usually studied by using different model petroleum hydrocarbons. However, generally the biodegradation of petroleum hydrocarbons occurs via several sequential reactions initiating by attack of microorganism on petroleum structure and formation of intermediate substances. The intermediate compounds are utilized by different microorganisms and lead to further degradation (Jafarinejad 2017).

In the first step of degradation pathway, the hydroxyl group is added to the end of alkane chain. This group can be added on the unsaturated ring of PAH and form alcohol as well. The length of the chain is then reduced by oxidation of compound to aldehyde and later carboxylic acid. Finally, CO_2, H_2O, and biomass are formed. Oxygen addition to hydrocarbons makes them more polar and water soluble, with more biodegradable and less toxic structure (Jafarinejad 2017).

During the degradation of aliphatic hydrocarbons such as n-alkanes, firstly alcohols are produced, which is sequentially oxidized and dehydrogenated to form primary alcohols and aldehydes and a monocarboxylic acid consequently. The carboxylic acids then undergo β-oxidation (Jain et al. 2011) and form fatty acids and acetyl coenzyme and release carbon dioxide. The limiting step is the addition of oxygen to the hydrocarbon, and once carboxylic acid is formed, it can be metabolized rapidly. In the case of branched isoprenoid alkanes such as pristine, the hydrocarbon undergoes oxidation and forms dicarboxylic acids. The presence of methyl branches increases the resistance of hydrocarbons to microbial attack (Atlas and Barsa 1992).

The degradation pathway for aromatic and PAHs is through hydroxylation of the ring by enzymes which are mono- or dioxygenase. Consequently, diol is formed, and the ring is cleaved and undergoes further degradation (Jain et al. 2011).

10.7 Biostimulation and Bioaugmentation

As mentioned, physicochemical conditions (temperature, pressure, pollutant surface area, oxygen content, nutrient availability, pH, salinity, oil composition, etc.) influence the natural bioremediation process. When applying bioremediation as a response to the oil spill, two main approaches are available which are biostimulation (enhancing the nutrients availability – mostly nitrogen and phosphorus – to initiate the growth and accelerate the biodegradation) and bioaugmentation (inoculation of microorganisms with enhanced ability to degrade petroleum hydrocarbons in order to facilitate the process). However, a novel approach is also available, which is bioaugmentation with genetically engineered microorganisms (bioaugmentation with GEMs) (Jafarinejad 2017) (Lahel et al. 2016). It was reported that the effects of bioaugmentation can be observed much faster than biostimulation (Pontes et al. 2013). However, the most promising approach is combination of biostimulation and bioaugmentation with addition of biosurfactants (Gonzalez and Sanchez 2011).

10.7.1 Biostimulation

Biostimulation is a nutrient-enhanced bioremediation process to improve the indigenous biodegradation rate of petroleum hydrocarbons especially organic pollutants by providing the limiting nutrient material to the polluted medium (Soleimani et al.

2013). The nutrients include carbon, nitrogen, and phosphorus and some other growth-limiting cosubstrates. Modification of the conditions including temperature and aeration can be also done during the biostimulation. All these activities are performed with the aim of acceleration of oil degrader's growth and activity. This approach can be called fertilization or nutrient enrichment. (Jafarinejad 2017).

The microbial metabolic activity is improved due to nutrient supply. Electron acceptors and donors can be also added to stimulate the oxidation and reduction mechanism. However, their addition must be under control. The provided nutrients must be available and be in contact with the microorganisms (Balba et al. 1998). The conditions for enhancing natural biodegradation can be adjusted by manipulating of different parameters such as application of fertilizers, nutrients, biosurfactants, and biopolymers. Manipulation of all these parameters with the aim of improving natural bioremediation can be considered as biostimulation (Lim et al. 2016).

Another practice that is used for improving the conditions especially aeration is bioventilation process which is application of oxygen to soil porous with the aim of enhancing microorganisms growth and metabolism of organic matter by providing aerobic conditions. It was observed that using bioventilation the rate of bioremediation increases to 85% from 64% in natural attenuation process (Lim et al. 2016).

In marine environment or generally open systems, addition of N and P is quite difficult. Therefore, uric acid is added instead, which is the waste product of animals (birds, reptiles, insects, etc.). Uric acid by low water solubility can attach to the petroleum hydrocarbons and can be used by bacteria as nitrogen source or both nitrogen and carbon source (Gonzalez and Sanchez 2011). It was observed that for light crude oil degradation, addition of nitrate is more effective than ammonia in seawater, while in the salt-marsh soil, addition of ammonia is more effective than nitrate. Fortunately, no adverse impact, such as algal blooms was observed by nitrogen addition (Jafarinejad 2017).

Good results have been obtained by using this approach on sediments of the cost contaminated after Exxon Valdez spill in Alaska, and the rate of biodegradation increased three to five times by addition of fertilizers, such as iron, phosphorus, and nitrogen (Martin et al. 2015).

10.7.2 Bioaugmentation

Bioaugmentation is an approach which is used when the native microbial populations are inadequate for degrading the pollutant mixtures such as petroleum. This is done when the population of hydrocarbon-degrading microorganism is low or there is a need to degrade particular hydrocarbon which cannot be degraded by indigenous microbes. As an example, polynuclear aromatic hydrocarbons are usually hard to be degraded (Jafarinejad 2017).

In this approach, microorganisms with enhanced biodegradation ability are added to the polluted environment to supplement the naturally available microbes.

Different methods are available for this approach. Commonly the nonindigenous microbes from other polluted environments are used to be added to the target site (Jafarinejad 2017). Alternatively, microbes from the target site are separated and mass cultured under laboratory conditions in bioreactors and are used as inoculum to the target site. This method is called *autochthonous bioaugmentation* and is referred to the cases where the bioaugmentation is done by the native microbes of the contaminated site after enrichment to be reapplied to the site (Lim et al. 2016). Seeding of microorganisms to the contaminated site can reduce the lag period to start the biodegradation. When the seeding is done by the enhanced indigenous organisms taken from the target site, the adaptation problem is avoided (Jafarinejad 2017). The criteria for selection of the added microbes are based on their physiology and metabolic ability (Lim et al. 2016).

Bioaugmentation was done successfully in bench scale under controlled conditions. However, it must be considered that conditions in real fields may be uncontrollable (Jafarinejad 2017). It has been suggested that primary laboratory tests for microorganism selection before in situ application of the microorganism can increase the chance of successful bioremediation. In the work of Szulc et al., the most effective consortium (*Pseudomonas fluorescens* and *Pseudomonas putida* mixed with *Aeromonas hydrophila* and *Alcaligenes xylosoxidans*, in addition to *Xanthomonas* sp., *Gordonia* sp., *Stenotrophomonas maltophilia*, and *Rhodococcus equi*) for bioaugmentation was selected in the laboratory based on the quantity of CO_2 and dehydrogenase activity (Szulc et al. 2014). Kim et al. proposed a gene-based diagnostic technique that can reduce the needed time for microorganism selection. The DNA diagnostic method via oligonucleotide microarray method was applied to detect and observe genes with desirable ability to degrade aliphatic and aromatic hydrocarbons. In this work, the bioremediation of contaminated site was performed in field tests by bioslurping (Kim et al. 2014).

Researchers claim that the commercial bacterial blends can be produced with customized properties for each specific site and type of pollution in spill, considering the specific nutritional needs and limitations. Large quantities of the microbial blend can be produced in laboratory and be stored for emergency cases for up to 3 years (Jafarinejad 2017).

10.8 Novel Approaches for Bioremediation of Oil Spills

Current research in bioremediation of oil spills is mostly focused on novel material addition for biostimulation, using genetically modified microorganisms for bioaugmentation and integration of different physicochemical and biological approaches for treatment of oil spills. The novel approaches in bioremediation of oil spills are explained in following.

10.8.1 Novel Material Addition

As mentioned earlier, bioremediation is done normally with the addition of fertilizers and nitrogen and phosphorus materials. In novel approaches biowastes, inorganic materials, polymeric materials, etc. are added to enhance the bioremediation. Biosurfactant addition is another material that recently gained attention in the studies on bioremediation of oil spills.

Biosurfactants are produced extracellularly or as part of the cell membrane by different microorganisms including yeasts, bacteria, and filamentous fungi. Microorganism activity in the case of biosurfactant is due to the production of extracellular biosurfactant (e.g., trehalose lipids produced by *Rhodococcus* species) or cellular biosurfactants (e.g., mycolic acids) which cause the microbial cells to be attached to hydrophobic phases. Wide structural diversity of biosurfactants is available, including lipopeptides, glycolipids, fatty acids, lipoproteins, phospholipids, neutral lipids, and polymeric biosurfactants (Ayed et al. 2015).

Two groups of biosurfactants are available, which are low-molecular-weight surface active materials with the ability to lower the tension (both surface and interfacial) efficiently and polymers with high molecular weight (bioemulsifiers) that are used for stabilization of emulsions (Bezza et al. 2015).

Biosurfactants in comparison with chemical surfactants have less toxicity, biodegradability, and ecological acceptability. Biosurfactants act more effective in different pH, temperature, and salinity in comparison to chemical ones (Bezza et al. 2015). The biosurfactant-producing microorganisms are interesting for bioremediation especially for biodegradation of hydrophobic compounds. Recently, the application of biosurfactant microorganisms gained attention in the research due to offering superior biodegradability and being environmentally friendly in comparison with synthetic surfactants (Szulc et al. 2014).

Most of petroleum hydrocarbons are insoluble in water. Considering the case of oil spill in aqueous environments, the petroleum oil droplets are dispersed naturally by wave action in water column. Emulsification agents can be used for emulsification of diverse oil components. The ratio of surface to volume is an influential factor in bioremediation since the biodegradation process occurs at the hydrocarbon-water interface. Biosurfactant role is reducing the interfacial tension available between oil and water and enhancing the droplets dispersion in water column (Montagnolli et al. 2015).

Surface active materials by increasing the solubility remove hydrophobic compounds from soil and contribute to their biodegradation. Hydrophobic and hydrophilic moieties available in amphiphilic molecules can interact with interfaces that have different polarities. This leads to reduction of interfacial and surface tension and increase of bioavailability, transfer rate, and solubility of hydrophobic and insoluble organic compounds (Bezza et al. 2015). Generally, biosurfactant role in bioremediation is reduction of surface tension, increasing the solubility of hydrocarbons and making them available to microorganism. The hydrophobicity of the bacterial cell surface can be also of influence, and this allows substrates which are hydrophobic to be more in contact with bacterial cells (Ayed et al. 2015).

Low-molecular-weight surface active materials include glycolipids, lipopeptides, and phospholipids. The most common surfactant is lipopeptides which contain both fatty acid moiety (hydrophobic) and peptide moiety (hydrophilic). The critical micelle concentration (CMC), proper emulsification properties, powerful surface activities, and outstanding foaming characteristics are among the characteristics of low-molecular-weight surface active materials. Lipopeptide's physicochemical properties make them stable at diverse temperatures and pH levels. Famous biosurfactant-producing bacteria are *Pseudomonas*, *Bacillus*, *Acinetobacter*, and *Mycobacterium* (Bezza et al. 2015). Rhamnolipids is one common surface active compound of microbial origin since the congeners-constituents of this bioemulsifier are well described and investigated for efficient application during soil flushing and mobilization of resistant contaminants. These qualities make rhamnolipids a potential agent for improving bioremediation of polluted terrestrial environments. However, in the work of Szulc et al., no significant change was observed in the treatment process (both non-bio- and bioaugmented treatment) of diesel-contaminated soil by addition of rhamnolipids in real field (Szulc et al. 2014).

Another novel biosurfactant was produced by *Paenibacillus dendritiformis* that was isolated from the soil of the plant contaminated with creosote. This biosurfactant was identified as lipopeptide. The produced biosurfactant was analyzed and showed an amino acid (Cys-Gly-Ala-Gly-Ile-Asn-Leu as sequence) with long chain fatty acid (522 Da molecular mass). With hexane this biosurfactant showed 74% emulsification index and with cyclohexane 82%. High pH, thermal and saline stability was observed as well. The ability of this biosurfactant was tested in the work of Bezza and Chirwa in batch experiments for enhancing the bioremediation of PAHs from heavy oil-contaminated sands (Bezza et al. 2015).

Bacillus amyloliquefaciens was also reported to be a strong biosurfactant-producing bacteria in *Landy medium* (semisynthetic medium). The surface tension decreased to less than 30 from 72 mN/m by this biosurfactant and has CMC of 100 mg/L. The biosurfactant showed better solubilization efficiency toward diesel oil than SDS and Tween 80. Ayed et al. have investigated the ability of biosurfactant that was produced by *Bacillus amyloliquefaciens* in lowering the surface tension, improving solubility, and enhancing biodegradation (Ben Ayed et al. 2015).

In the work of Montagnolli et al. biosurfactant produced by *Bacillus subtilis* was investigated for biodegradation of simulated wastewater contaminated with crude oil, diesel, and kerosene. Mathematical models were used for demonstrating and predicting the effect of biosurfactant on kinetics of biodegradation process. Higher yield of CO_2 output was observed in the assays containing biosurfactants (Montagnolli et al. 2015).

The work of Hernández-Espriú et al. addressed the application of biosurfactants obtained from plants including locust bean, guar, and mesquite seed gums for the bioremediation of the soil that was contaminated with diesel after a pipeline accidental spill. Natural gums can be used in variety of industrial applications for their emulsifying, microencapsulating, thickening, and stabilizing properties. The results showed that natural gums are promising biosurfactant in bioremediation of oil contaminated soil. The obtained efficiencies were 54.38% and 53.46% for Guar gum

and locust bean gum respectively which is higher than the efficiencies obtained by ionic and non-ionic surfactants. The best removal rate (82% for diesel) was obtained by application of a small amount of gum concentration (2 ppm) (Hernández-Espriú et al. 2013).

Compost addition can be considered as a method to supply nutrients to the medium. Therefore, several researchers added compost for improving the bioremediation (Gomez and Sartaj 2013; Bastida et al. 2016; Dadrasnia and Agamuthu 2014).

Bastida et al. conducted the bioremediation of hydrocarbon polluted soil in semi-arid areas where the soil nutrients and organic matter are poor. This makes the microbial development of soil problematic. The results showed enhanced (88%) removal of PAHs and alkanes after 50 days with compost, while the biodegradation without compost was not significant. Bioremediation in the presence of compost was conducted by *Sphingomonadales* and uncultured bacteria and led to secretion of catabolic enzymes such as 2-hydroxymuconic semialdehyde, cis-dihydrodiol dehydrogenase, and catechol 2,3-dioxygenases (Bastida et al. 2016).

Gomez and Sartaj performed combined biostimulation and bioaugmentation by inoculation of microbial consortia and addition of mature organic compost in cold environment. The bioremediation results were the best, having both consortia and compost in comparison with their individual use (Gomez and Sartaj 2013).

Besides application of nutrients and fertilizer to biostimulate the bioremediation process, some researchers suggested to use agricultural biowaste for biostimulation with organic matter. Rice husk, chicken manure, and other biowastes were used for this purpose (Dadrasnia and Agamuthu 2014; Adams et al. 2017). Manure addition have advantages including soil alteration, improving organic matter, increase of water holding capacity and advantageous biota (Adams et al. 2017). The application of biowaste in the soil-contaminated with diesel fuel showed enrichment value of $\delta^{13}C$ in treatments amended with organic waste (Dadrasnia and Agamuthu 2014).

In the work of Horel et al., the addition of organic nutrients plant material and fish tissue (*Spartina alterniflora* and *Chloroscombrus chrysurus*, respectively) was investigated for bioremediation of sandy beach sediments available in coastal region of Alabama, and the results were compared with the cases where inorganic nitrogen and phosphorus were added. The highest degradation rate was obtained by fish tissue which led to 104% increase of degradation rate. Inorganic nutrients addition increased the degradation rate 57%. Plant material only improved the degradation rate in low extent (7%) (Horel et al. 2015).

Dias et al. (2012) have compared the results obtained for bioremediation of soil with addition of different organic and inorganic materials. In their study, they have studied samples with inorganic salt, chemical surfactant (Brij700), fish meal, and a special commercial product. The inorganic salt was used as an example of component with solubility in water. Fish meal was a slow release source of N and P, and the used commercial product was OSEII (Oil Spill Eater International, Corp.) which is an oleophilic rich in nitrogen and phosphorus that can delay the washing process as reported. This commercial product is mentioned in EPA's National Contingency Plan for Oil Spills as a supplementary material that contains phosphorus, nitrogen,

carbon and some vitamins which are helpful for fast colonization of natural bacteria. Although the fish meal enhanced the bacterial growth and activity, it did not help the hydrocarbon removal. Organic salts evidence no significant decrease in the pollutant, while, commercial products caused around 50% increase in hydrocarbon removal after 45 days.

Ng et al. (2015) investigated the biodegradation of petrodiesel by using biodiesel obtained from *Jatropha*, soybean, and palm as an additive for biostimulation. Biodiesel addition enhanced the biodegradation rate of the mixture, respectively, 12.8%, 19.4%, and 17.5% (from different biomass sources). The efficiency of biodegradation was evaluated by CO_2 evaluation test. The enhancement was reported to be mostly related to co-metabolism and solvation. The co-metabolism effect of biodiesel is its potential to act as nutrient source with providing the energy for microorganisms that consume hydrocarbon and consequently increasing microbial activity. The solvation effect is due to increased exposure area that is caused by solubilizing effect that biodiesels has on petrodiesel. Petrodiesel when mixed with biodiesel enhances solvating and ease of dispersion which prevent the pollutants from integration into sediments and facilitates recovery.

Immobilization of the microorganisms used for bioaugmentation on a career is another effective method for enhancing biodegradation. The most common immobilization technique is formation of biofilm or entrapment and encapsulation of microorganisms using polymeric gels. Microbial immobilization in oil sorbents can produce series of synergetic sorption-biodegradation reaction. Alessandrello et al. immobilized coculture of *Pseudomonas monteilii P26* and *Gordonia* sp. *H19* on polyurethane foam and further used the immobilized cell for the removal of petroleum oil from artificial seawater. Polyurethane foam was selected as a carrier for being economic and readily available and presenting good buoyancy and oleophilic properties. In this work, different temperatures have been tested. The best oil removal was achieved at 30 °C with immobilized mixed biofilm on polyurethane foam after 7 days. The oil removal was due to both biological activity and sorption on the biofilm/carrier system. The immobilized cell can be also stored. Their storage at 4 °C enhanced oil bioremoval at low temperature even though bacterial viability of *P. monteilii* P26 in the biofilm decreased. They have concluded that bacterial acclimatization occurred during the storage improving their metabolic activity at low temperature (Alessandrello et al. 2017).

10.8.2 Genetically Modified Microorganisms

The first genetically engineered microorganism (GEM) was built in 1970 which got the name of "superbug" and was able to degrade oil. This was done with plasmid transfer to utilize some toxic hydrocarbons including hexane, octane, toluene, xylene, camphor, and naphthalene (Kulshreshtha 2013).

The development of GEMs became more important in the early 1980s after improvement of genetic engineering methods and thorough research on metabolic

capabilities of microorganisms. It was in 1981 that the first two strains which were modified genetically were patented. These two strains are *Pseudomonas aeruginosa* (NRRL B-5472) and *Pseudomonas putida* (NRRL B-5473) containing genes that give them the ability to degrade naphthalene, salicylate, and camphor. Two operons available in these strains (xylUWCMABN and xylXYZLTEGFJQKIHSR) are responsible for metabolism of toluene, *m*-ethyltoluene, and *m*- and paraxylene (Wasilkowski et al. 2012).

The limitation of natural microorganisms for bioremediation of contaminants is the slow degradation rate. Another limitation of natural attenuation is toxicity of some of organic pollutants for microorganisms in combination of complexity caused by diversity of pollutants. This is more severe about new man-made contaminants released into nature, since the microorganisms have not evolved the proper catabolic pathway for their degradation in such a short time (Chai et al. 2015). This is the main focus of genetic engineering and manipulation of microorganism for bioaugmentation with GEMs process. Recent advances in molecular biology promoted this area of research in the field of engineering microorganisms for specific bioremediation. During genetic modification, microorganisms are supplemented with new genetic properties to be capable of biodegradation of specific pollutants that are not degraded by natural microorganism proper and fast enough. Microbiological information in addition to knowledge on ecological and biochemical mechanisms are needed for combining various desirable metabolic characteristics of organisms and manipulation of important genetic parameters (Jafari et al. 2013). In order to develop genetically manipulated bacteria, there is a need for understanding the way that bacteria break down petroleum compound molecules for removal of the oil spills. For proper design of GEMs, information about interaction of microbes and contaminants, the genetic basis of the interactions, biochemical paths, operon arrangement, and molecular biology must be considered (Kulshreshtha 2013).

Researchers at the University of Texas, Austin, have revealed the genetic code of petroleum hydrocarbon degradation during the Deepwater Horizon oil spill. They have revealed that the ability of some bacteria for oil degradation is far greater than what was expected especially for aromatic hydrocarbon (as an example *Alcanivorax* was formerly considered to be incapable of oil degradation). In this research they have sequenced the DNA of the microbes that have oil degradation ability to uncover genetic characteristic of several bacterial species. The gene sequencing also revealed the method that the genetic potential of the microbial consortia increased (Dombrowski et al. 2016).

The construction of GEMs with enhanced ability for biodegradation of organic compounds is possible since the degradative mechanism, the enzymes, and the relevant genes are understood and biochemical reactions are explained thoroughly (Wasilkowski et al. 2012). The limitation of this method is on one hand the survival of GEMs in the environment and public acceptance on the other hand, which hinder their wide application (Jafarinejad 2017).

For the purpose of bioremediation, different genetic engineering methods are available including improving specificity and affinity of enzyme, metabolic pathway design, and its regulation, expansion of the range of substrate for existing path-

way, preventing the production of toxic intermediates which inhibit the path by redirection of carbon flux, enhancing of genetic stability of catabolic activities, identification of genetically modified bacteria in polluted environment by marker gene, and utilization of biosensor for monitoring specific chemical compound. The most common method for creation of GEMs is engineering of one gene or operons and construction of pathways and modification of the existing genetic sequence. For GEM construction, the first step is identification of microorganisms for modification with relevant genes (Kulshreshtha 2013; Chai et al. 2015). By genetic manipulation, rate-limiting steps in metabolic pathways are modified to increase the degradation rate. Incorporation of totally new metabolic pathways into bacterial strains is also possible. Genetic engineering can help for elaborating strategies to monitor, control, and assess the toxicity (Sayler and Ripp 2000). As an example, microbes are limited to aerobic catabolic and co-metabolic biodegradation pathway, and there are limitations for their application in anaerobic environments. By inserting oxygenase genes, this microorganism can undergo anaerobic pathways as well (Kulshreshtha 2013).

For multiplying or expressing specific genes, there is a need for a cloning vector which is commonly plasmids. Vectors are genetic molecules using for transfer of target genetic information to cell to be modified. In the new cell, they can replicate their chromosomal DNA independently. Vectors contain a set of diverse gene such as antibiotic resistance genes. Transposons are other type of genetic elements that act as vectors. Currently, the artificial plasmid vectors are used as well for construction of GEMs. Expression plasmids are also used widely since they facilitate production of desired protein in large quantity very quickly. Another genetic engineering tool for cut-and-paste techniques is enzymes including restriction endonuclease by cleaving DNA in a specific site and DNA ligases which facilitates the joining of DNA strands together and formation phosphodiester as backbone of DNA (Wasilkowski et al. 2012).

The object of genetic manipulation is mostly bacteria especially from genus *Pseudomonas*. These bacteria are available in most of environments and are potent degraders of toxic contaminants. They carry genes for metabolism of contaminants both in their chromosome and plasmids. This makes these microorganisms the main source for obtaining catabolic genes for genetic manipulation (Wasilkowski et al. 2012).

For construction of a proper GEM, there is a need to have a bank of genetic groups and encoding the properties to generate microorganism with improved degradation capabilities. One strategy for doing so is the logical integration of catabolic segments obtained from diverse organisms within one target strain (Jafari et al. 2013). The single constructed GEM has the capability of different microbial community due to insertion of different genes in it and can improve the efficiency and efficacy of the metabolic pathway (Wasilkowski et al. 2012). A successful example was used for bioremediation of a plant which was contaminated with polychlorinated biphenyls. In this case genetic engineering methods were applied to change biphenyl dioxygenase enzyme available in *Pseudomonas alcaligenes* KF707 and *Pseudomonas* sp. LB400 by modifying their substrate specificity. The substrate

range of these microorganisms were combined, and various biphenyl dioxygenase were created that can oxidize double ortho- and double para-substituted PCBs (Jafari et al. 2013).

Another strategy is protein engineering that is utilized for improving the stability of the enzyme specificity of substrate and the kinetic properties. This is done through site-directed mutagenesis or oligonucleotide-directed mutagenesis. For this molecular biology method, study of the molecule structure-function relationships and 3D structure of the enzyme or any other protein in protein family is needed to model the structure of the protein (Jafari et al. 2013). Different steps of metabolic pathway are triggered by translation and transcription of genes that lead to enzyme production. Therefore, hybrid gene clusters of GEMs change their enzymatic activity and enzyme substrate specifications (Kulshreshtha 2013).

The major limitation of protein design is that only the structure of few numbers of degradative enzymes is elucidated. By phenotypic selection, unconventional natural or induced mutants can happen. If not possible more efficient approaches are needed. The exchange of subunits or subunit sequences is a method to combine the best attributes of different enzymes. Production of hybrid genes is done by technology of recombinant DNA and in vitro mutagenesis (in which a mutation is generated in a part of cloned DNA). The hybrid genes then encode fusion proteins having improved properties and provide promoters for transcription and translational start sites to induce expression of enzyme (Jafari et al. 2013; Chai et al. 2015). Shuffling DNA sequences is another recently developed approach for obtaining novel proteins which is the random fragmentation and random reassembly. This leads to creation of a broad range of fusion proteins suitable for bioremediation applications (Jafari et al. 2013). Gene transfer encoding homologous (dissimilar) subunits, site-directed mutagenesis (SDM) of important amino acids, and DNA shuffling are among these methods (Chai et al. 2015).

The recombinant bacteria for metabolizing toxic pollutants are obtained in laboratory scale by transformation. Genetic transfer is the mechanism that is used for DNA transformation from a donor to recipient. The gene transfer is obtained by receive of free naked fragments of DNA from environment by the cell of recipient bacteria. The first step is insertion of DNA fragment into a vector and its introduction to the host cell. This is followed by production of multiple copies of a single gene and selection of recombinant DNA. DNA screening for desired biological properties is the final step. Another possibility is conjugation in which genetic material are transferred to another cell by direct contact. This process is done only in one direction (Wasilkowski et al. 2012).

Some modern molecular techniques are used for selection and identification of genetically modified microorganisms which are through detection of specific DNA or RNA sequences. These methods include fluorescent in situ hybridization (FISH, techniques to identify the positions of genes on chromosomes), polymerase chain reaction (PCR, laboratory technique to make billions copies of specific part of DNA), denaturing gradient gel electrophoresis (DGGE, applying a DNA or RNA sample to an electrophoresis gel containing denaturing agent), and terminal restriction fragment length polymorphism (T-RFLP, a technique for

describing microbial communities on the basis of the position of a restriction site) and amplified rDNA restriction analysis (ARDRA, extension of RFLP technique) (Wasilkowski et al. 2012).

Several efforts have been done to conduct genetic manipulation on microbes to enhance their oil chewing ability both on land and sea (Martin et al. 2015). The aim is creating microorganism that are more efficient than natural ones in degrading petroleum fractions. Some multiplasmid *P. putida* strain with the simultaneous ability to degrade light alkanes and aromatics has been created by genetic modification (Jafarinejad 2017).

The breakdown of crude oil components was tested with GEMs known as "metagenomic clones" to treat simulated seawater. Genetically modified microorganisms have DNA fragments cloned from the DNA of microbes extracted from oil-contaminated environments. Among them three metagenomic clones combined the metabolic pathways in a way that can be found in nature. They used metabolic machinery derived aerobic and anaerobic bacteria simultaneously. The results obtained for biodegradation of petroleum hydrocarbons by genetically modified bacteria were compared with the results obtained by bacterial strains isolated from reservoir-derived. For saturated hydrocarbons, 31% and 47% were obtained by two metagenomic clones and 99% with natural bacteria. For aromatic hydrocarbon, the degradation was more with metagenomic clones (94%) in comparison with natural strains (63–99%) (Dellagnezze et al. 2014).

Kim et al. have developed a DNA diagnostic method that enables the selection of contaminated sites which are suitable for bioremediation. In this work they have used an oligonucleotide microarray method and identified the genes that are suitable for degradation of aliphatic and aromatics. After that the bioremediation of the contaminated site was performed by applying bioslurping in the field. Bioslurping is an enhanced dewatering technology that is used for the bioremediation of soil and water. The advantages of this system include minimization of discharge of groundwater and soil (Kim et al. 2014).

Das et al. (2015) performed the genome sequence analysis for a strain with high contaminate degradation ability (*Pseudomonas aeruginosa* N002) isolated from the soil contaminated with crude oil. In this work gene sequencing was performed by shotgun sequencing. The catabolic genes encoding the enzymes contributing to hydrocarbon degradation pathways and expression include alkane monooxygenase of *Pseudomonas putida*, alkM from *Acinetobacter* sp. strain, alkane monooxygenase from *Rhodococcus* sp., catechol 2,3-dioxygenase of *P. putida*, naphthalene dioxygenase of *P. putida*, and pyrene dioxygenase from *Mycobacterium* sp. strain PYR-1.

Limitations of application of GEMs in the environment are due to the species classification ambiguities, probable gene transfer to other microorganisms and co-release of antibiotic resistance markers. The concerns about environment and public health safety limit the research with application of GEMs in real fields. Some regulation and limitation were established by US Environmental Protection Agency to control the release of GEMs in the environment (Sayler and Ripp 2000).

The investigation of GEMs application for bioremediation was done mostly in the laboratory experiments. However, for understanding the real effect of GEMs, long-term bioremediation in real field must be done. This is necessary for determining the overall effectiveness and their potential risk to ecosystem (Sayler and Ripp 2000). The survival of GEMs depends strongly on the environmental condition of the field such as clay content, pH, moisture, presence of competing microorganism, etc. (Urgun-Demirtas et al. 2006).

Pseudomonas fluorescens HK44 was the first GMM that was approved to be used for bioremediation in real field. This study was done with the aim of long-term bioremediation of naphthalene-contaminated soil. The used GEMs contained plasmid pUTK21 which made by inserting transposon Tn4431 into NAH7 plasmid obtained from *P. fluorescens* 5R. Simultaneous degradation of naphthalene and luminescent signal was due to the genes which promote pathway for naphthalene decomposition and gene cassette (lux) (Wasilkowski et al. 2012). The parental strain from which NK44 strain was derived was a strain isolated from gas plant facility that was severely polluted with PAHs. In this work a system was developed in which an environmental pollutant was sensed and the proper response was made through an easily detectable signal (bioluminescence) (Sayler and Ripp 2000).

Several authorities are reluctant to accept the release of genetically modified microorganisms due to their adverse environmental impact such as gene transfer. However, it must be noted that GEMs do not add new genes to the environment and are taken from another microorganisms, and usually the introduced engineered microorganism will not survive for a long time after exhaust of its specific substrate. On the other hand, transfer of gene materials among native organism is a common phenomenon. In addition, several methods are available for mitigation of the potential risk of genetically modified organisms (Jafari et al. 2013; Chai et al. 2015).

In general, a successful application of GEMs for bioremediation is based on establishment of capable microorganism for biodegradation and appropriate mechanism for their removal afterward (Kuhad and Singh 2013).

Some methods are available to reduce the potential risk of GEMs in the real field environments. One method is using some genetic barriers that restrict the recombinant bacteria survival and gene transfer in the environment. The restriction can be achieved by kind of transposons which are free from transposase gene or by elimination of conjugation gens from plasmid (Wasilkowski et al. 2012).

A novel strategy is construction of suicidal GEMs that can be achieved by addiction system with antisense RNA and proteic plasmid and application of degradative operons of bacteria. This novel GEMs makes microbes susceptible to death after finishing the degradation of contaminants and reduce their risk to human and environment. In the future, by having more information on microorganism, genomes, and biochemical mechanism, the development of suicidal GEMs would be the most efficient method of using GEMs in real fields (Kulshreshtha 2013).

10.8.3 Integrated Methods for Bioremediation of Oil Spills

As mentioned earlier the strategies to remediate the oil spills are based on physicochemical or biological technologies. These methods could be used individually or in integrated approach. Supplying electron donors and acceptors is a common approach that can enhance the bioremediation of petroleum hydrocarbons. This is mostly helpful for the degradation of halogenated compounds. Supplying electron acceptors stimulates the biodegradation of non-halogenated compounds. Common electron acceptors are hydrogen and acetate that are delivered directly or through passive dissolution by hollow fiber membranes. Organic substrates such as butyric, lactic, and humic acids as well as ethanol can be used for indirect supply of hydrogen. However, there is challenge in this approach which is the rapid consumption of reagents and their migration from the contaminated area. Therefore, there is need for constant reagent supplement, which is costly and problematic (Daghio et al. 2017).

In bioelectrochemical systems (BES) which is the integration of electrochemical and biological techniques, an electrical current is used both as electron donor and electron acceptor in bioremediation of oil spill by active bacteria (called also exoelectrogens, electricigens, or anode respiring) while they oxidize the substrates anaerobically (Balba et al. 1998; Lu et al. 2014). This technique is controllable and enables the real-time monitoring of the degradation process. Controlling the supply of electron donors is also helpful to avoid unwanted side reactions. For effective BES process, especially in the field applications, several aspects of system design, material selection, and radius of influence must be considered. Mode of action and operational parameters must be assigned effectively. For this process, the knowledge about the microbial process is limited in comparison with the knowledge about the mechanism of electron transfer. The effect that environmental parameters can have on the activity of pollutant-degrading microorganism is another limitation for real-field applications (Daghio et al. 2017; Mapelli et al. 2017). Knowing the microbial mechanism is helpful for understanding the two simultaneous activities taking place in the bioremediation which are the natural attenuation process with native electron acceptors in the environment, and exoelectrogen bacterial consortia that take advantages of the electrodes (Lu et al. 2014).

Having non-exhaustible electron acceptors and donors, this method does not consume large amount of energy and chemicals which makes the remediation process economical for long runs (Lu et al. 2014). In this process the microorganisms catalyze the oxidation reduction reactions near or on the surface of the electrodes. The system includes an anode and a cathode divided by a matrix. The microorganism can exchange electrons with the electrodes directly or indirectly by using a chemical compound as an electron shuttle. The chemical compound is secreted by the microorganisms such as *Pseudomonas* or added exogenously. The anode collects the electrons produced from the oxidation of organic compounds. In the benthic sediments or contaminated aquifer, the anode is buried and is electrically connected to a cathode which is located in the water. The collected electrons are transferred to the cathode via electrical connection and can be used to reduce the

oxygen in anaerobic water environment. Compounds available in oil spills such as alkanes and aromatic hydrocarbons could be removed by BES system effectively (Daghio et al. 2017). The biocatalyzing of oxidation reactions of highly concentrated organic compounds is reported to be thermodynamics favor reaction and leads to double benefits which are pollutants degradation and electricity production (H. Li et al. 2017).

The lack of electron acceptor is an important problem in the case of bioremediation of underwater sediments; therefore, application of BES is a promising alternative for the conventional remediation process to be applied to benthic microbial electrochemical system (Li et al. 2017). However, this method can be applied effectively for bioremediation of oil spills in soil and water too (Balba et al. 1998). The bottleneck of the process under anaerobic conditions such as benthic environments is the initiation of the degradation process. In aerobic conditions, the process is started by catalyzing the addition of hydroxyl groups by an oxygenase, which is a less efficient process in anaerobic conditions. In such cases, the anode apart from being electron acceptor contributes in initiation of the process by production of oxygen and modifying the pH. This ability depends on salinity, ion species and concentration, pH, temperature, and electrode properties. The reduction reactions at the cathodes are exploited for the reduction of oxidized compounds (Daghio et al. 2017). Inefficient mass transfer is another limitation of the BES techniques (Li et al. 2015). In the subsurface environment, usually graphite is used as electron acceptor in BES (Viggi et al. 2015).

This approach can be applied in a microbial fuel cell that is an electrochemical device to convert chemical energy into electricity using exoelectrogenic bacteria as biocatalysts. This approach was firstly applied for the wastewater treatment and further developed for recalcitrant compounds removal such as petroleum hydrocarbons (Adelaja et al. 2015). Simultaneous pollutant biodegradation (due to secondary reactions) and energy production can be achieved in this method. In microbial fuel cells, the electrons obtained by exoelectrogenic bacteria are transferred through external circuit from anode to cathode for oxygen reduction (Wang et al. 2012; Chandrasekhar and Venkata Mohan 2012). In this approach, non-exhaustible electron acceptors are used, and the necessity of aeration in the subsurface is eliminated. However, a semi-aerobic metabolic pathway on the cathode is sustained (Lu et al. 2014).

In the recent approaches, the application of electrodes colonized with mixed consortia has been used for their better stability and performance both in degradation and electricity generation (Venkidusamy et al. 2016).

For the microbial fuel cell application in the real field, there is a need to study the robustness of the system in different operating conditions. Temperature is effective, since low temperature inhibits the methanogenic bacteria growth. However, at low temperature electrogenesis is promoted, while high temperature improves the thermodynamics of the system and rate of substrate utilization and increase biokinetics by improving mass transfer and activation energy. The use of exogenous redox mediators enhances the electron transfer rate and improves the electrochemical performance of the system. The influencing parameters are toxicity of the redox mediator, the ratio of redox potential of the mediator to the redox potential of the substrate,

and permeability characteristics of the cell membrane for the molecules of redox mediator (Adelaja et al. 2015). However, the application of this system in real field has not been tested yet, and there are several facts to be verified according to different conditions and the actual scalability (Daghio et al. 2017).

A novel bioelectrochemical approach is a simpler approach called "Oil-Spill Snorkel" used for bioremediation of soil and sediments contaminated with petroleum hydrocarbons. This system is composed of a snorkel (which is the electrode made of conductive material) placed for providing electrochemical connection. The snorkel is the electrode (acting as both anode and cathode) which is a conductive rod which makes a bridge between aerobic and the anaerobic zones. However, in this method, the electricity cannot be harvested or monitored. The electrons derived from oxidation of contaminants are accepted by an anode electrode buried in sediments. These electrons are transferred through snorkel to the cathode where aerobic conditions are available. There, the reduction of oxygen is occurs to form water (Daghio et al. 2017; Viggi et al. 2015).

The snorkel provides link to connect an anoxic zone (polluted sediments) and the oxic zone (top oxygen-containing water). The bottom part of the snorkel buried in the target sediment is the anode acceptor. In this system the electric resistance available in conventional BES and microbial fuel cells resulting from the separate electrodes is eliminated. This way the bacterial community in the sediments can access the high redox potential electron acceptor (oxygen). This method was used by Viggi et al. for bioremediation of marine sediments contaminated with petroleum hydrocarbons (Viggi et al. 2015). The schematic figure of oil spill snorkel can be seen in Fig. 10.2.

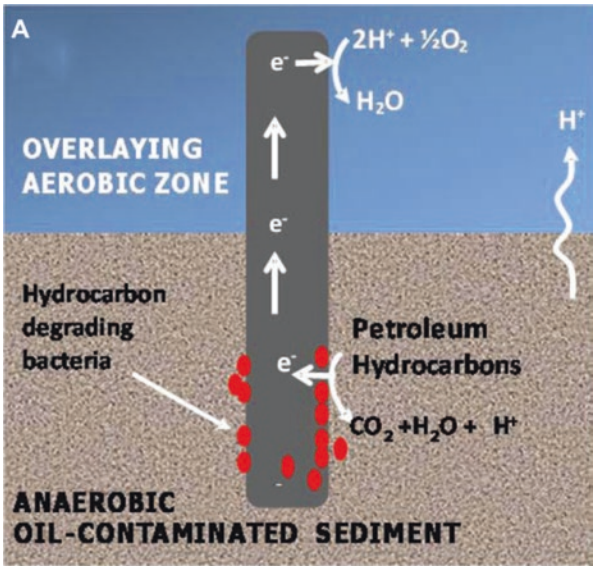

Fig. 10.2 Illustration of oil spill snorkel (Viggi et al. 2015)

For the electricity production, the type and amount of the contaminants can influence the potential current and power density produced (Li et al. 2017). The anodic solution conductivity was higher in more salinity condition. The internal resistance of microbial fuel cell was also decreased. However, the microbial activity and growth can be affected adversely by high salinity (Adelaja et al. 2015).

In the work of Cheng et al., the microbial fuel cell for bioremediation of oil spill in soil was used. In this work, the voltage of 190 mV and 24% total petroleum hydrocarbon removal was achieved in 66 days. The scanning electron microscopy images on the anode electrodes (carbon fibers) revealed the formation of biofilm which build the link between carbon fibers and can improve electron transmission (Cheng et al. 2017).

Li et al. used microbial fuel cell, for bioremediation of sediments contaminated by hydrocarbons. Sand was mixed with the contaminated soil to enlarge the pore size of soil in order to accelerate the ion and substrate transfer. Electricity generation and degradation rate were improved using this method (Li et al. 2015).

Bioelectrochemical remediation system in the work of Venkidusamy et al. was performed with pre-cultured anodes. The performance of enriched biofilm anodes was compared with the performance of freshly inoculated anode. It was reported that enrichment of anode had significant effects on the results obtained from microbial fuel cell both for contaminant removal and current generation (Venkidusamy et al. 2016).

The future studies in the field of BES must be focused on the physicochemical conditions that can lead the effective real-field application of this system. As an example, pH of the field can affect not only microbial activity but also the availability of the alternative electron acceptors which can affect the bioelectrochemical anode reduction reactions (Daghio et al. 2017).

A pilot-scale benthic microbial electrochemical system was built by Li et al. for bioremediation of polluted river sediments. The anode in this system was carbon mesh with honeycomb structure supports as anode, and the cathode was activated carbon. The river water was simulated with wastewater. Removal of polycyclic aromatic hydrocarbons reached 74%, and a maximum power density of 63 ± 3 mW m^2 was achieved. The power density decreased to 42 ± 2 mW m^2 due to cathode degradation and at the end of the operation reduced to 30 ± 3 mW m^2 due to substrate limitation (Li et al. 2017).

The effects of temperature, salinity, presence of redox mediators, and fed-batch system on the degradation efficiency and electrochemical functionalities were studied in the work of Adelaja et al. for bioremediation of mixture of petroleum hydrocarbon in a microbial fuel cell. The optimum condition was salinity of 2.5%w/v and temperature of 40 °C (Adelaja et al. 2015).

10.9 Conclusion

Oil spill occurrence is not a new problem and has been the issue for more than a century. This problem whether occurring in water or soil is a huge threat for ecosystem, fauna and flora. Bioremediation as an economical and environmentally friendly approach is based on microorganism's capabilities to degrade petroleum hydrocarbons. This method aims at biostimulation and bioaugmentation of the natural attenuation of the contaminants with indigenous microorganisms. In comparison with physicochemical methods (application of skimmers, booms, barriers and sorbents, dispersants, and controlled in situ burning), bioremediation is a more effective approach without disrupting the polluted environments. Although several aspects of this approach had been studied by different researchers and quite high hydrocarbon removal rate were reported specially in laboratory scale, the real-field applications are not developed thoroughly. Novel approaches for bioremediation including addition of novel materials, using GEMs, and integration of electrochemical strategies with biological methods are new fields of research for bioremediation of oil spills.

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Chapter 11
The Small-Scale Microbial Processes for Remediation of Sediments Contaminated with Hydrocarbons

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Abstract The accidents caused by the petroleum industry have caused several deleterious effects both in the environmental and economic fields, with the most affected being natural ecosystems. Among these, mangroves has been increasingly a center of great studies because of its great ecological, economic, and social importance and because it is one of the biosystems that suffers the greatest impacts. The use of biological processes to recover natural environments is gaining increasing importance throughout the world, especially in ecosystems affected by oil hydrocarbons. These methods are favored by being environmentally friendly, clear, lower cost, and easier to apply on a large scale and do not alter the balance of ecosystems. Currently one of the most applied biological techniques for the recovery of environments affected by petroleum activities is bioremediation, which consists of the use of microorganisms to decontaminate areas. The success of bioremediation is directly related to the physical and chemical properties of petroleum, the characteristics of the by-products generated by bioremediation processes, and the peculiarities of the affected ecosystems. Results of scientific research have corroborated that the decrease of hydrocarbon concentrations is directly related to the nutrient and microorganism rates present in the environment. However, this chapter presents a series of experiments with oil-contaminated sediments in laboratory scale, using the technique of biostimulation and bioaugmentation.

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11.1 Introduction

Petroleum and its derivatives are the main sources of energy and raw materials for production and are used in large quantities in all fields of life and work. Accidental spillage during the exploration, transportation, processing, storage, and use of oil and its derivatives leads to soil, sediment, and water pollution (Yanto and Tachibana 2013). The toxic effects of petroleum hydrocarbons are cumulative, while some are carcinogenic, mutagenic, or teratogenic and thus may endanger the health of future generations (Singh and Ward 2004; Jednak et al. 2017).

One of the biotechnologies that had notable worldwide success in the remediation of oil pollution is bioremediation. Bioremediation is a technology that uses the ability of microorganisms mainly to restore and preserve environmental quality for all life forms of an ecosystem, especially the human. The use of plants, bacteria, microalgae, and fungi for bioremediation processes has been reported lately, mainly regarding the use of fungi and bacteria due to their great genetic potential. These, in turn, can be isolated from highly contaminated environments because of their adaptive power to these conditions (Srivastava and Thakur 2006; Martins 2009).

The bioremediation technique involves the physical and chemical processes of mineralization and/or alteration of pollutants in less harmful proportions by various species of microorganisms. The process of natural attenuation is the most immediate form of bioremediation which includes monitoring the degradation of pollutants without any treatment. However, this procedure can be tested through biostimulation, which corresponds to the addition of nutrients and alternative acceptors of electrons (e.g., oxygen) to stimulate the growth of the microorganisms as well as the preservation of the appropriate environmental conditions (Leal et al. 2017). In order to evaluate the effect of the microorganisms on the microorganisms, it is necessary to evaluate the effect of the microorganisms on the microorganisms.

In order to use the biostimulation process, a pool of autochthonous microorganisms with potency in metabolizing the contaminants is necessary, because only the environmental conditions are not sufficient to obtain high rates of degradation (Ramaswami and Luthy 1997).

Scientific research cites that the biostimulation process potentiate the indices of degradation of contaminated solid matrices when the biotic and abiotic factors are controlled. Much of the research is based on the use of nutrients in the form of fertilizers such as NPK and OSMOCOTE (Vallejo et al. 2005).

NPK and OSMOCOTE have been widely used in biostimulation processes. Both NPK and OSMOCOTE have the same composition, ammonium phosphate, ammonium sulfate, and potassium chloride in the proportion of 10 (N):10 (P):10 (K); what differentiates OSMOCOTE from NPK is the form of release of these nutrients, since the OSMOCOTE release is attenuated because it is surrounded by a protective capsule (Lima 2010).

Research using NPK fertilizer showed positive results, with the removal of 100% of the n-alkanes comprised between the decane and eicosane, and 40% removal of polycyclic aromatic hydrocarbons was obtained with the combination of mixed culture + fertilizer, in the ratio of C:N (100:10) (Oliveira 2001).

Another technique used is bioaugmentation, which occurs by the addition of specific microorganisms in impacted regions, adapted in the laboratory to the environmental conditions. When using this technique, the evaluation of the microorganisms present in the environment is carried out, identifying the oil degraders. Next, the microbial growth of the species of interest is stimulated in the laboratory, and the microorganism pool is then injected into the contaminated site with the objective of increasing the microbial population responsible for oil degradation (Venosa et al. 1999; Rosa and Trigüis 2006).

The applied microorganisms must act in synergy with the native species, without interfering in the natural biogeochemical processes. Leavitt and Brown (1994) conducted a comparative study between biostimulation and bioassay for a case of soil treatment contaminated with crude oils, using in one case autochthonous microorganism and, on the other, a commercial crop with a recommended mixture of nutrients. They concluded in some studies that the use of local microorganisms (autochthons) is the best alternative when taking into account expenditure and efficiency (Mariano 2006).

Another way to treat oil-contaminated soils involves the use of bioreactors, a procedure recently used extensively. There are several factors that strengthen this assertion because it is a confined and controlled treatment that facilitates the microorganisms to accelerate the process of contamination degradation (Reichwald 2011).

Bioreactors facilitate the control of the biodegradation process of the pollutants in the soil, facilitating the acclimatization of the microbiota and its development. The use of bioreactors has emerged as a viable and decisive technology for the treatment of soils contaminated with organic compounds (Ururahy 1998).

The industrial development and consequent urban expansion in the region of São Francisco do Conde, Candeias, and Madre de Deus, Bahian municipalities located on the shores of the Bay of All Saints (BTS), Brazil, resulted in the reduction of large areas of mangroves due to the presence of several oil wells, with a historical record of blowout incidents, causing contamination by crude oil (petroleum) from local ecosystems.

Due to this scenario, the need for elaboration and testing using bioremediation for the recovery of these areas arises, since there are few studies in the scientific literature related to the theme of this research in the study area.

11.2 Remediation of Sediments Using Biostimulant NPK and OSMOCOTE

The use of biological processes began in 2018 with the treatment through biostimulation where two types of fertilizers were tested with NPK and OSMOCOTE. The main objective of the research was to monitor the degradation of n-alkanes in mangrove sediments contaminated by petroleum by means of mesocosmos experiments using NPK and OSMOCOTE as growth promoters of microorganisms.

We collected 72 samples of contaminated sediments from the Bay of All Saints (BTS) with the help of a witness. The pellet cores were then homogenized in a metal vessel. Part of the sediment was added with 0.5 g of NPK for each kg of pellet, another part was added to the same ratio of OSMOCOTE, and one part remained untreated (control). The experiments were mounted in glass vats (Fig. 11.1a), composed of eight cups (Fig. 11.1b) and two wooden holders (Fig. 11.1c).

The experiment lasted 90 days. Total hydrocarbons (TPH) were determined using the US EPA 8015B methodology; the nutrients monitored were concentrations of phosphorus (Grasshoff 1983), organic carbon (Walchley-Black (1947)), nitrate (Embrapa 1998), and ammonium (Walchley-Black (1947)).

Oils in advanced stages of degradation tend to have low values for petroleum hydrocarbons with consequent increase in polar compounds (NSO). The oil from the catchment basin, such as those found in the substrate of this mangrove, generally exhibits a range of 50–88% saturated hydrocarbons and from 2.8% to 35% for NSO compounds (Gaglianone and Trindade 1988). Reason that is inverted when the process of degradation occurs by the microorganisms, since these tend to metabolize the lighter fractions (saturated hidrocarbonetos), consequently increasing the fraction of heavier compounds (NSO).

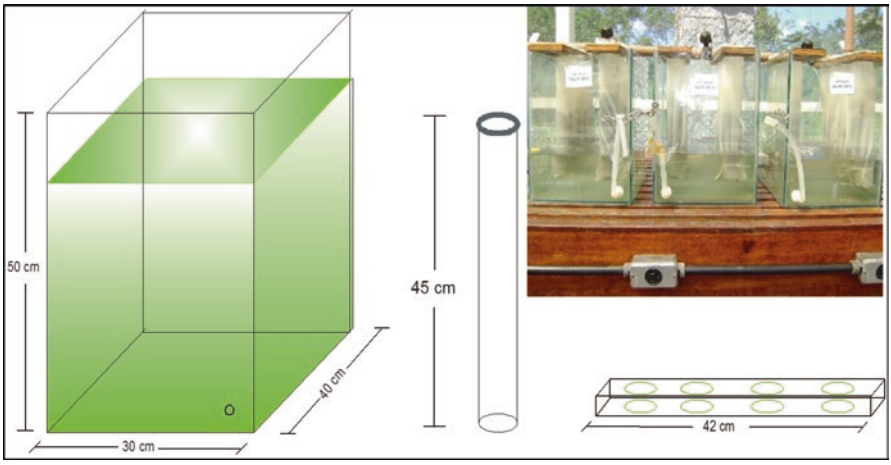

Fig. 11.1 Description of the simulation units. (Source: Lima 2010)

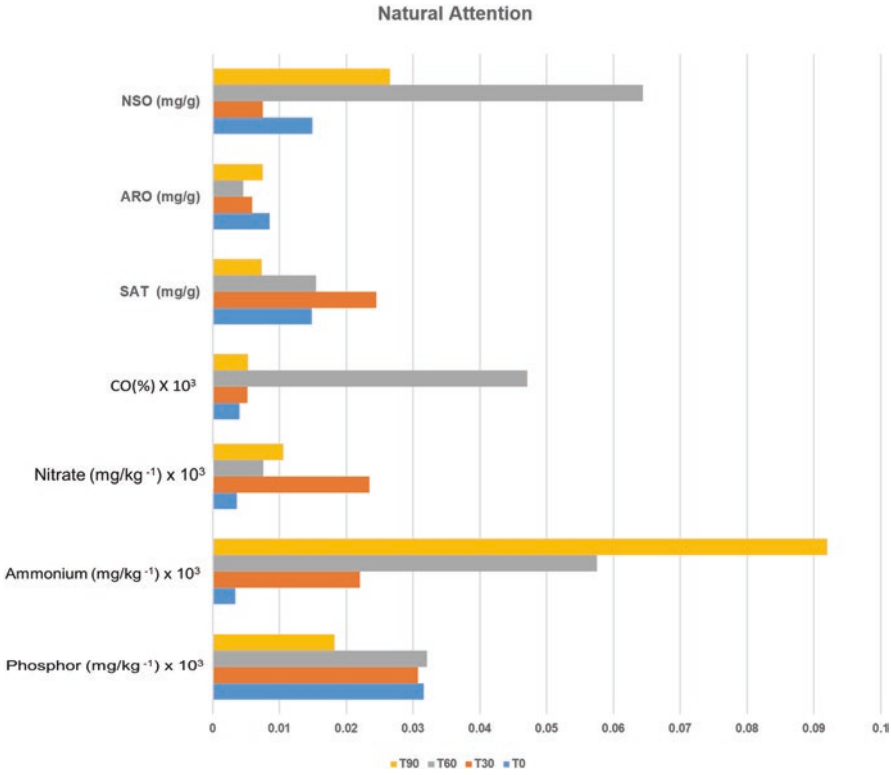

Fig. 11.2 Geochemical results at 0, 30, 60, and 90 days of experiment for natural attenuation. (Source: Lima 2010)

The extracts of the samples collected from the untreated units (natural attenuation) presented a decline in the richness of the lighter fractions (n-alkanes) and enrichment of the heavier fractions (NSO) (Lima et al. 2012). Mcmillen et al. (1993) in their studies already propose this biodegradation scale; n-alkanes are the first ones to be degraded, followed by aromatics and NSO (the most recalcitrant ones), thus confirming the results obtained in the present research (Fig. 11.2).

Rosa and Trigüis (2006) confirm, in their bioremediation experiments using seawater contaminated by hydrocarbons, this scale of degradation. Polar compounds because they are more persistent tend to accumulate, while n-alkanes are the first to be degraded, often by the action of weathering processes such as oxidation and evaporation.

Figure 11.3 shows the results of the analyses of extracts collected from the units treated with the NPK fertilizer, where the decline of n-alkanes was significant and can be attributed to the local microbiota stimulation predicted in other studies carried out with oil spills that reduced −75% TPH (Blenkinsopp et al. 1997; Lima et al. 2012; Roy et al. 2018).

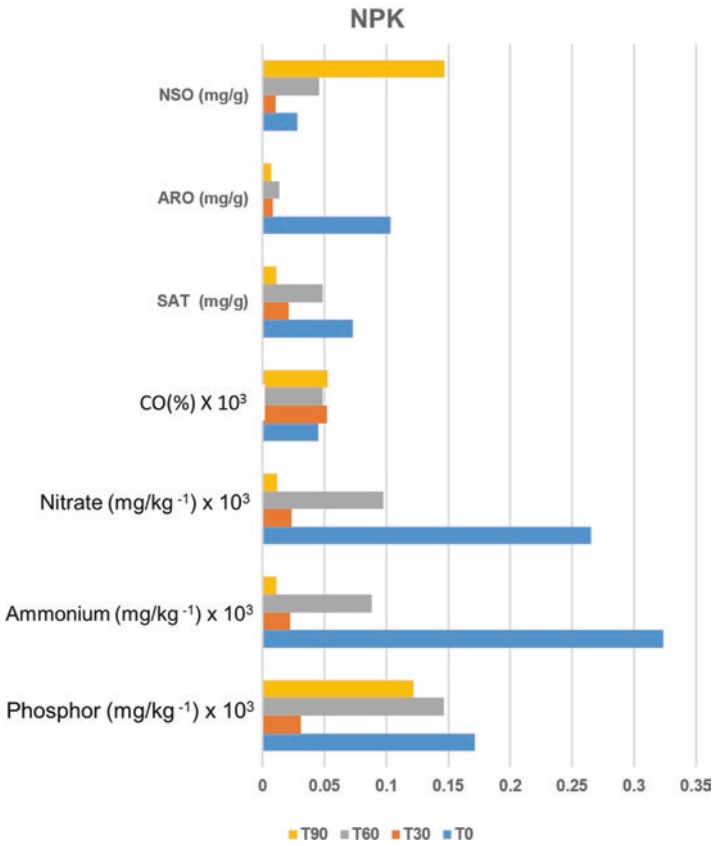

Fig. 11.3 Geochemical results at 0, 30, 60, and 90 days of experiment for NPK. (Source: Lima 2010)

NPK treatments were more efficient at the end of the 90-day experiment (Lima et al. 2012). It is already known that the stimulation with nutrients is of fundamental importance for the growth of microorganisms and consequently intensifies the biodegradation of the pollutant (Rosa and Trigüis 2006). Vallejo et al. (2005) in their studies report that biodegradation using inorganic nutrients showed higher degradation rates of TPHs. In addition, it was possible to observe a greater reduction of aromatics in NPK-treated samples when compared to NPK treatments (Lima et al. 2012).

Recent studies have shown that NPK treatments reduce the degradation rate of TPH by 43% when compared to control samples (32% decline), which contained only the oil and the natural microbiota (Brown et al. 2017). The most used nutrients for the recovery of areas degraded by hydrocarbons are nitrogen and phosphate, since they enter directly into the microbial metabolic pathway and their limitation directly affects the growth and consequently reduces the degradation process (Coulon et al. 2012).

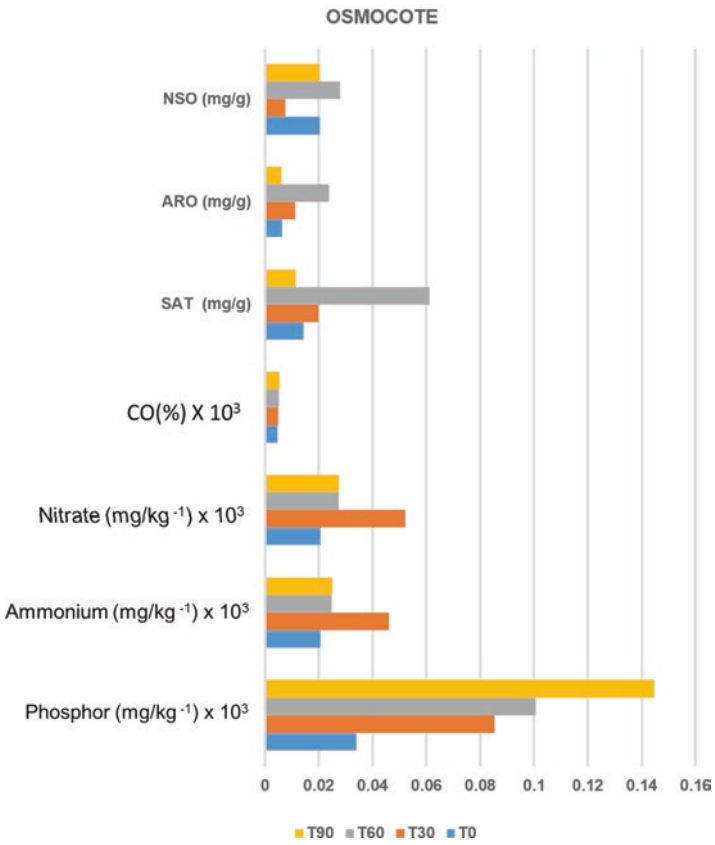

Fig. 11.4 Geochemical results at 0, 30, 60, and 90 days of experiment for OSMOCOTE. (Source: Lima 2010)

Samples treated with the OSMOCOTE fertilizer followed the same degradation scale already presented and discussed in the other control samples and those treated with NPK (Fig. 11.4) (Lima et al. 2012).

Treatment with OSMOCOTE showed a reduced efficiency when compared to the other treatments, and this can be attributed to the nutrient release form, which corresponds to a slower system and that needs a longer period to evaluate the level of degradation.

Unlike the NPK that has the advantage of solubilization and simple dissolution favoring the biodegradation processes. Therefore, it is extremely necessary to monitor phosphorus, ammonia, and nitrate nutrients to prove at what point in the experiment the nutrient levels are ideal for accelerated biodegradation, and it is also indicative of the right moment to add more nutrients to the system, giving continuity to the degradation of petroleum hydrocarbons.

Some authors defend the premise that the use of encapsulated fertilizers is the best way to provide nutrients in a continuous and gradual way, improving the

process of biodegradation of the oil; however the period for this will be longer (Olivieri et al., 1976; Lee and Tremblay 1993). This can be proven in this experiment, where the nutrient contents increased gradually and slowly throughout the experiment.

Comparing the results found, it was possible to conclude that the best treatment would be to use NPK, since a higher percentage of degradation of HTPs and consequently increase of NSO rate was seen. However, it would be extremely important to obtain toxicity data of this type of fertilizer.

11.3 Sediment Remediation Using Biostimulation and Bioaugmentation with Fungal Consortia

In 2013, the bioaccumulation technique was tested, which consisted in testing the efficiency of the fungal consortia immobilized in degrading the oil from the Recôncavo basin and the Campos basin, having as simulation units the glass vats (aquaria (AQ) composed of 24 test tubes and 2 wooden supports (Fig. 11.5).

The experiment was carried out in small-scale and laboratory conditions, where the sediment was sterilized and the leaf of mangrove and coconut fiber were tested as stimulators for the microorganisms (Table 11.1).

Two types of previously selected fungal consortia were tested, one with the potential to degrade the oil of the Recôncavo basin and another to degrade the Campos basin. Consortium 1 consisted of 29 fungal isolates and consortium 2 with 28 fungal isolates.

In each microcosm (test tube) were placed 10 capsules with the immobilized consortium. The samples were contaminated with 3% of oil from the catchment area and the same oil supply from the field basin. This calculation was related to the

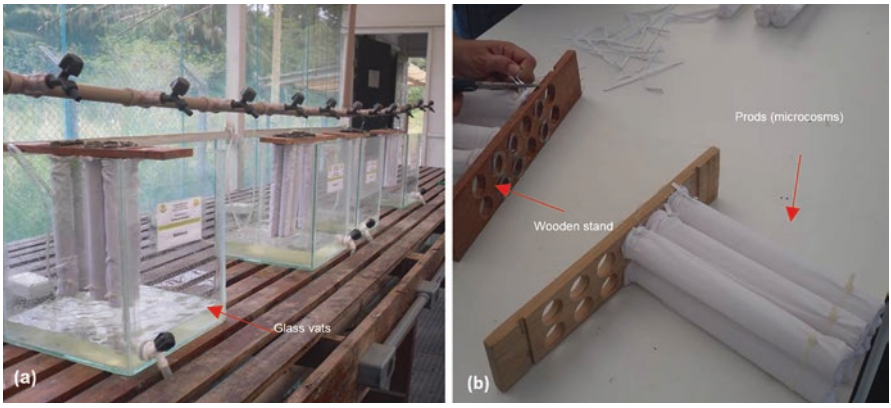

Fig. 11.5 Description of the simulation units. (Source: Lima 2014)

Table 11.1 Description of treatments

Treatments	Description of treatments
Unit 1, 2, and 3 (BRFO)	Sterilized sediment + BR oil + immobilized consortium (mangrove vegetation leaf as biostimulators)
Unit 4, 5, and 6 (BRFI)	Sterilized sediment + BR oil + immobilized consortium (coconut fiber as a biostimulator)
Unit 7, 8, and 9 (BCFO)	Sterilized sediment + BC oil + immobilized consortium (mangrove vegetation leaf as a biostimulator)
Unit 10, 11, and 12 (BCFI)	Sterilized sediment + BC oil + immobilized consortium type 1 (coconut fiber as a biostimulator)

Source: Lima (2014)

Fig. 11.6 Stages of the experiment. (**a**) Inoculation of the capsules, (**b**) simulation of the oil spill, (**c**) capped and sealed simulation unit, and (**d**) mounted bioaccumulation system. (Source: Lima 2014)

amount of sediment used in each microsmosm (test tube). To avoid contamination the aquariums were sealed with pvc film (Fig. 11.6).

All assembly procedures were performed in laminar flow. The water to simulate the tide was stored in gallons of 20 l. The water was sterilized at 121øC in an autoclave for 20 min. The tide simulation was monitored daily for 2 h, and in each aquarium was placed an oxygenation pump.

The results obtained in these experiments were integrated for better interpretation; the parameters used were the geochemical, microbiological, and chemical data of 0, 30, 60, and 90 days of experiment.

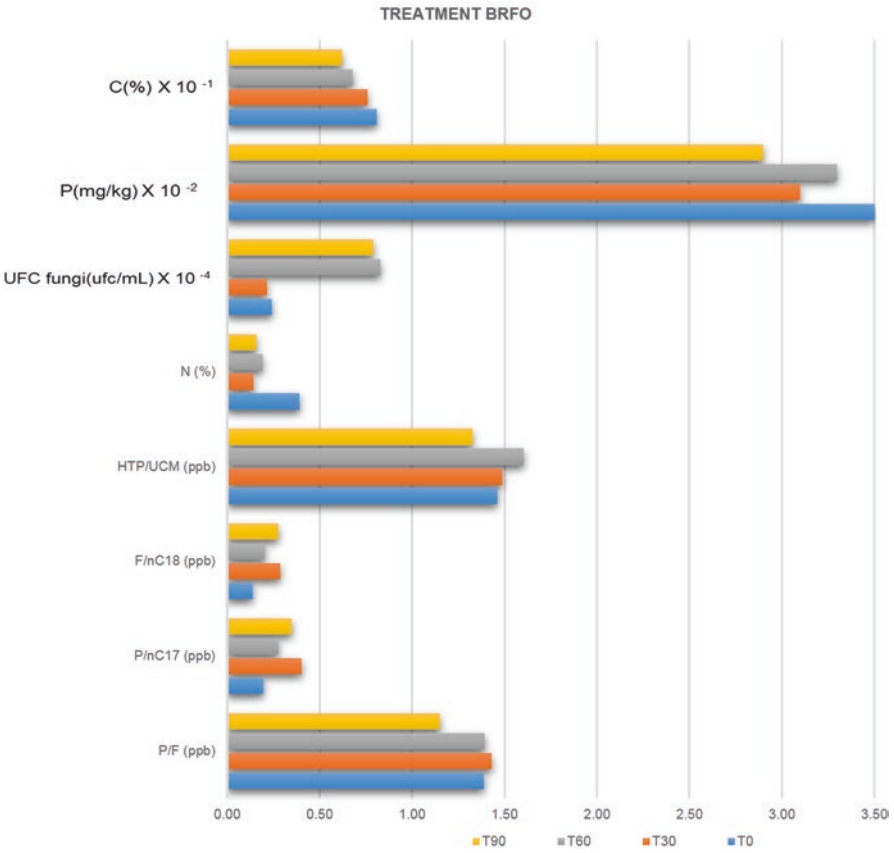

Fig. 11.7 Monitoring of the geochemical parameters in the BRFO samples. (Source: Lima 2014)

In Fig. 11.7 the data of the BRFO simulation unit are plotted. Nitrogen, phosphorus, and CO ratios declined during the 90-day experiment. The results of fungal colony-forming unit counts increased for 90 days. In contrast, the amount of fungal CFU increased during the 90 days. This fact can be justified by the metabolic preference of the consortiums, since they consumed nutrients that justify their growth.

The P/F ratios showed a slight increase in time 30, suggesting that the microbial degradation was small, being justified by the initial process of adaptation of the fungi to the carbon source present in the sediment. The HTP/UCM ratio showed a progressive increase up to 60, with a decrease in time 90.

The results show that biodegradation in this period probably decreased, where it may be linked to the reduction of the amount of CFU and reduction of nutrients at time 90. In T60, there is an increase of CFU with a respective increase in the content of N and P. This may have direct relationship with the type of treatment, since mangrove leaves were used and that probably caused foliar degradation and subsequent release of nutrients into the environment.

The geochemical reasons for saturated hydrocarbons such as PR/nC17, Ph/nC18, and Pr/Ph are used as tools in the diagnosis and identification of sources of pollution,

monitoring of degradation processes by the action of intemperic physical and biological processes, and geochemical interpretation of data oil spills (Onyema et al., 2013).

All the samples presented ratios >1 proving that there was degradation during the 90 days. The degradation process can be observed through the P/F ratio values that decreased over the 90 days.

In contrast the PR/nC17 and Ph/nC18 ratios increased when compared to time 0 with time 90 being more indicative of biodegradation, since these ratios are inversely proportional to the P/F ratio.

The increase of the ratio P/NC17 and F/nC18 is an indication of the action of microorganisms in oil, since, in biodegradation processes, the first compounds to be degraded are the linear alkanes (Hunt 1996; Killops and Killops 2005).

Integrating the data to the BRFI simulation units, it was observed that with 30 days of the experiment, the amount of CFU of the fungi increased, followed by a slight decrease in the amount of nitrogen and phosphorus, and the same happened with the samples treated with coconut fiber and BRFI; this is probably due to the preference for degradation following the same metabolic pathway (Fig. 11.8).

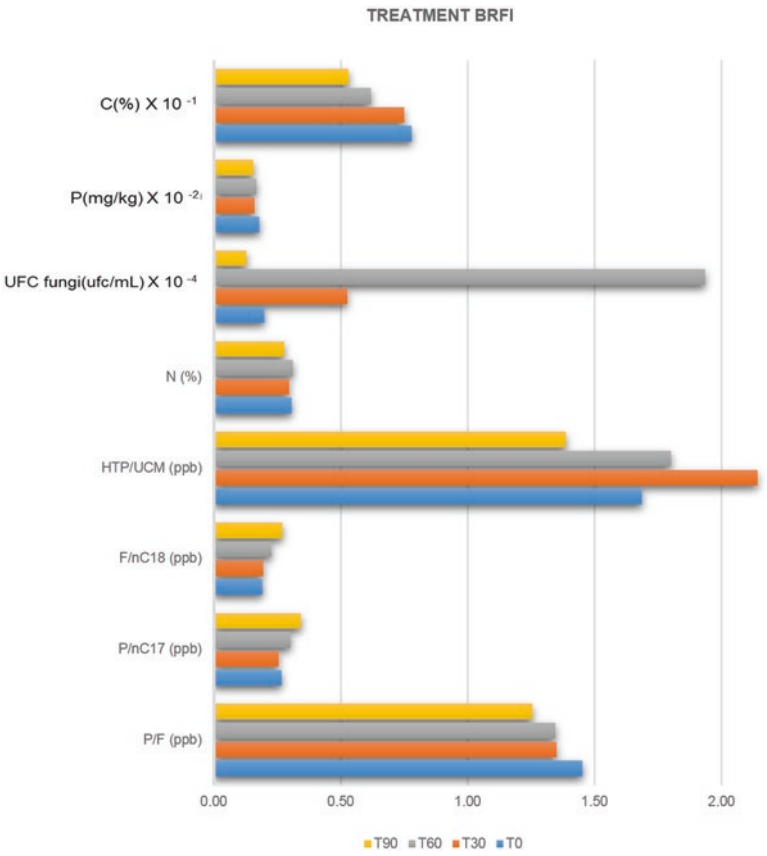

Fig. 11.8 Monitoring of the geochemical parameters in the BRFI samples. (Source: Lima 2014)

The decrease of the pristane/fitane and HTP/UCM proportions decreased over the 90 days, suggesting the occurrence of the biodegradation process.

However, the proportions P/nC17 and F/nC18 increased, proving once again the biodegradation process. After 90 days, the decrease of microorganisms was also observed, which did not necessarily compromise the biodegradation.

Regarding coconut fiber as a nutrient, it was not possible to reach any conclusion, since there was no significant increase of these nutrients in the system.

The microbial competence in the biodegradation of recalcitrant compounds should be linked to the presence of C, N, and P. However, since each microorganism depends on varying concentrations of these elements for its development, the need for deepening is justified, focusing directly on the conditions essential for the technique of bioremediation and consequently its success (Xia et al. 2006; Maciel et al. 2013).

Observing the results plotted in Figs. 11.9 and 11.10, it is possible to observe decay in the P/F ratio and an addition in the ratios P/nC17 and F/nC18. At 90 days this process continued to increase (Figs. 11.9 and 11.10).

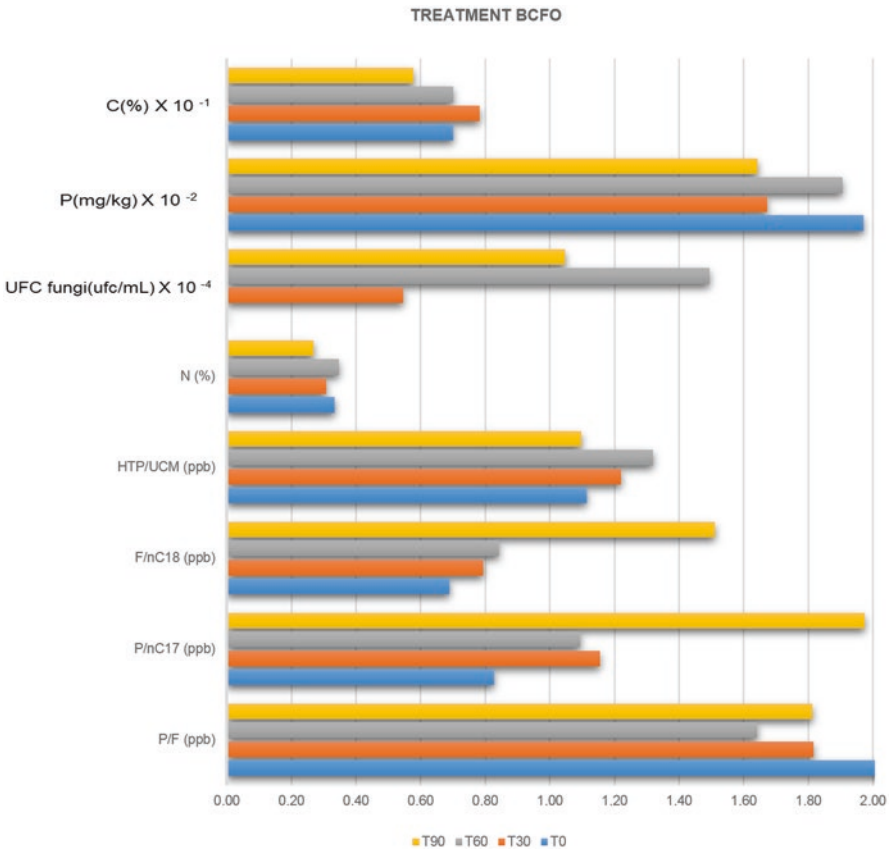

Fig. 11.9 Monitoring of the geochemical parameters in the BCFO samples. (Source: Lima 2014)

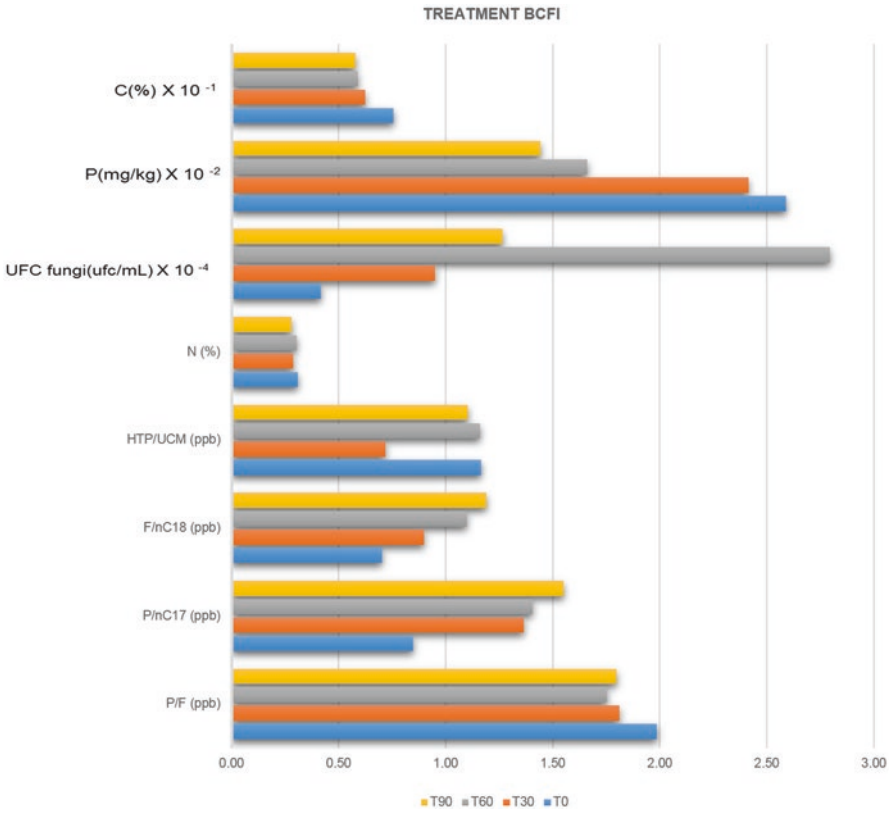

Fig. 11.10 Monitoring of the geochemical parameters in the BCFI samples. (Source: Lima 2014)

In the bioaugmentation process with fungal consortium, it was possible to observe that during the 90 days, the biodegradation of the two types of oil occurred; however, it will be necessary to study further the fungal isolates to accelerate the degradation process even further.

11.4 Sediment Remediation Using Fungal Consortia in Bioreactors

Also in 2013, new experiments were carried out, which consisted of the evaluation of the geochemical processes and the efficiency of the fungal consortia in the degradation of the Recôncavo basin oil in mangrove sediments confined to prototypes of bioreactors (Fig. 11.11). Prototypes of temporary immersion bioreactors were constructed, using glass jars and air filters, and interconnected by flexible tubes (Fig. 11.12).

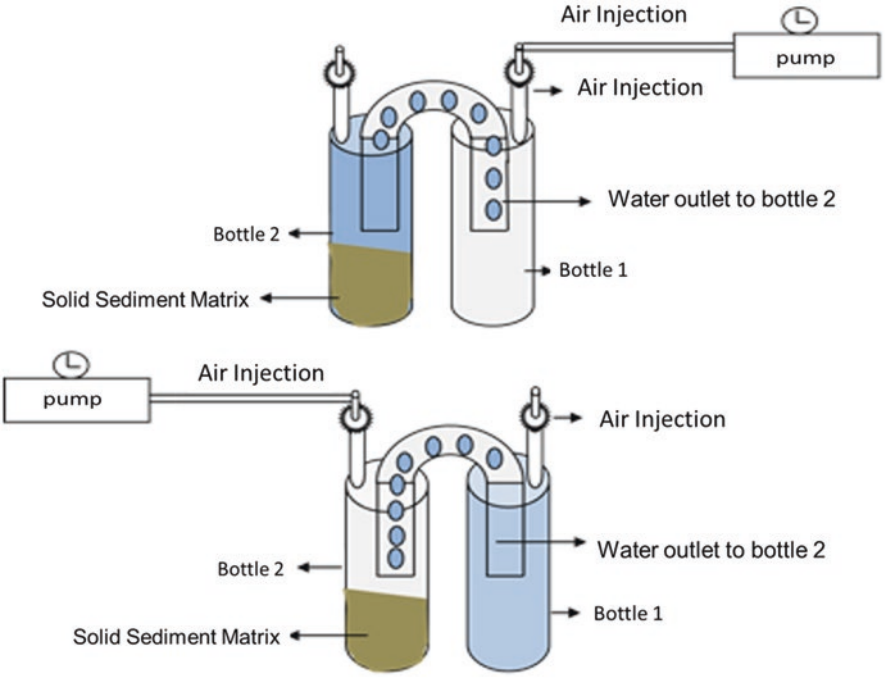

Fig. 11.11 Schematic of the temporary immersion bioreactor prototype developed for the experiment. (Source: Lima 2014)

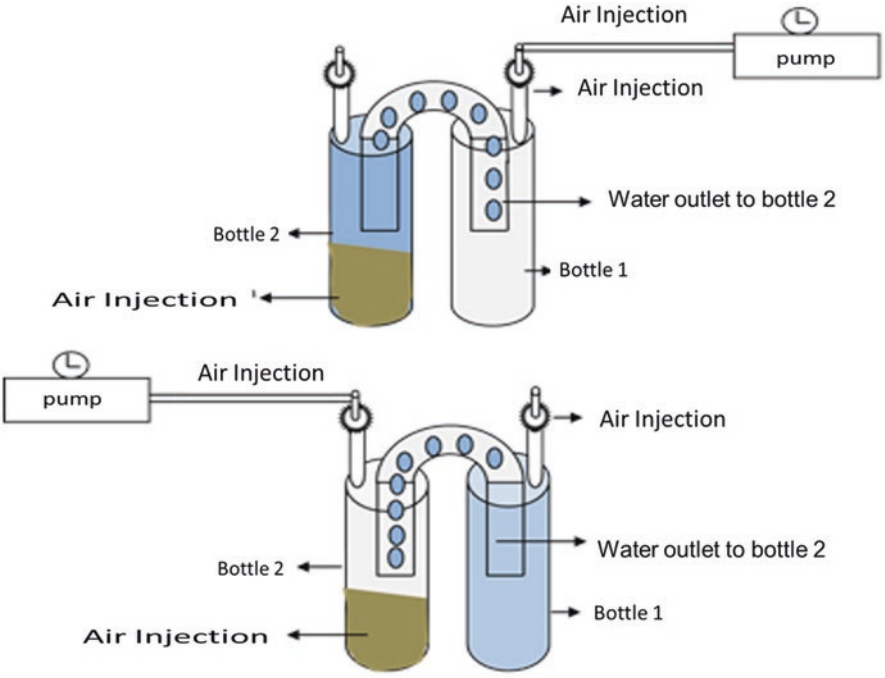

Fig. 11.12 Schematic of the temporary immersion bioreactor prototype developed for the experiment. (Source: Lima 2014)

Fig. 11.13 Assembling the experiment: (**a**) capsules with immobilized consortia, (**b**) simulating oil spill, (**c**) top view of the bioreactor, and (**d**) assembled prototype. (Source: Lima, 2014)

500 g of sediment + 3% of the oil of the Recôncavo basin + immobilized fungal consortium was placed in each flask.

Two bioreactors were used for incubation of the fungi studied and one bioreactor as control without fungi (Fig. 11.13).

The sediment samples were collected at intervals of 0, 30, 60, and 90 days. 100 g of sediment was collected; the sample was homogenized in a stainless steel container and separated into fractions for determinations of the following analytes: organic compounds, inorganic compounds, and count of fungal CFUs.

In the results it was possible to observe that in relation to the ratio of pristane/fitane, a decrease occurred during the 90 days. This proves the degradation of the oil of the Recôncavo basin. When comparing the results of the process in bioreactor 1 with the others, it can be seen that the pristane/fitane ratio was higher (Fig. 11.14).

All samples showed higher pristane/nC17 ratios in the T90, proving that degradation occurred during the 90 days. Comparing the graphs of Figs. 11.14, 11.15, and 11.16, it can be seen that in bioreactors 2 and 3, there was an increase in the pristane/nC17 ratio over the 90 days, unlike in the BIO 1 indicating a reduction of these values.

Similar behavior was observed in all bioreactors for the fitane/nC18 ratio. It was verified that even in the control bioreactor, degradation process occurred (Fig. 11.14).

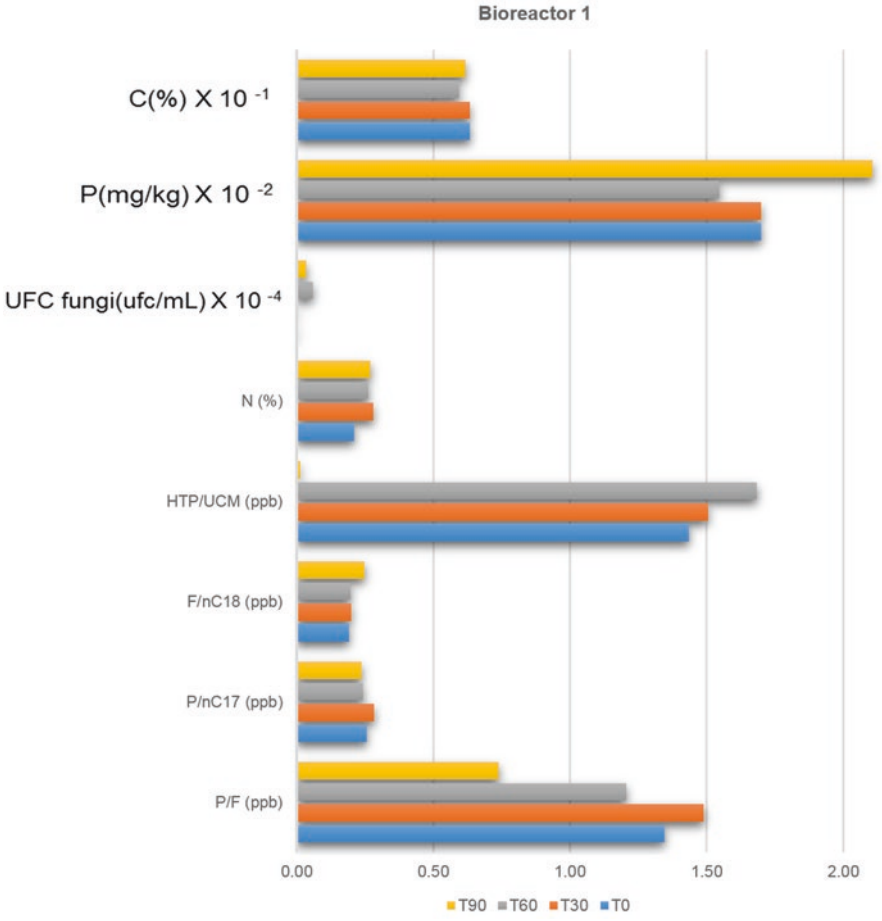

Fig. 11.14 Monitoring of the geochemical parameters in the bioreactor 1 (Source: Lima 2014)

The HTP/UCM ratio decreased with 90 days of the experiment and was indicative of degradation. Observing the graph of Fig. 11.16, the ratio increases at time 60 and then decreases. This fact can be directly related to the growth path of the fungi; it is observed that the quantification of the CFUs increases during this period; possibly the microorganisms must be in the phase of nutrient consumption for later degradation between T60 and T90.

Nitrogen concentrations and phosphorus salts can be used as an inorganic nutrient source to ensure that no nutrient limitation occurs. The coconut fiber used in the experiment was not efficient as a biostimulator (Fig. 11.16).

At time 90 the highest values for phosphorus were observed, showing that throughout the experiment, this remained at ideal concentrations for the degradation process. The graphs also show a fall in phosphorus levels at T30 and T60 times. This

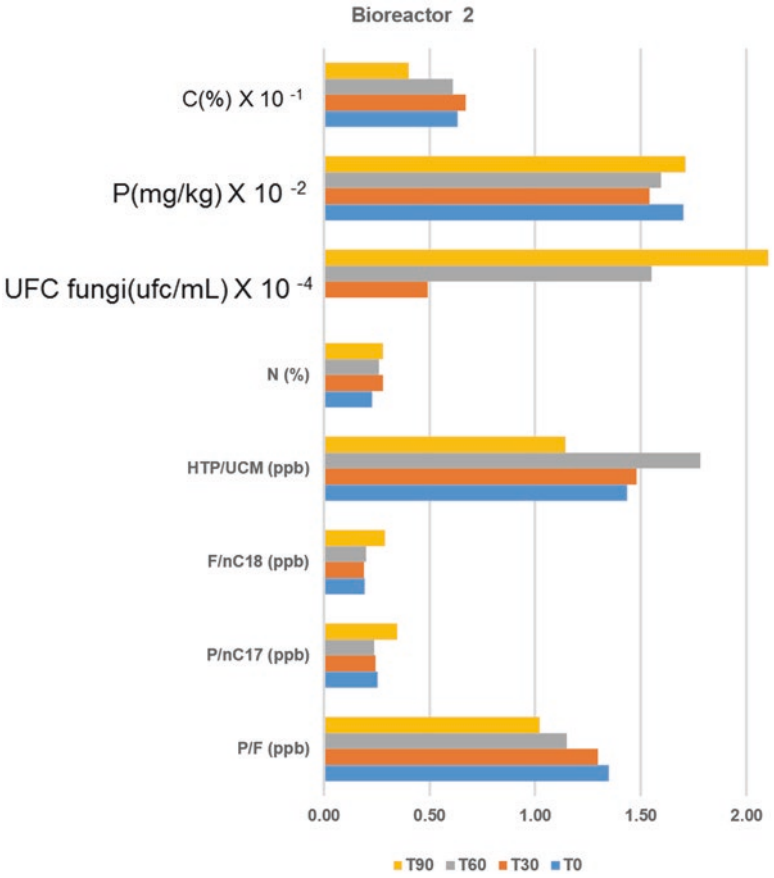

Fig. 11.15 Monitoring of the geochemical parameters in the bioreactor 2. (Source: Lima 2014)

can be justified by the increase of the fungal population in the bioreactor and increase in nutrient consumption for later degradation of the oil (Fig. 11.16).

The organic carbon present in the organic matter of the sediment was also used as a carbon source, and its decrease during the 90 days of the experiment can be observed.

The increase in the amount of viable heterotrophic fungi in soils may be a reflection of the adaptive abilities of these fungal isolates, even in the case of deliberate anthropogenic discharges in large quantities (Obayagbona and Enabulele, 2013). This can be verified in the present study, since by observing the graphs of Figs. 11.14, 11.15, and 11.16, it is possible to verify that a progressive increase occurred during the 90 days of experiment. By proving the efficiency of the encapsulation of the consortiums, it was confirmed that microorganisms can be released gradually into the microcosm.

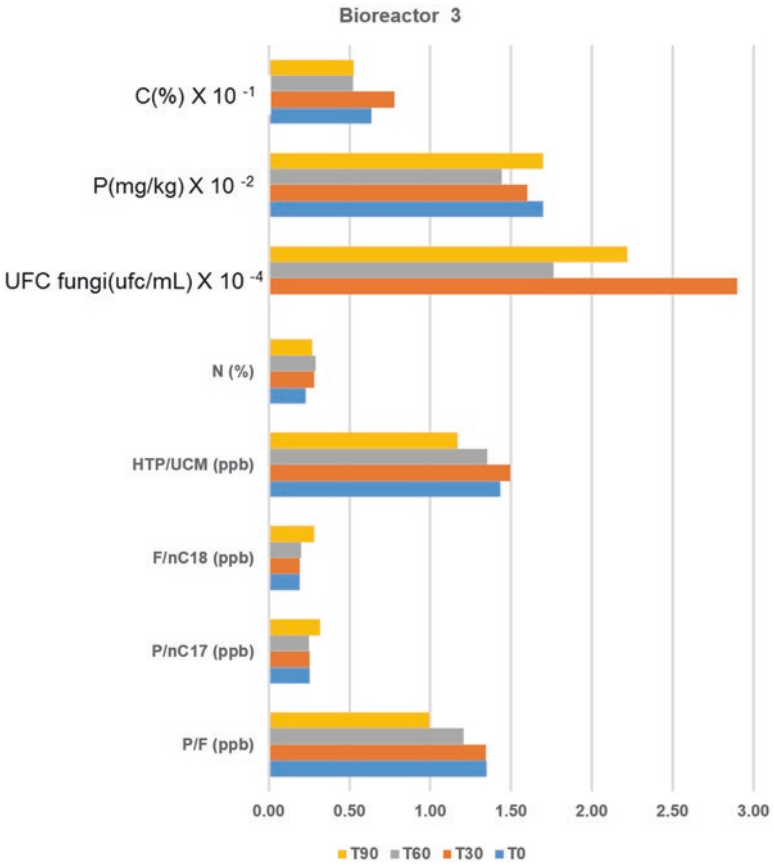

Fig. 11.16 Monitoring of the geochemical parameters in the bioreactor 3. (Source: Lima 2014)

With the experiment in bioreactor, it was possible to observe with greater control what possibly occurs in the process of degradation using the technique of bioaugmentation. Because it is a confined system, the parameters can be controlled, which is more difficult in open systems.

The reduction of n-alkanes from the oil of the Recôncavo basin was much more expressive. The pristane/fitane, P/nC17, F/nC18, and HTP/UCM ratios were also indications of the action of microorganisms in petroleum. This shows that when contaminated sediments are treated in confined locations, the results tend to be better.

11.5 Sediment Remediation Using Bioaugmentation with Fungal Consortia and Biostimulation with Coconut Fiber

Also in 2014, small experiments were set up to evaluate the biodegradation efficiency of the fractions of the Recôncavo basin oil, using fungal consortia associated with coconut fiber in mangrove sediments. Each experiment was set up according to Table 11.2, which evaluated the effect of nutrients and free cells and nutrients and immobilized cells, varying the amounts of coconut fiber and the amount of capsules.

For the assembly of the experiments, the sediment and the coconut fiber (quantities determined by the test code, described in Table 11.2) were weighed in pots.

These were capped, packaged, and decontaminated in autoclave at 121 ° C for 50 min, for the elimination of the microbiota in this sediment that occasionally could be present in the coconut fiber. After the pots arrived at room temperature in laminar flow, the pellet was homogenized. Capsules with the spore suspension were added in holes or covered by the sediment and the homogenously added cell suspension. Finally, the oil was added, simulating the spill.

The pots were capped, avoiding contact with the external microbiota (Fig. 11.17). Three experiments were carried out, with 32 biodegradability tests, to study the degradation in each independent fraction:

- Experiment 01 – Degradation of saturated hydrocarbons
- Experiment 02 – Degradation of aromatic hydrocarbons
- Experiment 03 – Degradation of NSO compounds

In each experiment two collections were performed: times 0 (assembly day) and 30 days. After assembly, the tests were then incubated in an incubator at 30 °C, except the tests corresponding to the 0 day time, which were collected as soon as the assembly was finished.

Table 11.2 Description of treatments

Experiment					
Test	Sediment (g)	Recôncavo basin oil (g)	Cell suspension (mL)	Coconut fiber (g)	Number of capsules
C1	100	–	–	–	–
C2	100	1,0	–	–	–
L1	100	1,0	0,2	1,0	–
L2	100	1,0	0,2	1,5	–
L3	100	1,0	0,2	3,0	–
E1	100	1,0	–	–	10
E2	100	1,0	–	–	30
E3	100	1,0	–	–	50

Source: Costa (2014) and Costa et al. (2016)

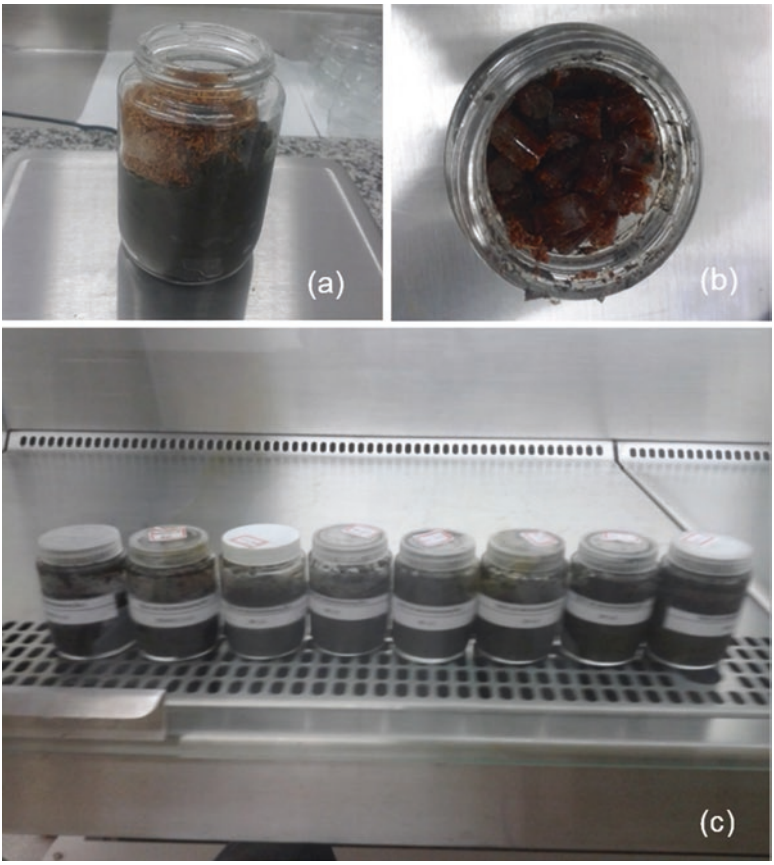

Fig. 11.17 Assembling the experiment: (**a**) glass pots with sediment and coconut fiber; (**b**) capsules with immobilized consortia; (**c**) assembled experiment. (Source: Costa et al. 2016)

The graphs in Fig. 11.18 represent the nutrient variations against the degradation of the fractions of the saturated hydrocarbons in experiment 01. In C1 and C2, there was an increase in the concentrations of all the nutrients, probably due to nonconsumption, since the microorganisms that existed in the experiment were eliminated. The C2 showed that the saturated fraction was degraded, even if there were no microorganisms; this can be explained by the intemperic processes that petroleum suffers. In L1, L2, and L3, there was an increase in the concentrations of phosphorus and organic carbon, while there was a decrease in the amounts of nitrate and ammonium. The saturated fraction presented an increase.

In E1 occurred the increase in all nutrient concentrations, and in the fraction of saturated, there was an insignificant increase. In E2, phosphorus and carbon nutrients decreased, ammonium increased, and saturation decreased. The nitrate and ammonium analyses for this test were not performed for 30 days, and it was not possible to infer the behavior over time.

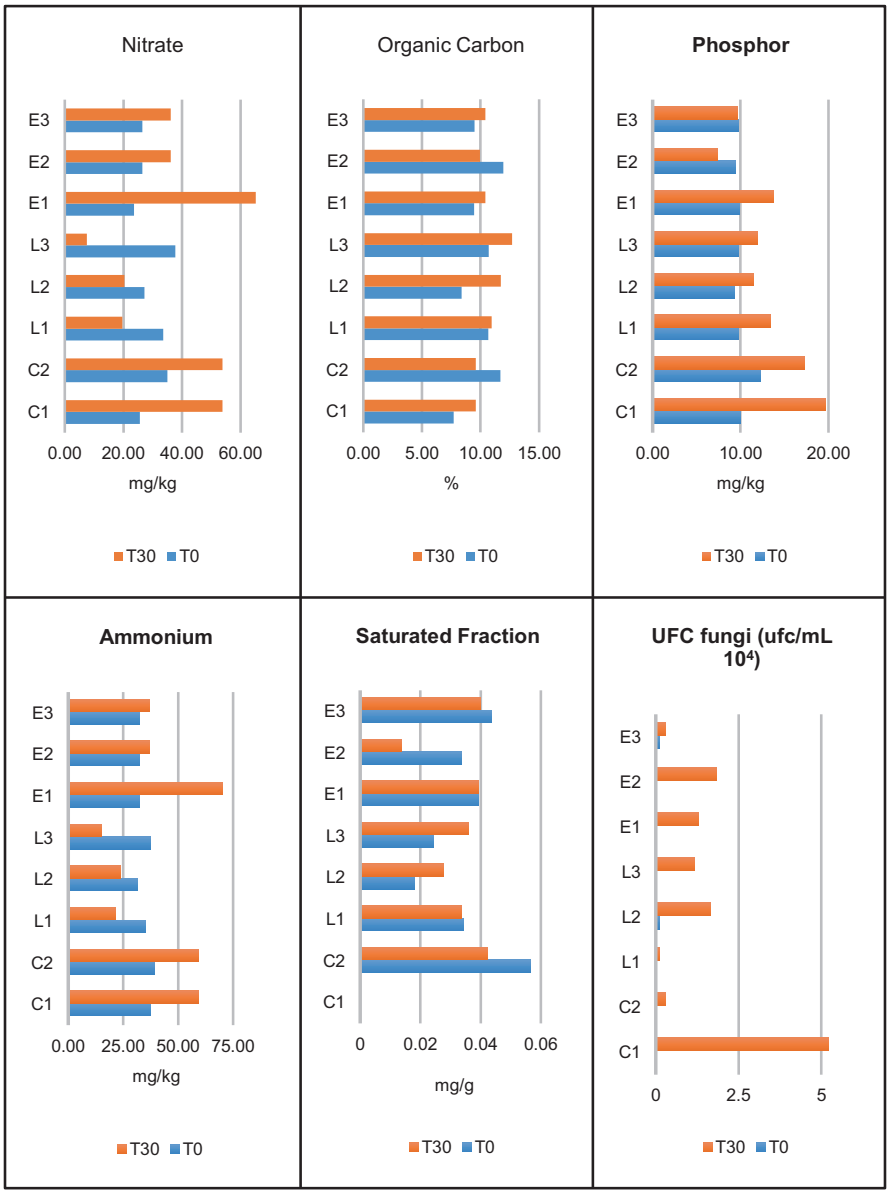

Fig. 11.18 Integration of biogeochemical data (nutrients, degradation, and fungal growth) for the 0th and 30th days for experiment 01. (Source: Costa et al. 2016)

At E3, there were only a decrease in the amount of phosphorus and an increase in the amount of organic carbon, nitrate, and ammonium. In all tests the increase in the number of fungal UFCs was observed.

From the integration of the nutrient and degradation data for the 0 and 30 days for the experiment 01, it was possible to infer that the nutrients, in the tests in general, showed a growth over the 30 days, accompanied by the increase of the amount of CFU and increasing the biodegradation of the saturated fraction. These are the indications that the available nutrients were used to establish the microbiota and the decline of this provided that these nutrients were made available again to the environment. Studies show that the counts of the total heterotrophic microorganisms present higher concentrations of microorganisms in the conditions with the addition of powdered coconut fiber, which can be directly related to the higher aeration provided by this material when added to the contaminated soil, favoring the growth of microorganisms (Santos 2007).

The addition of the saturated fraction to the L2 and L3 units may indicate that the heavier oil fractions have been degraded and added to the lighter ones.

The graphs in Fig. 11.19 represent the nutrient variations against the degradation of the aromatic oil fractions in experiment 02.

In the C1 and C2 tests, phosphorus and carbon nutrients decreased, while nitrate and ammonium increased. In L1, L2, L3, E1, and E2, there were a decrease in the amounts of phosphorus and carbon and an increase in the amounts of nitrate and ammonium. Only in L1 and L2, there was an increase of aromatic compounds, showing that the increase in the amount of free cells (L3) accelerated the bioremediation process.

At E3 there was only organic carbon fall. The other nutrients increased. Despite the degradation of the aromatic fraction, there was an increase in the number of fungal UFCs in all tests.

Observing the integration of the data of experiment 02, it was possible to conclude that the levels of organic carbon and phosphorus in the tests maintained the drop pattern, while the nitrogen species showed an increase, justifiable by the capacity of accumulation of nitrate and ammonium under conditions of low oxygenation and characteristic of mangrove sediment. The biodegradation of the aromatic compounds presented an increase, accompanied by the increase of CFU, but in one part of the tests presented opposite results, due to specific characteristics of the compounds, which end up delaying/hindering the degradation.

The graphs in Fig. 11.20 represent the nutrient variations against the degradation of the NSO fraction of the oil in experiment 03. In C1 only ammonium increased. In C2 there was increase of all nutrients and drop in the amount of NSO. In L1 there was a decrease in the amounts of phosphorus, ammonium, and nitrate. The increase occurred in the amounts of organic carbon and NSO compounds.

In L2, there was a decrease in phosphorus amounts. Ammonium, nitrate, and organic carbon increased. The amount of NSO compounds decreased.

In L3 the amounts of phosphorus and NSO compounds increased. The other nutrients presented a fall. In the E1 test, there was a decrease in all amounts of nutrients and in the NSO compounds.

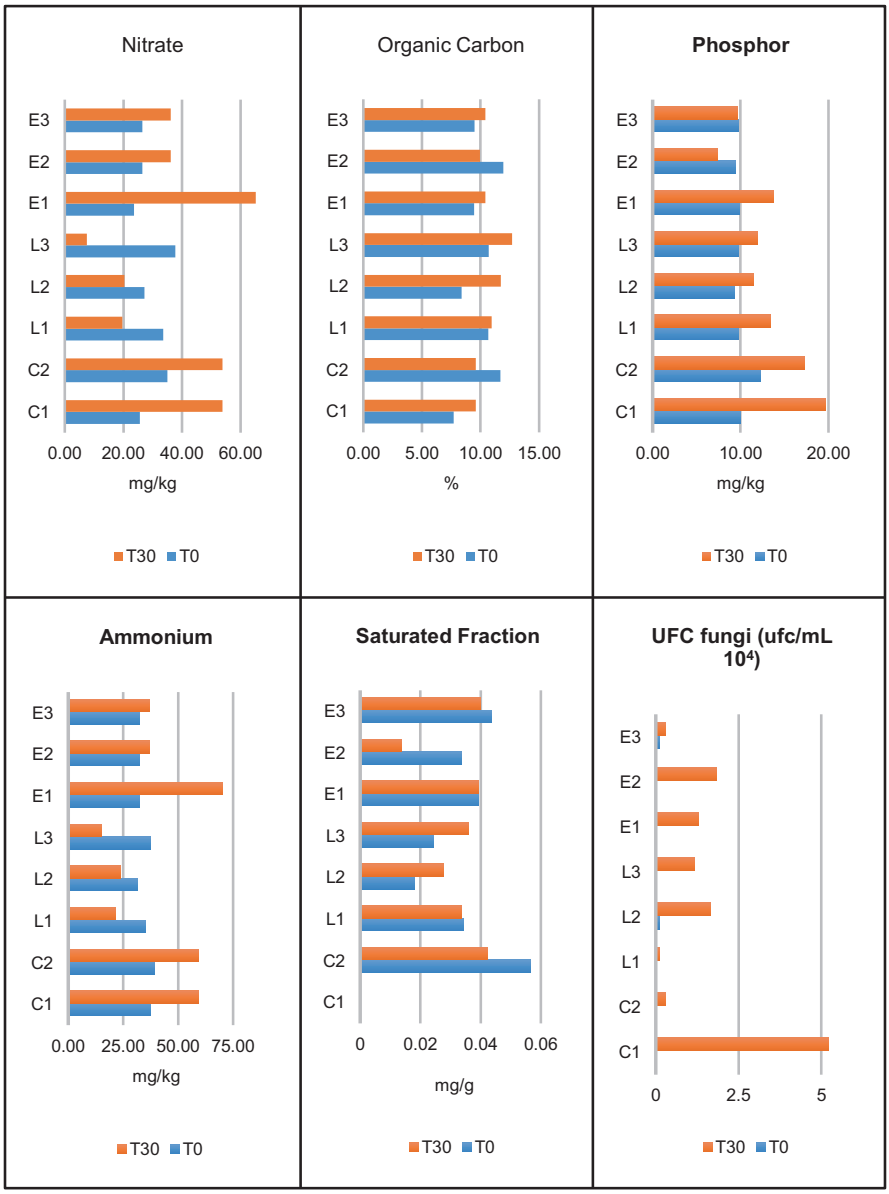

Fig. 11.19 Integration of biogeochemical data (nutrients, degradation, and fungal growth) for the 0th and 30th days for experiment 02. (Source: Costa et al. 2016)

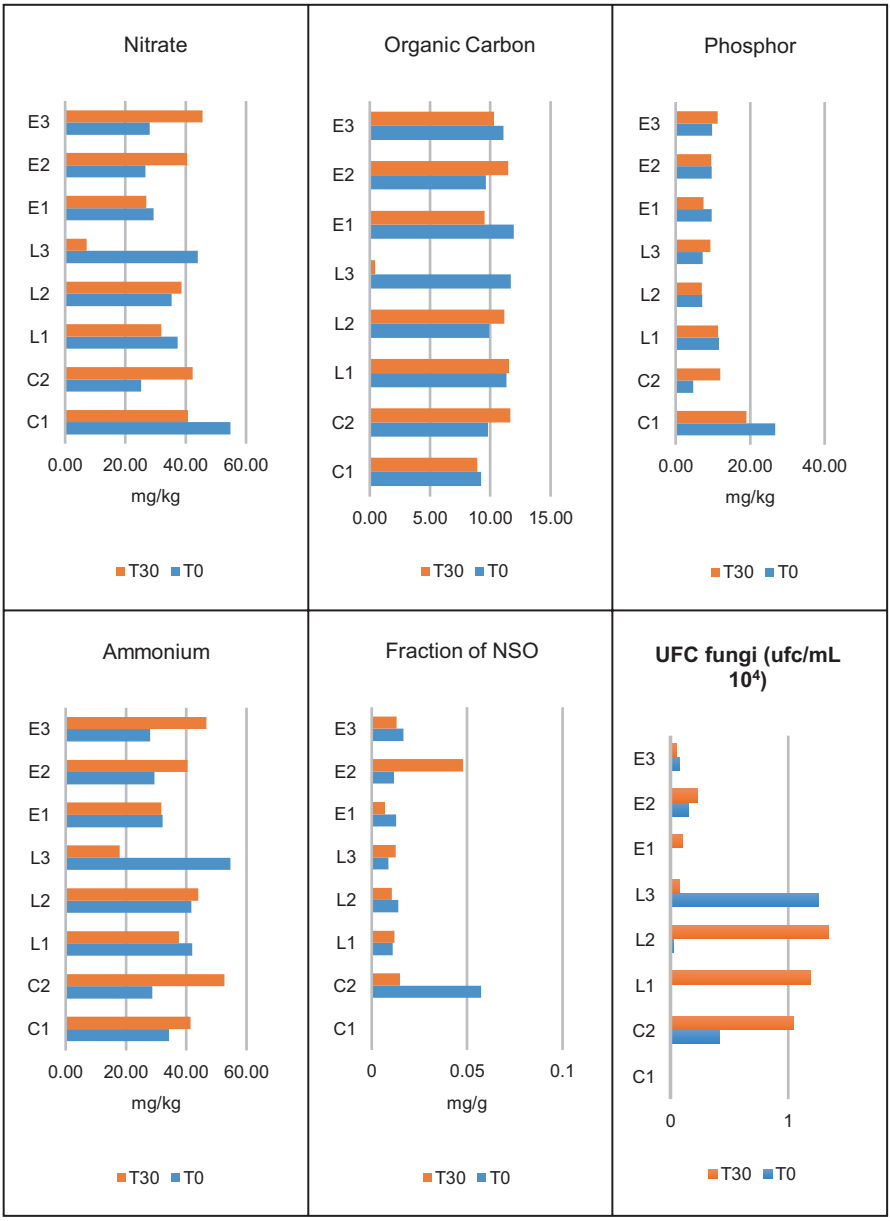

Fig. 11.20 Integration of biogeochemical data (nutrients and degradation) for 0 and 30 days for experiment 03. (Source: Costa et al. 2016)

In E2 only the phosphorus presented fall. Nitrate, ammonium, carbon, and NSO compounds increased. Already in E3 there was an increase in the amounts of phosphorus, ammonium, and nitrate. Organic carbon and NSO compounds dropped.

There was an increase in the number of CFUs in most of the tests, with the exception of L3.

Observing the integration of the data for the experiment 03, we noticed a heterogeneity in the data. The behavior expected by the nutrients over the 30 days was not observed, where the observed levels presented great variations. But what should be taken into account is the structuring property of the coconut fiber that probably facilitated the development of the fungi and consequently the degradation of the petroleum fractions. According to studies carried out by Santos (2007), the addition of coconut powder was responsible for a 110% removal, indicating that the use of this material encouraged the biodegradation of contaminating oil. In the present study, it was possible to prove the increase of biodegradation. However, it is known that the experiment time was not enough for the total degradation, but demonstrated a principle of biodegradation, even for high recalcitrance fraction (NSO).

Sediment Remediation Using Bioaugmentation with Fungal Consortia and Biostimulation with Mangrove Leaf

A third experiment in 2014 was set up to evaluate the efficiency of fungal and mangrove consortia as a nutrient in the degradation of the oil fractions (saturated, aromatic, and NSO) of the Campos basin in mangrove sediments.

Three experiments were performed to monitor the efficiency of degradation of the oil in the Campos basin with the addition of specific fungal consortia for each oil fraction (saturated, aromatic, and NSO) and mangrove as a nutrient under the same conditions. Each experiment was composed of 16 samples. Therefore, we had:

- Experiment 01: corresponds to the consortium of fungi with the potential to degrade the n-alkane compounds
- Experiment 02: fungi consortium with potential to degrade aromatic compounds
- Experiment 03: fungi consortium with potential to degrade polar compounds

For each experiment they were weighed in glass containers with 200 mL capacity, properly decontaminated, with 100 g of sediment, which in turn were packaged and sterilized in autoclave at 120 °C for 50 min, in order to eliminate the microbiological community existing in the sediment. With the aid of a laminar flow camera, the 16 pots were arranged and the pellet homogenized. After reaching room temperature, the mangrove leaf, fungal consortium, and oil were then added (Fig. 11.21).

Mangrove leaves, used in free form, were also sterilized as the sediment. The leaves used in the preparation of the capsules were sterilized in an oven at 120 °C for 30 min and exposed to ultraviolet radiation for 40 min with the same objective of the sediment. In the glass pots, the free leaves were placed and homogenized with the pellet. The capsules were carefully packed so that they did not break, in perforations made in the sediment, and covered.

Fig. 11.21 Assembling the experiment: (**a**) glass pots with sediment and mangrove leaf; (**b**) capsules with immobilized consortia; (**c**) assembled experiment. (Source: Palmeira 2014)

The fungal consortium solution with the ability to degrade the oil corresponding to each fraction was added in the free condition in the amount of 200 μL in the surface portion of the unhomogenized sediment and in the encapsulated condition 0.100 mL. Then, the oil was homogeneously placed on the surface of the sediment, simulating an oil spill in mangrove sediment. Experimental whites were also performed in order to guarantee quality control of the samples, which in turn was composed of only sediment or sediment and oil.

The collection periods occurred at time 0, which corresponds to the assembly day of the experiment and 30 days after assembly. The collection times, as well as the capsule amounts, of the leaf and fungal consortium concentrations were randomly chosen, based on an average of studies already performed; however, none of these were followed.

Table 11.3 Description of treatments

Sample	Treatment description
BS	Sediment
BSO	Sediment+oil
FFL1	Sediment+oil
FFL1,5	Sediment+1,5 g of leaf+suspension of fungus +oil
FFL3	Sedimento+3 g of leaf+suspension of fungus +oil
FFE10	Sedimento+10 leaf capsules with fungus+oil
FFE30	Sedimento+30 leaf capsules with fungus+oil
FFE50	Sedimento+50 leaf capsules with fungus+oil

Source: Palmeira (2014)

After assembly, the samples were incubated in a Logan incubator at a constant temperature of 30 °C, except time 0, which was collected and refrigerated. The collection consisted of the cooling at low temperatures of the samples at the established times, in order to interrupt the fungal growth in the experiment, until the other geochemical analyses were carried out. For the physicochemical and organic analyses, all samples were frozen and lyophilized in a Liotop L108 Lyophilizer in order to eliminate moisture. These samples were disaggregated with the aid of grade and pistil and sieved in stainless steel sieves of 2 mm mesh.

The fungus isolation was performed by Lima (2014), and the fungal consortium varied according to the fraction of the oil (saturated, aromatic, and NSO) that was wanted to observe, considering its greater degrading potential, following the study of Lima (2014).

The samples were identified according to Table 11.3, varying according to the consortium and time of collection.

In the present study, the results confirm the assertion of Safdari et al. (2018), which reports that microorganisms can effectively remediate highly contaminated soil with hydrocarbons and petroleum, especially when biostimulation and bioaugmentation processes are used together.

The analyzed nutrients presented a relation with the stages of fungal growth, being able to be seen with the increase or decrease of their consumption. This can be noticed in the integration of the geochemical data, as shown in Fig. 11.22.

In the results it was possible to observe a similar behavior of the nitrate/ammonium during the experiment, there was an increase of these nutrients probably due to the addition of the mangrove leaf, and the phosphorus presents a trend of growth over time but less expressive than the nitrate and ammonium.

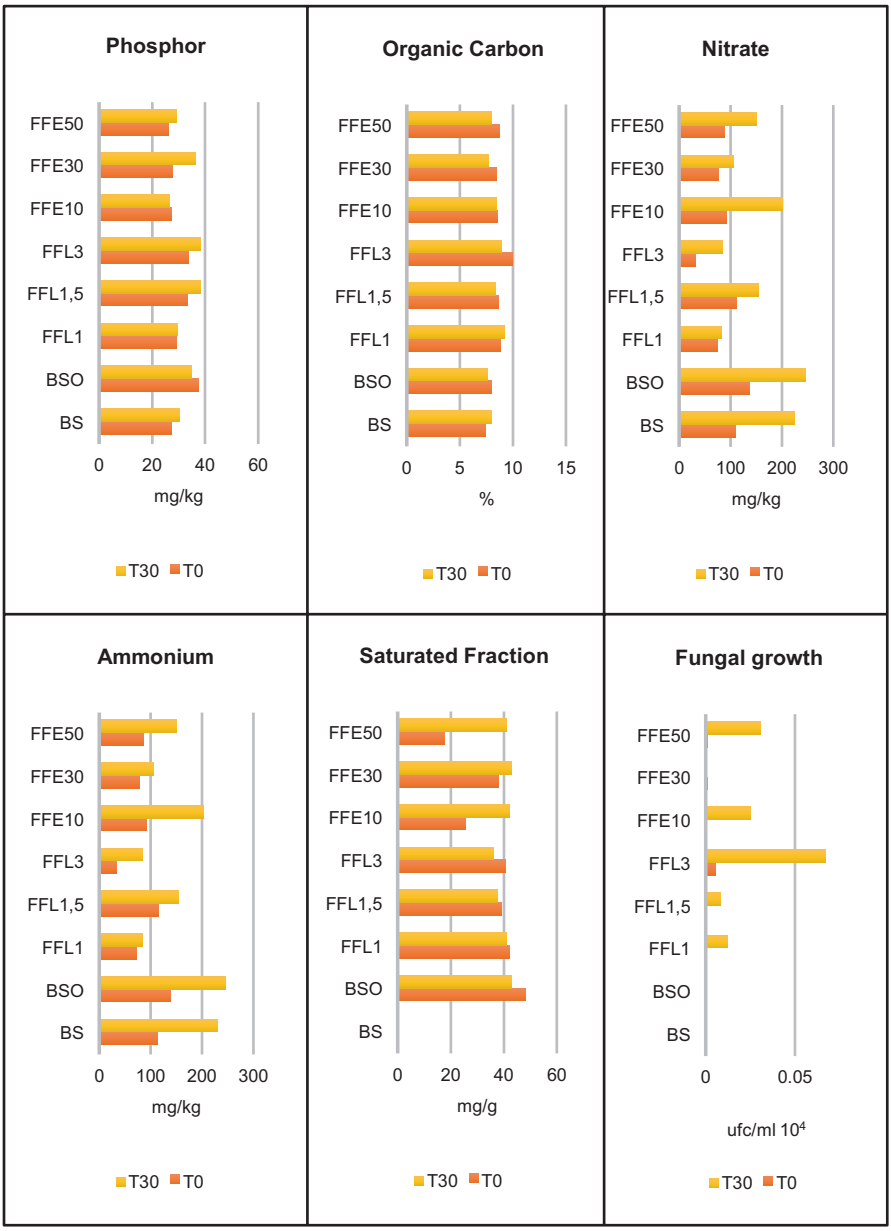

Fig. 11.22 Integration of biogeochemical data (nutrients and degradation) for the 0 and 30 days for experiment 01. (Source: Palmeira 2014)

What could have happened is that as this nutrient was available in the sediment, was also occurring the consumption by the microorganisms. Observing the graph of UFCs, this can be proven since the growth of cells is mainly where leaf and fungal suspension was added free form.

The degradation of the saturated fraction is related to the growth of the consortia. Observing the graph of Fig. 11.22, it is possible to observe that the degradation occurred in almost all units except for the units where they were treated with the capsules. What could have happened was the degradation of the fractions of aromatic and NSO and enrichment of the fraction of saturated. Another possibility for the occurrence is the process of encapsulation that may have hindered the release of nutrients and microorganisms, requiring a longer experiment time to prove their effectiveness.

In the experiment 02 whose objective was to observe the degradation of aromatics, it was possible to observe that the nitrate and ammonium ion have similarity to each other, being this similarity opposite to the organic carbon and phosphorus (Fig. 11.23).

In experiment 02, it was not possible to establish a relationship on the condition (free or encapsulated) of the mangrove leaf. Similarly to experiment 01, nutrients increased from T0 to T30, possibly for the same reasons.

As for the fraction of aromatics, no degradation was observed in any of the units; this enrichment can be attributed to the growth of the fungal isolates. As can be observed in the graph of Fig. 11.23, there was only growth over the 30 days in a treatment unit. This can be attributed to the toxicity of these aromatic compounds.

In the experiment 03 that had the objective of observing the degradation of the NSO fraction, it was possible to observe that the phosphorus, nitrate, and ammonia contents followed the same trend throughout the 30 days (Fig. 11.24). This increase of ammonia and nitrate can be attributed to the use of mangrove leaf as a nutrient additive. This was a positive point for the advance of the research with these microassays where it was possible to see that the mangrove leaf can be used as a biostimulator of the microorganisms, being also the material sustainable and less toxic as the fertilizers used in the diverse experiments.

For the three experiments, it was possible to make the following observations:

- It was not possible to establish a relationship on the condition (free or encapsulated) of the mangrove leaf on the efficiency of fungal growth.
- For experiments 01 and 02, it was possible to observe the increase of the nutrients in the initial phases of the growth of the fungal consortia and also in T30 that would correspond to the phase of their death.
- For the three experiments, nitrate and ammonium presented similar behavior as described in the literature for mangrove sediment.
- Nutrient contents in the three experiments are related to fungal growth (generating moments of higher concentration of nutrients due to the lower consumption and moments of lower concentration due to the higher consumption in the metabolic activities) and the increase by the mangrove leaf, which may have provided the nutrient elements over the experimental time, with the fact that nitrate and ammonium, for example, present high values in T30 does not necessarily imply that it was not consumed.

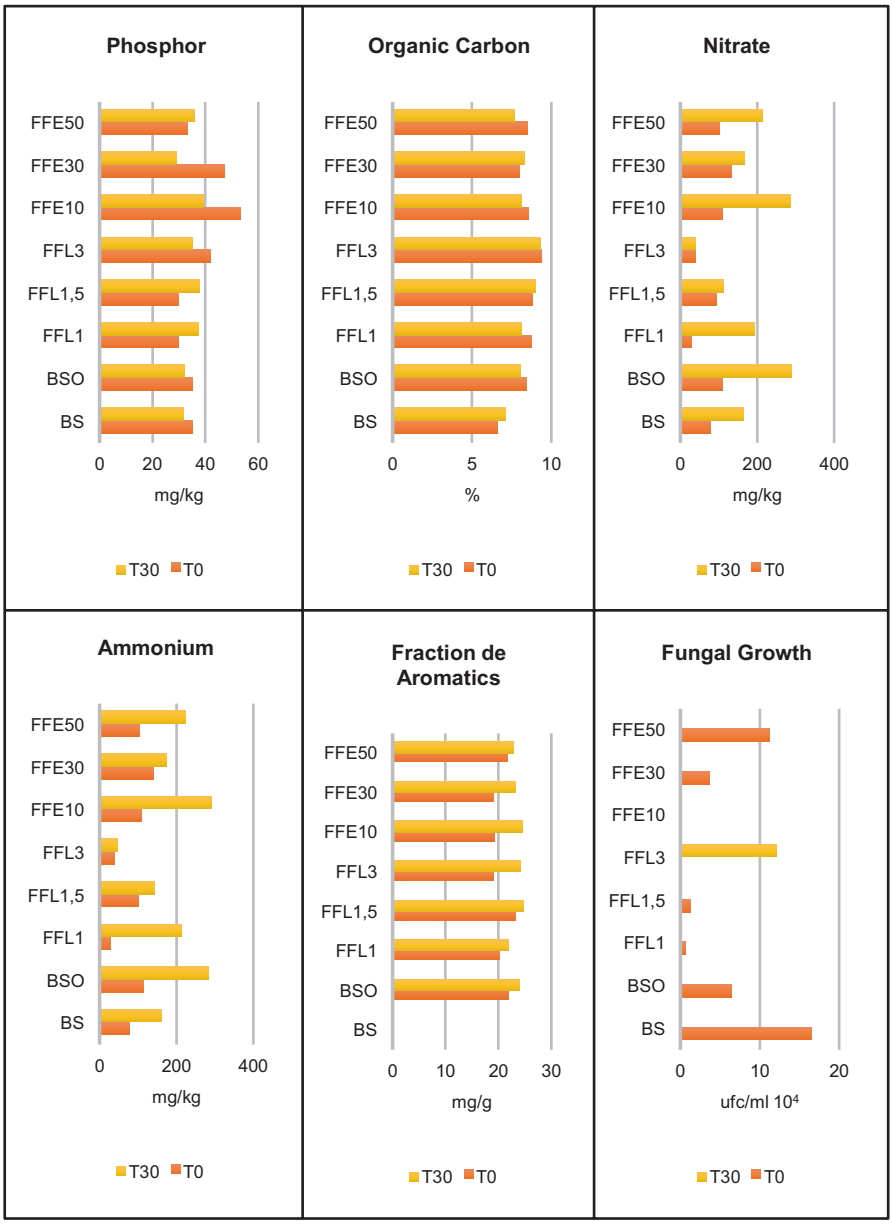

Fig. 11.23 Integration of biogeochemical data (nutrients and degradation) for 0 and 30 days for experiment 02. (Source: Palmeira 2014)

Fig. 11.24 Integration of biogeochemical data (nutrients and degradation) for 0 and 30 days for experiment 03. (Source: Palmeira 2014)

11.6 Sediment Remediation Using Biostimulation and Bioaugmentation with Mixed Consortia

In 2016 a mixed consortium (fungi and bacteria) was tested with the purpose of evaluating the potentiality of microbial consortia in the degradation of oil in mangrove sediments using prototype of temporary immersion bioreactor.

The degradation test was performed in a temporary immersion bioreactor system modified from the prototype developed by Lima (2014). The bioreactors consisted of two 2-liter-sealed glass vials, interconnected by flexible tubes through the lids, containing water in one of the vials and mangrove sediments in the other, both coupled to a vacuum pump (Fig. 11.25).

The immersion process was performed through the forced air injection system, injected with the help of a pump. When the pump was activated, air enters the bottle

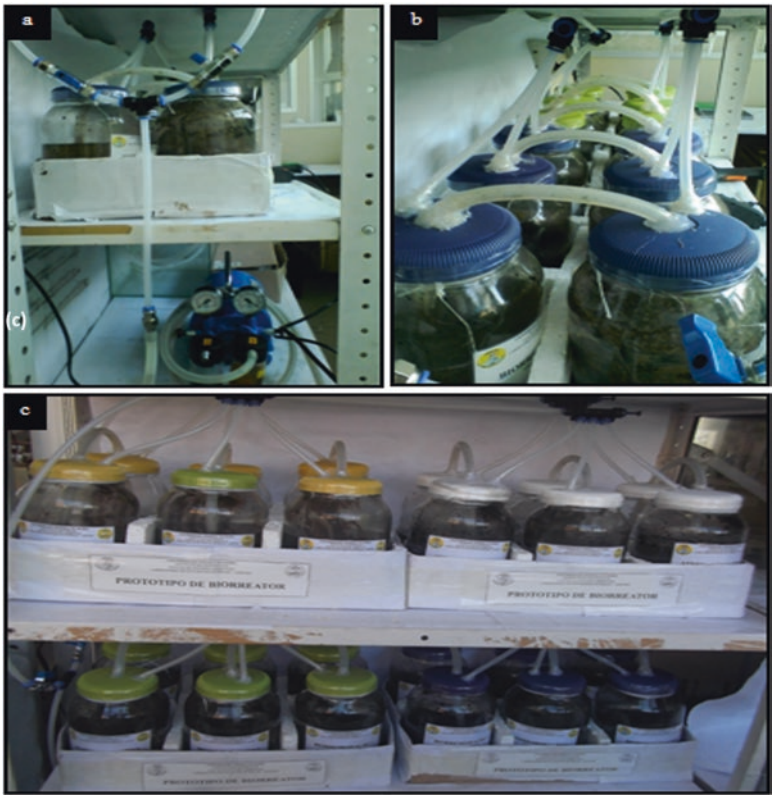

Fig. 11.25 Prototype of temporary immersion bioreactor. (**a**) Vacuum pump air injection system. (**b**) Tubes connecting bottle 1 to bottle 2. (**c**) Simulation units. (Source: Dantas 2016)

1 and, with increasing pressure, transfers the liquid medium to the vial 2 through the interconnected tubes, simulating the high tide. During this phase, the air that enters the system causes the formation of bubbles supplying the demand of oxygen required by the microorganisms. After the immersion phase, the pump was activated in the opposite system, and the water migrated from compartment 2 to 1 by the same tube, simulating the process of descent of the tide.

Three remediation models (triplicates of three tests: natural attenuation, bioremediation I, and bioremediation II) were set up to evaluate the potential for the degradation of total petroleum hydrocarbons, all in triplicate. According to the following design:

- Monitoring natural attenuation (intrinsic bioremediation): three bioreactors composed of reference sediment and the addition of 1% oil from the Recôncavo Baiano basin
- Bioremediation I: three bioreactors composed of reference sediment with the addition of 1% oil from the Recôncavo Baiano basin and mixed microbial consortium encapsulated with coconut fiber
- Bioremediation II: three bioreactors composed of reference sediments with the addition of 1% paraffinic oil from the Recôncavo basin and mixed microbial consortium encapsulated with mango leaves

The glass vials were sterilized at 121 °C in an autoclave for 15 min. Subsequently, in the laminar flow chamber, the sediment and 1% of paraffinic oil from the Recôncavo basin were added and subsequently homogenized. Then one capsule was added per 100 g of pellet. In vial 2 of the bioreactor was added mangrove water in a proportion that could cover the sediment of the opposite bottle according to Fig. 11.26.

The physicochemical parameters were analyzed by salinity, dissolved oxygen (DO), oxidation potential (Eh), hydrogen potential (pH), and water temperature, the last two being measured daily for 1 month. The geochemical monitoring consisted of extraction of the organic fraction in Soxhlet and later analysis by means of gas chromatography coupled to mass spectrometry.

The chemical monitoring consisted in the determination of nitrogen, phosphorus, and total organic carbon contents. Microbiological monitoring consisted of counting bacteria and fungi by the serial dilution method, after 24 h and 48 h, respectively. After incubation period, colony-forming units were counted (results expressed in CFU/mL).

The sediment samples were collected every 30 days, totaling 3 months of experiment. The sample consisted of the sampling of 170 g of sediment, where it was homogenized in a suitable sterilized container; later it was divided into fractions for the monitoring of chemical, geochemical, and microbiological parameters.

Figure 11.27 shows the data of the natural attenuation simulation unit. Concentrations of TOC and phosphorus were inversely proportional in 30 days of experiment. In this sense the phosphorus rate presented the highest peak in T30, and

Fig. 11.26 Montage of degradation test in bioreactor prototype. (**a**) Addition of mangrove sediment in flask 1. (**b**) Addition of saline water in flask 2. (**c**) Addition of 1% of oil. (**d**) Addition of capsules from the mixed microbial consortium. (Source: Dantas 2016)

the TOC values decreased. The amount of fungal CFUs remained practically constant until T30, where it started to increase. These were probably in the process of adjusting to changes in their habitat due to oil spill (acclimatization). In contrast, there were a drastic reduction of CFUs in 60 days of experiment and an increase with 90 days. They may be more sensitive to the contaminant. Total nitrogen data were below detection limit (<1 mg/kg).

Pristane and phytane are considered to be recalcitrant to biodegradation, since branched chain alkanes tend to exhibit lower degradability when compared to n-alkanes (Pereira et al. 2009), leading to an increase in pristane/nC17 concentrations and fitane/nC18 (Wang et al. 2013). Thus, the P/nC17 and F/nC18 ratios are widely used as indicators of biodegradation (Overton et al. 1981; Kennicutt 1998; Didyk Simoneit 1989; Barakat et al. (2001).

The P/nC17 and F/nC18 ratios showed similar response increasing over 90 days. Thus, it shows that there was preferential degradation of the linear alkanes nC17

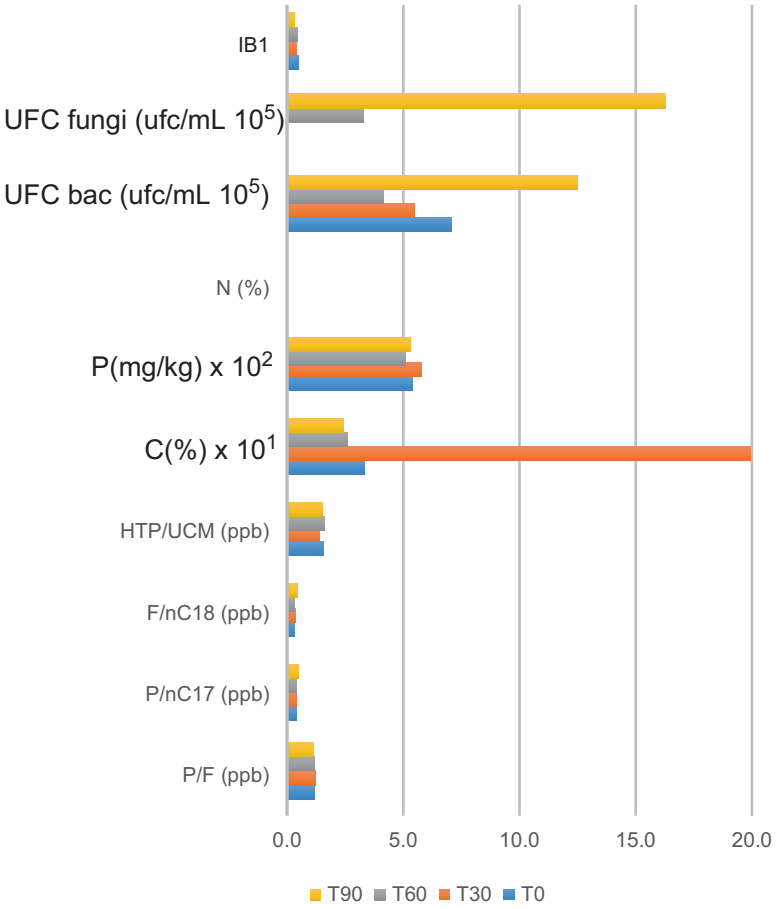

Fig. 11.27 Analysis of the biogeochemical parameters monitored in the natural attenuation simulation unit at 0, 30, 60, and 90 days of experiment. (Source: Dantas 2016)

and nC18. The P/F ratio did not differ significantly. The HTP/UCM ratio showed a reduction in T30 followed by an increase in time 60 and a further reduction in T90 (Fig. 11.27).

The IB1 index (biodegradation index) is commonly used to evaluate oil weathering as suggested by Barakat et al. (2001) and Reyes (2015). Neglecting the physical and chemical weathering because it is relatively smaller than the biological one in the bioreactor system, biodegradation can be evaluated. The even-odd relationship between light and heavy n-alkanes represented demonstrates the process of biodegradation, whose values decreased over time, rectifying the preference of lighter compounds mainly in the initial period of exposure to oil.

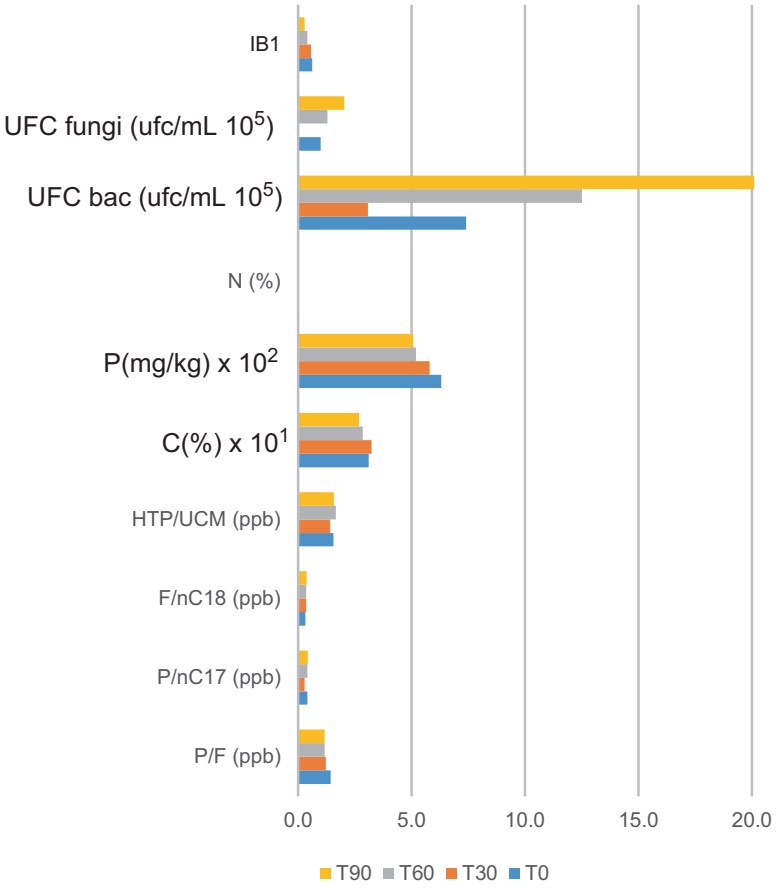

Fig. 11.28 Analysis of the biogeochemical parameters monitored in the bioremediation simulation unit I at 0, 30, 60, and 90 days of experiment. (Source: Dantas 2016)

In the bioremediation I, the phosphorus and organic carbon were consumed during the 90 days. This can be proved with the increase of fungal CFUs throughout the experiment.

In Fig. 11.28 we can see that between T0 and T90, the concentration of the P/nC17 ratio increased slightly. The F/nC18 ratio behaved similarly. These changes in the isoprenoid/n-alkane ratio confirmed the occurrence of the biodegradation process. The increase in the ratio can be justified by the slope of the microorganisms present in the sediment in consuming nC17 and conserving pristane, since unbranched hydrocarbons are easier to biodegrade.

The HTP/UCM ratio obtained similar values throughout the experiment (Fig. 11.28). Taking into account that the lighter hydrocarbons are preferably removed during the initial biodegradation, the appearance of unresolved complex mixture (UCM) and consequently the reduction of the HTP/UCM ratio are expected.

However compounds containing sulfur, nitrogen, and oxygen can also be biodegraded (Cruz and Marsaioli 2012), since macronutrients are essential for the development of microorganisms. The behavior of the ratio can be justified by the fact that the different microorganisms present in the sediment may have the ability to break down both the light hydrocarbon molecules and the NSO compounds and consequently contribute to the similarity of the ratio over the experimental time.

The IB1 index presented a decrease throughout the experiment, proving that there was biodegradation. This biodegradation may be related to the growth of the microorganisms that if observed in the graph had a considerable increase in the T90.

Bioremediation II presented values for nutrients similar to the other treatments (Fig. 11.29). Phosphorus and organic carbon were consumed over the 90 days. In contrast, there was only an increase in fungal CFUs at the end of the experiment. Bacterial cells decreased over the 90 days.

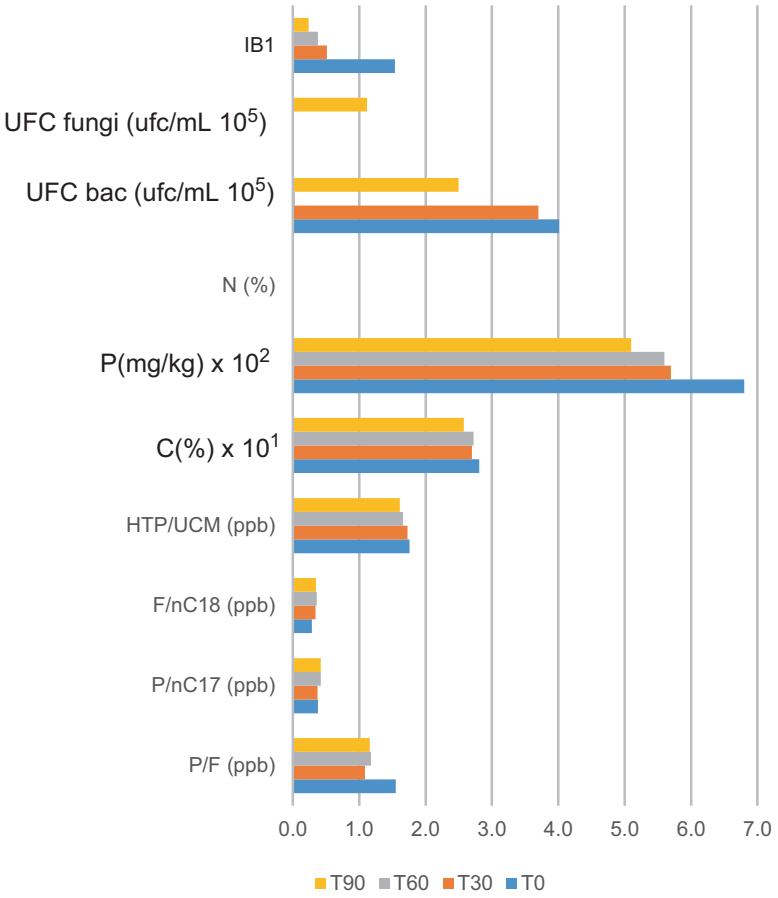

Fig. 11.29 Analysis of the biogeochemical parameters monitored in the bioremediation simulation unit II at 0, 30, 60, and 90 days of experiment. (Source: Dantas 2016)

The P/nC17 and F/nC18 ratio was uniform throughout the experiment, indicating that microorganisms were preferred in the mineralization of the lighter compounds. The P/F and HTP/UCM ratios decreased over 90 days, suggesting the occurrence of the biodegradation process. On the other hand, the ratios P/nC17 and F/nC18 increased, being indicative also of biodegradation. The growth of the microorganisms at the end of the experiment supposes that the degradation process can continue. The B1 indices decreased also being indicative of biodegradation (Fig. 11.29).

Studies have shown the efficiency of a mixed microbial consortium to degrade large amounts of hydrocarbons in soils, presumably due to increased co-metabolic degradation (Zafra et al. 2016).

Comparing the three experiments, the best results were obtained with bioremediation II treatment. This can be attributed to the use of mangrove leaf as a biostimulator, since it was possible to observe a greater availability of nutrients, despite a smaller growth of microorganisms. The bioremediation I where coconut fiber was used as a biostimulator favored the growth of microorganisms, probably due to its structuring property proven in other studies (Santos 2007). However, it did not favor faster biodegradation.

In the bioaccumulation process of the present study, using a mixed consortium, it was possible to observe that during the 90 days, biodegradation occurred, but it will be necessary to deepen with the help of molecular biology a better selection of the microorganisms used, focusing on the taxonomic and functional metagenomics to analyze the effects during the bioremediation process. It is important to better understand the metabolic pathways carried out by the microorganisms, in order to understand and with the intention to accelerate the process of biodegradation.

11.7 Future Perspectives

Petroleum biodegradation processes can be improved by deepening the research done and applications of new technologies. From the analysis of the results obtained and due to the complexity with the control of the process of bioremediation in mangrove environment, it is recommended new researches that have the objective to carry out further investigations on the metabolism and production of enzymes, together with the degradation studies, make the classical and molecular identification of lineages before assembling new consortia of microorganisms.

The use of new metagenomic techniques, associated with new-generation sequencing (NGS), makes it possible to characterize microbial communities in order to find genes that encode important enzymes for the biotechnological application and the elaboration of a reference library without prior culture, isolation of species, or cloning. These metagenomic studies applied in bioremediation include the evaluation of microbial diversity, selection of functional genes and metatranscriptomas and active microbial community, and detection of the presence of tolerant or intolerant taxa to the pollutants.

Biotechnology studies as an expression of functional genes during the remediation process have also been considered an efficient monitoring tool and allow to observe succession over time. This sequence of expression, when related to the degradation of petroleum hydrocarbons, is probably related to the succession of degradation that starts with the hydrocarbons of lighter chains.

The use of advanced molecular techniques in the study of the microbial community together with new bioinformatics tools, like those proposed in this scientific-technological research, constitutes an important advance in the field of geomicrobiology. Thus, the association of important topics such as oil studies, bioremediation, and metagenomics allows unpublished studies that contribute to strategic areas of science, still underexplored and developing, and to develop bioproducts with greater reliability in relation to their origin and efficiency.

However the path of the research carried out is directly related to the application of molecular biology techniques to prospect and identify genes of interest used in the identification and management of microbial communities and the expression and quantification of functional enzymes (functional selection), as well as to apply current sequencing platforms (NGS), real-time quantitative PCR (Q-PCR), and chromatography in metagenomic, transcriptomic, and metabolomic studies of microorganisms.

However, the deepening of research on the use of mangrove leaf as a nutrient for the microorganisms used in the bioaugmentation technique is of paramount importance. Current works are increasingly looking for an effective and sustainable technique, and the use of mangrove leaves is a new form of nutrient efficient and promising, thus making bioremediation a sustainable technique with lower costs than when using fertilizers as biostimulators. This is required in order to reduce the toxicity caused by certain chemical fertilizers, application of which results in negative effect on the environment.

Studies of coconut fiber should be explored in terms of their ability to absorb contaminants. This will consequently improve the biodegradation process when one thinks of the production of bioproducts containing an absorbent material, a biostimulator, and the biodegradator.

Microorganisms are increasingly being used in the area of industrial biotechnology. Biodegradation processes, in particular bioremediation, integrated into microbiology and applied to biotechnology, are likely to be one of the main research works, especially when combined with natural biostimulators.

It is in the interest of the oil industry to develop techniques and bioproducts to help recover areas affected by the damage caused by this fossil fuel.

Aiming to know the efficiency of microorganisms and natural additives as stimulants in accelerating the degradation of total oil in mangrove sediment, this research based on studies already carried out internationally obtained satisfactory and promising results, which will probably improve the applied techniques in the recovery of contaminated areas with oil.

11.8 Conclusions

The results obtained for the comparison tests between NPK and OSMOCOTE fertilizers indicated that there is NPK efficiency in the acceleration of the biodegradation process being a promising technique for area recovery.

The monitoring of nutrients over the 90 days showed that the treatments with NPK and OSMOCOTE were effective in relation to the continuous release of nitrogen and phosphorus to the system, but there was no expected cost, which can be justified by the nature of the oil found, since it was oil in an advanced stage of degradation. The levels of ammonia were much higher than those of nitrate, suggesting that this is the source of nitrogen favorable to the process.

The results of liquid chromatography showed a decrease of the n-alkane compounds, with consequent increase of polar compounds. When we compared the results of the treatments (reference, NPK and OSMOCOTE), the unit containing OSMOCOTE showed a similar tendency of reference units, but when compared to units with NPK, it was less efficient.

The application of immobilized consortia with the potential to degrade the two types of oil studied under laboratory conditions presented promising results for the understanding of the bioremediation process in mangrove sediments. In relation to the biostimulation process using the coconut fiber and the mangrove leaf powder immobilized together with the microbial consortium, it was not possible to observe a quantitative and qualitative significance regarding the release of nutrients. In contrast, it can be observed that, in some way, adequate nutrient contents were maintained during the 3 months, which would not occur if the system were not biostimulated. In relation to the bioaugmentation, it was possible to observe that the microorganisms probably follow the same route of degradation, even in different treatments. The results of the geochemical reasons also showed the degradation in the two types of oil.

The results obtained for the experiment in a bioreactor prototype showed better results. The reduction of n-alkanes from the oil of the Recôncavo basin was much more expressive. The pristane/fitane, P/nC17, F/nC18, and HTP/UCM ratios were strong indicators of biodegradation. This shows that contaminated sediments, when treated in confined spaces, tend to be better.

The use of coconut fiber when compared to the use of mangrove leaf as nutrient showed greater potential for mangrove leaf, and coconut fiber should be exploited in relation to its absorption power.

The combination of mixed bioassay and biostimulation techniques may favor degradation and reduce treatment time provided they are used correctly. More detailed studies are suggested in relation to the combination of bio-increase and biostimulation techniques, as well as to evaluate the possible generation of toxic coproducts to the mangrove ecosystem in the application of the capsules. It is necessary to carry out the molecular identification of the microorganisms that made up the consortium and to isolate the enzymes responsible for the degradation of hydrocarbons in order to obtain better interpretations regarding the biodegradation process.

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Chapter 12
Microbial Degradation of Petroleum Hydrocarbons

Ghada Abd-Elmonsef Mahmoud and Magdy Mohmed Khalil Bagy

Abstract Nowadays, petroleum hydrocarbon pollutants keep on being a genuine natural worry because of the managed development of petroleum oil extraction, related generation which ends up noticeably with ecological issue. The expanding in industrial progression causes expanding in a petroleum-essential product consistently to cover the human needs. Continuous growing, development and improvement of industrial exercises over the whole world make petroleum-based products the most significant issue in this century. Oil spills frequently happen by mishaps amid pumping, transportation and refining. Nearness of these petrochemicals in the environment makes huge risks to human health for their lethal, mutagenic, cancer-causing impacts and their capacity of aggregation in food chain. Researchers keep searching for sustainable remediation techniques for polluted sites. As of now physical and chemical remediation advances are by all accounts facing a few issues like transferring pollutants from one phase to another and not having the ability for complete removal of contaminants which turn into another problem. Among the varieties of the remediation techniques, microbial utilization of microorganisms in biodegradation processes demonstrated the achievement in degrading xenobiotic compounds contrasted with physico-chemical strategies in terms of money-related costs, efficiency, energy efficiency, versatility and simplicity to apply and seems to be the environment sound solution. The key factor for successful bioremediation involves selecting appropriate microbes with high capability of pollutant degradation. Microorganisms like fungi, bacteria and yeast are considered as promising dynamic remarkable microbes involved in biodegradation of petroleum aliphatic and aromatic hydrocarbons.

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Abbreviations

PAHs Polycyclic aromatic hydrocarbons
SNO Sulphur, nitrogen, oxygen
TPHs Total petroleum hydrocarbons

12.1 Introduction

More than 1500 million tons of petroleum oil are transported every year. Indeed, even with the best safeguard's strategies, a significant amount of petroleum oils and products are discharged into the environment, either because of operational releases or because of accidents. Thus extraction, transportation, refinement, leakage and transfer of petroleum become a critical environmental contamination issue around the world (Al-Baldawi et al. 2013; Masood et al. 2014; Deng et al. 2014; Bordoloi and Basumatary 2015; Hou et al. 2015; Priya et al. 2015). Petroleum hydrocarbons are composed of numerous carbon bonds that create exceptional multi-complex structures, and when they bind to others, it gives characteristic molecules, e.g. aliphatic alkanes, alkenes and polycyclic aromatic hydrocarbons (PAHs) of various proportions (Fig.12.1). These major components are announced for their harmful effect to the environment (Sarma et al. 2004; Steliga et al. 2012). Extensive quantities of different pollutants attacking soil or water make gigantic danger to the health of human and natural ecosystem, for example, pesticides, petroleum products, chlorophenols and heavy metals (Tang et al. 2014; Chen et al. 2015). These pollutants accumulate in the animal and plant tissues and can cause serious toxic, carcinogenic and mutagenic effects, with capability of accumulation in our food chain. Petroleum hydrocarbon accumulation in living organism's tissues can promote mutations and cancer development (Adeniyi and Afolabi 2002; Obuekwe et al. 2009; Steliga et al. 2012; Guarino et al. 2017). Additionally, soil regular contaminants cause dangerous and undesired effects on soil microorganisms, e.g. gasoline which showed a poisonous impact on different microorganisms in soils which prompted change in soil microbial population (Tejada et al. 2008). Because of the adverse effect of these

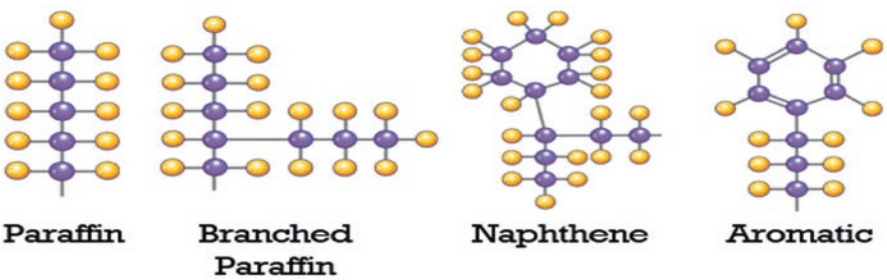


Fig. 12.1 Different structures of crude oil components

contaminants on both human health and environment, they are considered as priority ecological pollutants by the US Environmental Protection Agency (US EPA 1986).

Several studies occurred announcing the improvement strategies for remediation protocols to the environmental damage of petroleum spill salvage (Kadali et al. 2012). The most famous remediation technologies of petroleum hydrocarbon-polluted soils involve chemical and electrokinetic separation, soil flushing, electrochemical oxidation, stabilization of soil vapour, extraction and thermal treatments. These techniques are expensive and could convert pollutants from one phase to another, and also they could not accomplish the complete removal of the contaminants which become environmentally unsustainable (Hamdi et al. 2007; Cappello et al. 2015).

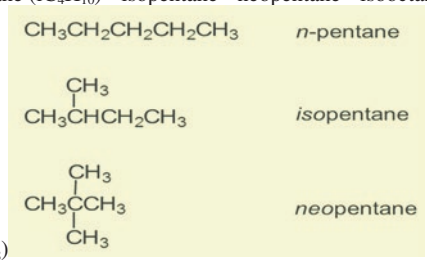
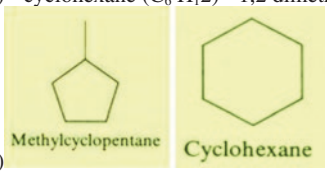
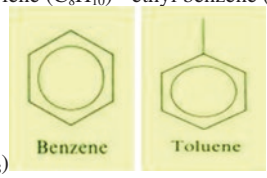
Bioremediation solves most of physical and chemical issues using the metabolic capability exploitation of different remarkable microorganisms. Biodegradation demonstrated that its success in degrading xenobiotic compounds contrasted with physico-chemical techniques is continually evolving because of energy efficiency, effortlessness, high efficiency, simple application and cost adequacy (Khan et al. 2016; Guarino et al. 2017). Increasing the effectiveness of petroleum products with hydrocarbon bioremediation particularly in soil remains a challenge after all decades. There are two common ways in bioremediation technologies: bioaugmentation by including microorganisms and biostimulation by presenting nutrient supplement or biosurfactant. Besides, successive bioremediation process not only depends on choosing microbes but also on studying the factors which impact the rates of microbial development and enzymatic capabilities consistently affecting the rates of petroleum oil hydrocarbon biodegradation. The tirelessness of petroleum oil contaminants relies upon the amount, the quality of the hydrocarbon blended in nature and on the properties of the influenced ecosystem. In one environment condition, petroleum hydrocarbons can hold indefinitely, whereas under another environmental arrangement condition, similar hydrocarbons can be totally biodegraded within couple of hours or days (Das and Mukherjee 2007; Atlas 1981; Whang et al. 2008). In this chapter we will focus on types of petroleum hydrocarbons, remediation strategies (especially bioremediation), different types of biodegraded microbes and various conditions affecting petroleum hydrocarbon biodegradation process.

12.2 Remediation Strategies of Petroleum Hydrocarbons

Petroleum hydrocarbons are made up of mixtures of hydrophobic and nonaqueous constituents, e.g. aromatics, n-alkane, resins and asphaltene fractions. Hazardous oil wastes contain a complex mixture of total petroleum hydrocarbons (TPHs) involving different aromatic and aliphatic nitrogen, oxygen, sulphur compounds and asphaltene (Bhattacharya et al. 2003; Wu et al. 2017) as shown in Table 12.1.

Most utilized remediation techniques for oil spill in marine environment were physical expulsion by skimmers, booms and absorbent matters; these assist in the removal of the petroleum oil from water surface layer but not complete removal;

Table 12.1 Petroleum oil composition

Major constituents	Types
Paraffins	Methane (CH_4) • ethane (C_2H_6) • propane (C_3H_8) • butane (C_4H_{10}) • pentane (C_5H_{12}) • hexane (C_6H_{14})
Isoparaffins	Isobutane (iC_4H_{10}) • isopentane • neopentane • isooctane (iC_8H_{18}) 
Olefins	Ethylene (C_2H_4) • propylene (C_3H_6)
Naphthenes	Cyclopentane (C_5H_{10}) • methyl cyclopentane (C_6H_{12}) • dimethyl cyclopentane (C_7H_{14}) • cyclohexane (C_6H_12) • 1,2 dimethylcyclohexane (C_8H_{16}) 
Aromatics	Benzene (C_6H_6) • toluene (C_7H_8) • xylene (C_8H_{10}) • ethyl benzene (C_8H_{10}) • cumene (C_8H_{10}) • naphthalene ($C_{10}H_8$) 
SNO compounds	Hydrogen sulphide, mercaptans, quinotine, pyridine, pyrrole, indole, carbazole, naphthenic acids, phenols

Google website and http://www.aip.com.au/industry/fact_refine.htm

chemical techniques, e.g. using solvents and surfactants, scattered petroleum oil into small droplets cause stress on marine ecosystem.

In petroleum hydrocarbon-contaminated soil, remediation techniques involve chemical and electrokinetic separation, electrochemical oxidation, soil vapour extraction, soil flushing, thermal treatments and stabilization (Hamdi et al. 2007; Cappello et al. 2015).

Remediation by chemical and physical technologies is unqualified or expensive and could not achieve their purpose. Every one of these techniques does not completely discard petroleum oil from the environment and could cause another poison-

ous side effect (Sheppard et al. 2014). Bioremediation, unlike physical and chemical strategies, is simple to apply and economical and considered as the most environmentally friendly solution for oil spill. Bioremediation goes for using the microbial potentials for cleaning up contaminated matrices via optimization of degradation conditions. It additionally gives the possibility for destroying or reducing other harmless pollutants using their natural biological activity which accelerates the biological degradation as well as precipitation of pollutants (Kharusi et al. 2016; Ma et al. 2016). Microbial community or single microorganism is considered a basic key part in hydrocarbon removal without affecting the stability of soil (Tejada et al. 2008). Biostimulation and bioaugmentation are two famous featured methodologies of bioremediation applied to treat the hydrocarbon-polluted environments. Biostimulation includes nutrient supplement to invigorate the hydrocarbon-degrading capacity of the inhibited microbial community. However bioaugmentation is a manner that enhances the disintegration rate through the presentation of particular microbial strains or various strains familiar to be dynamically utilized in polluted soil to degrade petroleum hydrocarbons (Wu et al. 2013, 2016, 2017; Priya et al. 2015).

Biostimulation of petroleum hydrocarbons using indigenous microorganisms stands out amongst the best biodegradation systems by which petroleum oil and other hydrocarbon pollutants are normally expelled from natural environment. The accomplishment of this operation relies upon the capacity of the method to create and preserve the promoting status for pollutant biodegradation in adequately high rating (Sihag et al. 2014; Guarino et al. 2017). The strategies to accelerate biostimulation in soil occur by optimizing the factors, for example, nutrients, oxygenation, pH, temperature, biosurfactant and inoculation, with enriched various microbial consortiums into the soil. This process also needs the presence of microorganisms with high metabolic aptitudes; however the associations between oil and aqueous phase (microorganisms) are vital for the accomplishment of bioremediation with consequental effects on natural biodegradation average of petroleum oil (Torlapati and Boufadel 2014; Ron and Rosenberg 2014; Guarino et al. 2017).

Bioaugmentation means improving polluted soil or sludge with compost mixture and introducing commercial microbes. Mostly bacteria and fungi were found in the reclamation of petroleum hydrocarbons or other organic pollutant-contaminated sites (Kriipsalu et al. 2007; Mancera-Lopez et al. 2008). Soil fertilization (composting) can increase soil organic matter content and fertility simultaneously other than bioremediation and thus is supposed to be one of the highest cost-effective strategies for bioremediation (Chen et al. 2015). Fungi seem to be the most functional degraders, when applying the composting process to decontaminate polycyclic aromatic hydrocarbon-polluted soils; the advantage of compost addition was supplementing nutrients and carbon sources to the soil, and it significantly upgraded the degradation levels of alkanes and petroleum hydrocarbons (Namkoong et al. 2002; Antizar-Ladislao et al. 2004). Bioaugmentation used with successful application in most soil environments however its need specific site requires and deep characterization of the soil system (Daghio et al. 2015).

12.3 Aerobic and Anaerobic Petroleum Hydrocarbon Bioremediation

In the regular habitat, petroleum hydrocarbons are biodegraded aerobically or anaerobically by bacteria, yeasts and fungi with varied degradation rates (Bagia et al. 2013; Guarino et al. 2017). The existence of mixed populations (consortia) demonstrating different and broad metabolic limits in oxygen presence or absence is considered basic for degradation of complex mixtures of petroleum hydrocarbons, for example, petroleum oil in soil, freshwater and marine environment. Several studies in detail mentioned petroleum hydrocarbon degradation capacity being related with the microbial degrader population (Krutz et al. 2005; Kauppi et al. 2011; Taccari et al. 2012; Khan et al. 2016; Wu et al. 2016).

Aerobic degradation is the highest prompt and complete method for the elimination of petroleum hydrocarbon hazards in the environment particularly aromatic types. Various enzymes and pathways in charge of petroleum hydrocarbon degradation have been established, e.g. cytochrome P450 family and alkane monooxygenase associated with petroleum elimination pathways (van Beilen et al. 2006; van Beilen and Funhoff 2007). The premier intracellular attack of aromatic hydrocarbons is oxidative operation by several impacted enzymes, for example, oxygenase and peroxidase enzyme systems, which incorporate oxygen presence. Oxidation of organic carbon, organic matter and petroleum contaminants using microorganisms in soil accelerates the existence of molecular oxygen as the oxidation operation mostly needs oxygenase enzymes (Atlas and Bartha 1987) and commonly occurs more rapidly when oxygen levels increase (Atlas and Cerniglia 1995). Under aerobic status, oxygenases start to attack benzene ring and set up for continuous ring cleavages (Yeung et al. 2013). Sierra and Renault (1995) showed that nitrogen mineralization of soil organic matter using microbes was stifled when CO_2 concentrations surpassed 4%. Another study recorded that biodegradation of petroleum oil in gasoline-polluted soil was quickest when microcosm oxygen consumption was 8% in O_2 concentrations. The degradation rate at 8% of O_2 was more than twice than what they saw at a near atmospheric O_2 level (18% O_2) (Zhou and Crawford 1995). As indicated by Wuerdemann et al. (1994), an oxygen level of 5% or less was restricted to biodegradation in soil from a former gaswork site.

Anaerobic degradation of hydrocarbons by microorganisms is considered as an ecological significance strategy for microbial degradation of petroleum hydrocarbons; it does not utilize the oxygenases as in aerobic organisms. Microbial degradation of aromatic benzoate, halobenzoates, polychlorinated biphenyls and chlorophenols occurs in zero oxygen levels (Boyd and Shelton 1984; Chen et al. 1988; Leahy and Colwell 1990). Under anaerobic conditions, microorganisms appeared to utilize different electron acceptors, for example, manganese, nitrate, sulphate and iron, substitute the molecular oxygen (for respiration) and oxidize different hydrocarbons in petroleum plumes of groundwater (Leahy and Colwell 1990; Azadpour-Keeley et al. 2001; Yeung et al. 2013). Without satisfactory quantities of O_2, anaerobic degradation of hydrocarbons, alkanes and aromatics from

respiratory denitrification occurs utilizing nitrate as electron acceptor. The nitrate is changed over to N_2 or N_2O gases amid this procedure, and carbon is changed over to CO_2 (Hutchins 1991; So and Young 2001; Burgin and Hamilton 2007). Anaerobic pathways such as prevailing deep soil profile, where O_2 dispersion is restricted. Aliphatic-aromatic hydrocarbon biodegradations are faster when sufficient soil O_2 is available, and this may regard to hydrocarbon compound structure. Anaerobic hydrocarbon degradation can also continue utilizing iron or sulphate followed by nitrate in biodegradation process (Zhou and Crawford 1995; Salminen et al. 2006; Hasinger et al. 2012).

12.4 Microbial Degradation of Petroleum Hydrocarbons

The key factor for effective bioremediation process is the appropriate microbial choice which had the ability to degrade contaminants without losing the microbial viability and competing other autochthonous microorganisms. *Alk* (C5-C12 *n*-alkanes), *nah* (naphthalene) and *xyl* (toluene) were the most known microbial catabolic pathways related to petroleum hydrocarbon degradation (Sayler et al., 1990; Whyte et al., 1997). Singular life forms can process just a constrained scope of hydrocarbon materials, so that gathering of different populations having a wide enzymatic capacity are required to eliminate complex mix of hydrocarbons like petroleum oil in soil, freshwater and marine environments (Floodgate 1984; Britton 1984; Whyte et al. 1997; Cappello et al. 2007; Tyagi et al. 2010; Dellagnezze et al. 2014; Priya et al. 2015). Microbes are ubiquitous in terrestrial and aquatic communities; the most heterotrophic community involved was hydrocarbon-utilizing bacteria and fungi which seems to be an assignment of the ecosystem and seems to be widespread in all environmental conditions, with detailed frequencies going from 6% to 82% for soil mycobiota, 0.13% to 50% for soil bacterial communities and 0.003% to 100% for marine bacterial communities (Atlas et al. 1980; Hollaway et al. 1980; Leahy and Colwell 1990; Yakimov et al. 2007; Foght 2008).

Bacterial communities are the most dynamic remarkable microbes in petroleum oil degradation and considered as highest oil spill (could feed exclusively on hydrocarbons) degraders in environment; its specific genes in connection with hydrocarbon disintegration assume an essential part in the degradation process of petroleum hazards (Habe and Omori 2003; Yakimov et al. 2007; Brooijmans et al. 2009). The interactions between bacterial cultures and petroleum-contaminated environments are knotted; the bacterial reaction to petroleum hydrocarbons creates powerful strategies for enhancing the degradation capacity. Using useful gene identification involved, directly or indirectly, in petroleum biodegradation (Hong et al. 2016). There are several genome sequences of bacteria with hydrocarbon elimination capacities, for example, *Alcanivorax borkumensis*, *Geobacillus thermodenitrificans*, *Desulfatibacillum alkenivorans*, *Polymorphum gilvum* SL003B-26A1T and *Pseudomonas aeruginosa* N002. Possible gene qualities and pathways connected to hydrocarbon degradation had been distinguished by the information from total

bacterial genome sequencing (Schneiker et al. 2006; Feng et al. 2007; Callaghan et al. 2012; Nie et al. 2014; Das et al. 2015; Hong et al. 2016). Few types of bacteria recovered from different petroleum-contaminated sites demonstrated their capacity to dispose the petroleum pollutants, e.g. *Acinetobacter lwoffii*, *Aeromonas hydrophila*, *Arthrobacter* sp., *Alcaligenes faecalis*, *Bacillus licheniformis*, *Bacillus marcescens*, *Corynebacterium* sp., *Flavobacterium* sp., *Klebsiella pneumonia*, *Klebsiella aerogenes*, *Moraxella* sp., *Micrococcus* sp., *Mycobacterium* sp., *Proteus vulgaris*, *Pseudomonas alcaligenes*, *P. putida*, *P. putrefaciens*, *P. aeruginosa* and *Vibrio* sp. (Jobson et al. 1972; Ghazali et al. 2004; Adebusoye et al. 2006; Sathishkumar et al. 2008; Mittal and Singh 2009; Hamzah et al. 2010; Khashayar and Mahsa 2010; Raza et al. 2010; Thenmozhi et al. 2011; Geetha et al. 2013; Sunita et al. 2013; Jesubunmi 2014).

Studies on bacterial degradation of petroleum hydrocarbons started many years ago; Jones and Eddington (1968) established that 11 fungal genera and 6 bacterial genera were in charge of hydrocarbon aerobic oxidation in polluted soil spots, and they recorded that fungi assumed an imperative part in hydrocarbon aerobic oxidizing actions of the soil samples compared with bacteria. Cook and Westlake (1974) recovered *Achromobacter* sp., *Acinetobacter* sp., *Alcaligenes* sp., *Flavobacterium* sp., *Pseudomonas* sp. and *Xanthomonas* sp. at 4 °C on crude oil. Thermophilic bacteria (*Thermomicrobium* sp.) demonstrated to be capable of hydrocarbon utilization. Cundell and Traxler (1974) recovered *Alcaligenes* sp., *Arthrobacter* sp., *Brevibacterium* sp., *Pseudomonas* sp., *Spirillum* sp. and *Xanthomonas* sp. from asphaltic flow in Alaska. Walker et al. (1975a) match the different capacities of bacteria, yeast and fungi to degrade hydrocarbons using *Candida* sp., *Sporobolomyces* sp., *Hansenula* sp., *Aureobasidium* sp., *Rhodotorula* sp., *Cladosporium* sp., *Penicillium* sp., *Aspergillus* sp., *Pseudomonas* sp., *Vibrio* sp., *Acinetobacter* sp., *Leucothrix* sp., *Nocardia* sp. and *Rhizobium* sp., and they found that bacteria and yeasts demonstrated decreasing capacities to utilize alkanes with expanding chain length, while fungi could not display special degradation for specific chain lengths. *Acinetobacter*, *Pseudomonas* and *Vibrio* species are recovered from crude oil-contaminated soil by Walker et al. (1976). In aquatic environments the most prevalent bacteria and yeast hydrocarbon-utilizing genera were *Pseudomonas*, *Achromobacter*, *Arthrobacter*, *Micrococcus*, *Nocardia*, *Vibrio*, *Acinetobacter*, *Brevibacterium*, *Corynebacterium*, *Flavobacterium*, *Candida*, *Rhodotorula* and *Sporobolomyces* (Bartha and Atlas 1977). Austin et al. (1977) tested 99 strains of petroleum-degrading bacteria and indicated that 85% of the strains belong to *Actinomycetes*, Enterobacteriaceae, coryneforms, *Klebsiella aerogenes*, *Micrococcus* spp., *Nocardia* spp., *Pseudomonas* spp. and *Sphaerotilus natans*. Crude oil biodegradation is observed with several microbes recovered from oil fields using crude oil as carbon source: *Halomonas shengliensis* sp., *Halomonas* sp. C2SS100, *Marinobacteraquaeolei*, *Streptomyces albiaxialis*, *Rhodococcus erythropolis* and *Dietzia maris* (Kuznetsov et al. 1992; Mnif et al. 2009; Huu et al. 1999; Wang et al. 2007; Zvyagintseva et al. 2001).

Bacteria related to genus *Paenibacillus*, recovered from the petroleum-contaminated sediment, could degrade PCA hydrocarbons (naphthalene and phen-

anthrene) as the only carbon source (Daane et al. 2002). Riis et al. (2003) announced that halotolerant bacteria belonging to *Cellulomonas*, *Bacillus*, *Dietzia* and *Halomonas* genera have the capacity to degrade crude oil as a sole carbon source. Ruberto et al. (2003) found that bioaugmentation of hydrocarbon-polluted Antarctic soils utilizing *Acinetobacter* sp. improved the bioremediation proficiency with 75% hydrocarbon removal. Daugulis and McCracken (2003) demonstrated the ability of *Sphingomonas* sp. to degrade polycyclic aromatic hydrocarbon. Petroleum-contaminated soil and water also recorded specific bacterial populations related to different genera, for example, *Acinetobacter*, *Achromobacter*, *Alcaligenes*, *Arthrobacter*, *Bacillus*, *Burkholderia*, *Brevibacterium*, *Corynebacterium*, *Dietzia*, *Flavobacterium*, *Methylobacterium*, *Nocardia*, *Sphingomonas*, *Pseudomonas*, *Rhodococcus* and *Vibrio* (Johnsen et al. 2005). Microbial consortium involving six types of bacteria belonging to *Bacillus*, *Pseudomonas*, *Klebsiella* and *Serratia* genera enhanced the petroleum oil biodegradation in soils with wastes of sugar cane bagasses (Trejo-Hernandez et al. 2007). *Acinetobacter* sp. could utilize n-alkanes of chain length C_{10}–C_{40} as the sole carbon source (Throne-Holst et al. 2007). *Bacillus* sp. recovered from oil product storage and distribution centre have the capability to expel crude oil, engine oil and other petroleum products (Sepahi et al. 2008). *Gordonia* sp., *Brevibacterium* sp., *Aeromicrobium* sp., *Dietzia* sp., *Burkholderia* sp. and *Mycobacterium* sp. recorded from petroleum-contaminated soil sites are estimated to be potential microorganisms for hydrocarbon biodegradation (Chaillan et al. 2004; Jain et al. 2010).

Biodegradable consortia made by three aquatic bacterial isolates recovered from a crude oil-contaminated groundwater of refinery plant were tested for their degradation abilities using different ratios, namely, *Pseudomonas aeruginosa*, *Rhodococcus* sp. M1. and *Rhodococcus* sp. Z, in the following proportions: (*P. aeruginosa*/*Rhodococcus* M1, 1:1), (*P. aeruginosa*/*Rhodococcus* ZH, 1:1), (*Rhodococcus* M1/*Rhodococcus* ZH, 1:1) and (*P. aeruginosa*/*Rhodococcus* ZH/*Rhodococcus* M1, 1:1:1); it was found that when fortifying the consortia with favoured nitrogen sources in minimal mineral medium for 7 days, these isolates biodegraded 97.6–99.9% of total petroleum oil (Malik and Ahmed 2012; Hamzah et al. 2013). Baelum et al. (2012) also demonstrated oil degradation capacity of *Gammaproteobacteria* in the oceans. The coculture between two bacteria (*Bacillus pumilus* and *Bacillus licheniformis*) and one yeast (*Candida viswanathii*) indicated capability to debase aliphatic and aromatic hydrocarbon fractions of petroleum oil with 96% degradation, accomplished after 28 days without including any supplements (Priya et al. 2015). *Bacillus licheniformis* is a potent efficient hydrocarbon-degrading bacterium recovered from crude oil-polluted soil in China and shows a great ability to degrade both short- and long-chain alkanes (Liu et al. 2016).

Fungi also have petroleum hydrocarbon degradation potential through the performance of chemical amendment and subsequently upgraded availability of pollutants. It possesses various non-specific enzymes that increase the degradation process due to the non-specificity of substrates (Pinedo-Rivilla et al. 2009). Diverse enzymes are utilized as basic pointers of mycoflora activities in polluted soil, including laccases, miscellaneous transferases, cytochrome P450 monooxygenases, nitroreduc-

tase and peroxidases (Cerniglia and Sutherland 2010). Filamentous fungi could utilize mycelia development and protraction and catabolic enzymes with less substrate specificity and low dependence on contaminants to be used as only development substrate (Harms et al. 2011). Fungi can also be utilized as a part of communities with bacteria, e.g. endo- or ectomycorrhizal associations for biodegradation of hydrocarbons (Khan et al. 2016). It plays an essential part in the aquatic ecosystems amid their capacity in removing pollutants from water specially; sediment granule polluted with petroleum oil is one of the common substrates to mycoflora which use their hydrocarbons as feeding carbon (Saraswathy and Hallberg 2002; Romero et al. 2010). The role of mycoflora in biodegradation process of different petroleum products has been widely tested, and the most familiar biodegrades fungal and yeast genera: *Aspergillus*, *Alternaria*, *Cephalosporium*, *Candida*, *Fusarium*, *Cladosporium*, *Gliocladium*, *Geotrichum*, *Paecilomyces*, *Mucor*, *Pleurotus*, *Penicillium*, *Rhizopus*, *Polyporus*, *Rhodotorula*, *Talaromyces*, *Saccharomyces* and *Torulopsis* (Saraswathy and Hallberg 2002; Gesinde et al. 2008).

Cladosporium resinae was from the first filamentous fungi with the ability to utilize petroleum products and was in charge of 20–40% of petroleum degradation. It is found in contaminant jet fuels; it can grow on various petroleum hydrocarbons and cause serious issues in aircraft industries (Cooney et al. 1968; Bailey et al. 1973; Hill and Thomas 1976; Hill 1978). Komagata et al. (1964) analysed about 500 yeasts for their hydrocarbon degradation capacity and discovered that only 56 could use hydrocarbons (*Candida*). Yeast isolates of *Candida*, *Rhodotorula*, *Saccharomyces*, *Rhodosporidium*, *Trichosporon* and *Sporobolomyces* are capable of utilizing various hydrocarbons (Ahearn et al. 1971; Cook et al. 1973). Ahearn and Meyers (1972) observed the increase of yeast communities especially in oil-polluted sediments in a 4-month period; however the population decreases in ocean in spite of oil occurrence. Cerniglia and Perry (1973) recorded that *Penicillium* and *Cunninghamella* spp. displayed as the greater hydrocarbon biodegrades than *Brevibacterium*, *Flavobacterium* and *Arthrobacter* spp. Llanos and Kjoller (1976) inspected variations in fungal soil communities after crude oil accumulation. Strains of *Acremonium*, *Fusarium*, *Gliocladium*, *Graphium*, *Mortierella*, *Penicillium*, *Paecilomyces*, *Sphaeropsidales* and *Trichoderma* are observed to be critical members of soil mycoflora fit for using petroleum oil hydrocarbons. Davies and Westlake (1979) examined 60 fungal strains for their capability to utilize crude oils of various compositions; the isolated oil-degrading fungi from soils were *Penicillium*, *Verticillium* spp., *Mortierella* sp., *Beauveria bassiana*, *Scolecobasidium obovatum*, *Phoma* spp. and *Tolypocladium inflatum*. Small yeast numbers are associated with crude oil observed in North Sea after Amoco Cadiz oil spill (Ahearn and Crow 1980). Mahmoud et al. (2015a) recovered *Absidia*, *Alternaria*, *Chrysosporium*, *Fusarium*, *Mucor*, *Stachybotrys* and *Trichoderma* (1%) crude oil Czapek's agar medium from Mazot and solar polluted soil samples aggregated from petroleum stations. Mahmoud et al. (2015a, b) isolated *Aspergillus terreus* from kerosene-polluted soil, and they found that it has a great potential for both lipase production

and crude oil degradation and also suggested that for researchers seeking for highly lipase-producing and crude oil-removing fungi, it can be advised to use hydrocarbon-polluted soils in isolation.

Yeast as showed above also has a great participation in microbial degradation of petroleum hydrocarbons, e.g. *Rhodotorula mucilaginosa*, *Candida lipolytica*, *Trichosporon mucoides* and *Geotrichum* sp., which are recovered from petroleum-polluted water and could degrade different petroleum fractions when tested (Bogusławska-Was and Da Browski 2001). *Candida catenulate* CM1 was used as amendments in petroleum-contaminated soil and composting with 2% (w/w) diesel as petroleum-degrading yeast, indicating higher disposal of petroleum hydrocarbon with degradation percentage of 84% (Baheri and Meysami 2002; Joo et al. 2008). *Amorphoteca*, *Neosartorya*, *Talaromyces*, *Graphium*, *Yarrowia*, *Candida* and *Pichia* obtained from petroleum-polluted soil sites also demonstrate to be promising microbes for petroleum hydrocarbon degradation (Chaillan et al. 2004). Obuekwe et al. (2005) isolated *Drechslera* sp., *Fusarium lateritium* and *Papulaspora* sp. as degrading crude oil fungi from a salt marsh; *Aspergillus*, *Amorphoteca*, *Cephalosporium*, *Neosartorya*, *Penicillium*, *Graphium*, *Talaromyces* and yeast genera; and *Yarrowia*, *Candida* and *Pichia* recovered from petroleum oil-polluted soil with the potential to degrade petroleum hydrocarbon (Chaillan et al. 2004; Singh 2006). Singh (2006) additionally revealed a gathering of *Cephalosporium*, *Aspergillus* and *Penicillium* which were also observed as possible crude oil hydrocarbon utilizers. Adenipekun (2008) announced that, in soils polluted with engine oil until 40%, *Pleurotus tuber-regium* increases the soil nutrient content reducing heavy metal concentration after 6 months of incubation. In a comparative report, Adenipekun and Isikhuemhen (2008) also found the capacity of *Lentinus squarrosulus*, white rot fungus, to enhance the nutrient concentrations and accumulation of Fe, Zn and Ni in appropriate concentration in the same oil engine-polluted soil. *Fusarium* sp. F092 could degrade chrysene and aliphatic fraction with degradation percentage of 35% of liquid culture contaminated with crude oil (Hidayat and Tachibana 2012).

Algae are considered as essential microbial consortium members in aquatic or terrestrial environments. However there is limited available evidence data about the algae degradation ability of petroleum pollutants in natural environment (O'Brien and Dixon 1976; Bossert and Bartha 1984). *Prototheca zopfii* chlorophyllous alga was able to use petroleum oil, various hydrocarbon substrates, n-isoalkanes and aromatic hydrocarbons considering it as a hydrocarbon-utilizing achlorophyllous alga (Walker et al. 1975b). Cerniglia et al. (1980) indicated the capability of seven *Cyanobacteria* to oxidize naphthalene, namely, *Agmenellum* sp., *Amphora* sp., *Anabaena* spp., *Aphanocapsa* sp., *Chlamydomonas* sp., *Chlorella* spp. and *Coccochloris* sp. Photoautotrophic marine algae consortia, e.g. blue green algae, green algae and diatoms, have the ability to utilize naphthalene with indications of cis-hydroxylation (pathways similar to fungi) by *Oscillatoria* and *Agmenellum* spp. (Narro et al. 1992a, b).

12.5 Factors Affecting Microbial Degradation of Petroleum Hydrocarbons

Biodegradation rates, biomass generation and destiny of petroleum hydrocarbons in natural environment are affected by various environmental parameters including oxygen accessibility, temperature, pH, water availability, nutrients and presence or concentration of pollutants (Brune and Bayer 2012; Guarino et al. 2017). One of the most standout factor impacts on the biodegradation process is the chemical composition of the petroleum-contaminated sites. Four major classes involved in petroleum hydrocarbon composition are aromatics, saturates, asphaltenes (phenols, esters, ketones, porphyrins and fatty acids) and resins (amides, carbazoles, pyridines, sulfoxides and quinolines) (Colwell and Walker 1977). When sludge become rich in microbial numbers and nutrient supplements, it increased the development of microbes and upgraded the biodegradation rates. Soils revised with oil sludge seem to have higher rate of oil-utilizing microorganisms than the rate in natural soil alone. Biodegradation rates are elevated for saturate fractions, followed by the simple aromatics, complex aromatics and various polar compounds which display extremely decreased rates of biodegradation (Walker et al. 1976; Fusey and Oudot 1984). Hydrocarbons could be also classified according to their ability to attack microbes into branched alkanes, n-alkanes, cyclic alkanes and low-molecular-weight aromatics. Additionally, the key differences between petroleum derivative degradation in soil and aquatic environment are concerning to movement and distribution of the spilled oil. Oil spill in terrestrial environments is distinguished by vertical movement of oil into the soil, instead of horizontal spreading linked with smooth oily formation. Penetration of oil into the soil particles blocks the evaporative capacity of different volatile hydrocarbons; consequently it could be very poisonous to microorganisms. Specific materials could reduce this toxicity rates by absorption; however this technique most likely forms different residue deposits in the soil causing another toxic effect on biological life (Bossert and Bartha 1984; Perry 1984).

Temperature plays a critical role in hydrocarbon biodegradation as it affects the chemical nature of the contaminants, physiology and microbial flora community variation. Hydrocarbon degradation passes in enormous temperatures ranges, but the biodegradation levels decrease with temperature lowering (Das and Chandran 2011). Low temperatures affect oil spill features, increased oil viscosity and physiological properties of microorganisms and decrease low MW toxic hydrocarbon volatility which cause retarding in biodegradation process and (Atlas 1985; Foght et al. 1996; Venosa and Zhu 2003). Highest biodegradation levels in soil are exhibited in the extent of 30–40 °C, in freshwater 20–30 °C and marine ecosystem 15–20 °C (Cooney 1984; Bartha and Bossert 1984; Das and Chandran 2011).

In aerobic respiration, the first part in aliphatic and aromatic hydrocarbon catabolism by microorganisms includes the oxidation of the materials by oxygenase enzymes with essential oxygen requirement. High-impact oxygen conditions are consequently fundamental for this microbial oxidation of various hydrocarbons in the natural ecosystem. States of oxygen restriction regularly do not exist in the

upper levels of water strands in both marine and freshwater ecosystems (Singer and Finnerty 1984; Perry 1984; Cerniglia 1984; Floodgate 1984; Cooney 1984; Leahy and Colwell 1990). Aquatic deposit sediments are anoxic with the exception of a thin layer at the above sediment surface. The accessibility of oxygen in soils is subject to rates of microbial oxygen utilization; the kind of soil, regardless of whether the soil is waterlogged; and the nearness of utilizable substrates which can prompt oxygen exhaustion. The centralization of oxygen has been recognized as the rate-restricting variable in the biodegradation of crude oil in soil and groundwater (Jamison et al. 1975; Hambrick et al. 1980; Bossert and Bartha 1984; Cooney 1984; von Wedel et al. 1988; Leahy and Colwell 1990).

Nutrients could be a limiting factor especially in fresh and marine environment biodegradation, e.g. phosphorus, nitrogen and iron (Bartha and Bossert 1984). When oil spill happened in marine or freshwater ecosystems, supplementary with carbon was observably raised, and the accessibility of nitrogen and phosphorus turns into a key parameter for oil biodegradation (Floodgate 1984; Atlas 1985). Also composting could increase both organic matter and the fertility of the soil besides bioremediation, and it could be considered as cost-effective methods in soil remediation (Chen et al. 2015). Utilizing of poultry manure in contaminated soil as natural fertilizer makes biodegradation process enhanced. On the other hand, excessive using of nutrients could inhibit the biodegradation activity (Okolo et al. 2005; Chaillan et al. 2006). Nitrogen and phosphorus availability affects the biological degradation of petroleum hydrocarbons in different types of habitats, for example, sediment, seawater, freshwater lakes, Arctic ponds and groundwater. Besides, it was observed that the decrease in nitrate, sulphate and phosphate affects the biodegradation process. Nitrogen and phosphorus addition in fertilizer forms, e.g. octyl phosphate, paraffinized urea and ferric octoate, increases the biodegradation of crude oil in seawater, ponds and lakes (Olivieri et al. 1976; Horowitz and Atlas 1980; Boehm and Fiest 1980; Leahy and Colwell 1990; Haritash and Kaushik 2009). Pressure effect on hydrocarbon biodegradation is considered as slightly limited factor as it related mostly to the deep-sea environment. Limited studies occurred in this point; Colwell and Walker (1977) revealed that petroleum products which could reach the deep-sea natural environment will degrade by the action of microbial populations in slow rates and this may lead to some hazard fractions of the crude oil and may remain in the deep ocean for years or decades.

PH effect on petroleum hydrocarbon biodegradation could be important for terrestrial ecosystems as soil pH is highly variable, extinct from 2.5 to 11, and most microbial communities favour neutral pH. Besides, extreme pH has an adverse effect on the degradation ability of microbial populations of hydrocarbons (Bossert and Bartha 1984; Atlas 1988). Hambrick et al. (1980) described the mineralization rates of microbes for octadecane which raised with pH increasing from 6.5 to 8.0. Verstraete et al. (1976) obtained double rates of gasoline biodegradation when setting the pH to 7.4. Similarly, Dibble and Bartha (1979) revealed that optimal pH for soil-sludge mineralization was of 7.8. Water availability could be a limited factor in terrestrial ecosystems for microbial growth and degradation. Dibble and Bartha (1979) indicated that the ideal biodegradation levels could range from 30% to 90%

water saturation when they studied the degradation of soil contaminated with petroleum oil sludge. Atlas (1981) also proposed that deposition of tar balls on beaches is considered as another issue for water availability which restricted the whole biodegradation process.

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Chapter 13
Microbial Bioremediation of Petroleum Hydrocarbon: An Overview

Debajit Borah

Abstract Increased environmental toxicity due to extensive use of petroleum-based products gradually proves itself as a major issue of global concern. The release of petroleum products to the environment may cause catastrophic effect on aquatic habitats as well as barrens of fertile soil. Petroleum oil basically contains VOCs (volatile organic compounds), paraffin, gases (methane, ethane, propane, butane, etc.), metal ions (iron, nickel, copper, vanadium, etc.), etc., out of which VOCs may cause severe health problems such as lung, liver and kidney disease. Bioremediation is a process of treatment of contaminated environment with the help of living organisms to bring back to its natural state. Treatment of hydrocarbon-contaminated sites may be accomplished with the help of indigenous microorganisms with diverse groups present in the soil by augmenting with necessary nutrients or by adding external necessary microorganisms. Further, as the petroleum hydrocarbon pollutant creates a stressful environment for growth, the bacterial species having potential to tolerate stress conditions would be an added advantage.

13.1 Introduction

Fossil fuel merchandise increases the probabilities of soil contamination that becomes one of the most important worldwide environmental issues. Statistical report released by the International Tanker Owners Pollution Federation (ITOPF) Ltd. shows incidences of oil spillage in sea since the years 1970–2015 which shows more than 700 tonnes of oil spillage which occurred in year 2015 itself (ITOPF 2016).

NAS report shows over ninetieth of oil spillage incidence is directly or indirectly as a result of human activities together with deliberate oil waste disposal (USEPA 2000; NAS 1985, 2005). Large-scale oil spills, and oil spill accidents, have received a great deal of attention worldwide, as a result of their destructive result on the

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surroundings. Oil spillage on water body ends up in the transience of thousands of aquatic animals and causes major reduction in population of the many aquatic organisms and lots of long-standing environmental impacts (Spies et al. 1996; Campbell and Cary 2001; Van Hamme et al. 2003). Soil contamination primarily arises as a result of petroleum dumping or due to the rupture of underground storage tanks, etc. (Briganti et al. 1997; Butler and Mason 1997; Chang 1998). Minor oil spills and oil contamination as a result of non-point sources like urban runoff, etc. are not any less a threat to public health though they need but received nominal attention in the past. Such non-point sources of pollution remain the largest threat to water body as stated by the reports published by the *National Water Quality Inventory, USA* (Etkin 1998; USEPA 1999, 2000). It may conjointly cause severe health risks to the employees involved in treating of oil spillage areas once exposed to hydrocarbon fumes, volatile organic compounds (VOCs), polycyclic aromatic hydrocarbons (PAHs), material from controlled burns, etc. (Bach et al. 2005; Biddle et al. 2006; Campbell and Cary 2001; Chang et al. 2002, 2005; Christopher and Christopher 2004).

13.2 Composition of Petroleum Oil

Petroleum is recovered principally through oil drilling, and it's refined and fractioned into a range of commercial products.

In true sense, fossil oil includes inflammable liquid consisting of a fancy mixture of hydrocarbons of varied molecular weights and alternative liquid organic compounds; however, in common usage it includes all liquid (e.g. pentane and heavier ones), gaseous (e.g. methane, ethane, propane, butane, etc.) and solid (e.g. paraffin) hydrocarbons (Speight 1999). The hydrocarbons in fossil oil are largely alkanes, cycloalkanes and numerous aromatic hydrocarbons, whereas the other organic compounds contain varied metals like iron, nickel, copper, etc. (Speight 1999). Precisely, the molecular composition of hydrocarbons may vary from formation to formation. Chemically, fossil oil contains paraffin (15–60%), hydrocarbon (30–60%), aromatics (3–30%) and mineral (6%); however, the relative proportion of fossil oil largely varies from oil to oil (Mabro 2006). Although the constituents of fossil oil may vary, the elementary compositions may be presented as shown in Table 13.1 (Hyne 2001).

Table 13.1 Elementary compositions of fossil oil

Name of the constituents	Percentage (%)
Carbon	83–87
Hydrogen	10–14
Oxygen	0.05–1.5
Nitrogen	0.1–2
Sulphur	0.05–6
Metals	<0.1

13.3 Petroleum Oil Pollution, Environment and Health

The illegal disposal of crude oil is also one of the major causes of environmental hazard with international ramifications (Blodgett 2001; Guermouche et al. 2015). The discharge of oil into the surrounding causes environmental concern and attracts the general public attention (Roling et al. 2002). Petroleum oil and PAHs (polyaromatic hydrocarbons) have a widespread impact on the body, as prolonged exposure to crude oil might induce liver and excretory organ diseases and bone marrow injury or might cause the event of cancer (Crebelli et al. 1995; Mandri and Lin 2007; Guermouche et al. 2015). Processed engine oil contains additional heavy metals and serious PAHs and therefore contributes a lot more too chronic hazards as well as mutagenicity and carcinogenicity as compared to unused engine oil (Boonchan et al. 2000).

Unlike the claims created by the oil-exploring industries concerning the security measures taken for safe unleash of treated effluents to the surroundings, the digital and print media besides restricted scientific study claims environmental problems associated with oil contamination within the region notably within the abandoned drilling sites (Das et al. 2004; Yenn et al. 2014).

Oil contamination of soil and water isn't solely a regional issue but a worldwide issue of concern. Not only soil, the aquatic system, notably the marines, is the foremost at risk of oil spillage (Cairns and Buikema 1984). Marine oil spillage may have an effect on organisms present therein by direct toxicity or by physical stress (Perry 1980). Oil spills usually will cause varied damages to the marsh vegetation. Oil spill in water body forms a surface slick whose elements will follow several pathways. Some might pass into the mass of water, and proof suggests they will persist for an extended time before their degradation by microorganisms within the water body. The slick sometimes becomes additional viscous and forms water-in-oil emulsion. Oil in water causes depletion of dissolved gases because of transformation of the organic element into inorganic compounds, loss of biodiversity and eutrophication. Toxicity in fishes includes blood disease, dermal dysplasia and pasteurellosis (Beeby 1993). It also affects plant physiology in terms of stem height, photosynthetic rate, overall plant biomass, germination, etc. leading to their death (Krebs and Tanner 1981; Onwurah 1999). Oil spill may increase the mortality rate of a population by damaging the reproductive capacity of the respective population (Hall et al. 2006; Tiido et al. 2006).

It conjointly affects soil fertility by altering the mineral and organic matter content, ion exchange capability, salinity, pH, etc. However, the size of impact depends on the number and kind of oil spilled (Onwurah et al. 2007). As petroleum oil creates anaerobic condition in the soil, coupled to water logging and acidic metabolites, the result is high accumulation of aluminium and manganese ions, which are toxic to plant growth (Onwurah et al. 2007). Petroleum oil hydrocarbons may cause DNA damage, resulting in carcinogenesis, mutagenesis and impairment of reproductive capacity (Short and Heintz 1997). The risk of drinking water contaminated by crude oil can be extrapolated from its effect on rats that developed haemorrhagic tendencies after exposure to water-soluble components of crude oil (Onwurah 2002). Volatile components of crude oil after a spill may lead to asthma, bronchitis and other liver and kidney diseases (Kaladumo 1996; Anozie and Onwurah 2001).

13.4 Countermeasures for Oil Pollution

Conventionally physical, chemical and biological ways are used for the treatment of oil spillage.

13.4.1 Physical Methods

Removal of oil spill in soil and water body may involve mechanical removal methods such as skimming, manual removal (wiping), water flushing, etc. According to the recent USEPA 2015 report, current mechanical methods other than open burning of petroleum oil typically recover up to only 10–15% of oil after a massive oil spill, whereas burning of oil may reduce up to 98% but with a massive air pollution (OTA 1990; NAS 2005; USEPA 2015). Although standard ways, like physical removal of spilled oil, usually are the primary response choice, they rarely succeed in complete clean-up of oil spills.

13.4.2 Chemical Methods

Chemical ways, notably dispersants (synthetic surfactants), are more often utilized in several countries as a response choice (USEPA 1999). However, chemical ways haven't been extensively used due to the disagreement concerning their effectiveness (recover no over 8% of usable oil) and also the issues of their toxicity and long-run environmental effects because of their toxic formulation (USEPA 1999; https://www.restorethegulf.gov/; Hemmer et al. 2011). Synthetic surfactants possess both hydrophilic and hydrophobic groups which form oil-surfactant micelles by decreasing tension between water and oil interface which may lead to further degradation of droplets of the micelles in the water body towards physical and microbial degradation (Hemmer et al. 2011).

13.4.3 Biological Methods

Biological strategies have emerged as promising technology nowadays, notably as a secondary treatment choice for the clean-up of oil spillage. Bioremediation has been outlined as a treatment method of contaminated environment with the assistance of living organisms to bring back to its original state (OTA 1991; Ivanova et al. 2015). This technology is based on the fact that a considerable amount of oil components are mostly biodegradable in nature (Atlas 1981; Atlas 1984; Prince

1993) and hence can be augmented by adding nutrients. Bioremediation has many potential benefits over standard technologies such as it is very cost-effective and eco-friendly in terms of its finished product.

The success rate of bioremediation process is dependent on the capacity to establish favourable conditions in the contaminated site which enhances the rate of hydrocarbon degradation. The vital demand for the success of bioremediation is the presence of indigenous microorganisms with the suitable metabolic activities for degradation of specific type of fossil oil.

There are diverge group of microorganisms known to be capable of degrading variety of target constituents present in oily sludge (Eriksson et al. 2002; Barathi and Vasudevan 2001; Mishra et al. 2001). Out of so many reported microorganisms, various strains of *Pseudomonas* sp. are mostly reported by many researchers as a potential fossil oil degrader (Fall et al. 1979; Johnson et al. 1996; Guermouche et al. 2015). Some of the field trial reports claim successful bioremediation of oil-contaminated nearby areas of abandoned drill sites located particularly in Gelakey, Amguri, Lakwa and Borholla regions of Assam, India, by augmenting contaminated sites with *Pseudomonas* strains designated as N3 and N4 along with plant species *Gmelina arborea, Tectona grandis*, *Michelia champaca* and *Azadirachta indica* (Yenn et al. 2014).

Apart from *Pseudomonas* sp., there are some other bacteria which include *Yokenella* sp., *Alcaligenes* spp., *Roseomonas* sp., *Stenotrophomonas* sp., *Acinetobacter* spp., *Flavobacterium* sp., *Streptococcus* sp., *Providencia* sp., *Sphingobacterium* sp., *Capnocytophaga* sp., *Moraxella* sp. and *Bacillus* sp.; some fungi, viz. *Alternaria* sp., *Aspergillus* sp., *Cladosporium* sp., *Eupenicillium* sp. and *Paecilomyces* sp.; etc. are known as potential hydrocarbon degraders (Rusansky et al. 1987; Sharidah et al. 2000; Bhattacharya et al. 2002; Juwarkar 2012; Bujang et al. 2013; Ameen et al. 2015). The component of media plays a vital role in microbial growth and proliferation. Hence, optimization and improvement of media components and growth parameters are crucial factors for obtaining enhanced rate of hydrocarbon degradation and are advocated by many researchers (Xia et al. 2006; Vieira et al. 2009; Dongfeng et al. 2011; Janani et al. 2014).

13.5 Microorganisms in Petroleum Degradation and Their Sources

Hydrocarbon bioremediation of contaminated sites by indigenous microbial population allows the conversion of toxic substances into less or nontoxic forms which might represent one of the primary mechanisms by which hydrocarbon products are removed from the contaminated environment inexpensively (Atlas 1981). The capability of microorganisms to emulsify hydrocarbon by producing surface-active agents is one of the most important characteristics of hydrocarbon-degrading

bacteria. Such surface-active agents cause dispersion of hydrocarbons in water emulsions leading to the formation of micro-droplets (micelles) which may be ingested by the microbial cells for further degradation with the help of certain enzymes. One such important enzyme involved in hydrocarbon degradation is oxygenase which converts complex chain of hydrocarbons into smaller and simpler forms which finally enters into peripheral metabolic cycles as shown in Figs. 13.1 and 13.2 (Hommel 1990; Cerniglia 1992; Yakimov et al. 1995; Adebusoye et al. 2008; Ibrahim et al. 2013). A number of microorganisms, viz. *Bacillus* sp., *Corynebacterium* sp., *Edwardsiella* sp., *Staphylococcus* sp., *Pseudomonas* sp., *Citrobacter* sp., *Micrococcus* sp., *Cladosporium* sp., *Acetobacterium* sp., *Mucor* sp., *Penicillus* sp., *Monosporium* sp., *Aspergillus* sp., *Alternaria* sp., *Mucor* sp., etc., are reported to be hydrocarbon degraders by various researchers (Fall et al. 1979; Okpokwasili and Odokuma 1986; Okpokwasili and Okorie 1988; Rusansky et al. 1987; Johnson et al. 1996; Campbell and Cary 2001; Bhattacharya et al. 2002;

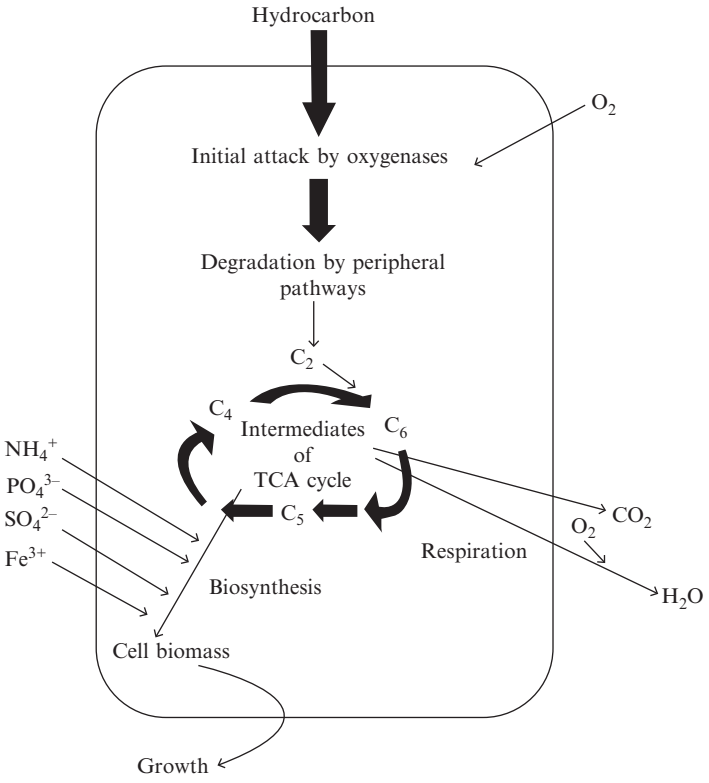

Fig. 13.1 Basic principle behind aerobic biodegradation of hydrocarbons by microorganisms. (Adopted from Das and Chandran 2011)

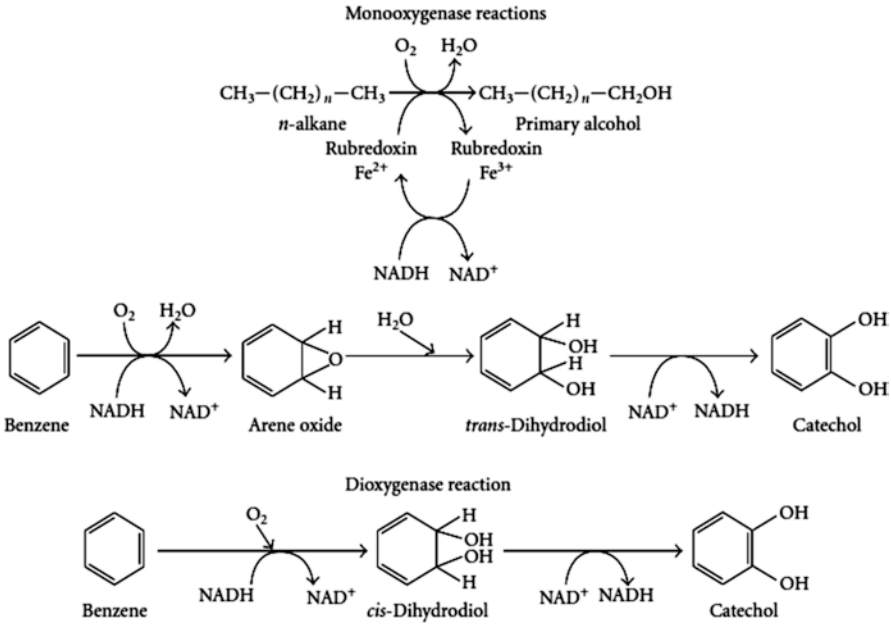

Fig. 13.2 Hydrocarbon degradation process by enzymatic action. (Adopted from Das and Chandran 2011)

Chang et al. 2002; Christopher and Christopher 2004; Chang et al. 2005; Bach et al. 2005; Biddle et al. 2006; Mandri and Lin 2007; Su et al. 2011; Luo et al. 2012; Yenn et al. 2014; Ameen et al. 2015; Jia et al. 2016).

Microorganisms, which are capable of hydrocarbon degrading, are believed to be capable of tolerating stress conditions to some extent, as hydrocarbon-contaminated sites itself provide hostile situations for the growth and proliferation of such microorganisms. Besides, very limited numbers of scientific reports dealing with microbial hydrocarbon degradation under stressed conditions are available (Sorensen et al. 2010; Tapilatu et al. 2010; Martino et al. 2012; Abed et al. 2014; Li et al. 2016). One such literature shows the existence of hydrocarbon degradation *Pseudomonas* sp. and *Variovorax* sp. at a temperature as low as 0 °C in fuel-contaminated soils of Greenland high Arctic region (Sorensen et al. 2010). Selection of such bacteria with stress-tolerant capability to degrade mono- or polyaromatic compounds by synthesizing biosurfactants and biopolymers that may play a certain role in enhanced stress tolerance could be a good approach to find a suitable bioaugmentation agent (Martino et al. 2012; Li et al. 2016). A detailed list of hydrocarbon-degrading microorganisms and their source of isolation is given in Table 13.2.

Table 13.2 Name of the microorganisms and their source of isolation along with the reported values of percentage hydrocarbon degradation are shown

Name of the microorganisms	Source	Hydrocarbon degradation (%)	References
Alcaligenes faecalis, *Candida tropicalis* (*the first report on the isolation of hydrocarbon-degrading microorganisms from Amazonian soil)	Artificially augmented Amazonian rainforest soil samples	–	Bastos et al. (2000)
Bacillus subtilis	Contaminated soil of Kuwait	–	Sharidah et al. (2000)
Alcanivorax borkumensis	Marine water	–	Golyshin et al. (2003)
Gordonia sp., *Aeromicrobium* sp., *Brevibacterium* sp., *Dietzia* sp., *Paecilomyces* sp., *Burkholderia* sp., *Yarrowia* sp., *Aspergillus* sp., *Fusarium* sp., *Penicillium* sp., *Neosartorya* sp., *Talaromyces* sp., *Graphium* sp., *Pichia* sp. and *Amorphoteca* sp.,	Contaminated soils of Indonesia	4.9–22%	Chaillan et al. (2004)
Microcosm of six microbes (names not mentioned)	Contaminated oil refinery plant in China	63.2 ± 20.1%	Ma et al. (2015)
Alkanindiges sp., *Arthrobacter* sp., *Pseudomonas* sp., *Mycobacterium* sp. and *Rhodococcus* sp.	Contaminated soils of Northern China	–	Sun et al. (2014)
Alternaria alternata, *Cladosporium* sp., *Aspergillus terreus*, *Eupenicillium hirayamae*, *Sphaerospermum* sp., *Paecilomyces variotii*	Contaminated mangrove sediments from Red Sea coast of Saudi Arabia	28–56%	Ameen et al. (2015)
Stenotrophomonas sp., *Pseudomonas* sp.	Dredged sediments of a river estuary in Italy	43–95%	Gregorio et al. (2016)
Polaromonas sp., *Sphingomonas* sp., *Alcaligenes* sp., *Caulobacter* sp. and *Variovorax* sp.	Uncontaminated Arctic soil	–	Eriksson et al. (2002)
Nocardia otitidiscaviarum	Contaminated desert soil of Iran	–	Zeinali et al. (2007)
Mycobacterium sp.	Soil	–	Miller et al. (2004)
Mycobacterium sp.	Uncontaminated Natural Park Soil of Schwa of Germany	–	Kim et al. (2005)
Acinetobacter sp., *Aeromonas* sp., *Alcaligenes* sp., *Bacillus* sp., *Kocuria* sp. (*Micrococcus*), *Ochrobactrum* sp., *Pseudomonas* sp. and *Xanthomonas* sp.	Contaminated Patagonian soil	0.028–100%	Peressutti et al. (2003)

(continued)

Table 13.2 (continued)

Name of the microorganisms	Source	Hydrocarbon degradation (%)	References
Rhodococcus sp. and *Pseudomonas* sp.	Contaminated soils of Kaluga, Kirov, Moscow	0–95%	Baryshnikova et al. (2001)
Not defined	Uncontaminated soils of Western Siberia (Arctic region)	3.8–51.2%	Belousova et al. (2002)
Alcanivorax borkumensis	North Sea, Atlantic Ocean, Mediterranean Sea, Sea of Japan, South China Sea and the Antarctic	80–90%	Golyshin et al. (2003)
Nocardia otitidiscaviarum	Soil contaminated with wastewater of a petroindustrial site in Iran	10–55%	Zeinali et al. (2007)
Afipia sp., *Janthinobacterium* sp., *Leptothrix* sp., *Massilia* sp., *Methylobacterium* sp., *Rhizobium* sp., *Sinorhizobium* sp. and *Thiobacillus* sp.	Uncontaminated soil of Arizona	88%	Bodour et al. (2003)
Mucor mucedo	Uncontaminated soils of Shenfu irrigation area, China	87%	Jia et al. (2016)
Haloarcula sp., *Haloferax* sp. (*the first report on the potential role of halophilic archaea belonging to the genera *Haloarcula* and *Haloferax*)	Uncontaminated pond water of Camargue, France	32–95%	Tapilatu et al. (2010)
Marinobacter sp., *Pseudomonas* sp., *Halomonas* sp., *Hahella* sp. and *Alcanivorax* sp.	Contaminated sediments from coastal region in Oman	67%	Abed et al. (2014)
Pseudomonas sp. and *Variovorax* sp.	Contaminated soils from Station Nord (St. Nord) in Greenland high Arctic region	70%	Sorensen et al. (2010)

13.6 Microbial Degradation of Petroleum Hydrocarbon

13.6.1 Enzymatic Pathways

Short-chain hydrocarbons are considered as the most biodegradable petroleum hydrocarbons. Hydrocarbons with C_5–C_{10} carbon numbers are homologues of solvents that tend to disrupt lipid membranes of hydrocarbon-degrading microorganisms, and hence, they are reported to have inhibitory action. Waxes, with alkane

of C_{20}–C_{40}, are solids with hydrophobic nature at room temperature and hence are less biodegradable in nature (Atlas and Bartha 1973). Literature suggests that the majority of organic pollutants completely or optimally degrade under aerobic condition. Enzymes such as oxygenases and peroxidases play a vital role in hydrocarbon degradation, and these are responsible for initial intracellular oxidation of organic pollutants. The organic pollutants are then converted into intermediates of central intermediary metabolism such as the Krebs cycle in a stepwise manner. Cell biomass are then synthesized by central precursor metabolites such as acetyl CoA, succinate, pyruvate, etc. Sugars are the essential source of carbon, which is required for different types of biosynthesis and growth of the microbes, and are synthesized by gluconeogenesis (Fritsche and Hofrichter 2000). Figures 13.1 and 13.2 show the initial action on hydrocarbon by oxygenases (Das and Chandran 2011).

13.6.2 Role of Biosurfactant in Petroleum Degradation

As discussed earlier, the ability to produce biosurfactants by microbes is the most important feature of a potential hydrocarbon degrader. Biosurfactants are a complex mixture of biomolecules such as proteins, exopolysaccharides, fatty acids, amino acids, glycolipids, etc. with hydrophobic and hydrophilic components that emulsify hydrocarbon by minimizing the interstitial surface tension which makes them suitable for bioremediation and microbial enhanced oil recovery process (Ibrahim et al. 2013). Thus, surfactant subsequently increases the aqueous solubility of oily contaminants and makes them available for microorganisms for further degradation (Karanth et al. 1999). On the other hand, synthetic surfactants such as SDS (sodium dodecyl sulphate), LAS (linear alkylbenzene sulphonate), Brij 30, Tween 80, etc. used for the treatment of oil contaminants are often toxic, which also act as an additional source of contamination (Bognolo 1998). Microbial surfactants have analogous properties to that of synthetic surfactants but are biodegradable in nature and can be produced at the contaminated site (in situ) (Cha 2000). Isolation of microorganisms with desired capabilities to emulsify and solubilize hydrophobic contaminants both ex situ and in situ is a major benefit over competitors in contaminated areas (Reddy and Singh 1982; Vecchioli et al. 1990; Chattre et al. 1996; Cassidy and Hudak 2001). Nowadays, these are not only important for bioremediation purpose but also area of research for microbial enhance oil recovery from wells. Such process involves the implementation of microbes directly or microbial surfactants in the well which helps in reducing the viscosity of oil to increase the free flow through the pipelines and also stabilizes fuel water–oil emulsions (Ghurye and Vipulanandan 1994; Makkar and Cameotra 1997; Bognolo 1998).

Contact angle of a fluid with a solid surface is an index used to examine the action of biosurfactant on the rock surface wettability (i.e. the interaction between fluid and solid surface) (Ibrahim et al. 2013). It is defined as the angle between the tangent to the periphery of the point of fluid contact with the solid and the surface of the solid in the direction where the droplet exists as shown in Fig. 13.3. Contact

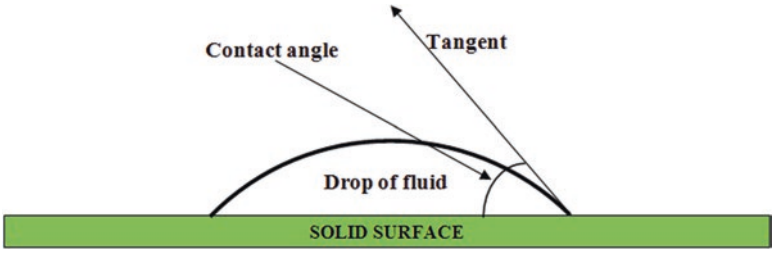

Fig. 13.3 The contact angle formed by a drop of fluid on a solid surface

angle less than 90° for surfactant-containing liquid is considered to be wet on solid surface, and a contact angle greater than 90° is said to be non-wetting (i.e. the liquid which does not wet the solid surface). The alteration in wettability has been projected as one of the mechanisms of MEOR (Alvarez et al. 2015).

Some of the field trials carried out by researchers in Poland, Holland, the Czech Republic, the United States, Romania and Hungary reported significant enhancement in oil recovery by MEOR process (Karanth et al. 1999). Apart from the cost-effective nature of biosurfactants, they are also widely used in food, pharmaceutical and cosmetic industry which makes it an important microbial product of commercial importance (Batista et al. 2006).

A review work carried out by Satpute et al. (2010) represents marine biosphere as a wealthy natural source of flora and fauna with functional commercial-grade bioactive compounds, biosurfactants/bioemulsifiers, etc. which may be used for the purpose of bioremediation. Marine microorganisms such as *Bacillus*, *Acinetobacter*, *Halomonas*, *Myroides*, *Pseudomonas*, *Corynebacterium*, *Arthrobacter* and *Alteromonas* sp. were reported for their potential application for the production of biosurfactants and exopolysaccharides. (Filonov et al. 2004; Coral and Karagoz 2005; Das and Mukherjee 2007; Simpson et al. 2011).

Various species of the genus *Pseudomonas* are probably the most widely studied hydrocarbon-degrading bacteria. Existing literature shows the role of various species of *Pseudomonas* isolated from contaminated sites in hydrocarbon degradation (Baryshnikova et al. 2001; Filonov et al. 2004; Shin et al. 2006; Niepceron et al. 2010; Aresta et al. 2010; Nie et al. 2010; Singh and Malik 2013; Li et al. 2016). Their source of isolation even ranges from hot springs (Perfumo et al. 2006), marine water (Satpute et al. 2010) and cow dung (Singh and Fulekar 2010) up to the Arctic region (Sorensen et al. 2010).

Some of the researchers studied the capability of various *Pseudomonas* strains in BTEX (benzene, toluene, ethylene and xylene), naphthalene and TPH degradation, surface tension reduction of petroleum oil, formation of biofilm on hydrocarbon-supplemented medium, formation of rhamnolipid, glycolipid-based surfactants, etc. (Baryshnikova et al. 2001; Perfumo et al. 2006; Kim and Jaffé 2008; Zhang et al. 2011; Cébron et al. 2011; John and Okpokwasili 2012; Martino et al. 2012; Li et al. 2013; Oyetibo et al. 2013; Pedetta et al. 2013; Dudášová et al. 2014;

Pacwa-Płociniczak et al. 2014; Ma et al. 2015). This report advocates their capability to achieve 20–100% of hydrocarbon degradation within 7–30 days of incubation. But physicochemical parameters such as pH, temperature, salinity, type of culture medium used, carbon source, etc. may influence the yield of biosurfactant production in industrial scale (Lang 2002; Batista et al. 2006).

13.7 Genes Involved in Petroleum Degradation

Molecular techniques not only play a vital role for the identification of petroleum degraders but are also largely used for the characterization and identification of different genes involved in hydrocarbon degradation. Genes responsible for hydrocarbon degradation may be present in either genomic or plasmid DNA or may be in both at the same time in a certain microorganism. Plasmid curing is a convenient tool for the determination of the involvement of plasmid-encoded genes in hydrocarbon degradation (Karpagam and Lalithakumari 1999; Liu et al. 2004; Vasudevan et al. 2007; Kumar and Gopal 2015; John and Okpokwasili 2012). Reports also show presence of certain genes in the genomic DNA of some microbes that are involved in hydrocarbon degradation (Kim et al. 2007; Quatrini et al. 2008; Weelink et al. 2009; Li et al. 2011; Sun et al. 2014).

In contrary to the above, there are also reports which show contribution of both genomic and plasmid DNA-encoded genes in the same organism in hydrocarbon degradation (Fondi et al. 2013). A brief list of important genes responsible for microbial hydrocarbon degradation is shown in Table 13.3.

13.8 Conclusions

Even though widespread research has been conducted on petroleum bioremediation during the last decade, the usefulness of these tools has only rarely been convincingly established, and on the commercially available bioremediation products, the literatures are nearly lacking with supportive evidence of success. Existing literatures chiefly demonstrated the assessment of factors affecting bioremediation under laboratory condition. Out of these, very few numbers of literature reports their implementation in field trials at pilot-scale convincingly demonstrated this technology. Only 27 commercially available bioremediation agents such as Inipol EAP22, BIOREN 1 and 2, Oil Spill Eater II® (OSE II), ENVIROZYME BR, BioCATalystIOS-500, Petro-Clean, IOS-500, Micro-Blaze, WMI-2000, etc. are listed on the NCP schedule of USEPA till March 2015 (USEPA 2015). But the scientific community is still trying to screen out more effective microorganisms of the same type or sometimes a consortium of different types of microbes for bioremediation of a hydrocarbon-contaminated site considering their advantages over commercially available synthetic bioremediation agents. In conclusion, as the petroleum

Table 13.3 A brief list of hydrocarbon-degrading genes and their source organisms

Name of the microbes	Genes involved	Encodes for	References
Rhodococcus sp., *Gordonia* sp.	alkB	Non-haem iron-containing alkane monooxygenases and hydroxylases	Kim et al. (2007), Quatrini et al. (2008)
Acinetobacter sp., *Staphylococcus haemolyticus*	C23O	Cytochrome C230	Onur et al. (2015)
Betaproteobacteria sp., *Sterolibacterium denitrificans*	bssA	α-Subunit of Bss enzyme	Weelink et al. (2009)
Proteobacteria sp., *Firmicutes* sp., *Acidobacteria* sp., *Actinobacteria* sp., *Deferribacteres* sp., *Bacteroidetes* sp., *Thauera chlorobenzoica*	bamA	6-oxocyclohex-1-ene-1-carbonyl-CoA hydrolase	Sun et al. (2014), Li et al. (2011)
Gammaproteobacteria sp.		Catechol	Sei and Fathepure (2009)
Acinetobacter sp., *Alcanivorax* sp.	almA	Flavin-binding monooxygenase	Wang and Shao (2012)
	arfA	Arthofactin	Roongsawang et al. (2003), Das et al. 2008
B. tequilensis, E. coli, B. subtilis, *Aeromonadaceae* sp., *Bacillaceae* sp., *Enterobacteriaceae* sp., *Gordoniaceae* sp., *Pseudomonadaceae* sp.	*sfp*	4′-phosphopantetheinyl transferase protein	Porob et al. (2013), Anburajan et al. (2015), Ndlovu et al. (2016)
	*sfp*O	Phosphopantetheinyl transferase	Anburajan et al. (2015)
Aeromonadaceae sp., *Bacillaceae* sp., *Enterobacteriaceae* sp., *Gordoniaceae* sp. and *Pseudomonadaceae* sp.	*rhl*B	Rhamnosyltransferase subunit B	Ndlovu et al. (2016)
Aeromonadaceae sp., *Bacillaceae* sp., *Enterobacteriaceae* sp., *Gordoniaceae* sp. and *Pseudomonadaceae* sp.	*bam*C	Bacillomycin C	Ndlovu et al. (2016)
Pseudomonas sp.	*rhl*	Rhamnolipid	Pacwa-Płociniczak et al. (2014)
A. xylosoxidans	bphA		Pacwa-Płociniczak et al. (2014)
Pseudomonas putida	ndoB	Iron–sulphur protein	Hamann et al. (1999)
Rhodococcus sp.	thmA		Kim et al. (2007)
Rhodococcus sp.	PrmA		Kim et al. (2007)

hydrocarbon pollutant creates a stressed environment for the growth and proliferation of indigenous microbial species, hence research on finding out novel hydrocarbon-degrading microorganisms with stress-tolerant potential would be an added advantage.

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Chapter 14
Microbial Degradation of Hydrocarbons in the Ecosystem

Anupreet Kaur

Abstract Hydrocarbon contamination from neurotoxic and carcinogenic pollutants is a major environmental problem. One of the best and most cost-effective technologies to clean up these pollutants is bioremediation. Bioremediation is a treatment that breaks down these hazardous chemicals into less toxic or nontoxic substances. In bioremediation, various types of microorganisms are used to decompose petroleum compounds into carbon dioxide, water, and inorganic compounds and complex organic contaminants into simpler organic compounds. This chapter presents an updated overview of petroleum hydrocarbon degradation by microorganisms in different ecosystems.

14.1 Introduction

Petroleum is a naturally occurring hydrocarbon material that consists of crude oil, condensate, and natural gas. In all, 50–80% of hydrocarbons are crude oil. Petroleum and its products can be leaked during exploration, production, refining, transportation, and storage. The seepage of crude oil has been estimated at 600,000 metric tons per year (Alexander 1995, 2000; Ivancev et al. 2004). Massive oil spills are an obvious source of damage to ecosystems and human health, as they clog pores, inhibit respiratory functions, and poison animals who ingest them (Jorgensen et al. 2000; Maletic 2010). The environmental release of crude oil causes a number of physical, chemical, and biological changes in human beings, animals, and plants (Balba et al. 1998; Brassington et al. 2007; Atlas 1984).

One of the best and most cost-effective technologies to clean up these pollutants is bioremediation. Bioremediation is a "treatment that uses naturally occurring organisms to break down hazardous substances into less toxic or nontoxic sub-

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stances (Vidali 2001)." In this technique, various microorganisms are used to generate enzymes that attack and degrade environmental contaminants into less toxic forms. This process often involves manipulating environmental parameters so that the microbial growth is encouraged and the degradation is accelerated. Bioremediation has several advantages over conventional pollution technologies (Roncevic 2007). Common advantages are as follows: it is cost effective and easy to carry out; use of biological inputs such as microbes makes this technique eco-friendly; and moreover, there are no side effects.

The microdegradation of hydrocarbon depends upon the composition of the microbial community and its adaptation to the environment. Marine water bacteria play an important role in biodegradation and fungi act prominently in fresh water. Hydrocarbon degradation involves selective enrichment and genetic changes, which increase the bioremediation-causing microorganisms. The communities that are exposed to the hydrocarbons biodegrade more hydrocarbon than newly exposed species.

14.2 Factors Influencing Petroleum Hydrocarbon Degradation

The bioremediation process requires various parameters to support microbial growth, including oxygen, inorganic nutrients, water, optimal temperature, and optimal pH (Bell 1973; Cooney et al. 1985; Dibble and Bartha 1979; Ward and Brock 1976; Fuchs et al. 2011). A number of factors can influence the biodegradation of petroleum hydrocarbons, as discussed in the following sections:

1. pH
2. Temperature
3. Nutrients
4. Pressure
5. Water activity
6. Solubility
7. Salinity
8. Bioavailability
9. Contaminant characteristics
10. Microorganism number and catabolism evolution

14.2.1 pH

pH is an important factor for the degradation of hydrocarbon in bioremediation. It is generally believed that a pH range of 4–10 is optimal for the favorable enzymatic activity of various microorganisms, such as bacteria, fungi, and algae. The degradation of hydrocarbons in sewage sludge is affected by the pH value, which can influence microbial enzymatic reactions. Sewage sludge generally has a neutral pH value. Although the degradation of most heterotrophic bacteria occurs at pH 7.0, fungi are tolerant to acidic environments.

14.2.2 Temperature

For the enzymatic activity of microorganisms, temperature plays an important role. Most degradation of hydrocarbon microorganisms is active in the temperature range of 20–35 °C.

To a certain extent, temperature determines the type of microorganisms that will be present for degradation. In general, degradation rates will be slower in cooler climates. As the temperature decreases, the viscosity of the oil increases. Therefore, the volatility of the lower-chain hydrocarbons decreases and solubility increases, making the oil more toxic and less appealing to degrading microbes.

14.2.3 Nutrients

In contaminated sites, organic carbon levels are often high due to the nature of the pollutants. The available nutrients can become rapidly depleted during microbial metabolism. It is well established that the availability of nitrogen and phosphorus limits the microbial degradation of hydrocarbon. The biodegradation of crude oil and hydrocarbons can be stimulated by adjusting the carbon/nitrogen/phosphorus ratio with the addition of nitrogen and phosphorus in the form of olephilic fertilizers, including paraffinized urea, octylphosphate, ferric octoate, paraffin-supported $MgNH_4PO_4$, and 2-ethyl hexyldipolyethlyene oxide phosphate. Various fertilizers can be used, such as urea-phosphate, N-P-K fertilizers, and ammonium and phosphate salts.

14.2.4 Pressure

Deep groundwater, deep sediments, and oilfields are influenced by high pressure. This parameter mostly effects deep-sea microorganisms and the degradation of hydrocarbons in the deep sea. Oil and hydrocarbons in the deep ocean environment degrade slowly, so they can persist for years or even decades. Here, hydrocarbon degradation depends on the survival of microorganisms at high pressure. Barophiles (piezophiles) are microorganisms that require high pressure for optimal growth.

14.2.5 Water Activity

Water and moisture are required for all biological processes to help transport nutrients. Water potential is a measure of the relative tendency of water to move from one area to another. In soil, the value of water potential is 0.98 compared with an aquatic

environment. Therefore, the degradation of hydrocarbons is limited in soils and terrestrial ecosystems.

14.2.6 Solubility

Hydrocarbons typically have a symmetrical distribution of charge. This property causes them to be nonpolar. Nonpolar substances do not dissolve in polar substances, so hydrocarbons are generally insoluble in water. In addition, organic compounds are non-electrolytes because they do not de-ionize in an aqueous solution.

14.2.7 Salinity

Many saline water environments are contaminated due to the seepage of hydrocarbons and other petroleum products. Normal microorganisms cannot survive in this saline environment, so the degradation of hydrocarbons is difficult issue. However, in the last decade, researchers have isolated many bacteria and Archaea with the phylogeny and metabolic capacity to degrade a variety of aliphatic and aromatic hydrocarbons of varying salinities. In such classes of microorganisms, members of the genera *Halomonas, Alcanivorax, Marinobacter, Haloferax, Haloarcula,* and *Halobacterium* can be used for the degradation of hydrocarbon.

14.2.8 Bioavailability

A hydrocarbon's physical, chemical, and biological interactions determine the exposure of plants and animals to chemicals associated with the environment. In bioavailability, the various physiological and microbiological factors affect the rate and extent of the biodegradation of hydrocarbons.

14.2.9 Contaminant Characteristics

Petroleum consists of a complex mixture of compounds that do not decompose at the same rate. The rate of decomposition depends on the chemical structure and concentration of the component. Alkanes decompose easily, whereas longer-chain alkanes are hydrophobic solids that are consequently difficult to decompose. Highly condensed aromatic hydrocarbons decompose and biodegrade slowly.

14.2.10 Microorganism Number and Catabolism Evolution

Microflora consist of a variety of different microorganisms, including bacteria, algae, fungi, and protozoa. These microflora have different attacking capacities to biodegrade the hydrocarbons. The catabolism of hydrocarbons by microorganisms depends upon their number, the activity of the microorganisms, and their molecular structure.

14.3 Microbial Degradation of Petroleum Hydrocarbons

Petroleum biodegradation depends on the microorganism's availability, number, and response to the hydrocarbon (Head et al. 2010). Bacteria and fungi are important microorganisms for the biodegradation of hydrocarbon.

Many degradation pathways are known for bacteria; the pathways including oxygen have been known for a long time and are the best understood. There are two types of microbial degradation, as described in the following sections: aerobic degradation and anaerobic degradation.

14.3.1 Aerobic Degradation

Aerobic degradation is characterized by the involvement of oxygen in the pathway (Pieper 2005; Rojo 2010). Most of the reactions on aliphatic and aromatic hydrocarbons are performed by mono-oxygenase, which add one oxygen to the hydrocarbon. Dioxygenase is able to add one or two oxygen atoms to aromatic molecules. Unbranched alkanes are degraded readily in the aquatic environment. This degradation works for small and larger carbon chains, but the shorter chains are degraded faster. Bacteria first convert most alkanes to alcohols. These alcohols can be catalyzed to aldehydes or carboxylic acids. In some rare cases, the alcohol is secondary, and the next step produces a ketone instead of a carboxylic acid. The carboxylic acid can be further degraded via β-oxidation, which produces a carbon chain with two carbon atoms less than the starting molecule and acetyl coenzyme A.

More branched alkenes are more resistant to bacterial degradation. This is because the carbon side group on the carbon chain makes most degradation mechanisms of the bacteria impossible. This group can be used for ring cleavage in a second step. If the cycloalkane is substituted, then the degradation is faster and an acidic group is likely to be inserted near the side group. Aerobic degradation is illustrated in Figs. 14.1 and 14.2.

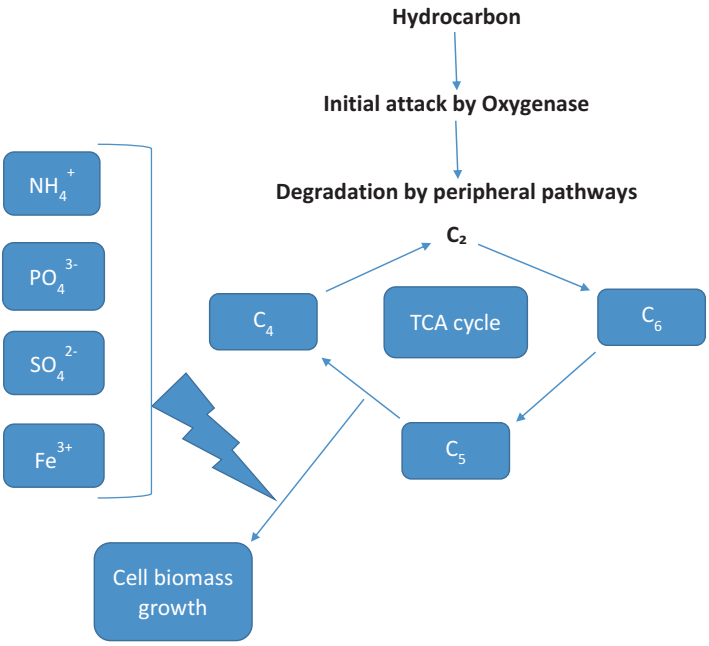

Fig. 14.1 Main principles of the aerobic degradation of hydrocarbons

14.3.2 Anaerobic Degradation

Hydrocarbons degraded by bacteria involve the reduction of nitrate, sulfate, and iron (Boll et al. 2002; Fuchs et al. 2008). Anaerobic bacteria are divided into two classes: strict and facultative anaerobes. Most of the sulfate-reducing bacteria are strict anaerobes. However, facultative anaerobes bacteria have a choice between hydrocarbon degradation with or without oxygen. Anaerobic degradation with sulfur or nitrogen uses bacteria. Alkanes and other aromatic contents of petroleum are degraded by bacteria to form fatty acids. In this degradation, the free radical mechanism of the main carbon chain starts the process. Anaerobic degradation is illustrated in Fig. 14.3.

14.4 Conclusions

The seepage of hydrocarbons in the petroleum industry is a major cause of environmental pollution. These petroleum components belong to the family of carcinogens and neurotoxin organic pollutants. Bioremediation is the only technology that can

Aerobic Routes

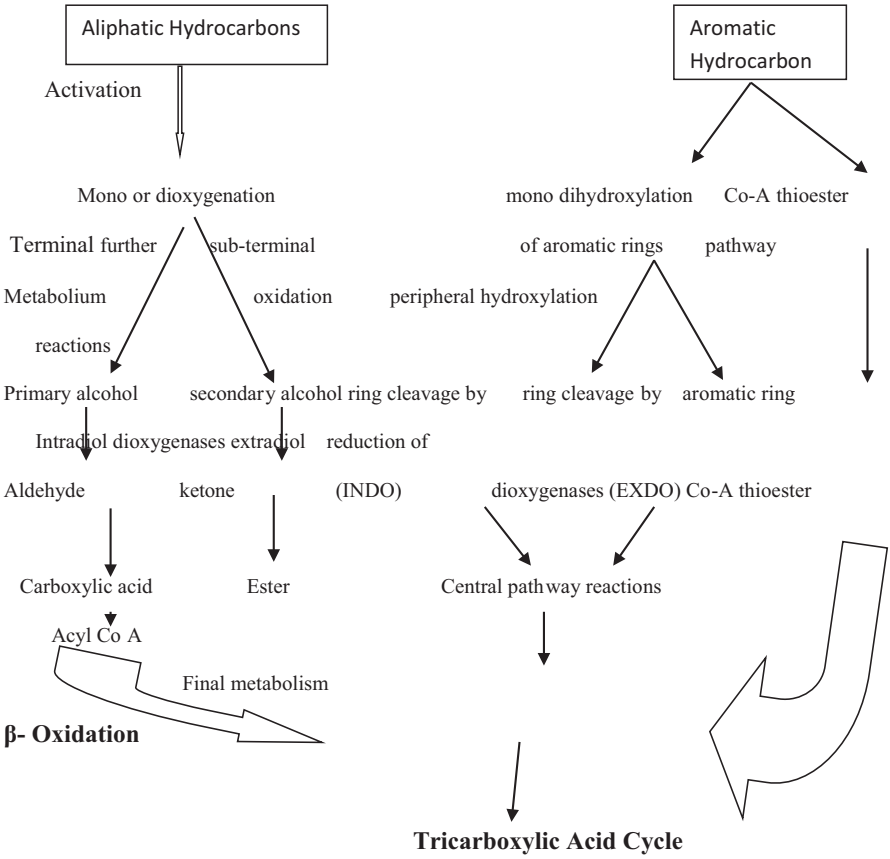

Fig. 14.2 Pathways for the aerobic bacterial degradation of hydrocarbon compounds. Two arrows represent more than one reaction

safely biodegrade these toxic substances. This process entails the bioremediation and complete mineralization of organic contaminants into carbon dioxide, water, inorganic compounds, and cell proteins or the transformation of complex organic contaminants to simpler organic compounds by microbes, such as fungi and bacteria. This biodegradation is possible only with microorganisms that have enzyme systems to degrade and use different hydrocarbons as a source of carbon and energy. The microbial degradation of petroleum in various environments depends on nutrients (e.g., nitrogen and phosphorus), salinity, pressure, oxygen, nutrient concentrations, moisture, and pH. Therefore, microbial degradation can be considered as a key component in the clean-up strategy for petroleum hydrocarbon remediation.

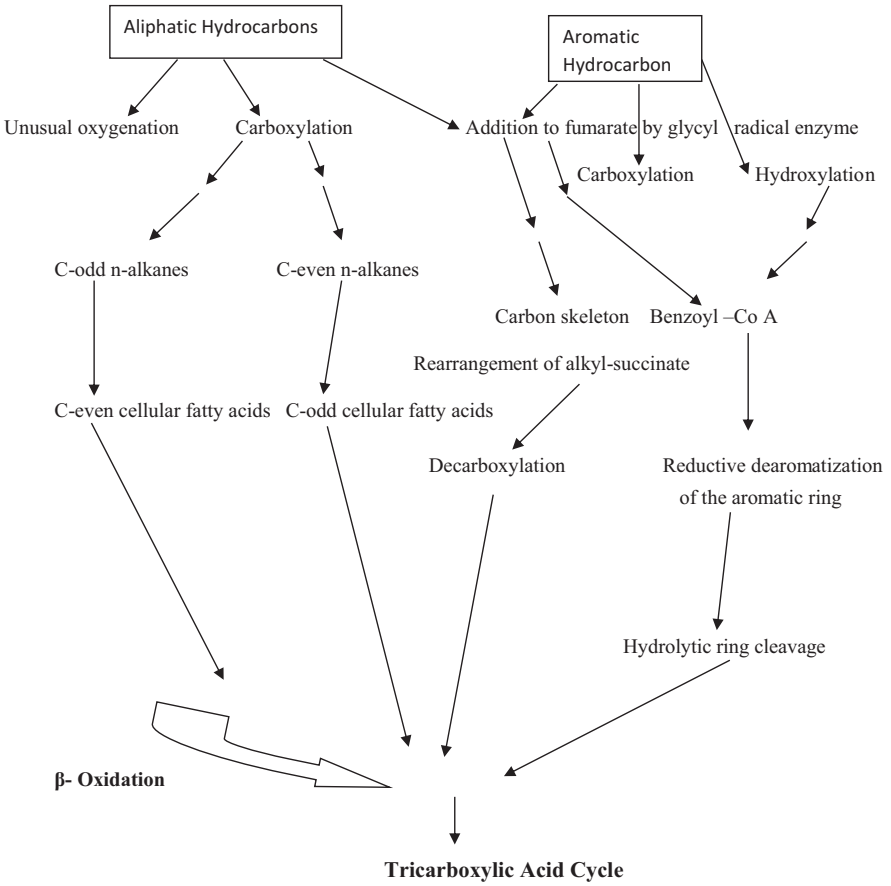

Fig. 14.3 Pathways for the anaerobic bacterial degradation of hydrocarbon compounds. Two arrows represent more than one reaction

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Chapter 15
Microbial Degradation of Hydrocarbons in the Environment: An Overview

Hussein I. Abdel-Shafy and Mona S. M. Mansour

Abstract Biodegradation of hydrocarbons is a cost-effective technique that is based on highly dispersed microbes in soil and water capable of biodegrading hydrocarbons. Degradation is an effective method for remediation of petro-hydrocarbons and causes changes in nature and concentration of petro-compounds. Biodegradation is classified as the most important tool for eliminating the toxicity and for removing the hydrocarbons in the different environments such as soil, water, and soil sediment that are polluted by hydrocarbons. The microorganisms employed in degradation process must be aboriginal in polluted sites. Investigators have recently discovered a large number of microbial groups from the sediment, water, and soil that have been polluted by crude petroleum oil. These microorganisms were able to transform hydrocarbons to energy and biomass as well as biological waste by-products. A variety of microorganisms have such capability of cleaning up and remediating locations that polluted by hydrocarbons. The microorganisms that biodegrade hydrocarbons are widely dispersed within surface water, sediments, and soil habitats. The importance of these microorganisms in biodegrading hydrocarbons and their other natural organic residues in aquatic ecosystems, soil, and sediment has long been recognized. Transformation of organic contaminants by these microbes naturally occurs because these organisms are able to use organic contaminants for their energy and carbon requirement as well as for their development and propagation. The capability of particular microbes to biodegrade the petro-hydrocarbons appears to be an acclimatization and is managed by several ecological factors. Primarily, the presence of hydrocarbons may also affect the microorganism community owing to its different chemical nature.

Petro-hydrocarbon biodegradation, by employing several microbial groups, is based largely on the structure of these communities as well as their adaptation in hydrocarbon contaminants. Bacterial and fungal strains are the main organisms for

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such crude oil biodegradation. Bacteria play a predominant role within the marine ecosystem, while fungi are more effective within both freshwater and earthen environment. The adaptive microorganism groups, which were formerly exposed to the petroleum hydrocarbon pollution, display much great degradation potential that exceeds other groups which has not exposed to such pollution. The microbial adaptation mechanism involves physical adjustment as well as some genetic changes, which leads to mutations. The mutation genes associated with the plasmid DNA could cause frequency increase in the plasmid-bearing microbes. In addition, seeding petroleum-polluted water and soil using microorganisms that feed on hydrocarbons exhibits significant success. Biodegradation of complex hydrocarbon pollutants needs a particular combination of more than one type of microbial group. This is mainly due to the fact that a single microorganism can only metabolize a limited amount of hydrocarbon substrates. Thus, the mixed cultivation (consortium) and the extensive enzymatic abilities of the microbes are strongly desired to enhance hydrocarbon degradation ratio**.**

15.1 Introduction

Nowadays, the hydrocarbon pollution, caused by several applications of petrochemical industries, is one of the environmental issues to a large extent. The petroleum product launch, caused by an accident, is a great environmental concern. Hydrocarbon compounds, either saturated or aromatic, widely spread within the environment. Such hydrocarbons show weak power of being reactive, chemically. Along several decades, it was believed that hydrocarbons were only biologically degraded in the existence of air or oxygen. However, through the last three decades, a huge number of microorganisms, which have the capability to biodegrade hydrocarbon compound directly under strict oxygen deficiency conditions, have been discovered. Hydrocarbons composed mainly from carbon and hydrogen. They are mostly polar, allowing very weak chemical reactions in ambient temperature due to deficiency of function groups. The chemical reactivity of hydrocarbons is mainly specified via type, existence, and order of the aromatic bonds (π bonds) in these compounds. Thus, these compounds are classified regarding their bonding types to the following categories: aliphatic and aromatic hydrocarbons. Furthermore, aliphatic hydrocarbon category is easily distinguished as straight chains, branched trains, and nonaromatic rings. Based on the structure, the aromatic hydrocarbons are classified as monocyclic, bicyclic, and polycyclic compounds. Among these classifications, alkylbenzenes are significant, in which the alkyl groups substituted one or more hydrogen atoms from the benzene ring. Availableness of these compounds is of fundamental importance for our industrialized civilization, due to the fact that hydrocarbons are fuels and initiator components of a wide area of the chemical preparations. Most or all of hydrocarbons are naturally subsist, as well as they can be easily synthesized from those that are naturally present. These compounds have been naturally formed through extended period of geochemical reactions of the very

old entombed biomass and the metabolism inside the living organisms. Thus, the environmental pollution and pollutants originating in human activities are not the only source of introducing the petroleum hydrocarbons to our planet. However, it can lead to rise of their buildup in the ecosystem (Widdel and Ralf Rabus 2001).

Petroleum is very intricate combination of several hydrocarbons. It includes variable combination of cyclic, linear, and branched alkanes, aromatic hydrocarbons (including mono-, bi-, and polycyclic compounds), and asphaltenes as well as resins (Fig. 15.1). The polycyclic aromatic hydrocarbon (PAH) compounds are mostly stable, toxic, and carcinogenic (Abdel-Shafy and Mansour 2016). Petroleum compounds, including alkanes, benzene, toluene, ethylbenzene, and xylenes besides few of PAHs, can be biologically degraded in an appropriate environmental conditions as well as under weak salinity of the marine ecosystem (Abdel-Shafy and Mansour 2016). Nevertheless, higher molecular PAHs, methyl tertiary butyl ether, polycyclic aromatic sulfur heterocyclic, a gasoline enhancer, and other constituents of petroleum products might not undergo the process of biodegradation. These compounds, which are not biodegradable, cause a great hazard in the location in which they exist. Therefore, petroleum hydrocarbons have been termed as the main environmental pollutants, because they harm their surrounding ecosystems. Groundwater, petroleum-polluted soil, as well as wastewater might include several contaminants such as salts, PAHs, organics, phenols, alcohols, acid, radionuclides, and heavy

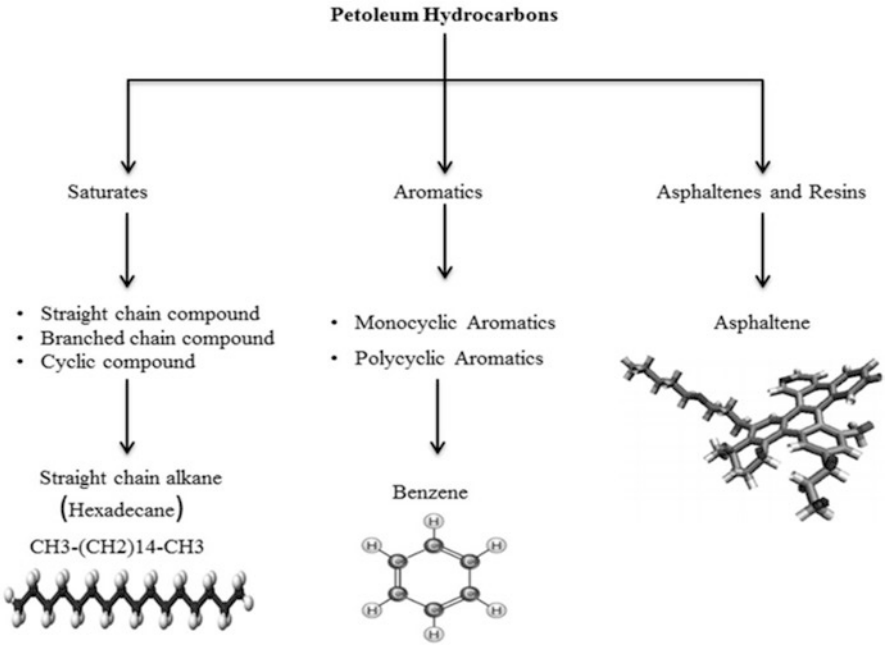

Fig. 15.1 Variable fractions of petroleum hydrocarbons. (Yasin et al. 2013)

metals (e.g., Hg, Cd, Pb, Cr, Cu, Zn, etc.) at widely varying concentrations (McGenity et al. 2012, Abdel-Shafy and Kamel 2016).

Conversely, hydrocarbon mixtures are classified as organic contaminants which have carcinogenic as well as neurotoxic effects. Primarily, burning or elimination of petroleum wastes in the unsafe landfills is environmentally prohibited; besides it is costly also as great quantities of these pollutants are discharged and are difficult to manage. Most of the techniques either mechanical or chemical being employed to degrade or eliminate petroleum hydrocarbons from the polluted locations are cost-ineffective and also have several side effects (Moursy and Abdel-Shafy 1984). On the contrary, bioremediation processes are the most appropriate technique to remove petroleum hydrocarbons from the polluted locations. They are inexpensive, as well as they can completely eliminate these wastes. These processes basically belong to the biodegradation technique. They are referring to an entire degradation for the organic pollutants of these wastes to CO_2, inorganic substances, water, as well as protein cells and, on the other hand, also transform the complicated organic pollutants into the simple forms by using the bioagents, the effective microorganisms. Several aboriginal microorganisms at water and soil are efficiently degrading petroleum hydrocarbon pollutants (Das and Chandran 2011).

In this respect, the microbial community in soil is greatly related to the hydrocarbon diversity. Therefore, the selection of the microorganisms, for the bioremediation of hydrocarbons, is very vital and important. Generally, the response of petroleum hydrocarbons to the soil microorganisms and their interaction with soil matrix define the fate of these pollutants. The biodegradation ratio of these pollutants is proportional with the capability of the microorganism as well as the chemical structure of these pollutants. It is important that the contaminated soils have their own special features and the necessary environmental parameters, including the activity of their inhabited microorganisms to achieve the bioremediation of petroleum hydrocarbons. Besides, there is no inhibition on the action of the microorganisms' metabolites in these soils, and also there are homeland hydrocarbons which are active and ready to be utilized by the microbes in soil. The biological degradation of petroleum hydrocarbons includes chemically, biochemically, and microbiologically molecular indicators which control the efficiency of the microorganisms' action. Several processes are utilized to monitor and to characterize the biological degradation of the petro-hydrocarbons in ecosystem such as a rapid plate reader based-method (microtiter), an easily applied protocol that gives information concerning the role of the microorganisms and their adaptations through space and time, molecular approaches like genetic fingerprinting, phospholipid fatty acid monitoring, metagenomics, microarray analysis, GC analysis, and respirometry (Chikere et al. 2011).

The bioremediation, of the petroleum hydrocarbons as well as the related pollutants, is a difficult and challenging technique. The transformations, even quantitatively or qualitatively for these pollutants, are based mainly on their kind as well as their quantity present in the environment. This includes free or dissolved oxygen, temperature difference, and dispersion of the petroleum physically as well as chemically. The bioremediation depends also on tumultuous conditions, as well as the

indigenous constituent of the microorganism groups. The optimal temperature of the petroleum to degrade varied between 20 °C and 35 °C. The microbial degradation of petroleum hydrocarbons occurs by targeting the aliphatic or the low molecular weight aromatic portions of the petroleum, followed by attacking the high molecular weight such as the aromatic, resin, and asphaltene fractions. The high molecular weight crude oil is classified as disobedient species; thus, they exhibit much weak values of bioremediation. However, their elimination has been reported in a few researches as considerable large values under the optimum conditions. Generally, the increase in temperature leads to increase the value of the biodegradation. Therefore, the biodegradation of petroleum hydrocarbons takes place very slowly for the very low-temperature ecosystem. Also the biodegradation of these compounds in the aquatic ecosystem is poor due to the limited presence of nutrients including phosphorus and nitrogen. Meanwhile, in the estuaries and the deep seas, the salinity and pressure are considered as key factors. The pH, oxygen, moisture, and the most important nutrient concentration are the controlling and predominant factors in defining the value of the petroleum hydrocarbon biodegradation inside the soil (Olajire and Essien 2014).

15.2 The Role of Microorganisms on the Biodegradation of Petroleum Hydrocarbon

It is well documented that microorganisms are capable of converting petroleum hydrocarbon into the required carbon and energy. Some bacteria in the polluted site are *Pseudomonas*, *Rhodococcus*, *Acinetobacter*, *Gordonia*, *Burkholderia*, *Enterobacteria*, *Sphingobium*, *Novosphingobium*, *Sphongomonas*, *Psychrobacillus*, and *Bacillus* (Chaudhary 2016). Hydrocarbons degrading bacteria and other microbes are widely dispersed in their soil homeland. Several investigators believe that some isolated bacteria from contaminated soil are effective in hydrocarbon degradation (Moursy and Abdel-Shafy 1983). *Thiobacter subterraneus*, *Alcaligenes* sp., and *Escherichia coli* were isolated and were found to be efficient for degrading phenanthrene and anthracene. Certain microorganisms primarily from the genera *Mycobacterium* and *Pseudomonas* were found to be able to degrade and transform polycyclic aromatic hydrocarbons under the presence of dissolved oxygen (Mrozik et al. 2003). As documented, anthracene is possible to totally degrade by *Nocardia*, *Sphingomonas*, *Beijerinckia*, *Rhodococcus*, as well as the dihydrodiol *Paracoccus* that is the initial oxygenate intermediary (Teng et al. 2010). It is evidently proposed some microbes specifically; Torulopsis bombicola, Pseudomonas aeruginosa as well as Bacillus subtilis which able to produce the biodegradation surfactants including surfactin, sophorolipid as well as rhamolipid. And they are able to enhance the biodegradation via making PAHs dissolve in the aquatic system. This enhances the PAHs suitability to biodegradation (Cottin and Merlin 2007). By a similar manner, biodegradation of the petroleum hydrocarbon by yeasts, molds, as well as

cyanobacteria was mentioned widely as an important means in such degradation (Chaillan et al. 2004).

Meanwhile, collections of microorganisms in soil are greatly influenced by the disorder of the hydrocarbons. Consequently, enrichment of the petroleum hydrocarbon selection to the microorganisms takes place. Thus, the interaction between petroleum hydrocarbon and soil microorganisms and the type of ecosystem that found only on biomes shape the fate of the contaminants according to both microbial degradative capabilities and their chemical nature. Microbial methods that are used to monitor the biodegradation of the petroleum hydrocarbon should contain molecular indicators that are chemical, biochemical, and microbiological. The purpose is to measure the activity rates of the microorganisms and to reach the level of the pollution reduction that is accepted. Although fungi and bacteria are widely reported to be the main agents in the petroleum hydrocarbons biodegradation, bacteria are much adaptable compared with fungi. Thus, bacteria can have a main function in the biodegradation of the petro-hydrocarbon. In addition, the degradation by protozoans, yeast, as well as algae is considered as significant means for the degradation of many of the petroleum hydrocarbon. The biodegradation via *Mycobacterium*, *Arthrobacter*, *Pseudomonas*, *Aspergillus*, *Rhodococcus*, *Chlorella*, *Penicillium*, *Candida*, and *Cyanobacteria* could be classified as some of the main components for the removal and remediation of the hydrocarbons from the environment (Pandey et al. 2016).

15.3 Biodegradation of Petroleum Hydrocarbons Using Bacteria

Several bacteria have a capability to biodegrade persistent carbon-based contaminants (Abdel-Shafy et al. 1988). Biodegradability of the petroleum hydrocarbons is able to be achieved via different microbes. It was reported that many bacteria have been found active for the biodegradation of PAHs as only carbon source. The widespread and well-known degradation of the PAHs including naphthalene, phenanthrene, acenaphthene, as well as anthracene via biochemical processes by bacteria was studied and reported (Abdel-Shafy and Mansour 2016). Primarily, the mechanism requirement of the degradation via bacteria, essentially, is an existence of dissolved oxygen in order to begin the degradation by enzymes for the PAH rings. In the first step, both monooxygenase (aliphatic) and dioxygenase (aromatic) act as catalyst in the oxidation processes. Firstly, the enzyme dioxygenase cracks an aromatic ring and then forms the first intermediate, namely, cis-dihydrodiols. These are classified as multicomponent enzymatic processes that contain several none protein compounds that are necessary for the functioning of the enzyme and metal ions, as an aid and cofactor (Peng et al. 2008). Lately, Amenu (2014) reported that *Pseudomonas* sp. S3 and F3 efficiently degrade naphthalene under optimal temperature and pH of 37 °C and 7, respectively. Biodegradation efficiency for F3 after 7 days of incubation was 61.11% of naphthalene. It was suggested by Pawar et al. (2013) that

the main bacterial genes responsible for the degradation of PAHs have been greatly homogenous with plasmid of *Pseudomonas putida* strain that exists in the gene responsible for the degradation of naphthalene (NAH7). The genus *Pseudomonas* considered among the famous agents that accountable for biodegradation of the PAHs that contain three and four ring. It was mentioned by Bamforth and Singleton (2005) that some of the bacterial sp. involved in biodegradation including *Bacillus* sp., *Pseudomonas* sp., *Acinetobacter calcoaceticus* and *Micrococcus* sp., *Nocardia erythropolis*, *Candida Antarctica*, *Ochrobactrum* sp., *Serratia marcescens*, *Acinetobacter* sp., *Alcaligenes odorans*, *Candida tropicalis*, and *Arthrobacter* sp. were used for alkane degradation. It was also reported by Bamforth and Singleton (2005) that the degradation of the mono-aromatic hydrocarbons was successfully achieved by *Pseudomonas* sp., *Brevibacillus* sp., *B. stearothermophilus*, *Bacillus* sp., *Corynebacterium* sp., *Vibrio* sp., *Ochrobactrum* sp., and *Achromobacter* sp.. Meanwhile, Bamforth and Singleton (2005) mentioned that *Alcaligenes odorans*, *Achromobacter* sp., *Mycobacterium* sp., *Sphingomonas paucimobilis*, *Mycobacterium flavescens*, *Pseudomonas* sp., *Arthrobacter* sp., *Bacillus* sp., *Rhodococcus* sp., *Xanthomonas* sp., *Alcaligenes*, as well as *Burkholderia cepacia* were also used for the degradation of poly-aromatic hydrocarbons. They also mentioned that members of *Vibrionaceae*, *Pseudomonas* sp., *Moraxella* sp., and *Enterobacteriaceae* were effective for resin degradation (Bamforth and Singleton 2005).

15.4 Fungi for the Biodegradation Petroleum Hydrocarbons

Several fungi possess the capability for degrading the recalcitrant organic contaminants (Haritash and Kaushik 2009). Also Spellman (2008) mentioned that fungi are able to undertake the metabolism of the organic matters that dissolved in the aquatic system, where they are considered as the main microorganisms responsible to decompose organic carbon within the ecosphere. Matavulj and Molitoris (2009), meanwhile, reached the conclusion that fungi, containing multiple extracellular enzyme combinations, degrade the natural organic matters using their systems of hyphae. Such systems of hyphae are, generally, capable for rapidly colonizing and penetrating the substances as well as transporting and distributing the substances that provides nourishment essential for growth and the maintenance of life to their vegetative part. The biodegradation of PAHs accomplished via using non-ligninolytic and ligninolytic fungal strains (Reyes-César et al. 2014, Bamforth and Singleton 2005). *Antrodia vaillantii* and *Pleurotus ostreatus*, fungi with white rots, produce the ligninolytic enzymes that responsible for the lignin oxidation process present in wood as well as any organic substances (Bamforth and Singleton 2005). It was reported by Haritash and Kaushik (2009) that the ligninolytic enzyme system consists of manganese-reliant peroxidases (MnP), laccases, and lignin peroxidases (LP). In addition, Hadibarata et al. (2013) studied the naphthalene degradation process using *Pleurotus eryngii*, a fungus with white rots. They found that this fungus cleaved the naphthalene bonds at both positions (C 1 and C 4) and gave 1,

4-naphthoquinone via the mechanism, including removal of oxygen atom from the molecule (deoxygenation). The obtained 1, 4-naphthaquinone converted to benzoic acid and then to catechol via combining the operations of carboxylic group removal and hydroxyl group addition. A study by Leitao (2009) confirmed that pyrene can be metabolized via strain SFU 403, which is a *Penicillium janthinellum* strain that isolated from oil-polluted soil. Furthermore, this biodegradation begins with forming quinones, dihydrodiols, diphenols, and monophenols. Pyrene is degraded to 1-pyrenol by addition of hydroxyl group. According to Wang and Zhao (2007), pyrene is degraded to 1-pyrenol and then 1, 6- and 1, 8-pyrenequinones. In further study, *Aspergillus terreus* was specified as the highest in ligninolytic enzyme production. And the extreme production of lignin peroxidase and manganese peroxidases occurred under the optimal physical conditions such as temperatures from 33.1 °C to 33.6 °C and pH from 4.1 to 5.8. By utilizing these physical optimal conditions, the degradation values of naphthalene and anthracene were 98.5% and 91%, respectively, in the soil under investigations (Ali et al. 2012).

15.5 Algae for the Biodegradation of Petroleum Hydrocarbons

Marine and freshwater macro- and microalgae are well known for their role in the accumulation of pollutants (Fayed and Abdel-Shafy 1985, Abdel-Shafy and Farghaly 1995, Fayed et al. 1983). Several researches confirmed the important role of *Scenedesmus platydiscus*, *Chlorella vulgaris*, *S. capricornutum*, and *S. quadricauda* as fresh algae, for the biodegradation of PAHs in ecosystem (Wang and Zhao 2007, Abdel-Shafy and Mansour 2016). Meanwhile, cyanobacteria, diatoms, and green algae, as prokaryotic and eukaryotic photoautotrophic marine algae, are famous for naphthalene metabolism via a series of metabolic products (Haritash and Kaushik 2009). Phenanthrene biodegradation via *Pseudomonas migulae* and *Sphingomonas yanoikuyae*, as algal-bacterial microcosms, was examined (Haritash and Kaushik 2009). Conversely, Ueno et al. (2008) investigated the efficiency of *Chlorella vulgaris*, *Scenedesmus platydiscus*, *S. quadricauda*, and *Selenastrum capricornutum* in degradation of pyrene, fluoranthene, and their mixture. In their study, PAHs were removed within 7 days of treatment, and this was achieved at the rates of 78% and 48% via using of *S. capricornutum* and *C. vulgaris*, respectively.

15.6 Yeast for Biodegradation of Petro-Hydrocarbons

Miranda et al. (2007) described that yeast are capable of utilizing aliphatic hydrocarbons as well as several petroleum products. *Rhodoturularubra aurantiaca*, *C. tropicalis*, *Candida lipolytica*, *Aureobasidium*, *Trichosporon*, *Rhodotorula aurantiaca*, as well as *C. ernobii* are the aliphatic hydrocarbons utilizing yeasts. They all

were capable of diesel oil degradation. Leelaruji et al. (2013) also added that var. melanogenum and *Aureobasidium pullulans* are classified as lipolytic yeast. They were efficient for the degradation of anthracene at rat of 37.3%, naphthalene at rat of 24.4%, benzo(a)pyrene at rat of 45.95%, and pyrene at rat of 27.3%, by producing of laccase. Furthermore, Hesham et al. (2006) reported that yeast strain (AEH) is efficient for the degradation of naphthalene of 5.36 mg L^{-1} in 2 days, chrysene of 1.54 mg L^{-1} in 10 days, as well as phenanthrene of 5.04 mg L^{-1} in 10 days. A combination of phenanthrene plus naphthalene of 4.20 and 3.79 mg L^{-1}, respectively, was degraded by AEH in 10 days. This strain can also degrade benzo(a)pyrene and chrysene of 1.91 and 3.37 mg L^{-1}, respectively, in 10 days. Further study showed that co-metabolic biodegradation of benzo (a) pyrene along with naphthalene in the environment can be easily used as a best source of carbon.

15.7 Protozoa for the Biodegradation of Petroleum Hydrocarbons

Protozoa has been identified as a bad agent of biodegradation compared with bacteria, algae, as well as fungi. On the other hand, its communities exhibited significant reduction of the numerical values of bacteria ready for removing of hydrocarbons. This indicates that its existence in the ecosystem does not have benefit in biodegradation (Stapleton and Singh 2002). However, the bacteria feed protozoa on the degradation of organic pollutants. Therefore, the reciprocal action between protozoa and bacteria will certainly effect on the biodegradation by these bacteria. A pattern for food series was constructed by Mattison et al. (2005) for examining the impact of flagellate *Heteromita globosa* from protozoa on the bacteria that leads to the degradation of benzene and methylbenzene. In addition, it was confirmed by Chen et al. (2007) that infusorian of protozoa speeds up heterogeneous compound degradation through the ecosystem, including PAHs. For example, the value of naphthalene degradation has been enhanced fourfold more than prior. Some potential assumptions were proposed by Chen et al. (2007) regarding the protozoa speeding up mechanisms for organic pollutant degradation. They include basically the following items:

- Enhancement of the nutrient circulation via mineralizing of the nutrient.
- Activation of bacteria that leads to rule the amount of antiquated cells feeds or leads to the secretion of the efficient matter.
- The feeding selections minimize the race on stuff and surface area. Therefore, consider it useful for outgrowth of bacteria involved in biodegradation.
- The disorders, occurring physically, lead to raise the oxygen content as well as the surface area of the compounds exposed to biodegradation.
- The biodegradation, occurring directly, leads to secretion of particular enzyme intricate in biodegradation process.
- The co-metabolic biodegradation gives bacteria the best source of energy and carbon.

15.8 Factors Affecting the Hydrocarbon Degradation via Microorganisms

15.8.1 Effect of Temperature on Hydrocarbon Biodegradation

Microorganisms, which degrade petroleum hydrocarbons, are extremely efficient at certain ranges of temperatures, which control enzyme-producing process. Furthermore, three types of produced enzymes within the optimal temperature ranges identified as the thermophiles (above 50 °C), mesophiles (15 °C–45 °C), and psychrophiles (below 20 °C). The majority of microorganisms have efficiency for degradation of petroleum at the temperature ranging between 20 °C and 35 °C (mesotherm), with which they give the maximum degradation values. Generally, temperature range determines the types of organisms, to a certain extent suitable to the process of biodegradation. Primarily, the biodegradation is slow within the cold water. As a result of low temperatures, the crude oil viscosity rises, the simple hydrocarbon volatility reduces, and the solubility of these hydrocarbons rises. This makes the crude oil highly toxic, as well as resistant to be degraded by microorganisms. The temperature of the seawater assigns between −2 °C and 35 °C. Furthermore, the temperature of more than 90% of the ocean water is less than 5 °C. The temperature of most biodegradation was recorded at this entire range. It was reported by das and Chandran (2011) that the lower of temperature from 25 °C to 5 °C leads to reduce the degradation values by around ten times. Slight biodegradation was recorded in the area of the Arctic sea ice as well as in the icy tundra, at insignificant values. The emission of heat is occurring throughout the operations of hydrocarbon degradation. Nevertheless, there is no automatic burning, but the temperature should be not more than 71 °C. This is the extreme temperature to sustain the existence of the microorganisms, and the majority of petroleum compounds are burning when exceeding this temperature. However, the chance remains very limited due to the volatilization of the hydrocarbon molecules (Pathak and Jaroli 2014). Figure 15.2 indicates that the great values of the biodegradation of hydrocarbon happened between 30 and 40 °C within the soil environments, within the range between 20 and 30 °C in some freshwater environments, and in the range 15–20 °C in marine environments (Das and Chandran 2011).

15.8.2 Effect of pH on Hydrocarbon Biodegradation

The choice of pH as a controlling factor is based on type of microbes that are utilized in the petroleum hydrocarbon degradation. The local microbes, involving some bacteria, fungi, as well as yeast, are sustained alive at pH 2. Bacteria, which greatly grow at pH exceeding 9 and slowly grow at pH value of 6.5, are called alkaliphiles. They were existed within the alkaline lakes in the pH range from 7.5 to 10.

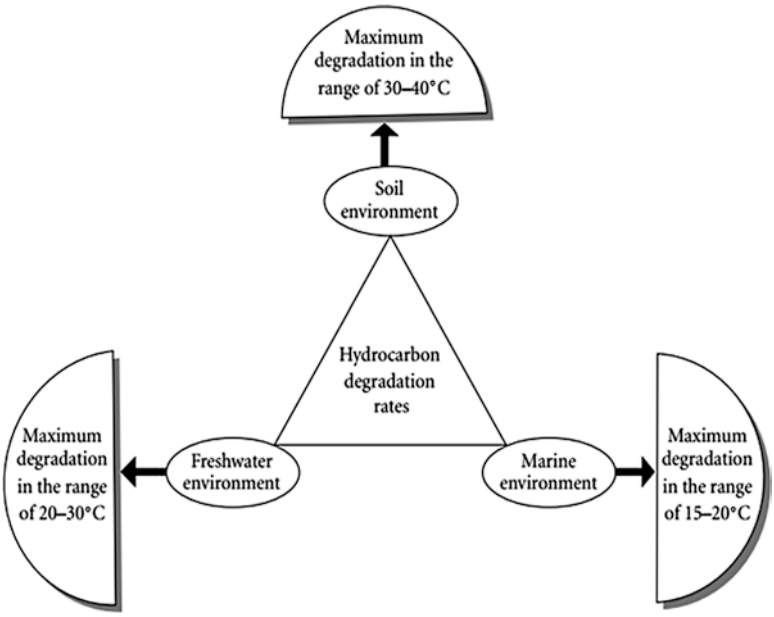

Fig. 15.2 Optimum temperature range of the biodegradation of the hydrocarbons within soil environments, freshwater environments, as well as marine environments. (Das and Chandran 2011)

Thus, the calcium-containing inorganic mineral such as carbonates and oxides or hydroxides was commonly added to the areas under investigation to increase the pH. Phenanthrene degradation at aquatic system with *Burkholderia cocovenenans*, isolated from the soil polluted by petroleum, is examined in pH values ranging from 5.5 to 7.5. The degradation rate of phenanthrene by bacteria was 40% after 16 days at pH 5.5. On the other hand, removal of phenanthrene was 80% at circumneutral pH values. Nevertheless, the pH of the growth medium is predominant factor on the efficiency of BA2 *Sphingomonas paucimobilis* strain. Biodegradation of anthracene and phenanthrene is considerably reduced at pH 5.2. Meanwhile, PAH biodegradation was observed at a highly acidic soil of pH 2 that polluted with coal tar. The local microbes, in soil that close to coal stack, are efficiently decrease the naphthalene amount to 50 % in more than 28 days duration. But when it comes with phenanthrene and anthracene, the removal rates were between 10 and 20%. It is interesting to mention that several isolated microorganisms, from the environment, were able to reduce aqueous medium pH from 9 to 6.5 in 24 h, as well as degrade naphthalene to use as a source of carbon. Instead, the efficiency of the microbes that are degrading the naphthalene such as *Pseudomonas fluorescens* (DSM6506) and *Pseudomonas frederiksbergensis* (DSM 13022) was strongly reduced due to the high pH values. Pathak and Jaroli (2014) were proposed that the microbes present in polluted regions have efficiency for PAHs metabolism at high pH.

15.8.3 Number, Activity, and Catabolism Evolution of the Microorganisms

The major parameters that effect on the petroleum hydrocarbons degradation rate via microbes including, the accessibility of microbes to the targeted pollutants that have capability for degrading by them, the numbers of the microbes that degrading these pollutants, the active action of those microbes, and finally the hydrocarbon pollutant molecular structure (Alan 2005). It is necessary for a successful biodegradation that the microbes must be improved their catabolism and this can be done as follow: inducing the specified active enzymes, improving novel metabolism by changing in genes, enriching the selective microorganisms that have capability for transforming the targeted hydrocarbon contaminants (Pawar 2015).

Hydrocarbons are well known for being an essential carbon and energy source, which are needed by the microbes for their outgrowth. Prior to the availability of carbon for utilizing, the high molecular weight petroleum hydrocarbons should be cracked to smaller ones, as the simple molecules are appropriate to utilize via the microorganisms for their growth. The species of microbes, over 70 species, have been well recognized which including organisms that biodegrading the petroleum hydrocarbons. Hydrocarbons of petroleum, discarded in the environment, have been successfully degraded via bacteria and fungi, where those microorganisms have been already present in the soil environment, freshwater environment, as well as marine environment. Numerous bacteria that degrade the hydrocarbons are *Acinetobacter*, *Achromobacter*, *Alcaligenes*, *Bacillus*, *Arthrobacter*, *Flavobacterium*, *Pseudomonas*, and *Nocardia*. They are mostly found in marine and soil environments. *Candida*, *Aureobasidium*, *Sporobolomyces*, and *Rhodotorula*, in between the registered fungi, commonly existed in the marine water. Meanwhile, *Mortierella* and *Trichoderma* have more commonly existed in the soil. Within the seawater and the marine waters, bacteria have been classified as prevalent microorganism for the degradation of the petroleum hydrocarbons. Fungi and bacteria participate in the petroleum hydrocarbon biodegradation within the soil. Their contribution percentage ranges from 20 to 80 in favor of bacteria. However, there is much less registration concerning the relative action of bacteria, yeasts, algae, protozoa, as well as fungi regarding the overall values of petroleum hydrocarbon degradation within freshwater. Microbes, consuming hydrocarbon of petroleum to be a food source, have been easily found with large amounts nearby the sites that polluted by petroleum, including infiltrate of natural oil, harbors, passageways of the shipping, stations of fuel, oilfields, as well as others of the related means. Comparatively, little microorganisms that are classified as petroleum hydrocarbon consumers exist within the maiden soil as well as in the large exposed sea. Very little of microorganisms stay alive within the crude oil, where the large quantity of petroleum exist underground for millions of years. However, the microbial communities will certainly differ throughout the samples, depending on the sites from which the samples have been collected. The harbors, contaminated by petroleum, are usually

including 1EO3 to IEI0A6 of microorganisms per ml. It is important to mention here that the water, containing no hydrocarbon contamination, has only 1% of bacterial communities composed of bacteria degrading the hydrocarbon. But the sites, contaminated by hydrocarbon, contain about 10% or more of the entire bacterial communities. Therefore, remediation of long-term roll military means (i.e., repository of fuel as well as harbors) might be more easy than this of a separated incident based on bioremediation technique, due to the raise in utilizing microbes for petroleum hydrocarbon degradation (Pathak and Jaroli 2014).

15.8.4 Effect of Microbial Consortium on Hydrocarbon Biodegradation

A consortium, used for hydrocarbon biodegradation, is two or more groups of living symbiotic microbes. The petroleum's main constituent is hydrocarbons with different lengths of aliphatic and aromatic carbon atoms. Generally, the enzymes, produced by microbes, attack the molecule of the hydrocarbons. It is well known that enzymes are sets of proteins responsible for conversion of petro-hydrocarbons into the more simple molecules. Some of these microorganisms are able to produce enzymes that have the ability to crack nearly all hydrocarbon types and sizes. Meanwhile, other microorganisms have been generating enzymes that are cracking and attacking certain size or kind of hydrocarbon molecules. As soon as petroleum hydrocarbons are cracked, the residual molecules of hydrocarbons need more enzymes to be cracked. The deficiency of the enzymes that are particular for cracking the residual hydrocarbon molecules, certainly, provides a barrier to complete the biodegradation. Thus the process will be stopped, till another one is generated into the combination. Such complicated sets of actions, through which the biodegradation takes place, are considered as a path of metabolism. There is not only one microbe type that has the ability to biodegrade so much variable petroleum hydrocarbon compounds. Therefore, numerous various enzymes as well as paths of metabolism have been needed for biodegradation of such considerable amount of hydrocarbon species, concerning substances of crude oil.

In the environment, in case of raw oil spills, particular microbes in that particular place will rapidly grow owing to enormous accessibility of biodegradable hydrocarbons. Such microorganism types with rapid growth might prevent the action of the other groups via decreasing the availability of the nutrients as well as oxygen inside these communities. While such hydrocarbons that degrade readily have been consumed, it will be a deficiency in the microbial enzyme that substantial for degrading the residual hydrocarbons and leads to death of these microorganisms. On the other hand, the other microbes that have the ability to degrade the residual hydrocarbon be willing to fast grow as well as prosper inside their communities. This cycle goes on, where the microbial types prosper, regress, and exceed due to the availability of the

degradation, after they will be die and so on (Pathak and Jaroli 2014). The microbes, existing in the crude oil and degrading the hydrocarbon compounds more easily, are commonly detected close to the surface water as well as the soil. Availability of hydrocarbons, close to water and soil surfaces, which is the main sources of oxygen, moisture, and food, leads to the existence of such microorganisms in such sites. The greatest number of microbial organisms, degrading the hydrocarbon compounds, required oxygen for their growth (aerobic). However, some microbes are anaerobic, i.e., they don't require oxygen (Pathak and Jaroli 2014).

15.8.5 Bioavailability of Hydrocarbons

The availability of petroleum hydrocarbons for biodegradation has been based on the physical properties, water-repellent properties, adsorption on soil particles, vaporization from the medium, as well as the ability of hydrocarbons to dissolve. All these factors significantly affect the amplitude of their degradation by microorganisms. Even though, all those factors affected on the hydrocarbon degradation by microbes have been sustained at optimum, there is proved that still remained hydrocarbons non degradable portion at the field. After hydrocarbons access to the soil environment, as an organic pollutant, they might be lost missed via degradation by microbes or via vaporization or via leaching. They might also be gathered by the soil micro- and macrobiota or adsorbed by the soil particles or be complexed in the organic matter portions of soil or minerals. The degradation values of hydrocarbons via microbes are based on different physicochemical procedures, including adsorption, diffusion, as well as dissolution (Brassington et al. 2007).

The overall transport of certain pollutant defines the biological availability of microbes. Biological availability points to the portion of organic substances within the soil that used or converted by the microorganisms. The biological availability of chemical substances is known as a proportion of the mass transport and lively organism substance actions in the soil. Major pollutants in the soil exhibit a behavior in two phases. Within the first phase of petroleum hydrocarbon degradation by microorganism, the degradation rates have been raised. The degradation by microbes has been mostly controlled via the kinetic of these biodegradation. Within second phase of hydrocarbon biodegradation, the degradation rate of hydrocarbon has been lower as it has been mostly restricted by slowly adsorption (Loeher et al. 2001). That is worth to mention that the degradation of hydrocarbons which polluted the soil is seriously affected by time due to the operations of weathering (i.e., reduction of the contaminant availability to the microbes). Operations of weathering point to physical, chemical, and biological operation outcome which have greatly affected the remaining petroleum hydrocarbon kinds into the soil environment (Maletic et al. 2011).

15.8.6 Salinity: Effect on the Biodegradation of Hydrocarbons

Microorganisms are known for being compatible with the broad extent salinity of the world oceans. A minor proof concluded that microorganisms are affected by another water saline environment rather than environment with hyperactive salinity, for example, saline water of petroleum reservoir. However, estuaries might give various situations due to the variation of their saltiness amounts according to different scales in comparison with the values of the seas and oceans. When the microorganisms are added to the marine environment for the biodegradation of hydrocarbon, it should recognize whether these microbes are convenient to the salinity level of this aquatic environment or not (Pathak and Jaroli 2014).

15.8.7 Effect of Petroleum Surface Area

Greatest amount of hydrocarbon degradation by microorganisms happens mostly in or close to the interface of air and water at marine environment and the interface of air and soil in soil environment. Thus, petroleum surface area value, faced to these interfaces, will impact and control the biodegradation rate. Therefore, as the surface area faced to air increases, the petroleum degrades faster. Transformation of the remaining petroleum hydrocarbons in the marine environment is related to the oil surface area. Pools of oil, or thick rafts, blankets, other high concentricity petroleum compounds, and lower surface area give a low biodegradation case. This is simply because the availability, of the active sites cracked by microorganisms, was decreased. Herein, petroleum behaves as blanket preventing the renewal of oxygen as well as nutrients for the microorganism. Thus, with the rise of concentricity, the substances that are easier to degrade are cracked by microorganisms and leave compounds highly reluctant at the back. The latter combine together to compose more reluctant substances, including star circles which own finite wetness contents as well as limited surface area. However, hydrocarbon concentration ranged between 1 and 100 μg/ml of water and 1 and 100 μg/g of soil (as dried mass) and is not considered as poisonous to the widespread bacteria and fungi (Potin et al. 2004).

15.8.8 Characteristics of the Contaminants

Generally, petroleum hydrocarbons consist of complicated substances mixed together. Every substance of hydrocarbons cannot be degraded by a ratio similar to others. Degradation rate, of petroleum hydrocarbon compounds by microorganisms, is based essentially on the chemical configuration of these compounds as

well as their concentration. The petroleum hydrocarbon compounds were classified to the following: saturated aromatic, resin, as well as asphaltenes. The n-alkanes, among the several petroleum products with average chain from C10 to C25, are most chosen compounds by the microbes as well as most easily biodegradable. On the contrary, petroleum products with short chain are much poisonous as well as much less degradable. Meanwhile, alkanes with long chain from C25 to C40 are water-repellent solids in the water medium. As a result of that, they are difficulty degraded because of the lack of dissolution of these compounds in water as well as the lack of biological availability. Similarly, the alkanes with branched chain as well as cyclic chain are biodegraded much slowly than the symmetric n-alkanes. On the other hand, highly condensed aromatic, tars, cycloparaffinic structures, asphaltic materials, and bitumen have higher boiling point, and they give the highest reluctance to the process of degradation by microorganism. Thus, it was proposed that such remaining materials from the biodegradation of petroleum are somewhat similar to humic materials (Brassington et al. 2007). It is important to mention here that the degradation rate of petroleum hydrocarbon substances by microbes depends on the solubility of these substances in the hosting medium. Thus, biodegradation rate of the petroleum hydrocarbon substances, extremely solubilized in the aqueous medium, is nearly proportionate to their concentricity. The reverse is true for the less aqueous hydrocarbons. Generally, petroleum content in the medium higher than 5% causes a noticeable reduce in the action of the microbes. Moreover, that rise concentricity of oil can be hindered carbon: nitrogen: phosphorus proportion and make restrictions on oxygen consumption (El-Tarabily 2002).

15.8.9 Availability of Dissolved Oxygen

The availability of dissolved oxygen is primarily essential to microorganism for respiration. Dissolved molecular oxygen is significantly utilized during all over pathways of the biodegradation. Usually, it takes between 3 and 4 ml of dissolved oxygen for converting of 1 ml of petroleum hydrocarbon substances into CO_2 and water. That is a comparatively great goal to reach because of rise in the concentricity of hydrocarbon in petroleum as well as very limited concentricity of the dissolved oxygen. Surface water including seas, oceans, lakes, as well as harbors basically has unlimited supplies of oxygen because of the continuous interface of air and water as well as the activity of wind and waves. Thus, surface waters are saturated with dissolved oxygen (Abdel-Shafy et al. 1994). On the contrary, dissolved oxygen decreases by depth, and the increase of the deepness leads to limit the rate of the biodegradation. When the oxygen supply is depleted within deepest water and sediment, the hydrocarbon substance biodegradation takes place without oxygen, anaerobically. Thus, petroleum hydrocarbon substances dispersing and reaching to the deepest seas and oceans, generally covered by sediment, take

very long time to be degraded by microorganisms. Certainly, the renewal of oxygen is hampered by heavy and large puddle of petroleum on the top of water surface because of blanketing contact interface of air and water. Generally, that issue is most probably to take place at harbors, quagmires, as well as the bays. These surface waters depend on the cleaning procedure supported by the tidal operation. In this respect, removal of petroleum mechanically should be employed in order to rise interfacial borders of air which essential in oxygen renewal. Oxygen availability for soil environments depends on soil sort, moisture quantity, as well as the biodegradation rate which have been happening. Because of the waves and the tidal operations that lead to renewal of aeration, the oxygen is becoming highly available and the water is becoming saturated at the surface of the shore. Also, availability of oxygen be great beside jetties, retaining walls, and pier frame opposed to the crashing waves. On the contrary, oxygen be limited in the soil having smooth granules, the shore forehead having few or nothing of the tidal operations, as well as the soil with the high depth. Thus, it has been seen that oxygen is the controlling factor of biological degradation rate of the hydrocarbon substances at deepen soil as well as groundwater. In order to reduce such main issue, the mechanical aeration should be provided to the groundwater or deep soil. Thus, oxygen is provided simply via aerating the water, pushing oxygen to a set of tubes that is concealed and punctured, injecting hydrogen peroxide, soil venting, air sparging, and providing the necessary amounts of oxygen (Pathak and Jaroli 2014).

It is well known that oxygen is among the essential and the main factors in the degradation of organic load by microorganisms. However, the utilized concentricity of oxygen is mainly depending on the chosen microorganism utilized in the environment. It has been reported that for the aerobic bacteria, stoichiometric, 3.1 mg per 1 ml of oxygen has been consumed in the biodegradation of 1 mg per 1 ml petroleum hydrocarbon substances excluding the entire mass of available aerobic bacteria (Das and Chandran 2011). Thus, the required oxygen has been changed by rising or reducing of the bacteria mass. Nevertheless, anaerobic bacteria have also confirmed their significant role in the biodegradation. Several various sorts of bacteria were examined and observed that they are very useful in degrading hydrocarbon compounds in freshwater, marine environments, and soil (Das and Chandran 2011). These hydrocarbon compounds include benzene, toluene, alkanes, etc.

15.8.10 Availability of Nutrients

It is confirmed that the organic contents, at the locations polluted by petroleum, are observed to be extremely great due to the fixed introducing to hydrocarbon pollutants. Due to great numbers of carbon in hydrocarbons as well as very little amount of other nutrient elements necessary for growth of microorganisms, rate

and value of biodegradation have been, thus, restrained via little accessibility to such nutritive elements, namely, phosphorus and nitrogen. Accordingly, the outgrowth of the bacteria that degrade hydrocarbon substances were highly promoted via the input of nitrogen as well as phosphorus for hydrocarbon degradation. For the remediation, the carbon: nitrogen: phosphorus ratio should be conserved at 120:10:1 (Rodriguez-Martinez 2006). The sorts as well as the amounts of the nutritive elements, existed within the degradation process, played the very significant part in reducing the hydrocarbon biodegradation rate. Several researches showed that the insufficient supply of nutritive elements resulted as the lower rate of hydrocarbon substance biodegradation. It is confirmed that the capability of microbial growth in the environmental mediums relies greatly on the capability of microorganism in profiting the accessible nutritive elements. The aerobic microbes profited from different sorts of nutritive elements such as phosphorus, nitrogen, as well as traces of potassium, calcium, sulfur, magnesium, iron, and manganese as micronutrients. Mainly, nitrogen and phosphorus classified as necessary nutritive elements, because of the deficiency of these two elements in the petroleum; the rate of the naturally occurring biodegradation will be certainly hindered. For example, seawater has predominantly lack of these two main nutrient elements because of their consumption by the microbes that are not degrading the petroleum rather than the microbes that are degrading petroleum. In addition, phosphorus converted to calcium phosphate precipitates in the sea-water environment (Tulevaa et al. 2005). It was stated that the amount of N and P of seawater varies from (0.1 to 1 mg/l) and (0 to 0.07 mg/l), respectively, depending on seasonal temperatures (Abdel-Shafy 1992). To compensate for such deficiency of these nutritive elements, fertilizing was utilized. Upgrading the soil by such process greatly enhances the nutrition of microbes, thus promoting their growing. The quantity, needed to biodegrade a nominated amount of petroleum hydrocarbons, is not so far fully known, although this point was studied (Pathak and Jaroli 2014).

15.9 Mechanism of Hydrocarbon Substance Degradation by Microorganisms

Generally, the biodegradation, that occur very fast and completely for the majority of organic contaminants, was achieved in the aerobic restraint. Figure 15.3 exhibits the major precept of the aerobic biodegradation of hydrocarbon substances (Fritsche and Hofrichter 2000). The first intracellular crack of organic contaminants in the environment was via an oxidation operation. The environmental paths of biodegradation of hydrocarbons transform such organic contaminants gradually to fragments through the centrally mediated metabolism such as the cycle of tricarboxylic acid. The biological synthesis of the cell mass occurs, primarily, from the metabolism of the centric precursors, for example, acetyl-CoA, pyruvate, and succinate. In

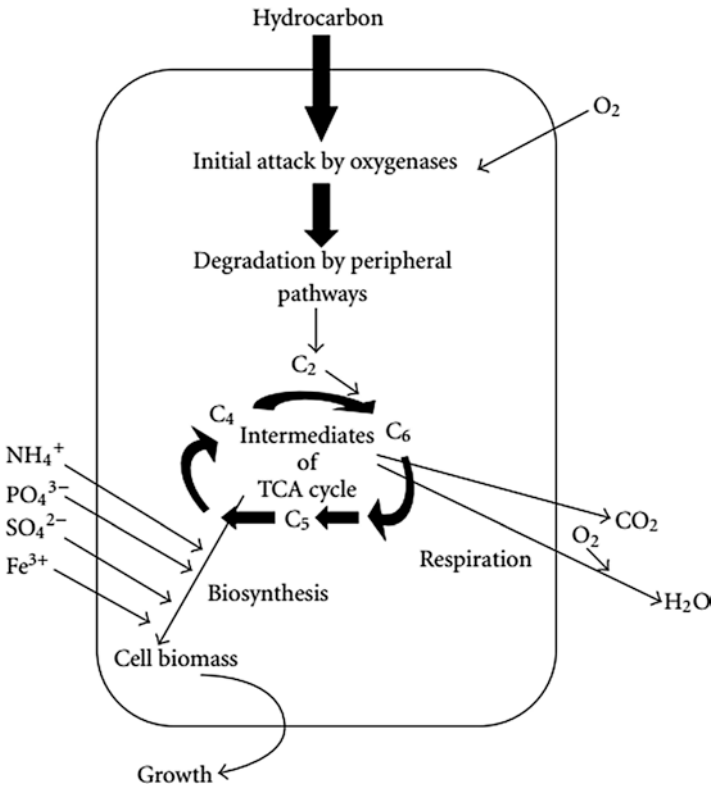

Fig. 15.3 Principle of aerobic biodegradation of hydrocarbons. (Fritsche and Hofrichter 2000)

addition, sugars, needed in different biological synthesis, are mainly synthesized by gluconeogenesis process (Fritsche and Hofrichter 2000).

15.10 Role of Enzymatic Reaction in Petroleum Hydrocarbon Biodegradation

It is imperative to mention that the biological degradation of petroleum hydrocarbon substances is able to mediate principally via particular enzymatic arrangement. Figure 15.4 exhibits the first crack of xenobiotic by oxygenase according to Fritsche and Hofrichter (2000). Additional mechanisms involved in this biodegradation were as follows:

- Linking the cells of microbes to the substrates
- Producing the bio-surfactants

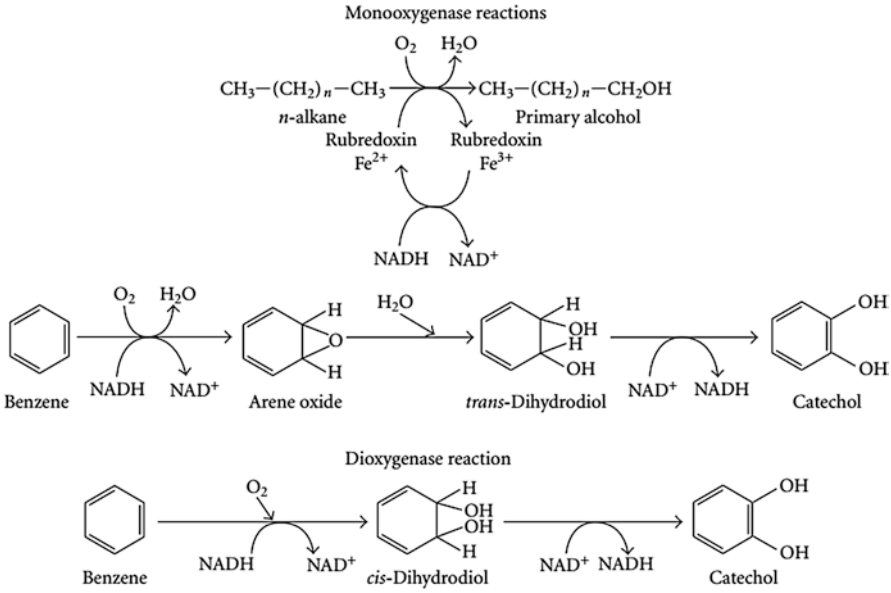

Fig. 15.4 Role of the enzymatic reactions and involvement in the processes of hydrocarbon biodegradation (Fritsche and Hofrichter 2000)

However, the degradation mechanism, related to the linking of cell with petroleum hydrocarbon droplet, is as yet unbeknown. Nevertheless, production of biosurfactants was fully investigated by Das and Chandran (2011).

It was reported by Van Beilen and Funhoff (2007) that cytochrome and alkane hydroxylase P450 are comprised of a superlative family of universal heme-thiolate monooxygenases. This family plays a vital role in biodegradation of petro-products fuel additives, chlorinated hydrocarbon substances, as well as several other substances. The process of oil biodegradation relies on enzyme efficiency, petroproduct chain length, and oxygen presence. Principally, such diversity of P450 was only detected in a few microbes. Furthermore, the arrangements for cytochrome P450 enzyme participated in the degradation of hydrocarbon substances (Van Beilen and Funhoff 2007). In this respect, the ability of many yeast types to utilize n-alkanes as well as other aliphatic hydrocarbon compounds, as energy and carbon source, mediated via the presence of various forms of cytochrome P450 for microsomes. Such enzymes of cytochrome P450 had been isolated from yeast, such as *Candida apicola*, *C. tropicalis*, as well as *C. maltosa*. Variety of systems, from alkane oxygenase in prokaryotes as well as eukaryotes, was examined by Van Beilen and Funhoff (2005). They mentioned that these alkane oxygenase systems were efficiently involved in the biodegradation of alkanes, under oxygenic conditions, such as the integration of membrane of di-iron alkane hydroxylases, enzymes

of cytochrome P450, solubilized monooxygenases of di-iron methane, as well as membrane of bounded copper-contained monooxygenases of methane (Van Beilen and Funhoff 2005).

15.11 Biodegradation of Petroleum Hydrocarbons by Bio-surfactants

According to Kiran et al. (2009), the bio-surfactants, a heterogeneous series of substances with chemically active surface, are generated by a broad diversity of microbes. These surfactant substances promoted mobility, solubility, as well as remediation of pollutants. In addition, the biodegradations are promoted by the action of bio-surfactants, mainly due to the increase of the biological availability of the contaminants. A successful biodegradation of petroleum sludge was achieved via using bio-surfactants (Cameotra and Singh 2008). In this investigation, a set of microbes, composing from *Pseudomonas aeruginosa* and *Rhodococcus erythropolis* isolated from polluted soil by petroleum sludge, had been employed. Such set of microbes have capability for degrading over 90% of hydrocarbon substances within only 6 weeks when cultured in aqueous. As the capability of such microbes, for degrading the sludge of hydrocarbon substances, had been examined by two field experiments, separately (Cameotra and Singh 2008). Furthermore, the effectiveness of biodegradation had been also investigated under the presence of two different factors, namely, a nutritive element blend and a petroleum bio-surfactant synthesis (Cameotra and Singh 2008). The employed bio-surfactant had been generated by an organ of microbe set that classified to be a blend of 11 congeners of the rhamnolipid. This set of microbes was able to degrade over 91% of petroleum hydrocarbon compounds in a polluted soil by petroleum sludge as 1% (v/v) within only 5 weeks. To enhance the removal rate, the separately utilizing, of any added ingredient along to the set of microbes, leads to from a 91 to 95% elimination of the amount of petroleum hydrocarbon substances within only 4 weeks, which was achieved by the crude bio-surfactant preparation as becoming a much efficient promotor for hydrocarbon biodegradation. Nevertheless, over 98% petroleum hydrocarbon substances removal was gained, while both added ingredients were blended together along to the studied set of microbes. Furthermore, results obtained demonstrated the efficient utilization of a petroleum bio-surfactant in the environment for hydrocarbon remediation. According to Pornsunthorntawee et al. (2008), *Pseudomonas* were the popular bacteria that have ability to utilize petroleum hydrocarbon substances to be a source of energy and carbon and also produce the bio-surfactants. *Pseudomonas* and *Pseudomonas aeruginosa* were also exceedingly investigated to produce the bio-surfactants of the glycolipid kind. Nevertheless, bio-surfactants of glycolipid kind were produced from several other microbes including *Pseudomonas chlororaphis* and *Pseudomonas putida*. The importance of the bio-surfactants is the rise of the surface area of petroleum; thus, the availability of

petroleum utilized by bacteria increased (Nikolopoulou and Kalogerakis 2009). Bio-surfactants, actually, have the ability to be strong emulsifiers which reduces the surface tension of the hydrocarbons as well as producing micelles. Thus, micro-droplets, enclosed on the hydrophobic cell surface of the microbes, grasped inside them and then degraded. Figure 15.5 exhibits the participation of rhamnolipid bio-surfactant generated by *Pseudomonas* sp., as well as the mechanism of micelles forming during the degradation of petroleum hydrocarbon substances (Fritsche and Hofrichter 2000). In addition to the things mentioned before, the Table 15.1 also describes the bio-surfactant production by numerous microorganisms (Das and Chandran 2011).

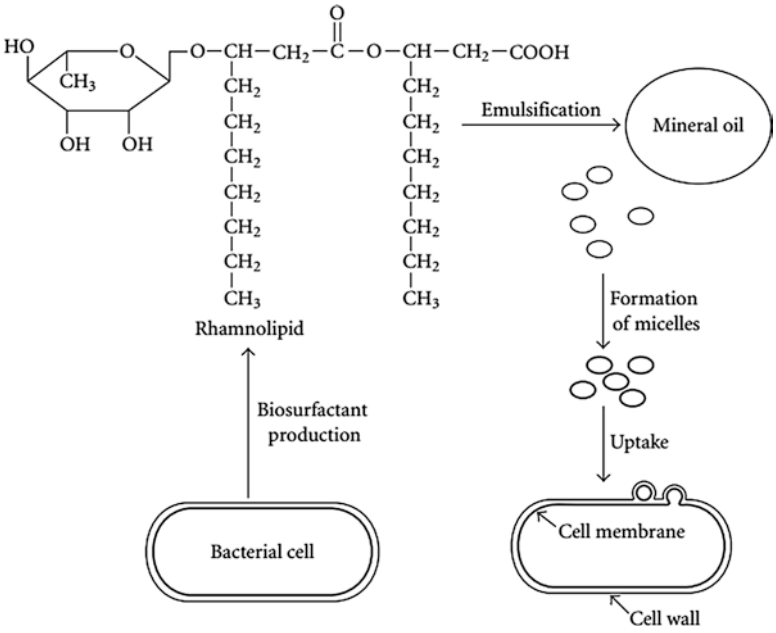

Fig. 15.5 Participating and demonstration of bio-surfactant (rhamnolipid) generated by *Pseudomonas* sp. during the degradation of petroleum hydrocarbon substances. (Fritsche and Hofrichter 2000)

Table 15.1 Bio-surfactants that are produced by numerous microorganisms

Bio-surfactants	Microbes	References
Sophorolipids	*Candida bombicola*	Daverey and Pakshirajan (2009)
Rhamnolipids	*Pseudomonas aeruginosa*	Kumar et al. (2008)
Lipomannan	*Candida tropicalis*	Muthuswamy et al. (2008)
Rhamnolipids	*Pseudomonas fluorescens*	Mahmound et al. (2008)
Surfactin	Bacillus subtilis	Youssef et al. (2007)
Glycolipid	*Aeromonas* spp.	Ilori et al. (2005)
Glycolipid	*Bacillus* sp.	Tabatabaee et al. (2005)

Das and Chandran (2011)

15.12 Degradation of Petroleum Hydrocarbon Substances via Cell Immobilization

Cell immobilization had been studied as well as examined for degrading various poisonous chemical substances. In the immobilization of cell, the targeted cell be immobilized in contrast with the enzyme immobilization where the enzyme attached to a solid prop such as calcium alginate or activated poly(vinyl alcohol) (PVA) or activated polyethylene imine (PEI). Furthermore, the immobilized whole cell system is feasible and reclaimable, so it be a cost-effective process. Diaz et al. (2002) documented that immobilized cells of bacteria promote the biodegradation rate of the petroleum more than liberated alive cells within a broad domain of saltiness. Immobilizing for whole cell is batching and continuing systems. Also, in the continuing system, the packed bed reactor is generally employed in biodegrade of petroleum hydrocarbon substances. Cunningham et al. (2004) investigated the use of PVA cryogelation, physically forming of hydrogel, be a trap medium in addition of the microorganisms indigenous to the site. Their study included constructing laboratory bio-piles to compare immobilized bio-augmentation with liquid culture bio-augmentation as well as bio-stimulation. The employed immobilized systems were very effective for degrading of diesel during 5 weeks. An experiment was proceeded by Rahman et al. (2006), in order to examine the capability of bacterial cells immobilized at alginate beads for biodegradation of petroleum hydrocarbon substances. Furthermore, outcomes obtained demonstrated that there was no reduction in the degradation efficiency by the selected set of microbes under frequent reuse. Thus, it was deduced the microbial cells immobilization is an assuring applied method for bioremediation of sites that contaminated by petroleum hydrocarbon substances.

15.13 Aerobic Biodegradation of Petroleum Hydrocarbons

Petroleum hydrocarbon substances were easily biodegraded under aerobic conditions. Algae, bacteria, as well as fungi were all capable of aerobic hydrocarbon biodegradation (Haritash and Kaushik 2009). Generally, hydrocarbons having double bonds (alkenes and alkanes) and the short carbon chain alkanes (hydrocarbons having single bond only) are the hydrocarbons which are easily biodegraded, followed by alkanes and lastly the aromatic hydrocarbons (Xue et al. 2015). Nevertheless, the rate of biodegradation varies widely based on numerous ecological parameters, and rate of biodegradation decreases as complexity in hydrocarbon increases. It has been reported that rate of biodegradation varies significantly owing to the reasons that composition of oil relies mainly on the petroleum sources and oil spill age. For instance, hydrocarbon biodegradation rate varies from 5% to 30%, during 28 days. After addition of nitrogen source, up to 100% biodegradation was

observed, since nitrogen fulfilled the nutritious requirement of some microbes (Röling et al. 2002). By employing potential fungal species, the rate of biodegradation ranged from ~ 30 to 100%, and this was achieved within 28 days. As discussed previously, oxygen is the main controlling factor in aerobic biological degradation. The availability of oxygen mainly relies on its ability to diffuse or move toward the environmental site and also available to the microbes, which are supposed to carry out bioremediation process. Meanwhile, the oxygen in addition to the environment can upsurge rate of biodegradation of many folds as compared to degradation occurring in less or no oxygen conditions (Zafra and Cortés-Espinosa 2015).

15.13.1 Aerobic Biodegradation of Alkenes and Alkanes

The alkanes having 14 carbons or less are easily volatilized, and those alkanes which contain carbon more than 14 are less volatile in nature (Chikere et al. 2011). Irrespective of alkenes and alkanes and with exception of alkanes having ring structure are hydrocarbons, which are easily biodegraded; it also includes biodegradation of 44 carbon containing alkanes (Abbasian et al. 2015). After addition of oxygen, both alkenes and alkanes are reasonably degraded. Rate of biodegradation depends upon the availability of oxygen. It was reported by Atlas and Philp (2005) that oxygenase enzyme provides molecular O_2 to petro-hydrocarbons and results in alcohol formation. The latter is finally degraded to carbon dioxide and water molecules via fatty acid formation, which consequently converted into acetyl-CoA (Abbasian et al. 2015).

15.13.2 Aerobic Biodegradation of Aromatic Hydrocarbons

It is well documented that aromatic hydrocarbons are more difficult to biodegradation process compared to short chain alkanes and alkenes owing to their higher toxic nature. However, they are easily biodegraded by many microorganisms' mainly bacterial and fungal species under aerobic conditions (Abdel-Shafy and Mansour 2016). Degradability of aromatic hydrocarbons decreased with increased molecular size and ring numbers. This is mainly because of augmented sorption capability and hydrophobicity (Chikere et al. 2011). The rate of degradation of toluene, ethylbenzene, benzene, and xylene (also BTEX) varies from 0.05 to 0.2 day^{-1} (Lawrence 2006). To degrade 1 mg/L of BTEX by oxidation, 3.1 mg/L dissolved oxygen (DO) is required. If the dissolved oxygen is less than 2 mg/L, biological degradation of BTEX slows down (Lawrence 2006). Generally, the pathway of the aromatic compound degradation commences with adding oxygen by enzymes like mono- and

dioxygenases (Baboshin and Golovleva 2012). This reaction results in important intermediary products, including phenol, benzyl alcohol, gentisate, protocatechuate, and catechol (Fuchs et al. 2011). According to Vaillancourt et al. (2006), these intermediate products lead to cleavage in rings, by different types of oxygenase enzymes, which results in production of carboxylic acid. The biodegradation process, thus, continues to form acetyl-CoA and succinyl-CoA that finally enter into the process of pivotal metabolism (Fuchs et al. 2011). Fungal biodegradation occurs by non-specific extracellular oxidizing enzymes that form radical intermediates. Several of these chemical reactions are alike to that performed by bacterial biodegradation (Haritash and Kaushik 2009).

15.14 Anaerobic Biodegradation of Petroleum Hydrocarbons

The biodegradation of hydrocarbon under aerobic situations is often faster as compared to the anaerobic biodegradation. This is mainly owing to less positive reactions energetic with alternate electrons acceptor. Nevertheless, both anaerobic and facultative archaea and bacteria are widely recognized because of their ability to biodegrade petroleum hydrocarbon substances exclusive of the oxygen presence. Such anaerobic microorganisms readily develop at petro-hydrocarbon spilled locations due to and resulted from the quick oxygen consumption. During anoxic petro-hydrocarbon biodegradation, the initial steps that involve the addition of oxidized functional groups to trigger the hydrocarbon molecules are limited. Normally the duration of anaerobic hydrocarbon biodegradation takes from few days to several months (Meckenstock and Mouttaki 2011). Despite such sluggish growth rate, an absolute biodegradation of variable kinds of petro-hydrocarbons happens without oxygen presence. For instance, biodegradation of PAHs took a long time, i.e., more than 90 days after inoculation. On the other hand, benzene biodegradation occurred within 120 weeks (Meckenstock et al. 2016). That have been mentioned by Jiménez et al. (2016) use of methanogens as biodegrading agents, resulted in degradation of linear alkanes within 7 months. The anaerobic microorganisms, generally, employ terminal electron acceptor in respiration process other than oxygen. Compounds like S, No_3, and CO_2 oxidized metals and also some organic complexes. Nevertheless, at a polluted location, these microorganisms incline to consume electron acceptor as in the following sequence: O_2, SO_4, Fe^{+++}, No_3, and hydrogen (Fig. 15.6) (Abbasian et al. 2015). In some cases, however, particular bacterial species of sulfate reducing or denitrifying microbes have been demonstrated to metabolize specific petro-hydrocarbon compounds entirely to water CO_2 gas. Nevertheless, it has been observed that syntrophy process has been found in anaerobic biodegradation of petro-hydrocarbons. Under anaerobic situations, the process of syntrophy is generally normal; here the O_2 utilization as terminal electron receptor was highly energetic and appropriate according to Morris et al. (2013).

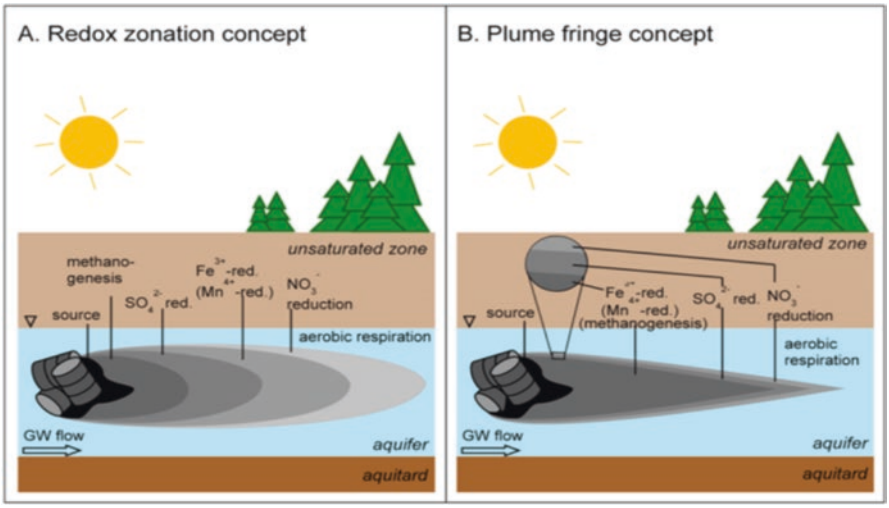

Fig. 15.6 Comparison between two concepts: (**a**) longitudinal redox zonation, (**b**) plume fringe. (Meckenstock et al. 2015)

The two concepts describe location apportionment for the receptors of electrons as well as aeration operation inside a hydrocarbon pollution plume. In the plume fringe, the following takes place inside the pulp of the hydrocarbon pollution plume (B): reduction of iron (III) trivalent, reduction of manganese (IV) tetravalent, as well as probability of simultaneous occurrence of methanogenesis (Meckenstock et al. 2015).

The process of syntrophy is completely essential for comprehensive anaerobic biodegradation to CH_4 and CO_2 gas. The methanogenic bacteria prefer to utilize easy substances such as hydrogen and acetate. In any given ecosystem, there are always a manifold syntrophy associations present. This association is established only on accessible substances and environmental surroundings (Fig. 15.7). According to Gieg et al. (2014), the anaerobic microorganisms use varied approaches to utilize petro-hydrocarbons minus air or molecular oxygen. The diverse strategy employed by microbes is simply separately discussed below. Each strategy is pertinent to both aromatic and aliphatic petro-hydrocarbon substances. The overall approach is to insert a molecule of oxygen into the hydrocarbon structure to so that it is easily consumed by potential microbes and the products are entered pathways of central metabolism. According to Gieg et al. (2014), most of the aromatic complexes are stimulated and channeled in the direction of the central anaerobic intermediates, such as benzoates, benzoyl-CoA, and coenzyme A.

In reactions 1, 2, and 3, hydrogen and acetate are utilized; this keeps the fermentation reactiondynamically favorable. When NO_3, SO_4, and Fe (the external electron acceptor) are not accessible, the methanogenic bacteria utilize hydrogen and consume acetate (Figure adapted from Gieg et al. 2014).

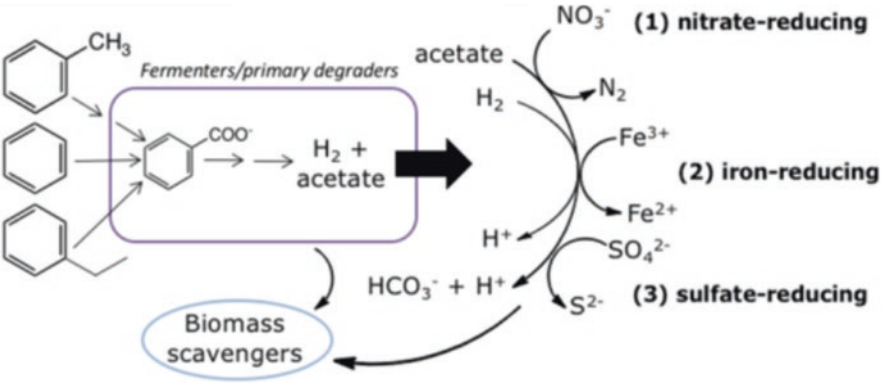

Fig. 15.7 Theoretical model of syntrophic anaerobic biodegradation of alkylbenzenes and benzene

15.15 Activation Methods for Anaerobic Biodegradation of Petroleum Hydrocarbons

Figure 15.8 exhibits the major procedures of activation within PAHs degradation. First step of this process, of the aerobically catabolism pathways for PAHs degradation, includes the ring-hydroxylating dioxygenases enzyme (RHD). Generally, in the anaerobic process of catabolism pathways for PAHs degradation, the steps of activation contain the following:

- Methylation via methyltransferases, a great set of enzymes that methylate their substrates
- Addition of succinate synthase, enzyme that contain a glycyl radical, to fumarate
- Carboxylation via carboxylase, enzyme that allow the production of new carbon-carbon bonds via inserting HCO_3- or CO_2 to the targeted substances

The pathways of metabolism were extensively reported by several investigators (Lu et al. 2011; Meckenstock and Mouttaki 2011; Heider and Schuhle 2013).

15.15.1 Fumarate Addition

The addition of fumarate by bacteria is to trigger alkanes and also alkyl-substituted aromatic hydrocarbons including methylnaphthalene, toluene, or xylene (Abbasian et al. 2015). Fumarate is a very usual cell metabolic substrate containing two groups of carboxylic acids and also contains double bonds. The double bonds in fumarate are added by subterminal or terminal methyl groups of alkylbenzene or alkane.

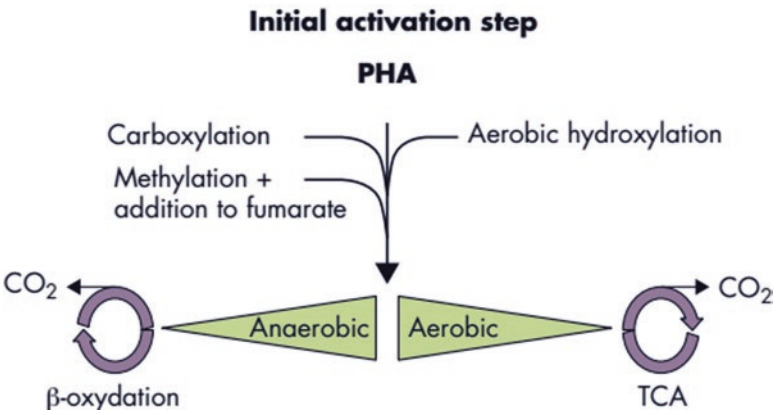

Fig. 15.8 Main activation processes in PAH's degradation. (Duran and Cravo-Laureau 2016)

In case of alkanes, this process was initiated using alkyl succinate synthase enzyme which leads in formation of transitional products such as 2-(1-methyl alkyl) succinate or 2-alkyl succinate. These abovementioned products are later biodegraded through reorganization of carbon, β-oxidation, and decarboxylation (Bian et al. 2015). The aromatic petro-hydrocarbons biodegraded by addition of fumarate include 2-methylnaphthalene, toluene, and xylene (Fuchs et al. 2011). This progression is, generally, carried out by benzyl succinate synthase enzyme. There are several bacteria, which require anaerobic conditions, such as nitrate and sulfate reducer bacterial strains, which alter toluene easily into to (R)-benzyl succinate via fumarate addition (Abbasian et al. 2015).

15.15.2 Oxygen-Independent Hydroxylation

According to several investigators, hydroxylation process, which does not depend on oxygen, can denitrify by those bacterial species which can metabolize ethylbenzene (Heider 2007; Fuchs et al. 2011). In such pathway, the hydroxyl group (-OH) is added to carbon 1 on the side chain by enzyme ethylbenzene dehydrogenase to form S-1 phenyl ethanol. This is later converted into acetophenone and later alteration to benzoyl – CoA and acetyl – CoA (Heider 2007). The first step of this pathway is to catalyze such contaminants by a molybdenum cofactor-containing hydroxylase (Boll et al. 2014). Flavocytochrome c hydrolase enzymes have also been implicated in oxidation of alkyl side chain oxidation to form their resembling alcohol molecules (Boll et al. 2014). It was reported by Callaghan (2013) that a typical hydroxylation mechanism has been suggested to act on subterminal carbons of alkane to produce alcohol molecule which is more vulnerable to oxidation process.

15.15.3 Carboxylation

To date, all discussed mechanisms apply to petro-hydrocarbons having alkyl group and do not apply naphthalene and benzene which are to unsubstituted aromatic petro-hydrocarbons. It is true that biodegradation of benzene is much slower under anaerobic conditions compared to that of xylenes or toluene (Lawrence 2006). Carboxylation is an important biodegradation mechanism for naphthalene and also hypothesized for benzene biodegradation under anaerobic conditions. In such course, carbon dioxide is directly added to the aromatic and aliphatic hydrocarbon substances (Abbasian et al. 2015). Such procedure is supposed to be to some degree similar to anaerobic phenol biodegradation mechanism; here phenol is first activated by exploiting ATP energy to phosphor-phenol before adding carboxyl acid to para-hydroxy-benzoate. Carboxylase enzymes are responsible for catalyzing the reactions that add a COOH group to their substrates (Meckenstock et al. 2016). Under reducing conditions of nitrate and iron, benzene activation was thought to occur by the process of carboxylation to benzoate (Meckenstock et al. 2016). Activation of benzene ring is rather problematic owing to mainly ring stability as well as the equally high detachment energy (Luo et al. 2014). It was mentioned by Mouttaki et al. (2012) that carboxylation of naphthalene has been established in raw cell extract. According to Boll et al. (2014) in general aromatic metabolism, aromatic ring carboxylase active sites were supposed to be analogous to UbiD carboxylases family.

15.16 Conclusion

Degradation, for petroleum hydrocarbons as well as other related pollutants by microorganisms within the ecosystem, is a complicated operation. The conversions of these pollutants quantitatively and qualitatively are based on origin as well as amount of the existed petroleum hydrocarbons and the variation of environment ambient and seasonally. This includes dissolved oxygen, temperature, and physical and/or chemical dispersion of oil.

Biodegradation of hydrocarbons is an alternate process of detoxication and removal of pollutants. Variable sets of microbes are participated in the hydrocarbon biodegradation operation. Generally, the microorganisms produce enzymes that attack the hydrocarbon molecule.

A part of bacteria as well as fungi, with white rot, offer an important role for the degradation of PAHs. Fungi contain essential enzymes such as peroxidases for lignin and manganese that have ability to transform and degrade the PAHs to the low-risk materials. Meanwhile, many set of algae own the ability for degrading a broad domain of PAHs. On the other hand, protozoa are not immediately participated in the operation of petroleum hydrocarbon degradation. Protozoa can influence the rate of this biodegradation. However, hydrocarbon biodegradation occurs more

rapidly under oxygenic conditions rather than anaerobic conditions. Oxygen, pH, moisture, and the most important nutrient concentration are the controlling and predominant factors in determining the rate of biodegradation in soil.

The primary concern in situ aerobic biodegradation is the presence of oxygen as well as mixing. This may be attained by several recognized approaches, including peroxide addition, sparging, and recirculation of groundwater. Nevertheless, aerobic biodegradation is not feasible and/or effective in all environmental conditions, especially soils with low permeability. In such circumstances, anaerobic biodegradation is favored for biodegradation. Significant thoughts for hydrocarbon biological degradation (light or heavy hydrocarbons) include the nature of hydrocarbons, microorganism population structure, availability of nutrients, pH, temperature, and permeability of soils. Thus, nutrients' addition, namely, N, P, and Fe, was very useful for microorganism community. Addition of actively degrading microbial cultures and the nutrient elements can certainly accelerate the biodegradation. This can help in the degradation of particularly recalcitrant hydrocarbons, namely, PAHs as well as benzene. Site conditions, at that respect, must first be assessed to predict as well as to ensure the microbial effectiveness for biodegradation. In addition, understanding the pathways of biodegradation and potential microorganisms is very important to (a) measure and enhance the hydrocarbon biodegradation and (b) track the rate of biological degradation location by observing functional gene biomarkers as well as the degradation intermediates.

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Chapter 16
Plant-Microbe Association for Bioremediation of Hydrocarbon Substrates

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Abstract Advancement in the standards of life quality along with awareness for environmental issues and the remediation of contaminated sites has attracted attention from society. Due to high cost of mechanical and chemical techniques for hydrocarbon remediation, the utilization of biological processes is gaining considerable attention. Plant-microbe association has been widely studied during the second half of the last century yet focusing on pathogenicity and plant-saprophytic associations like nitrogen fixation, improving soil nutrient cycles and plant growth, etc. However during the last decade, the emphasis has been shifted upon microbial communication with plants for remediation of hydrocarbon-contaminated sites. The efficacy of the remediation process mainly depends on the availability and performance of microbes having degradation genes responsible for enzymatic breakdown of organic contaminants as well as chemotaxis for hydrocarbons, biofilm production, cell surface hydrophobicity, and ability to produce biosurfactants. The rhizosphere and apoplast of the plants have been testified as the potential dwellings for microbes having degradation genes, but comparatively petite information is available about the degradation activities and metabolic pathways of endophytes. Diversity of biological systems warrants deep understanding of the mechanisms involved for utilizing plant-microbe association for bioremediation of hydrocarbons. This chapter focuses on an insight of the existing biological approaches for bioremediation of hydrocarbon-contaminated sites with emphasis upon required advancements in bioremediation and phytoremediation strategies to improve efficiency.

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16.1 Introduction

Carbon and hydrogen atoms constitute the backbone of organic compounds. The diversity of the organic world is based upon the variety in physical configuration and chemical properties of these atoms. They range from small-chain molecules and monoaromatics to long-chain compounds and polycyclic aromatic hydrocarbons (Kamath et al. 2004; Wang et al. 2016). Naturally these are the constituents of fossil fuels formed from the dead organisms buried millions of years ago. To meet the increasing energy demand for industrialization and transportation, exploitation of these natural resources is need of the time. However this extensive exploitation brings the problem of hydrocarbon contamination in biotic as well as abiotic spheres of life. Some of the known effects of long-term exposure to hydrocarbons include damage to the nervous system, respiratory system, and endocrine system (Locksley 2010). Therefore, remediation of contaminated sites is of prime importance.

Various treatment technologies in practice are physical removal of the top soil, thermal desorption, dilution, off-site treatment, controlled incineration, washing, advanced oxidation process, and photocatalysis, among others. However, these treatment options are associated with either cost-effectiveness issues or formation of secondary more harmful by-products/incomplete degradation (Xue et al. 2015). It is need of the hour to find eco-friendly and sustainable treatment options. For this purpose scientists are now focusing more on the biological treatment methods especially plants in association with microbiota. This theme requires extensive research to investigate the mechanism involved, and this is the main debate among the scientific community since the last two decades (Scoma et al. 2016). In this chapter, we will discuss the plant-bacterial association for bioremediation of hydrocarbons.

"Bioremediation" is a biological process of degrading, detoxifying, or transforming a harmful contaminant in the environment. The process involves biological machinery utilizing the natural processes for remediation of contaminated sites. Microorganisms utilize these toxic compounds as a source of carbon and energy and break down/detoxify them into less harmful ones (Azubuike et al. 2016). Bioremediation strategy has been extensively utilized for remediation of hydrocarbons from terrestrial as well as aquatic ecosystems (Almeida et al. 2013; Xue et al. 2015; Scoma et al. 2016; Wang et al. 2016; Azubuike et al. 2016). Bioremediation is sensitive process and is governed by various factors as summarized in Table 16.1.

Bioavailability (the amount of the contaminant freely available for degradation) of the petroleum hydrocarbon suggests the rate of biodegradation as proposed by Hamdi et al. (2007). It can be determined by chemically extracting the contaminant as well as biologically exposing the contaminant (Harmsen 2007). However, the bioavailability cannot always be linked with the bioremediation efficiency due to diverse genetic makeup and gene expression (Huesemann et al. 2004).

Bioavailability also depends upon hydrophobicity and movement of the contaminant across the microbial cell membrane. Microbial cell physiology suggests the mode of contaminant uptake. Abbasnezhad and team reported that cell physiology plays a very important role in biodegradation. If the microbial cell is efficient

Table 16.1 Factors affecting the bioremediation process

Factor category	Processes and activities
Biotic factors	Genetic makeup (gene regulation and expression)
	Metabolic capability (diversity and flexibility)
	Interaction with the contaminant (physicochemical properties for biodegradation)
	Interaction with other chemical compounds present in the system
	Interaction with other biotic life in the system (biofilms, chemotaxis)
	Toxicity profile
	Uptake mechanism
Abiotic factors	Contaminant concentration/toxicity profile
	Physical properties of the contaminant (molecular structure, physical state, hydrophobicity, etc.)
	Chemical properties of the contaminant (solubility, volatility, etc.)
	Bioavailability/interaction with soil or water medium
Environmental factors	pH
	Nutrient availability
	Availability of terminal electron acceptor groups
	Temperature profile
	Pressure
	Salinity

enough to adhere to the hydrophobic surface, it will positively affect growth and biodegradation process even for poorly water-soluble hydrocarbons (Abbasnezhad et al. 2011). However, Tzintzun-Camacho and coworkers revealed that microbes possessing the degradation capability can also regulate their cell surface hydrophobicity when grown on hydrocarbons (Tzintzun-Camacho et al. 2012).

Various bacterial and fungal groups are capable of synthesizing organics (amphiphilic, both water- and fat-loving compounds) utilizing their cell surface activity. These compounds are further classified on the basis of their molecular weight. The low molecular weight compounds (that decrease surface tension) include glycolipids, proteins, and lipopeptides which are termed as biosurfactants (Nguyen and Sabatini 2011; Banat et al. 2014; Dobler et al. 2016; Santos et al. 2016), while the high molecular weight compounds (that counterbalance oil in water suspension) include lipoproteins, polymers of polysaccharides, and lipopolysaccharides which are termed as bio-emulsifiers (Uzoigwe et al. 2015). Biosurfactants are suggested to enhance microbial growth on hydrocarbon contaminants by enhancing surface area or improving solubility among the soil micelles. Studies suggest increased hydrocarbon bioavailability upon biosurfactant application (Das et al. 2008; Das and Chandran 2010; Bordoloi and Konwa 2009; Pacwa-Plociniczak et al. 2011; Lawniczak et al. 2013).

Biofilms created by microbial communities also envelop themselves by producing polymeric structures. These structures can be considered as their evolved defense mechanism against harsh environmental conditions (Shemesh et al. 2010). It helps them to better degrade the hydrocarbons being in a well protective

environment by optimizing pH and solute concentration (Singh et al. 2006). Another important aspect which plays a key role in microbial degradation is chemotaxis. It is the intentional migration of microbes with reference to chemical gradient. The microbes move with the intent to find the conditions best suitable for growth and survival (Hazelbauer and Lai 2010; Krell et al. 2011). Chemotaxis has been reported as an important factor for biodegradation of hydrocarbons in terrestrial and aquatic ecosystems (Strobel et al. 2011) for sensing the particular organic compound and swim toward it like *n*-hexadecane (Nisenbaum et al. 2013), monocyclic aromatics, and polycyclic aromatic hydrocarbons (Xue et al. 2015; Scoma et al. 2016; Wang et al. 2016; Azubuike et al. 2016).

This adoptive strategy may permit the microbe to stabilize itself for hydrocarbon and to tolerate its toxicity. This can be positive (going toward) or negative (going away) chemotaxis from the chemical gradient (Azubuike et al. 2016). Hence, it is a regulatory mechanism that regulates the bioavailability by protecting from toxic effects. Sometimes chemotaxis genes are co-positioned with the genes responsible for degradation of hydrocarbons. Studies suggest that chemotaxis is a gene-dose-dependent phenomenon and microbial movement correlates with its metabolic pathway for hydrocarbon degradation (Smith et al. 2013).

16.2 Plant-Bacterial Associations

Rhizobacteria and endophytic bacteria have been reported for biodegradation capabilities of various hydrocarbons in contaminated soil and water. Rhizobacteria colonize in the rhizosphere and degrade the hydrocarbons present in the root zone, while endophytic bacteria degrade the contaminants being colonized in the internal tissues of plants and may reduce phytotoxicity and evapotranspiration of hydrocarbons (Germaine et al. 2009). Uptake of hydrocarbons and their accumulation in the plant body are comparatively less than plant-assisted biodegradation in the rhizosphere (Wang et al. 2012). Though hydrocarbons with low lipophilicity appear to be translocated to shoots via roots before the mineralization by rhizosphere bacteria, the hydrocarbons thus entered used to reside in the plant tissues for 2 days, and then endophytic bacteria mineralize them (Weyens et al. 2010).

16.2.1 Rhizobacteria and Hydrocarbon Degradation

Plant and bacterial association in terms of nitrogen fixation is well understood. Nitrogen-fixing bacteria in the plant roots fix the atmospheric nitrogen and provide them to the plants. Besides some bacteria have shown the nitrogen fixing as well as hydrocarbon degradation capabilities, and they could be an imperative option for hydrocarbon degradation in nitrogen-limited soils (Foght 2010). Apparently plants release organic compounds (enzymes, carbohydrates, organic acids, amino acids)

from their roots in the rhizosphere which increase soil microbial population. Many compounds released from the roots act as co-metabolite in the hydrocarbon mineralization by microbes or act as inducer for microbial genes responsible for hydrocarbon degradation (Toyama et al. 2011; Zhong et al. 2012).

Plant root exudates support the bacterial population having hydrocarbon degradation genes and improve their tolerance for higher concentration of hydrocarbons. Therefore improved phytoremediation of hydrocarbons might be due to the enhanced rhizosphere bacterial population, diversity, and activities (Germaine et al. 2009). Combinations of plant species and rhizosphere bacterial strains successful in hydrocarbon degradation are given in Table 16.2.

16.2.2 Endophytes for Hydrocarbon Degradation

After the first study by Siciliano et al. (2001) where endophytes were isolated from roots of the plants grown in hydrocarbon-contaminated soils, various studies have been carried out for isolation and characterization of endophytes from plants grown in hydrocarbon-contaminated soils. During these studies, it has been observed that concentration of hydrocarbons determines the population of hydrocarbon-degrading bacteria (Malfanova 2013). Most of the endophytic bacteria are also able to inhabit the rhizosphere. They may colonize in the plant body (roots, aerial parts) as well as in the rhizosphere. Their migration pathway from soil to the plant is estimated to be the transpiration stream (Germaine et al. 2009). Because of colonization of bacteria within the plant body, ample nutrients and space are available to endophytes as compared to rhizosphere bacteria, and they are also well protected from the changes in the physical environment (Schulz et al. 2012). And it has also been observed that certain rhizosphere bacteria are unable to degrade the extremely water-soluble hydrocarbons that may result in accumulation of the contaminants in the plant parts or to the atmosphere (Weyens et al. 2010).

During a recent study, it has been observed that endophytes were metabolically active in the entire body of plants grown in diesel-contaminated soil (Afzal et al. 2012). About 4% cultured endophytes were introduced into *Festuca arundinacea* and *Trifolium fragiferum* grown on an old hydrocarbon-contaminated soil by Siciliano et al. (2001). They observed that endophytes can transmit their hydrocarbon-degrading genes to the indigenous bacterial populations inhabiting the plant. Some endophytic bacteria are also reported to reduce phytotoxicity caused by organic pollutants via production of various enzymes and chemicals like siderophores, organic acids, ACC (1 aminocyclopropane -1-1 carboxylate) deaminase, iron chelators, and various other degrading enzymes (Sheng et al. 2008; Li et al. 2008).

Germaine et al. (2006) studied the interaction of endophytes capable of degrading herbicide for phytoremediation of 2,4-dichlorophenoxyacetic acid. They observed that endophytic bacteria efficiently colonized the plant tissues and any signs of phytotoxicity were not observed. Weyens and coworkers in 2010 investigated the capabilities of engineered bacterial endophyte *Burkholderia cepacia*

Table 16.2 Overview of the rhizosphere bacteria involved in hydrocarbon degradation

Plant species	Bacterial strain	Properties	References
Lolium multiflorum	*Mycobacterium gilvum*	Hydrocarbon degradation	Guo et al. (2017)
	Members from Pseudomonadales, Actinobacteria, Caulobacterales, Rhizobiales, and Xanthomonadales	Phenanthrene degradation	Thomas and Cebron (2016)
	P. putida PCL 1444	Naphthalene-degrading bacteria	Kuiper et al. (2001)
Lolium multiflorum var. Taurus	*Pantoea* sp. BTRH79	Hydrocarbon degradation and ACC deaminase activity	Afzal et al. (2012)
	Acinetobacter sp.	Hydrocarbon degradation	Yu et al. (2011)
	Pantoea sp. BTRH79 and *Pseudomonas* sp. ITRH76		Yousaf et al. (2010a, b)
	Rhodococcus sp. ITRH43		Andria et al. (2009)
Lotus corniculatus var. Leo	*Pantoea* sp. BTRH79 and *Pseudomonas* sp. ITRH77	Hydrocarbon degradation	Yousaf et al. (2010a, b)
Medicago sativa var. Leo	*R. meliloti* ACCC 17519	Hydrocarbon degradation	Teng et al. (2011)
	Azospirillum brasilense SR81	Hydrocarbon degradation and *IAA production	Muratova et al. (2010)
Hordeum sativum L.	*Pseudomonas putida* KT2440	Hydrocarbon degradation	Child et al. (2007a)
	Mycobacterium sp. KMS		Child et al. (2007b)
	P. aureofaciens, P. fluorescens		Anokhina et al. (2004)
Zea mays L.	*Pseudomonas* sp. UG14Lr	Hydrocarbon degradation	Chouychai et al. (2012)
	Rhizobacterium gordonia S2RP-17	Hydrocarbon degradation and ACC deaminase activity, auxin production	Hong et al. (2011)
	Pseudomonas putida MUB1	Hydrocarbon degradation	Chouychai et al. (2009)
Oryza sativa L.	*Acinetobacter* sp.	Hydrocarbon degradation	Li et al. (2008)
Sorghum bicolor L. Moench	*Sinorhizobium meliloti*	Hydrocarbon degradation and auxin production	Golubev et al. (2011)
	Sinorhizobium meliloti P221	Phenanthrene degradation, IAA production	Muratova et al. (2009)

(continued)

Table 16.2 (continued)

Plant species	Bacterial strain	Properties	References
F. arundinacea var. Inferno	*A. brasilense* Cd, *Enterobacter cloacae* CAL2, *P. putida* UW3, *P. putida*, *Flavobacterium* sp., *P. aeruginosa*	Hydrocarbon degradation and ACC deaminase activity	Huang et al. (2004)
Triticum spp.	*Pseudomonas* sp. GF3	Phenanthrene degradation	Sheng and Gong (2006)
	A. lipoferum	Hydrocarbon degradation and IAA production	Muratova et al. (2005)
T. repens L.	*R. leguminosarum*	Hydrocarbon degradation	Johnson et al. (2004)
Secale cereale L.	*Azospirillum brasilense* SR80	Hydrocarbon degradation and IAA production	Muratova et al. (2010)

**IAA* indole-3-acetic acid

VM1468 having (a) the pTOM-Bu61 plasmid, coding for constitutive TCE (trichloroethylene) degradation for co-contamination by toxic metal as well as organic pollutant. They observed that Ni uptake was enhanced after inoculation with endophytic bacteria, while phytotoxicity and evapotranspiration showed a decreasing trend. Yousaf et al. (2011) compared the efficiency of bacterial endophyte *Enterobacter ludwigii* capable of hydrocarbon degradation along with ACC deaminase activity for promotion of plant growth with the strain only possessing alkane degradation capability. It was estimated that *E. ludwigii* was effective in plant growth as well as hydrocarbon degradation as compared to the strain with only hydrocarbon degradation potential.

In another study, Hardoim et al. (2008) demonstrated that ACC deaminase released by endophytic bacteria actively reduces plant stress induced by contamination. ACC being the ethylene precursor break downs into α-ketobutyrate and ammonia (NH_3), decreasing ethylene production. Some endophytic bacteria are also reported for indirectly enhancing plant growth by production of ACC deaminase to control ethylene level in plants (Zhang et al. 2012a, b). Some plant species and endophytic bacterial strains successful in hydrocarbon degradation are listed in Table 16.3.

Overall, it can be appraised that endophytes have the potential to enhance plant growth and adaptability in contaminated environments by several mechanisms (Kathi and Khan 2011). Other beneficial effects of endophytes on plants have also been observed, e.g., opening and closure of stomata, alteration of the root morphology, enhanced mineral uptake, and modification of degradation processes (Compant et al. 2005). Plant growth promotion (PGP)-related ACC deaminase activity of bacterial endophytes has been estimated to be the major contributor in improving plant growth and adaptability in contaminated environments by alleviation of stress due to pollutants (Weyens et al. 2009). The beneficial characteristics of endophytes not only enhance plant growth, it also improves phytoremediation efficiency. Therefore

Table 16.3 Overview of endophytic bacteria involved in hydrocarbon degradation

Plant species	Bacterial strain	Properties	References
Achillea millefolium, *Solidago canadensis*, *Trifolium aureum*, and *Dactylis glomerata*	*Microbacterium foliorum* and *Plantibacter flavus*	Hydrocarbon degradation	Lumactud et al. (2016)
Medicago sativa var. Leo	*E. ludwigii*	Hydrocarbon degradation and ACC deaminase activity	Yousaf et al. (2011)
Lotus corniculatus var. Leo	*E. ludwigii*	Hydrocarbon degradation and ACC deaminase activity	Yousaf et al. (2011)
	Pantoea sp. ITSI10, *Pseudomonas* sp. ITRI16	Hydrocarbon degradation	Yousaf et al. (2010a)
Lolium multiflorum var. Taurus	*Pseudomonas* sp. ITR153, MixR175	Hydrocarbon degradation	Afzal et al. (2011, 2012)
	E. ludwigii	Hydrocarbon degradation and ACC deaminase activity	Yousaf et al. (2011)
	Pantoea sp. ITSI10, *Pseudomonas* sp. ITRI15	Hydrocarbon degradation	Yousaf et al. (2010b)
	Pseudomonas sp. ITR153	Hydrocarbon degradation	Andria et al. (2009)
Zea mays L.	*Burkholderia cepacia* FX3	Toluene degradation	Wang et al. (2010)
	Enterobacter sp. a2J1	Pyrene degradation, IAA, siderophore production, and inorganic phosphate solubilization	Sheng et al. (2008)
Pisum sativum	*P. putida*	Naphthalene degradation	Germaine et.al. (2009)
Populus spp.	*P. putida* w619-TCE	Trichloroethylene degradation	Weyens et al. (2010)
Triticum spp.	*Burkholderia cepacia* FX2	Toluene degradation	Wang et al. (2010)
	Enterobacter sp. a2J1	Pyrene degradation, IAA, siderophore production, and inorganic phosphate solubilization	Sheng et al. (2008)
Lupinus luteus	*Burkholderia cepacia* VM1468	Trichloroethylene degradation and Ni resistance	Weyens et al. (2010)

analysis of such characteristics of endophytes for phytoremediation has gained importance. Many endophytic bacteria isolated from plants growing in different contaminated environments have shown the potential to degrade organic contaminants and enhanced phytoremediation efficiency (Yousaf et al. 2011).

Siciliano et al. (2001) observed for the first time that endophytes of the plants growing in the soil contaminated with hydrocarbons would have the potential to phytoremediate the organic contaminants being rich in alkane-degrading genes, while

Lodewyckx et al. (2001) reported that endophytes from yellow pine improved the phytoremediation efficiency of inoculated plant. Van Aken et al. (2004) isolated endophytic bacteria from *Populus nigra* and *P. deltoids* and characterize them for degradation of nitroaromatic compounds. Later that year they utilized an endophytic species (*Methylobacterium* sp.) for degradation of explosive chemicals (octahydro-1,3,5,7-tetranitro-1,3,5-tetrazocine, hexahydro-1,3,5-trinitro-1,3,5-triazine, and 2,4,6-trinitrotoluene) and observed much efficient phytoremediation efficiency (Van Aken et al. 2004). Plants inoculated by *B. cepacia* showed reduced toluene volatilization phytotoxicity (Barac et al. 2004). Germaine et al. (2006) observed the interaction of *Pisum sativum* with an endophyte for mineralization of 2,4-dichlorophenoxyacetic acid. The endophyte was not only efficient in colonization inside the plant body, but it also reduced the phytotoxicity suggesting the effectiveness of utilizing plant-endophyte interaction in remediation of contaminated sites.

Diversity of bacterial endophytes in a contaminated site (benzene, toluene, ethylbenzene, and xylene) was observed by Moore et al. (2006). They reported that majority of the bacterial endophytes were capable of degrading BTEX. Afterward the same site was investigated by Barac et al. in 2009. They found out that degradation capability of endophytic bacteria disappeared, when the concentration of BTEX decreased below the detection limit. *Pseudomonas putida* VM1441 was studied to be efficient in colonizing inside the plant body. Plant inoculated with *P. putida* VM1441 showed less phytotoxicity and 40% more naphthalene degradation as compared to uninoculated plants (Germaine et al. 2009). Similarly *P. putida* W619-TCE was observed to reduce trichloroethylene phytotoxicity and improved plant growth (Weyens et al. 2010). Wild-type endophytic strain *P. putida* W619 is also reported for improving plant growth and decreased stomatal resistance (Weyens et al. 2012).

A bacterial endophytic strain *Achromobacter xylosoxidans* F3B was observed to decrease evapotranspiration with improved phytoremediation of aromatic compounds (Ho et al. 2012). Kathi and Khan (2011) revealed the advantages of plant endophyte companionship for phytoremediation that inoculation with endophytic strain capable of hydrocarbon degradation, diversity, and improved expression of degradation genes and inoculation strategies along with soil type affect the process.

16.3 Role of Enzymes in Hydrocarbon Degradation

Several bacterial strains carrying genes for hydrocarbon degradation have been found in the rhizosphere and the plant apoplast (Afzal et al. 2011), though hydrocarbon degradation capabilities of endophytes have been rarely analyzed (Afzal et al. 2012). Bacterial genes (cytochrome P450), alkane hydroxylase (CYP 153), and alkane monooxygenase (*alk*b) responsible for hydrocarbon degradation have been isolated from the plants (Yousaf et al. 2011; Becerra-Castro et al. 2011). The efficiency of plant-bacterial association largely depends upon the endurance of

exogenous bacteria and their metabolic efficiency in terms of hydrocarbon degradation. Hydrocarbon removal efficiency in the soil depends upon the plant-associated bacteria, while its remediation is coupled with rhizospheric and endophytic bacteria (Muratova et al. 2008). Gene abundance and gene expression responsible for hydrocarbon degradation can provide direct evidence for the efficiency of bacteria (Juhanson et al. 2009).

The conventional enrichment culture techniques along with new "-omics" technology have illustrated that bacteria are metabolically more active for remediation of contaminated sites. Living beings can produce a wide range of metabolic compounds approximately >1 million. Most of these compounds have been discovered in the plants. Microorganisms are also a major source of biologically active compounds responsible for various functions. Microorganisms are reported to impact the performance and survival of other living beings as well (Demain and Sanchez 2009). It has been estimated that metabolic compounds identified so far constitute only a little proportion of the existing metabolic diversity, because a very small number of bacteria have been cultivated till date, as evidenced by different soil studies (like Sanger sequencing of clone libraries, next-generation sequencing, and DNA:DNA hybridization) (Raaijmakers and Mazzola 2012). Although *Actinobacteria* especially genus *Streptomyces* is considered as the enormously rich source of secondary active metabolic compounds (Zin et al. 2007; Qin et al. 2011), knowledge about the potential for more rare and exotic actinobacterial taxa is less established (Li et al. 2012). Similar inferences might also be applicable for large proportion of other less well-studied bacterial taxa. Also certain niches, among various other bacteria living especially inside the plant body, are not very thoroughly investigated. Endophytes can inhabit various different environments therefore of prime interest for exploitation. The focus of the investigations should be plant-bacterial endophyte interaction as a non-phytopathogenic association for phytoremediation.

Advancements in biotechnology have opened new doors for remediation of contaminated sites in eco-friendly manner. In spite of the fact that bacterial endophytic strains have the potential to enhance phytoremediation capacity of plants, the metabolic information is far from saturated. Furthermore, it is also necessary that host plants should be able to host efficient organic pollutant-degrading microflora. The ability of endophytes to inhabit inside plant tissues and show metabolic activity was assessed by Andria et al. (2009) using quantitative PCR. They inoculated the alkane-degrading strains of bacterial endophytes in *Lolium multiflorum* (Italian ryegrass) growing in diesel-contaminated soil. They observed that endophytes were efficient in inhabiting the rhizosphere and especially inside plant tissues. Endophytes showed higher level of *alkb* gene expression inside the plants. Afzal et al. (2011) utilized the similar approach to study the colonization efficiency and metabolic activity of endophytes in selected plant species. They utilized *L. multiflorum* var. Taurus, *Lotus corniculatus* var. Leo, and *Medicago sativa* var. Harpe to study the gene abundance

and expression of endophytes. They observed that endophytes can better phytoremediate the organic pollutants reducing phytotoxicity and evapotranspiration (Afzal et al. 2011).

Quantitative polymerase chain reaction (qPCR) is efficient to determine the abundance of *alk*b gene in the plants and their rhizosphere and also to investigate its expression for phytoremediation. Recent studies about the abundance and expression of CYP153 and *alk*b suggest that bacterial populations carrying these genes can colonize in the rhizosphere and plant apoplast and efficiently degrade hydrocarbons (Afzal et al. 2011). However, much investigation about the efficiency of different bacterial strains and their metabolic efficiencies and specific plant species and their growth stages is needed. Also the research about plant growth promotion (directly/indirectly) in relation to hydrocarbon degradation is required. Nevertheless some studies did suggest active enzyme production for plant growth promotion as discussed below.

Production and efficiency of different phytohormones especially auxins were investigated to compare its role with the different effects from PGPB (de Garcia Salamone et al. 2006). Nitrogenase enzyme present in plant-associated bacteria reduces the atmospheric nitrogen to ammonia and makes it usable for plants (Doty 2008). Phosphatase enzyme can degrade the organic contaminants containing phosphates, or bacteria can increase availability of inorganic phosphate by releasing organic acids (Vessey 2003). Several bacteria have the efficiency to produce special organic compounds like siderophores that reduce Fe^{+3} to Fe^{+2} that can be easily absorbed by plants (Katiyar and Goel 2004). Some microbes in the soil environment are reported to produce biosurfactants that increase bioavailability of organic contaminants. It has also been observed that individual bacterial strains don't work efficiently; however, a consortium of different bacterial strains possessing several varying degradation pathways may perform well in hydrocarbon-contaminated soils (Balcom and Crowley 2010; Schulz et al. 2012).

Utilization of bacterial strains carrying both genes for hydrocarbon degradation and plant growth is advantageous over the bacteria, with only individual genes for plant growth or hydrocarbon degradation. Certain bacteria possess 1-aminocyclopr opane-1-carboxylate (ACC) deaminase activity that increases plant growth by reducing organic contaminant stress (Afzal et al. 2012). Research on bacterial ACC deaminase activity reveals that it improves plant growth, especially root growth and development leading toward extensive root system, more root exudates, and more bacterial population in the rhizosphere. Also ACC deaminase reduces ethylene produced by the plants under stress and hence reduces stress symptoms enhancing plant growth (Glick 2010). This potential of bacterial ACC deaminase activity suggests that application of such bacteria can be useful in decreasing stress on the plants. It has been reported that bacteria carrying hydrocarbon degradation and ACC deaminase genes show more biomass production and hydrocarbon removal than the bacteria only having one type of gene expression (Afzal et al. 2012).

16.4 Influence of Soil Characteristics on Hydrocarbon Degradation

Soil's physicochemical properties (pH, texture, particle size, structure, cation exchange capacity, and organic matter) affect plant survival and growth, solubility and availability of organic contaminants, endurance and colonization of bacterial populations, and their metabolic activities to biodegrade the hydrocarbons (Schulz et al. 2012). Hydrocarbons have the strong affinity to bind with clay minerals and humic substances. It has been observed that bacterial colonization and gene expression are affected by soil types. Loamy soil supports greater microbial population, gene abundance, and expression than sandy and loamy sand soil (Afzal et al. 2011), while soil salinity in the rhizosphere is reported to affect the functional structure of hydrocarbon-degrading genes (Zhong et al. 2012; Afzal et al. 2012).

Due to the presence of hydrocarbons, the bioavailability of minerals in the soil decreases. Higher concentration of organic pollutants can limit uptake of iron (Fe), phosphorous (P), and nitrogen (N) directly affecting plant growth (Basumatary et al. 2012). It has been investigated that soil moisture content and quantity of available P have significant impact on phytoremediation of hydrocarbons. Higher concentration of available P and soil moisture show maximum hydrocarbon degradation as compared to soil deficiency in P or water availability (Jidere et al. 2012). Increased plant growth and hydrocarbon degradation are investigated to be linked with induced provision of NPK fertilizers or organic manure though hydrocarbon-contaminated soils which were mostly found to be deficient in bioavailable nutrients (Song et al. 2012). PGPB mineralize the nutrients present in soil, utilize their enzymes to degrade organic contaminants, and enhance plant growth (Weyens et al. 2010).

Fungal endophytes have been investigated for their potential to produce biologically active compounds. However, bacterial endophytes and their association with plants for metabolic potential are not very well constructed. A number of studies reveal that endophytic bacteria first colonize in the rhizosphere and then gain entry into the plant, where they can reside in different plant parts like stem, leaves, reproductive organs, etc. (Reinhold-Hurek and Hurek 2011). Endophytes are required to be capable enough to reside a specific plant environment. Therefore, their degradation potential probably will be different from rhizospheric counterparts. Competition inside the rhizosphere among bacteria and other microorganisms is greater; hence rhizosphere microflora is expected to produce a wide range of antibiotic and antinematodal chemicals. In comparison, the obligate endophytic bacteria experience less competition, hence less metabolic diversity, but are likely to produce other specific metabolites required for association with the host plant (Sturz et al. 2000).

16.5 Genes Involved in Hydrocarbon Degradation

Among all the factors discussed above for improving bioremediation efficiency, the presence of active catabolic machinery is of prime importance. The presence of right set of genes and production of necessary enzymes responsible for hydrocarbon degradation are essential for bioremediation to proceed. Optimum oxygen, temperature, and pH conditions required for that particular enzyme to function are of subsequent importance. Hydrocarbon degradation in the presence of oxygen is preferred over the reaction to occur in oxygen-free environment. In aerobic conditions, alkanes are oxidized to CO_2 and H_2O molecules. Microbial hydrocarbon degradation potentially increases in the following order: asphaltenes < polyaromatic hydrocarbons < cyclic alkanes <monoaromatic hydrocarbons < low molecular weight aromatic hydrocarbons < branched alkene < branched alkanes < straight chain alkanes (Tyagi et al. 2011).

Various studies reported the presence of numerous alkane-degrading genes in the microbial populations. However, these genes only express under certain conditions, sometimes only when other substrates are not present (Rojo 2009; Wang and Shao 2013). Under aerobic conditions, oxygenase enzymes introduce oxygen atoms into hydrocarbons, where monooxygenases introduce one, while dioxygenases introduce two oxygen atoms to the substrate (activation stage; Fig. 16.1).

Anaerobic metabolism involves utilization of different terminal electron acceptors (van Hamme et al. 2003). Aerobic metabolism of hydrocarbons is advantageous and rapid because of availability of oxygen as electron acceptors. Finally acetyl-CoA is produced by the oxidation of saturated aliphatic hydrocarbons (Cao et al. 2009). Acetyl-CoA is further catabolized in tricarboxylic acid cycle (also known as TCA, citric acid, or Krebs cycle), along with production of electrons in electron transport chain (ETC). This chain repeatedly degrades the hydrocarbons and fully oxidizes then to CO_2 (Madigan et al. 2010). Benzene, xylene, naphthalene, and toluene (aromatic hydrocarbons) can also be metabolized aerobically. Metabolism of these compounds generally assists as the first phase in the production of catechol or structurally similar chemical. After catechol production, it can be catabolized which produces such compounds that can be introduced in TCA cycle and finally degraded to CO_2 (Cao et al. 2009; Madigan et al. 2010). The process for degradation of aliphatic and aromatic hydrocarbon is illustrated in Fig. 16.1.

Alkane hydroxylases (alkane-degrading enzymes) have been further subdivided into three categories by van Beilen and Funhoff (2007): methane to butane (C1–C4, oxidized by enzymes like methane monooxygenase), pentane to hexadecane (C5–C16, oxidized by cytochrome P450/integral membrane nonheme iron enzymes), and longer alkane (C17+, oxidized by essentially unknown enzyme systems).They also provided the composition, ranges of the substrates, cofactors involved, and existence of main groups of alkane hydroxylases like soluble methane monooxygenase (sMMO),

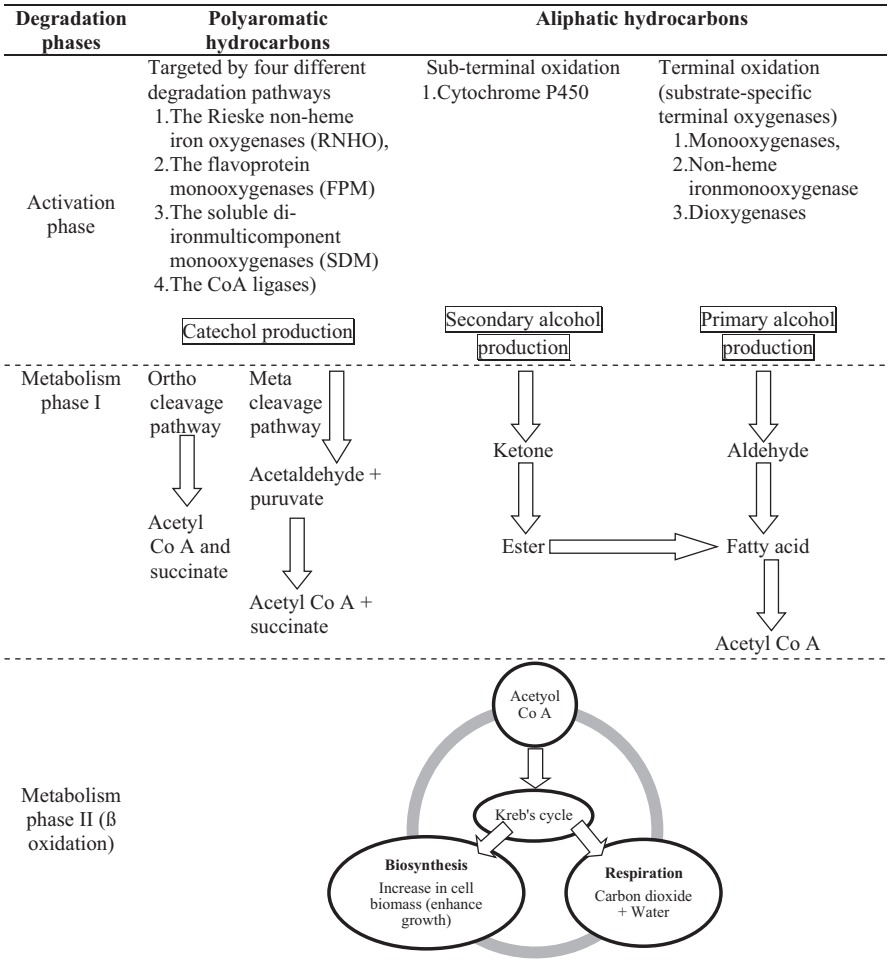

Fig. 16.1 Potential degradation pathway for aliphatic and polyaromatic compounds

Alkb-related alkane hydroxylases, particulate methane monooxygenases (pMMO), bacterial P450 oxygenase system, dioxygenase (CYP153, class I), and eukaryotic P450 (CYP52, class II). They explained that bacterial strains capable of degrading alkanes can have different multiple alkane hydroxylases which leads to consumption of different ranges of substrate (van Beilen et al. 1994, 2001).

Expression of multiple Alk hydroxylase genes was observed in *Pseudomonas* spp. (RR1 and PAO1). These strains showed the presence of AlkB1 and AlkB2 genes oxidizing C_{16}–C_{24} and C_{12}–C_{20}, respectively. It was observed that in the presence of alkanes ranging between C_{10} and C_{22}, both the genes were expressed where AlkB1 expressed almost double to AlkB2. They also observed that AlkB2 expressed more at the start of exponential phase, while AlkB1 expressed in the late exponen-

tial phase. Their expression decreased in stationary phase (Marin et al. 2003). When *Alcanivorax borkumensis* was targeted for the presence of hydrocarbon-degrading genes, it was revealed that the organism possesses both the two alkane hydroxylase and three cytochrome P450 genes. AlkB1 was found to be oxidizing C_5–C_{12}, while AlkB2 was capable of oxidizing C_8–C_{16}. Optimum expression was observed upon exposure to C_{10}–$C_{16.}$ Their expression decreased in stationary phase (van Beilen et al. 2004; Sabirova et al. 2006; Schneiker et al. 2006). They concluded that AlkS activates the AlkB1 gene upon exposure to n-alkanes. Expression of AlkS was more upon exposure to hexadecane instead of pyruvate (van Beilen et al. 2004; Sabirova et al. 2006). Schneiker and team found a regulator belonging to AraC family present near P450-1; however they did not study its function in regulating the P450-1 (Schneiker et al. 2006). Funhoff and team observed the presence of Cypr (an AraC family regulator) active for regulating CYP153 gene. CYP153 gene is responsible for encoding alkane hydroxylases belonging to P450 genes in *Dietzia* sp. (Funhoff et al. 2006; Liang et al. 2016).

A. borkumensis is reported to have a gene encoding for a regulator protein similar to TetR, which is present downstream of AlkB1 gene. Another gene encoding for a regulator protein GntR is found upstream of the AlkB2 gene. However, the function of these genes in hydrocarbon degradation still needs to be investigated (Wang and Shao 2013). Tani and team reported the presence of AlkMa and AlkMb genes responsible for hydrocarbon degradation in *Acinetobacter* sp. AlkMa was activated by AlkRa upon exposure to alkanes >C_{22}, while AlkMb was activated by AlkRb upon exposure to C_{16}–C_{12} (Tani et al. 2001). Other important systems which facilitate hydrocarbon degradation include product repression and controlling catabolite repression. BMO gene expression is inactivated by a metabolite of propane oxidation (Doughty et al. 2006) which represses hydrocarbon degradation by inhibiting the BMO catalytic site (Doughty et al. 2007). However, Kurth and team suggested the activation of BMO gene expression by BmoR (a putative sigma 54-transcriptional regulator) which identifies by-products produced during hydrocarbon degradation (Kurth et al. 2008).

Catabolite repression includes repression of preferred hydrocarbon metabolism. For example, alkBFGHJKL (alkS) operon is responsible for encoding enzymes active for altering alkanes to CoA (acetyl coenzyme-A). alkST is responsible for encoding alkT (rubredoxin reductase) and positive regulator for alkS. alkS and alkST are located end to end disconnected by 9.7Kb DNA. In this ladder there exists another gene alkN. This gene encodes for a transducer protein which upon methylation acts for alkane chemotaxis. Upon exposure to alkanes, alkS triggers alkST gene expression via PalkS2 promoter. Higher alkS leads to alkBFGHJKL gene expression using PalkbB promoter. On the other hand, actively multiplying cells in a rich medium deactivates the PalkB and PalkS2 even upon exposure to alkanes (Canosa et al. 2000). It is worth mentioning here that of all the genes discussed above, the role of alkL gene is still unknown though it is thought to be associated with transport.

16.6 Degradation Mechanisms

Hydroxylation of alkyl group is usually the first phase in the metabolism of organic compounds which is mediated by oxygenases. The alkane hydroxylase which metabolizes the terminal carbon consists of three subunits that also include membrane-bound hydroxylase subunit encoded by *Alkb*. CYP153 may also metabolize hydrocarbon in similar manner. One of the most studied alkane metabolic pathways was provided by van Hamme and his coworkers for *Pseudomonas putida* Gpo1, encoded by OCT plasmid. They described the pathway for alkane metabolism in gram-negative bacteria and discussed the loci of Alk genes and functional products. They highlighted that transformation of an alkane to alcohol is initiated by a membrane monooxygenase, rubredoxin reductase, and soluble rubredoxin (van Hamme et al. 2003).

Aromatic rings also undergo hydroxylation for their degradation, but it first requires opening of the hydroxylated aromatic ring; this process is catalyzed by aromatic ring cleavage dioxygenases. The three main forms of the enzyme are gentisate/homogentisate, extradiol, and intradiol. These three are substrate specific and can be distinguished by their positions along the fission of the ring relative to the hydroxyl group (Harayama 1997; Cao et al. 2009). Catechol dioxygenase is the enzymatic class containing iron and participates in the metabolism of aerobic aromatic hydrocarbons. Catechol dioxygenases are capable of catalyzing the addition of molecular O_2 atoms to catechol (1,2-dihydroxybenzene) and its derivative compounds followed by the breakdown of aromatic ring (van Hamme et al. 2003; Cao et al. 2009; Madigan et al. 2010).

Usually during anaerobic degradation, aromatic compounds are transformed into benzoyl-CoA. Benzoyl-CoA is further targeted by benzoyl-CoA reductase. Directly influenced by environmental conditions, various different types of terminal electron acceptors can be utilized like Fe (III), sulfate, nitrate, etc., but generally the metabolic pathway leads to benzoyl-CoA (Hosoda et al. 2005; Cao et al. 2009). The process for degradation of aliphatic and aromatic hydrocarbon is illustrated in Fig. 16.1.

Endophytes having suitable catabolic pathways for organic molecules encoded on transposons or plasmids potentially are the efficient candidates for phytoremediation. Horizontal gene transfer is estimated to be the possible way of transferring the mobile elements of such catabolic pathways across the endophytes (Taghavi et al. 2005; Weyens et al. 2009; Yousaf et al. 2010a, b).

Application of genetic engineering has also broadened our knowledge about the phytoremediation of organic contaminants and mechanism. Engineered endophytes (with pollutant-resistant/pollutant-degrading genes) have been used to study phytoremediation efficiency. These studies also reveal that plant transpiration stream helps in the uptake of organic contaminants in the rhizosphere (Newman and Reynolds 2004; Doty 2008; Soleimani et al. 2010). Degradation of these contaminants may take place in the rhizosphere, inside the plant, or in both. Organic contaminants can be translocated from the root symplast to the aerial parts of the plants

via apoplast (xylem). Inside the shoot these can be degraded or sequestered by endophytes (Weyens et al. 2009). Though plants can utilize organic contaminants, they do not use them as a source of energy and carbon. So plant-associated endophytes efficiently mineralize these contaminants. Endophytes are reported to be efficient in colonization inside the plants as well as metabolically active in degradation of organic contaminants (Weyens et al. 2009; Yousaf et al. 2011; Kathi and Khan 2011).

Most of the endophytic bacteria facultatively colonize the plant body and face competition in the rhizosphere before entering inside the plants. Therefore they might be able to produce a wide diversity of metabolites responsible for active defense as well as for interaction with the host plant. Hence it can be elucidated that these compounds not only function in defense but also play a major role in inter- and intraspecies signaling processes (Fajardo and Martinez 2008).

16.7 Future Prospects

Utilization of hydrocarbons for industrial development is linked with contamination of biotic and abiotic spheres of life. To overcome the problem, it is important to improve existing bioremediation and phytoremediation approaches. And treatment of more toxic compounds should be emphasized. Table 16.4 provides the main focus areas and experimental approaches for plant-bacterial association for improving remediation of hydrocarbon-contaminated soils and water.

Some perspective advancements in bioremediation and phytoremediation approaches involving bacteria to improve efficiency have been discussed here.

16.7.1 Improving Available Bioremediation Approaches

As discussed above bioremediation processes are affected by various factors like augmentation with active microbial population, provision of growth media for microbial growth, and environmental factors. Currently scientists are focusing on optimizing these parameters for improved efficiency. Likely optimized C:N:P ratio (Álvarez et al. 2015) will automatically reduce use of fertilizers, changing nitrate, sulfate, iron, and carbon dioxide as terminal electron acceptor instead of oxygen in anaerobic bioremediation process which will reduce cost in comparison to aerobic process (Siddique et al. 2011; Hasinger et al. 2012; Franco et al. 2014) and mathematical modeling to study the effects of biosurfactants on hydrocarbon removal (Geng et al. 2014; Pham et al. 2014; Gao et al. 2014; Montagnolli et al. 2015). However, the areas that need special consideration include novel strategies with improved biodegradation rate and hydrocarbon removal especially for bio-refractory contaminants and critical analysis for underlying mechanisms with computational studies for optimized operating conditions.

Table 16.4 Possible strategies and experimental approaches for plant-bacterial association for improved phytoremediation

Target component	Focus areas	Experimental approaches
Selection of active biodegraders	a) Single species	Native vs. non-native population
	b) Microbial Consortia	Biodegradation potential
		PGP potential
		Microbial interaction (quorum sensing)
		Horizontal gene transfer
		Gene expression
		Metabolic pathways
		Toxicity assessment
		Association with plant
		Field trials
Selection of host plant	a) Bacterial communities	Native vs. non-native plants
	b) Fungal communities	Inoculation methods
		Transgenic plants
		Association with microbiota
		Phytotoxicity assessment
		Field trials
Rhizosphere ecology	a) Root exudates	Quality and quantity of root exudates
	b) Microbial community	Metagenomics
	c) Selection of strains/consortia for inoculation	PGP potential
	d) Environmental factors	Biodegradation potential
		Transgenic plants
		Antimicrobial activity
		Carrying capacity
		Soil type
		pH
		Temperature

16.7.2 Advancements in Biotechnology and Bioengineering

Bioremediation has been considered as the most eco-friendly treatment option since decades. Though it is the best on-site treatment solution, it is not universal due to diverse pollutant as well as microbial properties. It is widely accepted that success of on-site bioremediation strategy depends upon inoculation with functional (approved after laboratory experiments) microbial population. Szulc and team studied the effect of bioaugmentation and addition of biosurfactants for bioremediation of diesel-contaminated soil. They used two separate setups for laboratory- and field-scale analysis. Soil type used in both the experiments was the same. The experiment lasted for 1 year. During the lab experiment, bacterial strains used included

Aeromonas sp., *Alcaligenes xylosoxidans*, *Gordonia* sp., *Pseudomonas fluorescens*, *P. putida*, *Rhodococcus equi*, *S. maltophilia*, and *Xanthomonas* sp. In the next stage, active microbial community was tested for diesel degradation (1% w/w) in the field. Results suggested enhanced bioremediation efficiency upon addition of active microbial consortia (89%) as compared to the control (without addition) to be 53% (Szulc et al. 2014). This suggests that a preliminary laboratory-scale investigation may enhance the on-site bioremediation efficiency.

Another diagnostic approach to select the most efficient bacterial strains was provided by Kim et al. (2014). They devised an oligonucleotide microarray diagnostic method. The success depends upon selecting bioremediation sites by identifying bacterial strains responsible for particular hydrocarbon degradation. The implementation of this method yielded noticeable results reducing trial and error. The researchers usually target efficiency of bacterial community for hydrocarbon degradation. However, it is required to study the effect of hydrocarbon toxicity on microbial consortia for further improving bioremediation efficiency. Smulek and team (2015) observed association between cell surface of *Rahnella* sp. EK12 and diesel surfactants. Growing cells of *Rahnella* sp. EK12 were in long-term contact with diesel. Analysis revealed considerable modifications after exposure to surfactants in hydrophobicity, fatty acid composition, and zeta potential. These modifications were linked with changes in the genetic code responsible for producing capsules. Study proved that long-term exposure to hydrocarbons affects the microbial genetic material.

Bastida and team (2016) studied the effect of crude oil on bacterial biomass, activity, and community composition. They corresponded to biostimulation efficiency with and without compost addition. The analysis showed insignificant biodegradation activity without compost as compared to 88% degradation efficiency in compost-added soil. Metaproteomic analysis described the association between structural and functional properties of microbial community upon exposure to hydrocarbon contamination. The study further enhanced the idea of improved bioremediation efficiency by considering bacterial resistance and toxicity profile. Therefore it is necessary to carry out genomic analysis of microbial community prior to their use for bioremediation. Also there is a need to investigate associated factors effecting bioremediation process.

16.7.3 Advances in Phytoremediation

Care should be taken to investigate plant bacterial associations especially for recalcitrant because of their potential effects on metabolism. Microbial hydrocarbon degradation potential is attributed to the presence and expression of functional genetic and enzymatic machinery. This potential can be enhanced by exploiting plant microbial association for hydrocarbon degradation.

On the other hand, this association will positively affect plant growth by synthesizing plant growth hormones, using biosurfactants as biocatalysts, increasing

bioavailability of essential nutrients, and suppressing ethylene production. Bioremediation and phytoremediation are considered the best remediation technologies for treatment of hydrocarbon; however extensive studies are required to elucidate the treatment outcomes.

Advances in genomic analysis (metagenomic, metatranscriptomic, metaproteomic, and metabolomic) are a way forward to analyze complex community structure and interactions among the biotic and abiotic spheres of life (Bell et al. 2014; Kaul et al. 2016). Genomic analysis paves the way to envision biodegradative potential of environmental components and on-site degradation efficiency, identifying suitable inoculants, elucidate degradation pathways, and assess enzymatic machinery's efficiency for hydrocarbon degradation (Uhlik et al. 2013; Sierra-Garcia et al. 2014). However, laboratory and field studies are required for optimum utilization of plant bacterial association with enhanced remediation efficiency.

16.7.4 Addition of Eco-Friendly Nutrients/Fertilizers

Studies suggest that addition of nutrients to optimize microbial growth has positive influence in bioremediation efficiency especially aerobic bioremediation. However, this approach is associated with algal bloom formation due to runoff or leaching. This leads to deterioration of water quality. A possible solution to the problem has been proposed to use organic fertilizers instead of inorganic. This organic fertilizer could be derived from plants or animals. Horel and team (2015) compared the effect of two organic fertilizers on hydrocarbon bioremediation. They used tissues from *Spartina alterniflora* (a plant) and *Chloroscombrus chrysurus* (a fish). They observed 104% increase in degradation efficiency upon addition of *C. chrysurus*, while addition of inorganic nutrients (P and N) enhanced 57% as compared to 7% by *S. alterniflora* in 42 days in sandy soils.

Less toxic, temperature- and alkali-resistant biosurfactants having high solubility for hydrocarbon are primarily in demand for bioremediation. *Streptomyces* spp., *Achromobacter* spp., *Pseudomonas* spp., *Bacillus* sp., *Rhodococcus* spp., *Arthrobacter* spp., *Acinetobacter* spp., and *Brevibacterium* spp. are mostly used for biosurfactant production during bioremediation (Petrikov et al. 2013; Xia et al. 2014; Ayed et al. 2015; Bezza and Chandran 2015). Recent studies should focus on looking for improved biosurfactants with enhanced bioremediation efficiency covering a wide temperature range, pH, and saline conditions.

16.7.5 Field-Level Applications

Various field studies reported recently were based on comparisons between use of different microbial communities, their application rate, and percentage of fertilizers used (Venosa et al. 2010; Beškoski et al. 2011; Gomez and Sartaj 2013; Akbari and

Ghoshal 2014; Ferradji et al. 2014; Meyer et al. 2014; Gomez and Sartaj 2014; Yadav and Yadav 2017) with hydrocarbon removal efficiency ranging between 50% and 82%. Advanced field experiments using combination of bioaugmentation and biostimulation, nanoremediation (use of nanofertilizers), and use of organic fertilizers especially in cold climatic conditions are required for further strengthening the applicability of bioremediation.

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Chapter 17
Bioaugmentation of Petroleum Hydrocarbon in Contaminated Soil: A Review

Z. Nur Zaida and M. T. Piakong

Abstract The release of petroleum hydrocarbon products into the soil due to rapid industrialization and accidental spills poses a serious threat to soil as well as groundwater. These compounds are known as carcinogen and neurotic organic pollutants which cause serious risk to human health and ecosystem. The introduced of bioaugmentation is one of the promising strategy, inexpensive and clean in situ bioremediation due to noninvasive and the selected of hydrocarbon-degrading microorganisms added into the soil can accelerate the degradation capacity of organic pollutants together with indigenous microbial population in the soil. This technique produced a maximum biodegradative capacity of organic pollutants in the soil. To demonstrate the potential of bioaugmentation in soil contaminated with petroleum hydrocarbon, many researchers studied the parameters to determine the optimal degradation conditions. In this chapter, we reviewed the experimental findings and the process of bioaugmentation of hydrocarbon-polluted soil by several selected bacterial strains that were isolated from previous studies around the world.

This review focuses on the parameters affecting the bioaugmentation process by abiotic and biotic factors. The various environmental parameters monitored are temperature, moisture pH, oxygen, and nutrient levels. Other factors include the contention between indigenous and exogenous microorganisms in utilizing carbon sources and the effects of antagonistic and synergistic interactions, and these interactions potentially change the number of cells augmented in the soil. Recent studies show that mixed cultures were more successful in degrading hydrocarbons than the single strains. Therefore the best technique and approach for bioaugmentation of soil contaminated with petroleum hydrocarbons will be highlighted and discussed in this chapter.

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17.1 Introduction

Petroleum hydrocarbon is one of the most important substances consumed by people around the world that originally come from crude oil. It is a complex mixture of hydrocarbons that occurs in the sedimentary rocks in the form of gases (natural gas), liquids (crude oil), semisolids (bitumen), or solids (wax or asphaltite).

Generally, crude oil has been used as the raw material in manufacturing petroleum product. The composition of crude oil varies considerably from source to source. Each of the petroleum products has different compositions and properties that influence its fate and transport in the subsurface of environment (Hayden et al. 1994). The increase of demand from consumers has forced rapidly the industry production of chemicals and in turn the improper disposal and leaking during storage and accidental spills resulting in many cases reported around the world.

The main sources of these toxic substances are from oil refineries, wood preservatives, gas station, and agrochemical, petrochemical, and pharmaceutical industries (Wang et al. 1998). The release of hydrocarbons due to human activities into the environment whether directly or indirectly has caused water and soil pollution (Holliger et al. 1997). Soil pollution is caused by disposal of petroleum products such as benzene, toluene, and xylene which are of main concerns as these products can lead to significant effect on water quality and threaten human health, plants, and animals. There were estimated 1.6 million USTs (underground storage tanks) and 37,000 hazardous tanks in 1992 as reported by the US Environmental Protection Agency (EPA). Among these, there were 320,000 USTs leaking, and 1000 tanks are confirmed produced each week (Cole 1994). This process of storage and leaking has posed tremendous petroleum hydrocarbon contamination in soil and groundwater.

Aromatic compounds are one of the environmental concerns as these compounds are relatively soluble in water and therefore have a high potential mobility within groundwater systems. Most aromatic compound is recalcitrant and has high hydrophobicity making them persistent and difficult to be degraded (Budavari 1996). The disposal of petroleum hydrocarbon could lead to various toxic effects; these include benzene, toluene, and xylene (present in gasoline) which can affect the human central nervous system when exposed to high level of concentrations. This compound will cause headache, nuisance, trouble, pain, and drowsiness (USEPA 2008). Other petroleum compound such as hexane can cause irritation of the eyes and throat and dermatitis. Individuals are most likely to be exposed to hexane in workplace causing mild CNS depression and sometimes numbness in the feet and legs and in severe cases can lead to paralysis (Chen et al. 2012). The causes of groundwater pollution by these hydrocarbon compounds are the fact that this fraction will float in the water and form the thin surface layer; in turn the heavier compounds will accumulate in the sediment or soil in the bottom of the water. This may affect the benthic organisms especially feeding fish and organisms (USEPA 2008). Thus this leads to groundwater as well as river pollution in which these bodies of water are the major sources of drinking water by people. The quality of water also deteriorates in terms of taste and odor at very low concentration of petroleum hydrocarbon (Rahman

et al. 2002). Apart from that, seaside flora and some fauna such as birds and bivalve mollusks often are affected by the spills (Aguilera et al. 2010).

There are various methods applied for the removal of petroleum hydrocarbon pollutants from the soils (Reddy and Saichek 2003; RAAG 2000; Khan and Husain 2002). The advanced technologies that are commonly used for soil remediation include chemical and mechanical dispersion, oxidation, evaporation, incineration, and stabilization/solidification (USEPA 2004). However, these technologies are expensive and noninvasive and can lead to severe impacts to environment and incomplete degradation of contaminants. Bioremediation, the use of microorganisms to detoxify or remove pollutants, offers a promising technology due to its environment-friendly, relatively cost-effective, and low-maintenance equipment (April et al. 2000). Besides its cost-effectiveness, this technique served complete solution which may lead to maximum mineralization of the pollutant.

Bioremediation also is one of the methods that offer green technology solution to the problem of hydrocarbon and heavy metal contamination of soil (Grishchenkov et al. 2000; Gogoi et al. 2003; Townsend et al. 2004; Lakha et al. 2005). For this process to be effective, microorganisms used must enzymatically attack the pollutants and convert them to harmless or simple products. The standard process can only be effective along with environmental conditions that support microbial growth and activity. Therefore the manipulation of environmental parameters is very important to allow the maximum biodegradation capacity of microbes in the soil (Vidali 2001).

It is important to note that microbiological activity is affected by a number of environmental parameters, such as energy sources, nutrients, pH, temperature, and soil moisture content. These parameters determine the utilization of hydrocarbons by microorganisms and adaptation of this microorganism to the available compounds (Boopathy 2000). The most widely used study for in situ bioremediation approach includes landfarming, biostimulation, bioventing, and bioaugmentation (Powell et al. 2007). In this paper, attention has been focused on one of the bioremediation techniques, the bioaugmentation of petroleum hydrocarbon in contaminated soil, and this review expands the case study on bioaugmentation and the factors influencing bioaugmentation of petroleum hydrocarbon in soil.

17.2 Bioaugmentation of Petroleum Hydrocarbon

Bioaugmentation is a technique used in bioremediation to enhance the microbial population by adding selected microbial cultures at a site to improve contaminant cleanup and enhance the degradation time needed (Alvarez and Illman 2006). The purpose of bioaugmentation is to supplement the existing microbial community in order to improve its functionality (Vogel 1996; Iwamoto and Nasu 2001; El Fantroussi and Agathos 2005). In some cases, natural populations in the soil are not capable to degrade a wide range of petroleum hydrocarbon (Leahy et al. 1990). Therefore, bioaugmentation has been applied to seek the successful remediation site

Table 17.1 Advantages and limitations of bioaugmentation study on petroleum hydrocarbon (Carter and Jewell 1993; Edwards and Cox 1997)

Advantages	Limitations
Cheaper cost (low maintenance in terms of labor work)	Microbes need favorable environmental condition to survive in the target area
This process can be done in situ	Bacteria cannot metabolize every kind of waste
Environment-friendly techniques whereas indigenous microbes are used to treat the pollutant instead of using chemical additions. It gives minimal harmful impact on the environment	It is a long-term process
In situ process reduces the potential to create a large mess during transportation (noninvasive)	Microbes generate their own waste products
It is a natural process where microbes will continue to clean up the area	Compete between oil degrading microorganisms in soil and augmented strains resulting in failure of bioaugmentation
Microbes break oil down into simple carbon compounds that are used to make the sugars, fats, and proteins needed for growth	Daily monitoring on physical, chemical, and biological factors to achieve maximum biodegradation process

by accelerating the activity of the organisms under the right environmental conditions (Boopathy 2000). The detail on advantages and limitations of bioaugmentation process is showed in Table 17.1.

It is important to note that different microorganism species have their different biodegradation capabilities in degrading hydrocarbons. Due to this fact, the selection of microorganism for bioremediation study is very important for successful remediation. In order to accomplish this, screening and evaluation for biodegradation of potential bacteria are usually established before further bioremediation studies. The concept of introduction of nonindigenous or cultured microorganisms has been seen as a solution that overcomes some of the most common contaminated soil. Previous study by Vecchioli et al. (1990) had demonstrated that biodegradation of polluted soils with petroleum hydrocarbons may be enhanced by indigenous bacteria inoculation. The selection of proper culture with best characteristics such as easy to culture, fast growth, and having high capabilities to withstand high toxicity levels of contaminants is the best for bioremediation. Moreover, the selected microorganisms also must be well characterized in order to express catabolically superior toxin-degrading enzymes and highly resistant to environment stress. Figure 17.1 showed the illustration of basic biodegradation of hydrocarbon in soil by selected microbial cultures in the environment.

Previous study has reported that the best cultures for bioremediation are the ones who can survive in various environmental conditions which can maintain their genetic stability and viability plus have the potential in degrading most the petroleum components (Gentry et al. 2004). This finding is in line with Throne-Holst et al. (2007) who demonstrated that single culture of *Acinetobacter* sp. has the potential in degrading n-alkanes of chain length C_{10}–C_{40} as a source of energy and

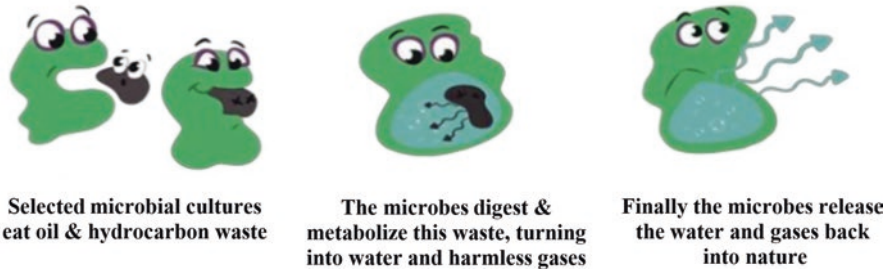

Fig. 17.1 Illustration of biodegradation of oil in soil by selected microbial cultures in the environment. (Sources: NCEPI)

growth (Throne-Holst et al. 2007). Gentry et al. (2004) found that the use of strains that produce biosurfactants is more accessible to degrade the pollutants with various PAH compounds. Many studies on bioaugmentation have showed that gram-negative bacteria belonging to species *Pseudomonas*, *Flavobacterium*, *Sphingomonas*, and *Achromobacter* (El Fantroussi and Agathos 2005) have been used to degrade petroleum hydrocarbons. Meanwhile other potential gram-positive bacteria species such as *Mycobacterium*, *Bacillus*, and *Rhodococcus* were found to utilize well the carbon sources.

It should be noted that fungi such as *Aspergillus*, *Penicillium*, and *Verticillium* also have been used in bioaugmentation purposes. Studies by Ying et al. (2010) illustrated that bioaugmentation of PAH-contaminated soil with *Paracoccus* sp. strain HPD-2 had resulted in 23.2% degradation of total PAH compounds in soil after a 28-day period. He observed that the PAH compound was reduced from 9942 to 7638 μg kg^{-1} dry soil. He also discovered that the percentage degradations of 3-, 4-, and 5(+6)-ring PAH compounds are 35.1%, 20.7%, and 24.3%, respectively. Table 17.2 showed the discovered selected microorganisms used in bioaugmentation of petroleum hydrocarbon in the contaminated environment.

17.3 Bioaugmentation with Single Strain

The use of specific oil-degrading strains in a single culture to remediate oil-contaminated soil has been one of the most powerful tools for bioremediation. Basically the isolated single strains or enriched cultures have been selected by previous screening under various concentrations of pollutants (Hosokawa et al. 2009). Previous study reported that the use of single culture with adapted biochemical potentials showed a great performance in degrading hydrocarbon compounds (Devinny and Chang 2000). For example, the application of pure culture of *Pseudomonas putida* ZWL73 in soil contaminated with 4-chloronitrobenzene (4CNB) showed the highest degradation of 4CNB in soil microcosms (Niu et al. 2008). Previous study by Dams et al. (2007) reported that the use of *Sphingobium*

Table 17.2 Selected microorganism used in bioaugmentation of petroleum hydrocarbon in soil

Microorganisms (genus or species)	Remark	References
Pseudomonas fluorescens	Microorganism used in degradation of crude oil within 13 months	Peressutti et al. (2003)
Pseudomonas alcaligenes		
Alcaligenes xylosoxidans		
Burkholderia cepacia		
Acinetobacter lwoffii		
Pseudomonas stutzeri		
Acinetobacter baumannii		
Pseudomonas vesicularis		
Pseudomonas aeruginosa		
Achromobacter	Main oil degradation bacteria used in marine and soil environment	Leahy et al. (1990)
Arthrobacter		
Bacillus		
Flavobacterium		
Amorphoteca	Most common fungi used in bioaugmentation of oil	Chaillan et al. (2004), Singh (2006)
Neozartoya		
Cephalosporium		
Penicillium		
Graphium Aspergillus		

chlorophenolicum for pentachlorophenol (PCP) remediation has showed that after 2 weeks of incubation, 80% of added PCP was degraded, whereas in non-inoculated soil about 5% was utilized. This finding indicates that the augmented strains of *Sphingobium chlorophenolicum* performed faster PCP degradation as compared to non-inoculated soil.

Our laboratory-scale experiment assisted by aerated static pile (ASP) was conducted to compare the ability of five single strains *C. tropicalis* RETL-Cr1, *C. violaceum* MAB-Cr1, *P. aeruginosa* BAS-Cr1, *S. paucimobilis* RETOS-Cr1, and *S. maltophilia* RAS-Cr1 in degrading oil sludge at different concentration levels (5% and 10%). The findings demonstrated that the usage of *P. aeruginosa* BAS-Cr1 was best performed in degrading oil sludge at 5% and 10% concentration with more than 80% degradation of TPH within 42 days of treatment (Zaida and Piakong (2017)). Table 17.3 showed the selected single microorganisms used in bioaugmentation of contaminated soil.

17.4 Bioaugmentation with Microbial Consortia

Another approach of bioaugmentation involves the use of microbial consortia to remove the target pollutants. It has been reported that a combination of two or more cultures is known to have synergistic effects and have high potential to be good degraders of many hydrocarbon compounds especially PAH fraction. Previous

Table 17.3 Selected single microorganisms that are commonly used in bioaugmentation of soil contaminated with aromatic compound

Microorganisms (single strains)	Contaminated/treated	References
Comamonas testosterone BR60	Crude oil, PAHs	Gentry et al. (2001)
Arthrobacter chlorophenolicus A6L	4-Chlorophenol	Jernberg and Jansson (2002)
Absidia cylindrospora	Fluorene	Garon et al. (2004)
Pseudomonas sp. ST41	Marine gas oil	Stallwood et al. (2005)
Pseudomonas aeruginosa WatG	Diesel oil	Ueno et al. (2006)
Sphingobium chlorophenolicum ATCC	Pentachlorophenol	Dams et al. (2007)
Burkholderia sp. FDS-1	Fenitrothion	Hong et al. (2007)
A*spergillus* sp. LEBM2	Phenol	Santos et al. (2009)
Gordonia sp. BS29	Aliphatic and aromatic hydrocarbon	Franzetti et al. (2009)
Pseudomonas putida ZWL73	4-Chloronitrobenzene	Niu et al. (2008)
Trichocladium	HWM-PAHs (4–7 rings)	Silva et al. (2009)
Pseudomonas aeruginosa BAS-Cr1	Oil sludge	Zaida and Piakong (2017)
Candida tropicalis RETL-Cr1		
Chromobacterium violaceum MAB-Cr1		
Stenotrophomonas maltophilia RAS-Cr1		
Sphingomonas paucimobilis ReTOS-Cr1		

study has showed that application of mixed cultures (consortia) is more powerful than single strains by the fact that the intermediates of a catabolic pathway of one strain may be further degraded by other strains possessing suitable catabolic pathway (Heinaru et al. 2005). As mentioned by Bento et al. (2005), the addition of a bacterium consortium isolated from the Long Beach soil had shown degradation capacity of 73–75% of the light (C_{12}–C_{23}) and heavy (C_{23}–C_{40}) fractions of total petroleum hydrocarbons (TPH) present in the soil. On the other hand, 46–49% removal was obtained as a result of biodegradation by natural attenuation. This finding concludes that the addition of bacterium consortium into the soil performed the highest degradation by 1.5-fold higher than the natural attenuation.

Jacques et al. (2008) reported the capacity of various species consortia of *Mycobacterium fortuitum*, *Bacillus cereus*, *Microbacterium* sp., *Gordonia polyisoprenivorans*, *Microbacteriaceae* bacterium, and *Fusarium oxysporum* to degrade and mineralize anthracene, phenanthrene, and pyrene in soil. The results illustrated that each PAH compounds is degraded from 96% to 99% of initial doses (250, 500, and 1000 mg/kg) by this consortium within a 70-day period. The results also showed that this consortium can mineralize the different concentrations of PAH mixture by 70%, while non-bioaugmentation soil did not show essential utilization of PAHs. This finding indicates that microbial consortium was more effective as compared to bacterial and fungal isolates inoculated separately to the soil. This result also in line with

Yu et al. (2005) showed that biodegradation of a mixture of fluorene, phenanthrene, and pyrene by a bacterial consortium is made up of three strains of *Rhodococcus* sp., *Acinetobacter* sp., and *Pseudomonas* sp. The results also showed that the addition of this consortium into sediments significantly enhanced the efficiency of fluorene and phenanthrene biodegradation but not pyrene. The biodegradation efficiency was found at 97% and 99% for phenanthrene and fluorene, respectively. However about 10% of pyrene was degraded after 2 weeks of incubations.

Our studies using microbial consortium assisted by aerated static pile showed that the combination of *Pseudomonas aeruginosa* BAS-Cr1, *Sphingomonas paucimobilis* ReTOS-Cr1, and *Stenotrophomonas maltophilia* RAS-Cr1 degraded 68% of oil sludge within 56 days of treatment as compared to 22% degradation in a control plot. This consortium proved to be better than treatment using single strain *Stenotrophomonas maltophilia* RAS-Cr1 with only 65% of degradation. The assistance of aerated static pile in a bioreactor was pronounced to supply more oxygen in the soil, thus enhancing the rate of oil degradation. Analysis of hydrocarbon compounds also confirmed that most of the alkanes have been degraded up to 99% especially using microbial consortium. The ratios of n-C_{17}/Pr and n-C_{18}/Ph in microbial consortium were decreased from 3.16 to 0.49, respectively. It can be summarized that n-alkanes degrade faster than isoprenoids (branched alkanes) because isoprenoids are relatively resistant to biodegradation. Table 17.4 shows the selected consortia microorganisms used in bioaugmentation of soil contaminated with polyaromatic hydrocarbon (PAH) compounds.

Table 17.4 Selected consortia microorganisms used in bioaugmentation of soil contaminated with polyaromatic hydrocarbon (PAH) compounds

Microorganisms (consortia strains)	Contaminated/treated	References
Rhodococcus sp., *Acinetobacter* sp., *Pseudomonas* sp.	PAHs (fluorene, phenanthrene, pyrene)	Yu et al. (2005)
Bacillus subtilis DM-04, *Pseudomonas aeruginosa M* and *NM*	Crude petroleum oil hydrocarbons	Das and Mukherjee (2007)
Mycobacterium fortuitum, *Bacillus cereus*, *Microbacterium* sp., *Gordonia polyisoprenivorans*, *Microbacteriaceae* bacterium	PAHs (anthracene, phenanthrene, pyrene)	Jacques et al. (2008)
Rhizopus sp., *Penicillium funiculosum*, *Aspergillus sydowii*	Petroleum hydrocarbons	Mancera-Lo'pez et al. (2008)
Bacillus strains B1F, B5Q, and B3G, *Chromobacterium* sp., *Enterobacter agglomerans* sp., *Acremonium* sp., *Aspergillus* sp., *Verticillium* sp.	Mixture of PAHs (naphthalene, phenanthrene, anthracene, pyrene, dibenzo anthracene	Silva et al. (2009)
Pseudomonas aeruginosa BAS-Cr1, *Sphingomonas paucimobilis* ReTOS-Cr1, *Stenotrophomonas maltophilia* RAS-Cr1	Oil sludge	Zaida and Piakong (2017)

17.5 Factors Influencing Petroleum Hydrocarbon Degradations in the Environment

There are a number of limiting factors that influence biodegradation of hydrocarbon in soil. These include the presence of basic components such as nutrients (nitrogen and phosphorus), soil pH, soil moisture content, temperature, and the bioavailability of the hydrocarbons treated. Figure 17.2 illustrates various factors that influence the rate of biodegradation in the environment.

17.5.1 Temperature

Temperature is a degree or intensity of heat present in the soil. In biodegradation of hydrocarbons, temperature plays an important role by directly affecting the chemistry of the pollutants such as physiology and diversity of the microbial flora. Previous study has showed that a high temperature persuades a high degradation rate of biological degradation process in the soil (Sims and Bass 1984). According to Gunkel (1967), low temperature will lead to slow rate of microbial activity; thus this consciously affects the utilization of hydrocarbon by microbes in the soil. Parr et al. (1994) asserted that a range of 20–35 °C is the most optimal temperature for the growth of microorganisms. At this range majority of the microorganisms achieve maximum degradation of hydrocarbon products. This has been proved by Hong et al. (2007), where inoculation of *Burkholderia* sp. to treat fenitrothion (nitrophenolic pesticide) has showed that 30 °C was the most optimal temperature recorded as compared to 10 °C and 50 °C condition. The findings were supported by Malina

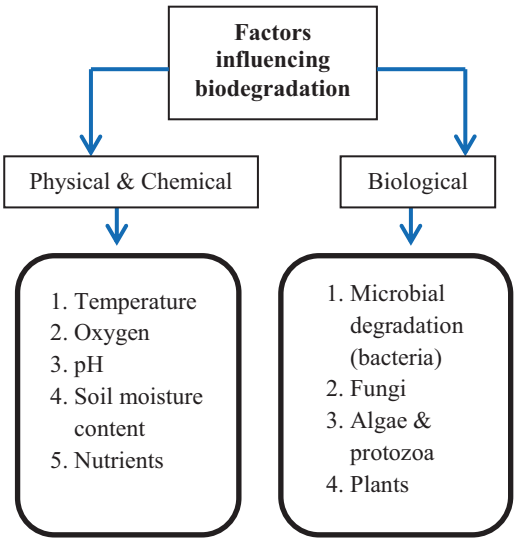

Fig. 17.2 Physical, chemical, and biological factors that influence the rate of biodegradation in soil

et al. (1999), demonstrating that degradation of toluene and decane was reduced when exposed at low temperature of 20–10 °C.

17.5.2 Nutrients

Nutrients are one of the important life supports for microbial growth. Previous studies have showed that nutrients (nitrogen and phosphorus) are the factors that mostly influence the rate of biodegradation of oil in soil. In fact many researchers demonstrated that the bioaugmentation to biodegrade hydrocarbon is more effective with the presence of nutrients in soil. These nutrients are found to be a limiting factor, thus affecting the biodegradation process. The C/N ratio recommended by the US Environmental Protection Agency ranges from 10:1 to 100:1 for bioremediation of petroleum compounds leaking from underground storage. This broad range of values suggests that the need for nitrogen depends on the environmental conditions as well as type of contaminants and area, the microorganisms present, and nitrogen source needed (Leys et al. 2005). The optimum amounts of nitrogen were found to increase the cell growth of microorganisms, decrease the acclimation phase, and sustain fast the activity of microorganism (Ferguson et al. 2003; Leys et al. 2005).

On the other hand, excessive nutrient concentrations can also retard the biodegradation activity of microorganisms in soil (Choi et al. 2002). The inhibition of microbial activity at high nutrient levels can be associated with increased salinity in soil caused by the nutrients. Moreover, microbial adaptation time can increase due to soil salinity caused by high level of NH_4^+ and NO_3^-. These findings have been reported by Oudot et al. (1998) and Chaineau et al. (2005), who found the negative effects of NPK levels on degradation of aromatic compounds.

17.5.3 Soil Moisture Content

Soil moisture can be defined as the amount of water content in the material of soil. As water is the main component of biological and ecological process, the amount of water present in the soil is very important for remediation study. A microorganism is requiring enough water for its growth and diffusion of nutrients along the biodegradation process. Generally, soil type is the main factor influencing the soil water content. The optimum aerobic degradation in the field capacity mostly occurs in soil with moisture of 50–80% (Pramer and Bartha 1972). However, when soil moisture content is below than 10%, the degradation capacity will drop due to low bioactivity of microorganisms in the soil (Testa and Winegardner 1991).

Previous study has showed the survival of *Achromobacter piechaudii* TBPZ and degradation of tribromophenol (TBP) in soil with 25% and 50% water content; however when exposed to 10% of moisture content, the degradation was lower than the preference conditions (Ronen et al. 2000). He also mentioned that the low deg-

radation process is due to the limitation of substrate supply and adverse physiological effects associated with cell dehydration in the soil. On the other hand, the excess moisture would significantly reduce soil gas permeability and thus limit biodegradation (Leeson and Hinchee 1996b). Soil water content above the water holding capacity of the soil had an inverse effect on biodegradation of petroleum hydrocarbons. High soil water content can reduce effective gas diffusion and air-filled porosity, which resultantly makes oxygen diffusion limited for aerobic biodegradation (Borresen and Rike 2007). In turn, insufficient oxygen levels can reduce the rates of biodegradation in higher levels of moisture content.

17.5.4 pH Values

Microorganisms require a specific range of soil pH to stay alive and proliferate. The presence of ion hydrogen in the soil will demonstrate the acidity and alkalinity of the soil. Previous studies have reported that some microorganisms can survive in a wide range of pH, but others are sensitive to small variations. The effective range of soil pH recommended by Dupon et al. (1991) is 5.5–8.5, and the USEPA (1995) suggests the optimum ranges of pH for successful bioremediation are from pHs 6 to 8. The soil pH must be of concern in order to enhance microbial activity to increase the degradation process.

Deviation of pH from the optimum range may result in reduced microbial population, because each species displays optimum growth at a particular pH. However, microbial respiration based on oxygen utilization has been observed in soils where the pH was slightly below 5 or above 9 (Leeson and Hinchee 1996a). Good levels of pH also depend on the type of contaminants. Correspondingly, Hambrick III et al. (1980) found an increased rate of octadecane mineralization when the pH was increased from 6.7 to 8; however naphthalene mineralization rate remained unchanged at this range. The optimum soil pH for hydrocarbon biodegradation also depends on the type of microbial species present. Nevertheless the majority of the bacteria grow well at a neutral pH (Dibble and Bartha 1979; Leahy et al. 1990). Verstraete et al. (1975) studied biodegradation of gasoline-contaminated soil at pH range of 4.5–8.5 and found optimal microbial activity at a pH of 7.4 and a considerable inhibition at pH 8.5. Dibble and Bartha (1979) also reported optimum pH range of 7.5–7.8 for oil sludge biodegradation.

17.5.5 Oxygen

Oxygen availability is one of the critical factors for aerobic degradation (Floodgate 1984). Microorganisms in soil and groundwater use oxygen to fuel cellular activity and degrade or transform hydrocarbons or other contaminants (Davis et al. 2005). The first attack on hydrocarbons by microorganisms always requires the action of an

oxygenase enzyme which catalyzes biochemical reactions in the presence of oxygen (Leahy et al. 1990; Okoh 2006). Likewise, a very quick and effective way to monitor aerobic biodegradation is the determination of the oxygen consumption rate.

Dineen et al. (1990) found that the basic amount of oxygen to degrade hydrocarbon is 3.1 g of oxygen for 1.0 g of hydrocarbon. In surface soil, oxygen is mostly accessible, but in the subsurface, where oxygen diffusion is restrictive, aerobic biodegradation may become uncertain (Song et al. 1990). Limiting oxygen diffusion in soil with depth also gradually decreases oxygen level in soil with increasing subsurface depth. Franzmann et al. (1999) determined that oxygen concentrations at the soil surface were around 20%, decreasing to 14% at 0.25 m below surface and to 1.2% at 0.75 m below surface. Similarly, the oxygen level dropped and remained constant at 1% at 1 m below surface. At this point, biodegradation of benzene was completely anaerobic. Oxygen concentrations between 2 and 5% are the minimum range for aerobic biodegradation, while Leeson and Hinchee (1996a) found lower rates of biodegradation particularly when oxygen level was below 10%.

In order to maintain aerobic biodegradation activities, there are several ways to supply additional oxygen to a contaminated site. Several techniques applied are air injection or extraction during bioventing, the addition of pure oxygen, soil tilling, and mechanical rotation. Using hydrogen peroxide and calcium peroxide which are recommended chemicals is an alternate method to add oxygen (Fiorenza et al. 1991; Fagan 1994). However, microbial activity will be in active conditions when exposed to excessive airflow rates in soil. This is due to the excessive airflow which caused low moisture content in soil resulting in drying out of the soil for microbial activity (Pedersen and Curtis 1991). Table 17.5 showed the summary of various parameters that affect the rates of biodegradation in soil.

17.6 Microbial Degradation

17.6.1 Bacteria

Bacteria are one of the key players in bioremediation. Previous study has showed that many bacteria have the ability to transform and degrade many types of pollution in different pollutant matrixes (Saval 1998). These several bacteria can survive in contaminated habitat due to its metabolic capability of utilizing its resources and can occupy a suitable niche. Moreover, some contaminants like hydrocarbons are often potential sources of energy for their growth. This has been proved by Yakimov et al. (2007) who reported that several bacteria are even known to feed exclusively on hydrocarbons as their sole carbon source. According to Scragg (2005), natural soil usually contains microorganism in the range 10^4–10^7 CF/g soil. However, for bioremediation purposes, the number of degrading microorganisms should not be lower than 10^3 per gram of soil. The amount of CFU/g soil lower than 10^3 indicates the presence of toxic concentrations of organic or inorganic contaminants. As

Table 17.5 Summary of various physical factors that affect the rate of biodegradation

Parameter	Condition	References
Structure and composition of hydrocarbon	Hydrocarbon attacks by microbes are in descending orders of the following compounds of n-alkanes> branched alkane> low molecular weight aromatics> cyclic alkanes	Fusey and Oudot (1984)
Temperature	With increase in temperature, the rate of biodegradation also decreases because of decreasing enzymatic activity	Atlas and Bartha (1973)
	Highest degradation rate occurs in the range of 30–40 °C (soil), 20–30 °C (freshwater), 15–20 °C (marine)	
Oxygen	The oxygen availability in soil depends on the rates of O_2 consumption by microbes and the type of soil with utilizable substrates which lead to oxygen depletion	Pelletier et al. (2004) and Delille et al. (2004)
Nutrients	Nutrient is important for microbial growth and enzyme activity in soil	Choi et al. (2002) and Chaillan et al. (2006)
Acidity and alkalinity	pH neutral is favored by most heterotrophic bacteria and fungi	Bossert and Bartha (1984) and Atlas (1988)
Soil moisture	The optimal rates of biodegradation of oil sludge in soil at 30–90% water saturation	Dibble and Bartha (1979)

mentioned by Margesin et al. (2003), the best selected microorganism for bioremediation must have the characteristics of developing catabolic activity, inducting specific enzymes, developing new metabolic capabilities through genetic changes, and selecting enrichment of organisms that are able to transform the target contaminant to the simple compounds.

17.6.2 Fungi

Fungi are one of the main degraders of hydrocarbons. They use another mechanism for degradation than bacteria. In some cases, they may be able to degrade the hydrocarbon compound faster than degrading bacteria. Fungi are commonly used for the degradation of five-ring PAHs, which are poorly degraded by bacteria. According to Field et al. (1992), fungi secrete extracellular oxidizing enzymes for degradation of lignin. These enzymes are known to be able to make reactive peroxide from oxygen (Barr and Aust 1994). White rot fungi are one of the species that are able to degrade lignin. Lignin is a complex random molecule containing a lot of aromatic groups. White rot fungi are known to degrade lignin, making them possible candidates for PAH degradation. The degrading enzymes lignin peroxidase and manganese peroxidase have shown to be able to degrade some model lignin compounds.

Studies by McFarland et al. (1992) reported that peroxides showed to be involved in degradation of PAH to quinones. However, fungi did not degrade hydrocarbons completely to CO_2 as bacteria do. According to Field et al. (1992), the highest conversion of hydrocarbons by fungi was only 19%. Instead they form a range of degradation products which are solved in the aqueous phase or become bound to organic fraction into the soil. For degradation of benzopyrene, it was found that nearly all degradation products were bound to the compost fraction used in the experiment. The degradation rate of benzopyrene was found to be double of the degradation rate in a culture without the fungi (McFarland et al. 1992).

17.6.3 Algae

Algae also have the possibility to degrade hydrocarbons specifically PAHs. They use the eukaryotic mechanism similar to fungi. These mechanisms make use of a dioxygenase enzyme which leads to cis-trans hydroxyl groups. Algae are dependent on light to be able to degrade PAHs. The metabolites are depending on the kind of light radiation the algae irradiated with. The production of quinines was related to the intensity of light, and therefore the toxicity of PAHs to algae was also related to the light intensity (Warshawsky et al. 1995).

Some green algae were very effective; they degraded all PAHs in 5 of 6 days for low concentrations, but other green, yellow, and blue green algae were less effective (Warshawsky et al. 1995). It was shown that the difference in degradation rate was enormous for different algae but also had to take consideration that the fate of the PAHs was different. PAHs can be degraded or accumulated in the algae as biomass. Some algae only accumulate, while other degrades nearly all PAHs. This is shown in an experiment of degradation of benzopyrene by Muñoz et al. (2003) who stated that over 90% of the benzopyrene was found in brown algae biomass.

17.7 Plants

Phytotechnologies with an increasing development during the last two decades involve the use of plants to remove, transfer, stabilize, and destroy organic or inorganic contamination in groundwater, surface water, and soil. These technologies have been applied widely because of their advantages, that is, environment-friendly, cost-effective, energy-efficient, and aesthetically pleasing method of remediating sites with low to moderate level of contamination. Moreover, this method also operates easily and safely in processes of harvesting the plants for the extraction of absorbed contaminants such as metals that cannot be easily biodegraded for recycling (Maine et al. 2001; Malik 2007).

Previous study by the Alabama Department of Environmental Management involved about 1500 cubic yards of soil where 70% of the baseline samples con-

tained over 100 ppm of total petroleum hydrocarbon (TPH). The results showed that after 1 year of vegetative cover, approximately 83% of the samples were found to contain less than 10 ppm of TPH.

Other studies by Nedunuri et al. (2000) reported that the initial TPH-contaminated site with crude oil and diesel fuel had reduced from 1700 to 16,000 mg/kg by applied phytoremediation strategies involving various plant species. Among plants used for petroleum degradation, *Cordia subcordata*, *Thespesia populnea*, and *Scaevola sericea* are known to have potential in tolerating field conditions and facilitating cleanup of soils contaminated by diesel fuel (Miya and Firestone 2001). He also reported that high amount of hydrophobic contaminants such as TPH, BTEX, and PAHs was found binding and transforming in fine roots of the surface soil. Firestone and Miya (2001) also reported that, the most significant mechanism for removal of diesel range organic in vegetated contaminated soil are occur in rhizosphere part of plants. This is due to the fact that PAH compounds are highly hydrophobic and their sorption can decrease the bioavailability of plant uptake and phytotransformation in soil.

17.8 Hydrocarbon Biodegradation Mechanism and Products

Biodegradation is the process by which microorganisms transform or mineralize organic contaminants into less toxic compound. Organic chemical can be degraded by two biodegradation mechanisms which are aerobic (with oxygen) or anaerobic (without oxygen).

17.8.1 Aerobic Degradation

Aerobic degradation is characterized by involvement of oxygen in the pathway. Generally this process involved the breakdown of organic contaminants by microorganisms when oxygen is present. The organic contaminants are rapidly degraded under aerobic conditions by aerobic bacteria called as aerobes. The degradation starts with intracellular attack of organic pollutants known as oxidative process. Later the activation continued along the incorporation of oxygen called as enzymatic key reaction catalyzed by oxygenases and peroxidases. The process converts organic pollutants to intermediate metabolites such as tricarboxylic acid cycle intermediates. The biosynthesis of cell biomass occurs from the central precursor metabolites, for example, acetyl-CoA, succinate, and pyruvate. For degradation of aromatic hydrocarbons, the process is converted to the natural intermediates known as catechol and protocatechuate. Some gram-negative bacteria have the plasmids (TOL plasmid) that encode enzymes for degradation of aromatic hydrocarbons. This process mainly involves hydroxylation catalyzed by dioxygenase. Other mechanisms involved the attachment of microbial cells to the substrates to produce biosurfactants (Hommel 1990). The uptake mechanism linked to the attachment of cell

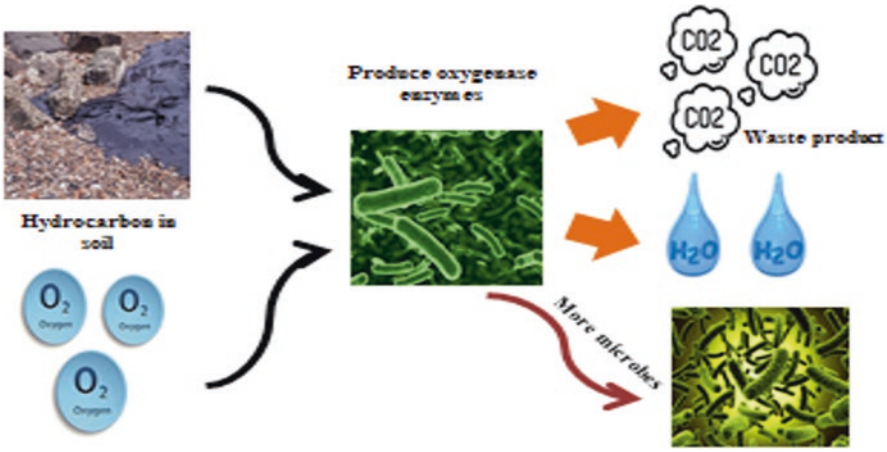

Fig. 17.3 The principle involved in aerobic degradation of hydrocarbon by microorganisms

to oil droplet is still unknown, but production of biosurfactants has been well studied. Figure 17.3 shows the initial attack on xenobiotics by oxygenases.

17.8.2 Anaerobic Degradation

Anaerobic biodegradation occurs when the anaerobic microbes are dominant over the aerobic microbes, and this study usually is used to treat wastewater sludge. The metabolic pathways behind anaerobic alkane biodegradation are not well understood. Most of the reports related to the anaerobic mineralization of aliphatic hydrocarbons showed that several alkylbenzenes, alkanes, or alkenes are anaerobically utilized as substrates by several species of denitrifying, iron-reducing, and sulfate-reducing bacteria. Another group of anaerobic hydrocarbon-degrading bacteria are "proton reducers" that depend on syntrophic associations with methanogens. For two alkylbenzenes, toluene and ethylbenzene, details of the biochemical pathways involved in anaerobic mineralization are known. These hydrocarbons are initially attacked by novel, formerly unknown reactions and oxidized further to benzoyl-CoA, a common intermediate in anaerobic catabolism of many aromatic compounds.

17.9 Bioaugmentation of Petroleum Hydrocarbon in Soil (Case Studies)

The application of bioaugmentation with various scales of treatment and types of contaminants done by previous studies is shown in Table 17.6. The case study on degradation of diesel hydrocarbon by Sharma et al. (2014) using bioreactor had

Table 17.6 Specific bioaugmentation of petroleum hydrocarbon case studies

Scale of treatment	Type of contaminant	Duration of research	Microorganisms used	Initial TPH	TPH reduction	Remarks	References
Lab scale (bioreactor)	Diesel oil	30 days	Single culture *P. aeruginosa*	*NS	66%	They found a new strain having a significant rate of diesel degradation capability showing 66% of degradation in 30 days of incubation periods	Sharma et al. (2014)
Prepared bed (field scale)	Oil sludge	160 days	Single culture (Rhedor)	129,600 mg/kg^{-1}	52.75%	Addition of extraneous microorganism can improve the bioremediation process as compared to without extraneous microorganism	De-qing (2007)
Lab scale	Contaminated soil from Greek oil refinery site	190 days	Single culture *P. aeruginosa*	*NS	51% of n-alkanes and 44% of aliphatic hydrocarbon	The addition of *P. aeruginosa* reduced biodegradation rate as compared to control	Karamalidis et al. (2010)
Field scale	Oil refinery	10 months	Bacteria consortium	*NS	96.51%	Degradation of n-alkanes and aromatic compounds occurs during bioremediation time	Mandal et al. (2012)
Lab scale	Crude oil	35 days	*Acinetobacter baumannii* T30C	4200 mg/kg^{-1}	43%	The bioaugmentation of *A. baumannii* T30C into the soil is not significant in the reduction of TPH since the results are not much higher than the control	Chang et al. (2011)
Lab scale	Oil sludge	2 months	Bacteria consortium	100 g/kg	79%	The result proved that the addition of preselected microbial consortium was best performed in oil sludge degradation within 3 months	Zaida and Piakong (2017)

(continued)

Table 17.6 (continued)

Scale of treatment	Type of contaminant	Duration of research	Microorganisms used	Initial TPH	TPH reduction	Remarks	References
Lab scale	Petroleum-contaminated soil in Zichang County in China	10 weeks	*Acinetobacter* strain SZ-1 KF453955	46,600 mg/kg^{-1}	34%	The TPH degradation was enhanced by bioaugmentation of *Acinetobacter* strain SZ-1 KF453955 after 6 weeks of treatment period	Manli et al. (2016)
Bench scale	Soil contaminated with TPH and PAH	195 days	Bacteria consortium of 927 different strains	*NS	69%	The addition of exogenous microbial strains significantly enhanced the biodegradation rate as compared to soil with nutrient amendment alone	Ellis et al. (2012)
Lab scale	Oil sludge	90 days	Bacteria consortium	*NS	76%	The increase of oil degradation occurs after the addition of selected bacterial consortium in oil sludge-contaminated soil	Vasudeva (2001)

demonstrated that the highest degradation (66%) was observed in bioreactor E with inoculation of *P. aeruginosa* after 30 days of incubation period. It was noted that the addition of nutrient was mandatory for enhancing bacterial growth and degradation potential in soil. He also noted that lipase activity was found to be the best parameters for testing hydrocarbon degradation.

Another relevant study conducted by Chang et al. (2011) has reported that microbial degradation of Tapis crude oil-contaminated soil by *Acinetobacter baumannii* T30C was found with low degradation after 35 days of incubation. The results indicate that the depletion of nutrient levels affects the biodegradation performances in the soil. Chang et al. (2011) also suggested that the addition of nutrients was necessary for enhancing the bacterial growth and degradation activity except in cases where the indigenous identifiable petroleum degraders were too small and necessitate introduction of biodegrading strains.

Fatima et al. (2003) has studied the biodegradation of soil contaminated with diesel oil by adding hydrocarbon-degrading bacterial consortium (*Bacillus cereus*, *Bacillus sphaericus*, *Bacillus fusiformis*, *Bacillus pumilus*, *Acinetobacter junii*, and *Pseudomonas* sp.). She reported that the highest rate of degradation of the light fraction TPH (C_{12}–C_{23}) occurs in the first 2 weeks of incubation in soil. After 12 weeks of incubation, the results showed that the greatest percentage of degradation of the light fraction was 75% followed by heavy fraction with 73%. It was reported that addition of nutrients had the least effect on the degradation of both light and heavy fraction of TPH in both contaminated soils.

Experiment conducted by Vasudevan and Rajaram (2001) showed that soil contaminated with oil sludge plus wheat bran and bacterial consortium is recorded to have the highest percentage degradation with 76% within 90 days of incubation period. An increase of oil degradation percentage was noticed with an increase in the bacterial population from (1×10^9) to (6×10^{13}). The author summarized that the tillage operation and bulking agent (wheat bran) seemed to influence the degradation of the hydrocarbons. This concluded that the addition of bulking agent and bacterial consortium in contaminated soil gives synergistic effect toward the oil degradation study.

The bioremediation of hydrocarbon in contaminated soil by mixed cultures of hydrocarbon-degrading bacteria was investigated by Farinazleen et al. (2004). She found that Consortium 1 and Consortium 2 consisted of three and six bacterial strains that have the capability to degrade n-alkanes. The highest degradation of n-alkanes was more pronounced in the soil sample that inoculated with Consortium 2 (*Bacillus* sp., *P. aeruginosa*, and *Micrococcus* sp.). The maximum reduction also was observed in tetradecane compound as compared to others.

17.10 Conclusion

The use of bioaugmentation technology in the environment contaminated with petroleum hydrocarbon has showed a positive effect toward the remediate soil. However, the selection of proper microbial strains should be highly focused in

order to achieve the target. The most effective elimination of hydrocarbon in the environment is based on the selection of oil-degrading microorganisms that were able to survive in high concentration of pollutants. In fact, the ability of inoculants to survive in thrive condition poses a great potential to remediate contaminated soil. A part of that, physical, chemical, and biological factor also plays an important role for bioremediation purposes. Therefore in considering the fact above, the aforementioned bioaugmentation strategies are the best approach to remediate contaminated soil.

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Chapter 18
Petroleum Microbiology Under Extreme Conditions

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Abstract Petroleum contamination of environmental matrices is a pervasive, global problem. Crude oil exploration, processing, handling and transport release significant amounts of petroleum hydrocarbons into the environment. Many petroleum compounds are known or suspected carcinogens, mutagens and teratogens and therefore, pose significant risks to human and ecosystem health. Petroleum hydrocarbon pollution constitutes an enormous challenge when areas with suboptimal environmental conditions are contaminated. This is because these regions are characterized by the occurrence of delicate ecosystems and because remedial efforts tend to be frustrated, owing to the unfavourable climatic and environmental conditions. Due to extensive petroleum exploration in some of these areas, petroleum hydrocarbon contamination occurs frequently, degrading the environment. Efficacious, sustainable abatement strategies are therefore, necessary to mitigate contamination.

Over time, several treatment schemes and strategies for the replenishment of petroleum-contaminated sites have been designed, optimized and implemented. Many conventional techniques and technologies however, have significant limitations. This has prompted research into environmentally friendly and cost-effective clean-up alternatives. Bioremediation is an appealing option, which has been the subject of extensive research and has been adopted in many parts of the world because of its (comparative) low cost, minimal environmental impacts and public acceptance. Here, the general sources of petroleum hydrocarbons into the environment are explored as well as the effects of physicochemical and environmental factors on the transport, microbiology and overall fate of petroleum hydrocarbons in environmental matrices. The potential of petroleum hydrocarbon biodegradation under extreme environmental conditions is considered with an emphasis on the effects of unfavourable salinity, temperature, moisture, oxygen, nutrient, pressure and pH conditions. The roles of extremophiles in petroleum hydrocarbon biodegradation in extreme environments are also discussed. The influence of biosurfactants and the capacity of extremophiles to produce these under extreme environmental conditions are discussed as

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well as the relevance of bioaugmentation and biostimulation. Bioavailability, which influences the overall rate and efficiency of bioremediation protocols, is also considered.

18.1 Introduction

Petroleum is a heterogeneous mixture of simple and complex hydrocarbons (Atlas 1981). Petroleum hydrocarbons can be classified into the saturates or aliphatics (including n-alkanes, branched alkanes and cycloalkanes), the aromatics, the (polar) asphaltenes (phenols, fatty acids, ketones, esters and porphyrins) and the resins (pyridines, quinolines, carbazoles, sulphoxides and amides) (Atlas 1981; Leahy and Colwell 1990). Organometallo constituents such as vanadium and nickel as well as nitrogen, sulphur and oxygen in some amount may also be found occurring in petroleum (Van Hamme et al. 2003). The different constituents vary in physicochemical properties depending on the source and formation and based on relative proportions of heavy molecular weight constituents present, are classified as light, medium or heavy (Varjani 2017). Petroleum hydrocarbons and products (such as gasoline, kerosene, diesel) are introduced into the environment during offshore and onshore petroleum industrial activities including exploration, extraction, production, refining, transportation, processing and storage (Varjani 2017). Other anthropogenic sources include industrial and municipal discharges, accidental spills, incomplete combustion of fossil fuels and so on (Peixoto et al. 2011; Varjani 2017). Many of these compounds, particularly the high molecular weight ones, are hydrophobic, stable and therefore, recalcitrant, remaining in environmental matrices for protracted periods (Atlas 1981). They are also confirmed or potential carcinogens, mutagens and teratogens; thus they pose substantial health risks to humans and ecosystems (Varjani 2017).

Conventional approaches and strategies, including physical and chemical technologies, have been optimized and utilized to ameliorate petroleum contamination and restore polluted sites (Castaldini 2008). Drawbacks, such as technological complexity, high cost and a general lack of public acceptance, however, limit the efficacy of some of these. Many of the techniques are invasive and merely relocate the contamination problem, often requiring further management (Castaldini 2008). Experts reckon that it is more practical to adopt a sustainable approach that is cheaper and more environment friendly, which will completely mineralize the pollutants or transform them into innocuous substances (Lundstedt 2003; Castaldini 2008).

Bioremediation is a biological approach that is an appealing alternative because it has been demonstrated to be relatively cost-effective, environmentally friendly, generally accepted and in many documented cases, more effective (Bamforth and Singleton 2005). Environmental factors such as temperature, pH, moisture, salinity and pressure are pivotal in the removal of petroleum hydrocarbons from contaminated sites (Martínez Álvarez et al. 2017). Other relevant factors include the scale of pollution (e.g. the volume of oil spilled), character-

istics of the impacted area, duration of the contamination (spills), financial circumstances, perceived risks and regulatory stipulations (Filler et al. 2008).

18.2 The Effects of Physicochemical Characteristics on Microbiology of Petroleum Hydrocarbons

The composition of discharged petroleum hydrocarbons varies significantly, depending on the source, generation mechanisms, weathering of the product over time, differential movement of the components in the environment (or migration) as well as trapping and possible alteration (Heath et al. 1993; Head et al. 2003). Physicochemical properties differ depending on the constituent and percentage or concentration of crude or refined oil that is spilled (Speight and El-Gendy 2018d). Variations in composition and properties among different crude oil constituents and refined products influence the rate of biodegradation of oil and its component fractions (Leahy and Colwell 1990). There are different constituents of petroleum with varying molecular structures – could be straight or branched chain, single or condensed rings or aromatic rings (Speight and El-Gendy 2018a). Some common constituents include alcohol, ether, simple alkanes/alkenes, cycloalkanes, chlorinated aliphatics, ethyl alkanes/alkenes, monocyclic aromatic hydrocarbons [such as benzene, toluene, ethylbenzene and xylene (BTEX)] and polycyclic aromatic hydrocarbons (e.g. anthracene, phenanthrene and benzo(a)pyrene) (Atlas 1981). Physicochemical properties differ – molecular weight, water solubility, specific gravity, vapour pressure, diffusivity, organic carbon/water partition coefficient, octanol/water partition coefficient, marine life bioconcentration/bioaccumulation factors, biota-sediment accumulation potential and surface water half-life. These factors influence their transport and fate in environmental matrices (Van Stempvoort and Biggar 2008). Susceptibility to microbial attack differs considerably among hydrocarbons depending on molecular weight and structure. The HMW compounds are generally more hydrophobic (Bamforth and Singleton 2005). Hydrocarbons ranging from C_{10} to C_{26} and LMW aromatics are usually more readily degraded (Atlas 1995), whereas more complex compounds such as those comprising of fused aromatic (benzene) rings as well as a system of hydrophobic and lipophilic double bonds in their structures are generally more resistant to biodegradation (Van Stempvoort and Biggar 2008). Biodegradability of oil components generally decreases in the following order: n-alkanes, branched-chain alkanes, branched alkenes, low molecular weight n-alkenes, aromatics, monoaromatics, cyclic alkanes, PAHs and asphaltenes (Schmidt and Schaechter 2012). Biodegradation rates are highest for unsaturated compounds, followed by the light aromatics, with high molecular aromatics and polar compounds (asphaltenes and resins) exhibiting low rates of degradation (Oudot 1984; Chandra et al. 2013). For example, aliphatic compounds, which are less hydrophobic, are more prone to degradation compared to polycyclic aromatic hydrocarbons, which are not only more hydrophobic but

also have higher sorption capacity (Haritash and Kaushik 2009). Also, it is apparent that abiotic losses of lighter oils are higher compared to heavier oils. They are also more susceptible to biodegradation compared to heavier oils (Atlas 1995).

Solubility affects constituent migration in surface water, soils and groundwater (Lu et al. 2012). Similarly, volatility influences the mobility and recalcitrance of organic and inorganic constituents. Vapour pressure and solubility affect constituents' volatilization potential or ability to partition from the aqueous phase to the vapour phase. All of these factors have direct impacts on the adsorption capacity – potential to adsorb to soil organic matter or sediment particulate matter (Atlas 1981). These directly influence migration through contaminated matrices such as soil and aquifer as well as migration (or partitioning) from surface water to sediments, bioavailability and overall fate and transport of petroleum hydrocarbons both near and far from the original point of release in the environment (Atlas 1981). These factors also influence the overall toxicity of the oil, the consequent deleterious effects on the indigenous microbial community as well as the toxicity that reaches human and ecological receptors (Atlas 1981).

18.3 Fate of Petroleum Hydrocarbons in the Environment

Some of the likely processes and pathways, to which released petroleum hydrocarbons may be subjected to, include volatilization, photo-oxidation, chemical oxidation, emulsification, adsorption onto particulate/organic matter, leaching and microbial degradation (Wild and Jones 1995; Pantsyrnaya et al. 2011). Major pathways of petroleum hydrocarbons in the aquatic environment include dissolution, adsorption onto suspended solids and subsequent sedimentation, biotic and abiotic degradation, uptake by aquatic organisms and accumulation (Pantsyrnaya et al. 2011). These processes and pathways are ultimately responsible for or contribute to their removal.

Petroleum contamination of terrestrial and aquatic ecosystems is quite common (Logeshwaran et al. 2018). There are, however, key differences in remediation in these different ecosystems. The main issues are related to the spreading and movement of the oil as well as the incidence of particulate/organic matter, which significantly influences the behaviour of the pollutants and therefore, its susceptibility to and rate and efficiency of microbial degradation (Leahy and Colwell 1990). Dispersion of hydrocarbons in the water column in the form of oil-in-water emulsions increases the surface area of the oil and thus its availability for microbial attack. This improved dispersion and emulsification enhances degradation rates in aquatic ecosystems, whereas the potential for adsorption to particulates in terrestrial ecosystems has negative implications for the efficacy of degradation (Leahy and Colwell 1990; John and Okpokwasili 2012). In the case of terrestrial oil spills, the oil typically moves vertically into the soil as opposed to horizontal distribution associated with slick formation (Leahy and Colwell 1990). Percolation of oil into the soil minimizes evaporative losses of volatile hydrocarbons, which can be toxic to microorganisms (Sherry et al. 2014). Particulate matter can reduce,

by absorption, the effective toxicity of the constituents of petroleum, but absorption and adsorption of hydrocarbons to humic substances probably contribute to the formation of persistent residues (Leahy and Colwell 1990). The chemical structure of the hydrocarbons also matters, because generally, the higher molecular weight compounds are more hydrophobic and toxic and persist longer in environmental matrices (Bamforth and Singleton 2005).

The remediation strategies designed to ameliorate petroleum hydrocarbon pollution are generally classified into three main categories including physical (e.g. excavation, retrieval and off-site disposal, dredging, dry excavation of sediments, thermal treatment, capping technique and incineration), chemical (chemical oxidation, photocatalysis and solvent extraction, amongst others) and biological (bioremediation – transformation and mineralization) (Singh 2006). Physical treatment systems may be used in conjunction with attenuation approaches or in the case of polluted groundwater, can take the form of permeable reactive barriers that transform pollutants into environmentally acceptable forms (Tong and Yuan 2012).

Some common traditional oil spill clean-up techniques include the use of controlled burns, skimmers, vacuum pumping, low pressure flush, other manual mechanical techniques and the use of gelling agents, amongst others. These techniques will however, merely relocate the contaminants from the impacted environment to a different location or create further pollution (Castaldini 2008). Expensive, complicated technology, energy inefficiency and general public scepticism are other significant limitations of available conventional techniques (Castaldini 2008). It is more sensible to adopt protocols that are not only effective but also more environmentally sound and sustainable.

Bioremediation harnesses the biodegradative capabilities of living organisms to either completely destroy petroleum hydrocarbons or transform them into harmless forms (Vidali 2001; Lundstedt 2003; Castaldini 2008). It uses living organisms, including plants, animals but primarily microorganisms, to remove or neutralize petroleum contaminants (Barret et al. 2010; Langenbach 2013). This could be via the adoption of a natural degradation pathway or through the stimulation (and optimization) of recombinant strains to use hydrocarbons as carbon or energy sources (Lu et al. 2012). Bioremediation can occur on its own through natural attenuation (intrinsic bioremediation) but in most cases could take a long time (Brown et al. 2017). As such, various bioremediation strategies have been developed to promote the microbial metabolism of contaminants by manipulating several variables (Langenbach 2013). Some of these strategies include biostimulation (stimulating viable native microbial population), bioaugmentation (artificial introduction of viable populations), biosorption (dead microbial biomass), bioaccumulation (live cells), phytoremediation (plants) as well as rhizoremediation (plant and microbe interaction) (Sharma 2012). Other common examples of bioremediation technologies are land farming, bioventing, bioleaching, composting and the use of bioreactors amongst others (Vidali 2001; Chadrankant and Shwetha 2011). Laboratory and field trials, documenting successful reduction of petroleum contaminant levels, abound in the literature. In situ and ex situ applications have been optimized, and all of these have proven that bioremediation technologies are not invasive, are energy efficient and are eco-friendly (Sharma 2012; Castaldini 2008).

The rate, efficiency and overall success of petroleum hydrocarbon bioremediation depend on a variety of biotic and abiotic factors (Leahy and Colwell 1990). The presence and activity of efficient petroleum-degrading microbiota in the contaminated environment and their adaptive response to the presence of hydrocarbons, the physiological capabilities of these populations, and existing abiotic circumstances that may influence the growth and proliferation of the hydrocarbonoclastic strains (i.e. how conducive the environment is) affect the success of bioremediation efforts (Atlas 1981; Leahy and Colwell 1990; Chadrankant and Shwetha 2011). Ratios of various structural classes of hydrocarbons present, properties of the biological systems involved, competitiveness, degree of acclimation and accessibility of the contaminants are also crucial. The bioavailability of the substrate, cellular transport properties, concentration and accessibility of nutrients are equally important conditions (Van Hamme et al. 2003; Alegbeleye 2015).

Several life forms, capable of proficiently degrading various classes of hydrocarbons, have been characterized (Shukla et al. 2014). However, microorganisms are better candidates for bioremediation because of their ubiquitous distribution in normal and extreme environments, fast biomass growth, easy manipulation and high diversity of catabolic enzymes (Sharma 2012; Patil and Rao 2014). A myriad of prokaryotic and eukaryotic organisms are capable of metabolizing petroleum hydrocarbons, and these are dominated by *Eubacteria*, comprising 7 of the 24 current major phyla (Prince et al. 2010; Margesin 2017). Overall, site-specific circumstances can be highly variable and can have profound effects on the efficiency of selected treatment strategy (Prince et al. 2010). Extreme environmental conditions may constrain hydrocarbon degradation, rendering bioremediation in certain environments problematic (Van Hamme et al. 2003). Extreme, sporadic fluctuations and variations have been reported in some hydrocarbon-contaminated regions. In the Antarctic, for example, drastic temperature changes such as from −15 °C to nearly 30 °C within 3 h have been reported (Cowan and Tow 2004).

Isolation, identification and characterization of microorganisms capable of proficiently degrading the vast range of petroleum substrates/contaminants in locations with harsh environmental conditions has been recognized as fundamental and vital to the success of clean-up. Strategies, which maximize rates and efficiency of microbial growth and utilization of petroleum hydrocarbons under such peculiar environmental conditions, as well as improved microbial access for hydrocarbon contaminants, and transformations have been designed and optimized over the last several decades (Van Hamme et al. 2003). Members of bacteria, yeasts, fungi and algae have been isolated for these purposes (Buzzini and Margesin 2014; Maiangwa et al. 2015). The development of molecular tools and strategies has further enhanced our understanding of microbial community structure and hydrocarbonoclastic capacity (Varjani 2017). Numerous studies have explored and described genes, community composition, complexity of interactions, trophic levels, guilds, functional diversity, enzymes and transformation steps for some hydrocarbons both under ambient and extreme environmental conditions (Van Hamme et al. 2003). The biochemistry, genetics and pathways of hydrocarbon degradation have been provided in some cases (Bosch et al. 1999; Pinyakong et al. 2003; Liang et al. 2006; Sierra-García et al. 2014; Bian et al.

2015). Studies that have illustrated the hydrocarbon metabolic capacities of extremophilic microorganisms are available (Whyte et al. 1996; Abed et al. 2014). Typical microbial cellular and physiological responses to petroleum hydrocarbon pollutants such as cell surface alterations, adaptive mechanisms for accession and uptake and efflux of these substrates in optimal environmental conditions have been extensively explored; however, a lot remains unknown about metabolism of petroleum compounds in extreme environments (Van Hamme et al. 2003).

18.4 The Concept of Extremophiles and Their Implications for Bioremediation

Based on the characteristics of biomolecules and requirements for biochemical reactions, there are specific physicochemical limitations to cellular processes (Dion and Nautiyal 2007). There are maximum and minimum environmental requirements, above and below which it becomes very difficult for microorganisms to survive (Shelford 1913). Environmental conditions that are near limits for cell functioning are regarded as 'extreme', and these are typically damaging to biomolecules or limiting for enzyme activities (Torsvik and Øvreås 2008).

Extremophiles can be grouped according to the conditions to which they are adapted or thrive. They are classified as thermo (high temperature), psychro (low temperature), hyperthermo (very high temperatures), halo (high salt concentration), acido or alkali (extreme low or high pH), ero (low water activity) and baro (under pressure) (Torsvik and Øvreås 2008; Rampelotto 2013). The suffix -phile is used for those that thrive under the extreme conditions (require such for optimum metabolic activities) and troph or tolerant for those that tolerate the extreme condition (Torsvik and Øvreås 2008). In other words, extremophiles are adapted to and are limited by very narrow sets of environmental conditions, and they require the extreme conditions for their metabolic processes, whereas extremotrophic or extremotolerant organisms are more flexible as they can survive and proliferate under a wider set of environmental parameters. That is to say, they tolerate extreme environmental conditions but will ideally cope better under moderate 'normal' conditions (Rampelotto 2013).

A broader but less conventional definition includes organisms, which can tolerate pollution such as heavy metals, ionizing radiation and organic or other toxic compounds (Torsvik and Øvreås 2008). Research indicates that the taxonomic diversity of microbial populations in 'extreme' environments is low (Dong and Yu 2007). This is consistent with ecological principles related to biochemical requirements described previously that these extreme environments are inhabited by less diverse communities. However, many microbial species are stretching the boundaries for their life processes thereby evolving to at least subsist under these otherwise stressful conditions. Others have been documented to thrive and proliferate under these harsh conditions (Peeples 2014; Varshney et al. 2015). Many times, two or

more extreme variables may prevail within the same environment as independent or interrelated conditions (Birch 2017). For instance, many hot springs are acidic and alkaline at the same time and usually have high metal concentrations; the deep ocean is generally cold, with oligotrophic (very low nutrient content) and high hydrostatic pressure conditions; and also, several hypersaline lakes are very alkaline (Glazier 2014; Fazi et al. 2017). Therefore, many extremophiles are normally poly-extremophiles, being adapted to survive in habitats with diverse extreme physico-chemical parameters (Torsvik and Øvreås 2008; Rampelotto 2013). In some areas such as the Antarctic deserts, several harsh environmental factors interact, such as low temperature, low annual precipitation and strong desiccating winds (Dion and Nautiyal 2007). For instance, extreme temperature occurs concomitantly with extreme water stress in the deserts in Antarctica although in some areas and during restricted periods melting snow may generate some water (Kennedy 1993; Dion and Nautiyal 2007).

An organism may therefore, belong to multiple categories and, for example, be considered as both psychro- and xerophile (Seckbach et al. 2013). Microorganisms adapted to more than one extreme variable have better potential for biological decontamination of extreme environments where a wide range of extreme conditions may prevail simultaneously (Margesin and Schinner 2001).

Petroleum contamination of extreme environments characterized by low or elevated temperature, too acidic or too alkaline pH and high salinity or high pressure has been widely documented (Darvishi et al. 2011; Fathepure 2014; Al-Sarawi et al. 2015; Logeshwaran et al. 2018). High-temperature soils, permafrost soils, the Arctic and Antarctic, rainforests, abyssal regions, marine ecosystems such as the deep sea muds and sediments, deep ocean hydrothermal vent systems, hot springs and muds, salt evaporation ponds, crystalline rocks, ancient sedimentary rocks, hypersaline lakes, dry deserts and many others are contaminated continuously or periodically by petroleum hydrocarbons (Margesin and Schinner 2001). Various life forms which inhabit specific environments based on their biotic and abiotic characteristics, adapt/survive and in fact thrive under these so-called extreme environmental circumstances can however, be found in these diverse environments (Peixoto et al. 2011). Although optimal microbiological processes cannot be guaranteed under these conditions (Fathepure 2014), many extremophiles have been reported to metabolize petroleum compounds in these environments.

18.5 Sources and Occurrence of Petroleum Hydrocarbons in Extreme Environments

Oil exploration and production activities in extreme environments pose risks from oil discharges, leaks or spills, by-products such as natural gas condensates, transport and storage of petroleum and petroleum-derived products and so on (Van Stempvoort and Biggar 2008; Brakstad 2008). Mining activities and chemical and metallurgic industries in close proximity to these regions represent major risks as well.

18.6 Effects of Petroleum Contamination on Microbial Biodiversity

Many petroleum hydrocarbons are recalcitrant, bioaccumulative and toxic to living organisms and ecosystems (Hong et al. 2014). Their occurrences in environmental matrices cause ecological disturbances and alter biodiversity (Mapelli et al. 2017). After contamination events, microbial diversity may be significantly altered owing to sensitivity to different classes of petroleum pollutants (Yang et al. 2009). Petroleum hydrocarbon pollution events have been seen to induce microbial succession in affected ecosystems, associated with HC composition and quantity, as well as the delicate nature of microbial existence (Beazley et al. 2012; Mapelli et al. 2017). Selective pressure on microbial communities subsequent to pollution incidents such as oil spills has been observed (Beazley et al. 2012). In pristine environments, hydrocarbonoclastic group may account for only about 0.1% of the microbial community but could make up 100% of the viable microbial diversity in an oil-polluted ecosystem (Yang et al. 2009). Post-spill (Deepwater Horizon) samples from the Gulf of Mexico near Mississippi (United States) contained mostly oil-degrading organisms resulting from a drastic decrease in diversity (Atlas and Hazen 2011). An initial surge in *Oceanospirillales* and Pseudomonads population (in May 2010), right after the spill, which occurred in April 2010, was observed. A shift in dominance to *Colwellia*, *Cycloclasticus*, *Pseudoalteromonas* and methylotrophs, which persisted until August 2010, was subsequently detected (Mapelli et al. 2017). The actual physiological drivers for the observed shift in microbial community remain controvertible; however, studies and reports have strongly associated this with the spill. Selective pressure exerted on microbial communities by the large increase of petroleum and other petroleum derivatives selects for the survival and proliferation of organisms that can use petroleum and derivatives as energy, electron and/or carbon sources (Beazley et al. 2012). To design appropriate remediation protocols, it is important to understand the impact of petroleum contamination on microbial diversity – the cooperation, competition and succession of microorganisms post oil spill.

18.7 Petroleum Microbiology Under Extreme Environmental Conditions

18.7.1 Salinity

There is a close correlation between osmotic stress and water stress because solutes have a remarkable effect on water activity (Dion and Nautiyal 2007). High salinity is common in restricted habitats, and affected ecosystems especially soils are usually characterized by uneven temporal and spatial water distribution (Dion and Nautiyal 2007). These fluctuations are stressors for microorganisms, as they have to

simultaneously adapt to desiccation and high salt concentrations. High levels of petroleum hydrocarbons have been detected in many hypersaline habitats such as salt flats, natural saline lakes, oilfields, saline industrial effluents, estuaries, beaches, inland lakes, rockpools, desert rain pools, sabkhas, other kinds of salt marshes and so on. Some of these habitats are ubiquitous features of arid and semiarid regions of the world with high evaporation rates (Central Asia, the Arabian Peninsula and Australia) that have been subjected to extensive crude oil contamination (Fowler et al. 1993; Al-Mueini et al. 2007; Al-Mailem et al. 2013; Fathepure 2014). Oilfields are especially problematic because there are a significant number of them worldwide and the high salinity is caused by salty brackish (produced) water generated during oil and gas extraction (Fathepure 2014). Disposal or recycling is expensive and challenging because of high salt levels (about 1000–250,000 mg/l), presence of oil and grease, toxic chemicals, heavy metals as well as certain radioactive materials (Cuadros-Orellana et al. 2006; Bonfá et al. 2011), although there are significant advancements in disposal technology as >95% of all produced waters are reinjected these days, as opposed to the old practice of releasing to the surface (Kuwayama et al. 2013). However, many small-/moderate-scale operators continue to release substantial amounts of produced water into the environment especially accidentally such as via leaky tanks and flow lines. Moreover, inappropriate management in the past has created environmental problems that persist until now in certain regions (Varjani 2017).

Positive correlations between salinity and rates of hydrocarbon mineralization have been demonstrated by several studies (Kerr and Capone 1988; Leahy and Colwell 1990; Van Hamme et al. 2003; Qin et al. 2012)**.** High salt concentrations can lead to low oxygen and hydrocarbon solubility, disrupt cell membrane function and microbial tertiary protein structure, denature enzymes and dehydrate cells, thereby limiting microbial metabolic rates (Whitehouse 1984; Dupraz and Visscher 2005; Pernetti and Palma 2005). Microbial species of the domains Archaea, Bacteria and Eucarya have been isolated and characterized, and their metabolic capacity to degrade a myriad of aliphatic and aromatic hydrocarbons under varying salt concentrations has been demonstrated (Oren et al. 1992; Margesin and Schinner 2001). Several studies have illustrated microbial capacity to use crude oil constituents as carbon sources under moderate- to slightly high-salinity environmental conditions (Díaz et al. 2000; Abed et al. 2006).

There are however, conflicting reports in literature regarding microbial metabolic rates and salt conditions. Some studies indicate that halophilic and halotolerant species are exclusively required for successful bioremediation in high-saline environments, for example, discussed by Fathepure (2014), while some others suggest that salinity does not significantly affect microbial hydrocarbon degradation rates (Kerr and Capone 1988). There are other indications that increased salinity may enhance hydrocarbon biodegradation (Díaz et al. 2000; Yang et al. 2000), while hypersalinity has been shown to reduce microbial metabolism of hydrocarbons (Dupraz and Visscher 2005). For example, hydrocarbon metabolism rates decreased with increasing levels of salinity (3.3–28.4%) in hypersaline evaporation ponds of the Great Salt Lake, Utah, United States (Ward and Brock 1978), and more efficient

PAH degradation in a medium containing 0% NaCl than in a 5% NaCl medium has been reported (Minai et al. 2012).

Halophilic and halotolerant microorganisms, which have very versatile metabolic capacities, have been identified (Oren 2011). Halophiles including prokaryotes and eukaryotes, oxygenic and anoxygenic phototrophs, aerobic heterotrophs, fermenters, denitrifiers, sulphate reducers, methanogens, heterotrophic and methanogenic Archaea, photosynthetic, lithotrophic and heterotrophic bacteria, as well as photosynthetic and heterotrophic eukaryotes have been documented in literature to degrade various types of hydrocarbons (Oren 2002, 2011). Extremely halophilic prokaryotes can tolerate very low water potential and thrive at saturated NaCl concentration ($0.75a_w$). Salt solubility and not cell physiology determines this limit (Dion and Nautiyal 2007). Bacterial diversity decreases with an increase in salinity compared to Archaeal groups which generally increase (Jiang et al. 2007; Valenzuela-Encinas et al. 2008); therefore, Archaea is usually more predominant in high-salinity environments (Maturrano et al. 2006). This may be due to their different salt tolerance capabilities. Most halophilic bacteria can survive at moderate-salinity conditions of up to 2.5 M salt concentrations (Ventosa et al. 1998), but halophilic Archaea can survive up to salt saturation (Oren 2008; Mirete et al. 2015). Due to the differences in salt requirements, halophilic bacteria and Archaea tend to occupy different salinity niches with the former being mostly predominant in low salinity and the latter being dominant in high-salinity environments (Oren 2013). A succession of proteobacterial groups due to salinity has been reported by Wu et al. (2006) who studied the bacterioplankton (free-living) community composition along a salinity gradient of high mountain lakes located on the Tibetan Plateau, China.

Halophilic and halotolerant microorganisms adopt strategies such as the accumulation of small molecules (osmolytes or compatible solutes) in the cytoplasm to counter external osmotic pressure (Kempf and Bremer 1998; Dion and Nautiyal 2007). This is known as organic-osmolyte strategy, and it involves the accumulation of organic compatible solutes – zwitterions (e.g. proline, glycine betaine, ectoine, methylamines and derivatives) or nonionic molecules (polyols, carbohydrates, neutral peptides, as well as amino acids and derivatives) (Dion and Nautiyal 2007). This process is compatible since there is no actual need for the change of intracellular proteins and is quite common among the domain Bacteria and Eukarya and some methanogenic Archaea. Some other halophiles and halotolerant species maintain an osmotic balance by accumulating high salt concentrations via a strategy known as 'salt-in-cytoplasm strategy', but this requires salt adaptation of the intracellular enzymatic machinery and is therefore, energetically demanding (Oren et al. 1992). A minority of the known halophiles, including Halobacteriales of the domain Archaea and Halanaerobiales of the domain Bacteria, use this mechanism. Eubacteria's intracellular salt concentration is low, and the enzymes involved in biodegradation may be conventional (i.e. not salt dependent), and this confers an advantage on this group compared to Archaea (Oren et al. 1992).

Members of genera *Cellulomonas*, *Bacillus*, *Dietzia*, *Halomonas*, *Haloarcula*, *Haloferax*, *Halobacterium*, *Alcanivorax*, *Marinobacter*, *Streptomyces*, *Rhodococcus*, *Gordonia* and *Pseudomonas* are very popular in the literature (Huu et al. 1999;

Zvyagintseva et al. 2001; Wang et al. 2007; Mnif et al. 2009; Fathepure 2014). Some of these microorganisms have been isolated from environments with high salt concentrations such as the Cormorant oilfields in the North Sea, sediments associated with mangrove roots, oilfields, production water, oil and stratal waters and other saline environments (Borzenkov et al. 2006). These microorganisms have been reported to degrade aliphatic and aromatic crude oil hydrocarbons under salinity conditions ranging from 0 to as high as 30% NaCl (Díaz et al. 2000).

Specific examples of studies include microorganisms isolated from Argentine saline soils which were demonstrated to biodegrade diesel fuel (Riis et al. 2003). The degradation of hydrocarbons, crude oil, diesel oil, naphthalene, hexadecane, pyrene, dibenzothiophene, salicylate, catechol and phenanthrene as sole sources of carbon in a 0–10% salinity treatment medium by a *Bacillus* strain has also been documented (Kumar et al. 2007). An actinomycete, *Amycolicicoccus subflavus*, isolated from an oily sludge at Daqing oilfield in China degraded crude oil in the presence of 1–12% NaCl (Wang et al. 2010). Its genetic capacity to metabolize short-chain and long-chain *n*-alkanes like propane and C_{10}–C_{36} alkanes as sole carbon sources was subsequently demonstrated (Nie et al. 2013). *Marinobacter sedimentalis* and *M. falvimaris* have been isolated from soil and pond water collected from hypersaline sabkhas [coastal salt marshes (18–20% salinity)] in Kuwait (Al-Mailem et al. 2013). *Pseudomonas* sp. strain C450R and *Halomonas* sp. strain C2SS100 degraded 93–96% of the aliphatic fraction of crude oil (C_{13}–C_{29}), while producing biosurfactants in the presence of 5–10% NaCl (Mnif et al. 2011). Extreme halophiles that require at least 1 M NaCl (approximately 6% w/v) for survival and can grow optimally at NaCl concentrations above 3 M have been identified and described (Kushner 1978).

Other recent studies seem to place the optimum salinity requirements at neutral. The degradation rates of several hydrocarbons under a range of salinities 0, 35, 50, 80, 120 and 160 $g.L^{-1}$ were assessed by Abed et al. (2006). Microbial mats from an Arabian Gulf area chronically exposed to oil spills were investigated, and almost 100% of initial phenanthrene and dibenzothiophene were degraded at 35 $g.L^{-1}$. The best degradation results for pristine (approximately 75%) and n-octadecane (around 85%) occurred between salinities of 35 and 80 $g.L^{-1}$. Another study reported a 30% increase in degradation rate of petroleum hydrocarbons with a decrease in salinity from 2.86% to 0.10% (Díaz et al. 2002). Hydrocarbon biodegradation capacity of a mangrove microbial consortium immobilized onto polypropylene fibres with treatment salinity ranging from 0 to 180 g L^{-1} was assessed by Díaz et al. (2002). Less than 40% alkane biodegradation was achieved in the 0 $g.L^{-1}$ NaCl medium, about 50% at 20 $g.L^{-1}$ and 65% at 40 $g.L^{-1}$. At higher salt concentrations (60–140 $g.L^{-1}$), alkane biodegradation rates were between 50–60% and reduced to less than 30% at 180 $g.L^{-1}$.

To bioremediate high-salt environments where there are no proficient halophilic or halotolerant strains, there are two possible strategies to override salinity. One is reducing the salt concentrations by dilution or irrigation with fresh water or diluted seawater to lower the salinity or the removal of salt by reverse osmosis, ion exchange or electrodialysis. The other is manipulating the bacterial species to function in a

high salt concentration matrix (Oren et al. 1992; Rhykerd et al. 1995) by producing genetically engineered halophilic oil-degrading bacteria (Kapley et al. 1999), bioaugmentation with foreign consortia or stimulating metabolic/biochemical activity of indigenous species (biostimulation) (Al-Hadhrami et al. 1996). Although expensive, successful bioremediation of extreme environments has been achieved using these strategies. For example, the irrigation of polluted sediments in Kuwait, even though it altered microbial community, facilitated hydrocarbon bioremediation (Radwan et al. 1995; Al-Daher et al. 1998; Balba et al. 1998). Due to the cost implications of diluting high salt concentrations, the use of halophilic or halotolerant organisms seems to be a more promising option.

18.7.2 Temperature

Temperature significantly influences the rate and efficiency of petroleum hydrocarbon biodegradation (Atlas 1981). It has a marked influence on the diversity, physiology and metabolic capacity of microorganisms as well as the physicochemical characteristics (such as viscosity and solubility) of the hydrocarbons (Leahy and Colwell 1990; Foght and McFarlane 1999; Margesin and Schinner 2001). Seasonal shifts in the composition of microbial communities which affected hydrocarbon metabolism rates and efficiency at various temperature values have been reported (Atlas 1981). Temperature directly influences the solubility of many petroleum hydrocarbons, which dictates the degree of spreading and partly determines the surface area of oil available for colonization by hydrocarbon-degrading microorganisms (Yang et al. 2009). An increase in temperature leads to increased solubility, which in turn improves the bioavailability and mass transfer of the hydrocarbon substrates to microbial cells. There are, however, certain exceptions to this principle; for example, some small alkane constituents of petroleum oil are more soluble at 0 °C than at 25 °C (Polak and Lu 1973). Temperature influences solubility of oxygen, which decreases with increasing temperature and reduces the metabolic activities of aerobic species. It also influences availability of nutrients and other electron acceptors (Margesin 2017). It affects the equilibrium (partitioning) and kinetic rate constraints as illustrated by van't Hoff Isochore and Arrhenius equations, respectively. In addition, it influences the rate of abiotic losses such as through evaporation, volatilization, dispersion and oxidation (Atlas 1981). For example, in hot environments, volatile hydrocarbon fractions of crude oil have been observed to evaporate rapidly, leaving longer-chain aliphatic and aromatic constituents, which are generally more hydrophobic and persistent (Abed et al. 2006), although, in some cases, these volatile fractions were characterized as toxic and evaporated slowly, inhibiting microbial degradation of these oils at elevated temperatures (Atlas 1975, 1981; Floodgate 1984; Ubalua 2011).

At low temperatures, there is increased oil viscosity and the volatilization of short-chain alkanes is reduced, water solubility is reduced, and these may delay the onset of biodegradation as these conditions are typically not optimal to trigger

biodegradation (Leahy and Colwell 1990; Atlas 1991; Margesin and Schinner 2001). Rates of degradation are generally observed to decrease with decreasing temperature, and this has been attributed to decreased enzymatic activity, or the 'Q_{10}' effect (Atlas and Bartha 1972; Gibbs et al. 1975). Higher temperatures increase the rates of hydrocarbon metabolism, with optimal mineralization typically in the range of 30–40 °C, above which toxicity of hydrocarbons to microbial cell membrane likely increases (Leahy and Colwell 1990).

Hydrocarbon biodegradation has been shown to occur over a wide range of temperatures, and psychrophilic, mesophilic and thermophilic hydrocarbon-utilizing microorganisms have been isolated and characterized (Atlas 1981; Margesin and Schinner 2001). It is however, noteworthy that a more abundant diversity of hydrocarbonoclastic microorganisms is prevalent at temperatures between 25 and 30 °C (Olliver and Magot 2005). Price and Sowers (2004) assessed the relationship between temperature and metabolic rates in different environments. Three categories of metabolic rates were distinguished: (a) rates sufficiently high to permit growth, (b) intermediate rates sufficient for maintenance of cell functions, but too low for growth, and (c) basal rates sufficient for cell survival as well as repair of damaged macromolecules, but otherwise permitting only cell dormancy. Minimum temperature required for metabolism was not established, but at low temperatures, extremely low metabolic rates were observed.

The effects of temperature are interactive with other factors such as the hydrocarbon composition of a petroleum mixture and the composition of the microbial community (Atlas 1981). For example, hydrocarbons such as isoprenoids, phytanes and pristanes seem to be generally more resistant to bacterial attack at low temperature (Atlas 1991).

18.7.3 Oxygen

Oxygen is an important, rate-limiting factor in bioremediation (Atlas 1995). It is a terminal electron acceptor for aerobic microorganisms and therefore, aids microbial growth (Logeshwaran et al. 2018). A good number of documented microbial degraders are aerobic, many of which have been demonstrated to decompose petroleum hydrocarbons, form biofilms and produce biosurfactants, slime and other biomolecules that aid pollution metabolism. In the major hydrocarbon degradation pathways, oxygenases and molecular oxygen are important participants (Aydin et al. 2017). Aerobic processes mostly generate a considerably greater potential energy yield per unit of substrate and tend to occur appreciably more rapidly (Yang et al. 2009). Moreover, most of the fully optimized bioremediation strategies are based on aerobic processes.

Oxygen may however, be severed as the terminal electron acceptor in metabolism and oxygen limitation sets in (Yang et al. 2009). One possible scenario is that the pollutant may stimulate the indigenous microbial community, resulting in accelerated aerobic metabolism and a consequent depletion of available molecular oxy-

gen. Replenishment rate of depleted oxygen is usually comparatively slow, and so, anaerobic zones are formed within and close to the contaminated site (Bamforth and Singleton 2005). Under such oxygen-deficient conditions, anaerobic biodegradation may be triggered. Unlike aerobic biodegradation, anaerobic microorganisms utilize other available substrates such as nitrate, sulphate, iron, manganese and carbon dioxide as their electron acceptors to break down hydrocarbons often producing carbon dioxide and methane as the final products (Gan et al. 2009). Alternatively, some anaerobic microorganisms can degrade organic contaminants by fermentation in which case the organic contaminants act as the electron acceptors (Gan et al. 2009; Ukiwe et al. 2013). Another possible scenario is when contamination is high, in which case anaerobic biodegradation may be enforced restricting oxygen diffusion due to organic matter pore saturation or clogging of aggregates (Gan et al. 2009). In cold regions, oxygen supply is a common constraint where oxygen is usually scarce and diffusion can be partly or completely blocked (Yang et al. 2009). There is no air phase in groundwater, and thus the availability of oxygen as an electron acceptor is greatly diminished, limited by the low aqueous solubility of oxygen (estimated to be 12 mg/L at 5 °C). This typically results in anoxic conditions within petroleum-contaminated plumes in groundwater.

As such, anaerobic biodegradation is appealing for the remediation of accidental oil spills and contaminated areas with anoxic conditions such as waterlogged and underground soils, sediments and aquifers (Bamforth and Singleton 2005; Prince 2010; Karigar and Rao 2011). Although anaerobic biodegradation proceeds at a much slower rate, it is an important process that has prospects to clean up anaerobic zones since a large aeration area is not necessary and this may minimize cost (Bamforth and Singleton 2005). BTEX compounds have been degraded under various anaerobic conditions (Krumholz et al. 1996; Leahy and Colwell 1990). BTEX biodegradation in a biofilm system under nitrate-reducing conditions (Arcangeli and Arvin 1994) and biodegradation of toluene in iron-reducing aquifer zones have been reported (Albrechtsen and Christensen 1994).

18.7.4 Moisture

Living cells require liquid water for survival and metabolism (Margesin 2017). Physically, in many different environments such as soil, water acts as an agent of transport by mass flow as well as a medium through which substrates and other reactants diffuse to and from reaction sites (Paul 2014). Microbial motility and spacial proliferation as well as substrate transport are other functions of water in soil (Smiles 1988). Chemically, it acts as a solvent in important biochemical reactions (Paul 2014).

There is a persuasive body of evidence indicating that microorganisms can survive harsh environmental conditions such as sub-optimal temperature provided liquid water is available. Moisture and water activity is more critical for microbial life in terrestrial ecosystems compared to aquatic ecosystems (Leahy and Colwell

1990). Hydrocarbon metabolism in terrestrial environments may be limited by water availability for microbial growth and metabolism because the water activity or potential (a_w) of soils ranges between 0.0 and 0.99 a for most bacteria and above 0.86 a for most actinomycetes and fungi in contrast to aquatic environments, in which water activity is stable at around 0.98 (Leahy and Colwell 1990; Dion and Nautiyal 2007). In water-deprived soils, sometimes, a hydrocarbon-mediated reduction in the water-holding capacity of soils may be induced to provide moisture necessary for microbial metabolism and not inhibit degradation. Dibble and Bartha (1979) illustrated this in a study, in which optimal oil sludge degradation rates in soil were observed at 30–90% water saturation. No inhibition of degradation at the lower values was reported. Soil fungal species have a higher capacity to tolerate water stress compared to prokaryotes (Zumsteg et al. 2013; Romaní et al. 2017). Hyphal growth, which provides cross dried pores and procures water from smaller pores that can hold water longer, is an advantage (Killham 1994; Dion and Nautiyal 2007). Some organisms, for example, lichens, can even survive on water vapour rather than liquid water (Kappen et al. 1995; Esseen et al. 2017). Dry soils are particularly prone to large diurnal temperature fluctuations compared to wet soils because of the high specific heat (Paul 2014). Spatial patterns and high spatial heterogeneity are typical in dry soil ecosystems. The microorganisms in such habitats, therefore, have to adapt to severe thermal contrasts, strong UV and light intensities and inadequate nutrients in addition to negligible precipitation rates (Mykytczuk et al. 2013). Many extreme environments such as polar and alpine habitats, desert regions, etc. experience very dry conditions and low precipitation (Mykytczuk et al. 2013; Goordial et al. 2016). However, some microorganisms are able to adapt and thrive under these low moisture conditions (Margesin and Schinner 2001). Xerotolerant microorganisms may counterbalance low water potential in the environment by accumulating highly soluble small molecules in the cytoplasm (Kempf and Bremer 1998). They accumulate solutes (which may be organic or inorganic molecules)- such as;- amino acids, polyols, carbohydrates, quaternary ammonium compounds which enables them to withstand water and even salt stress (Killham 1994; Kempf and Bremer 1998). These are termed compatible solutes or osmoprotectants, some of which may be constitutively produced and others induced. This results in decreased internal water potential and can influence and modulate specific enzyme activities, but they do not inhibit the overall metabolism capacity of the cells (Dion and Nautiyal 2007). Although energy intensive, osmoregulation is another strategy that enables microorganisms particularly in soils to conserve intracellular enzyme activities under water stress conditions (Dion and Nautiyal 2007). Another strategy against desiccation, which is usually adopted by prokaryotes, is the production of extracellular polysaccharides which retain water (Wright et al. 2005). Formation of microaggregates of cells where elevated water activities are retained may further protect microorganisms from desiccation (Or et al. 2007). Actinomycetes are particularly well osmoregulated, as their cell membranes are selectively permeable, restricting salt ions to cells' exterior while retaining organic solutes within the cells. Like fungi, they can differentiate into dormant cells that are resistant to drying (Dose et al. 2001). Photosynthetic cyanobacteria, which are the

primary inhabitants in many dry habitats (Wynn-Williams 2000; Dong and Yu 2007), commonly live a few millimetres below the surface of translucent rocks, such as quartz (Schlesinger et al. 2003), sandstone pebbles (Wynn-Williams 2000), halite (Wierzchos et al. 2006) and gypsum (Dong et al. 2007). These microhabitats support microbial life because they provide adequate supply of CO_2, N_2 and light, which aid photosynthesis and N_2 fixation on the one hand and offer protection from intolerable levels of irradiation, high temperature and arid surface conditions on the other (Cockell and Raven 2004; Dong and Yu 2007). Heterotrophic bacteria are also ubiquitous in desert environments, and their abundance has been linked to mean annual precipitation (Lacap et al. 2011; Maier et al. 2018). In the hyper-arid core region of the Atacama Desert, the heterotrophic groups seem to prefer the soil subsurface (25–30 cm in depth) as opposed to the more hostile surface (Drees et al. 2006; Navarro-González et al. 2003).

In deserts, microbiota have been observed to inhabit pores in sandstones, and some tend to form biological soil crusts (Dion and Nautiyal 2007). In the Ross Desert, an Antarctic cold desert, cryptoendolithic microorganisms grow in the near-surface layer of porous sandstone rocks, where the microclimate is less hostile (Friedmann 1982, 1986). They depend on the unsteady interactions between biological and environmental factors for survival, and alterations that create unfavourable conditions may terminate them (Friedmann and Weed 1987). It has been suggested that tar balls deposited on beaches may represent another situation in which available water limits hydrocarbon biodegradation (Atlas 1977).

On the other extreme, coastal areas are submerged with seawater during tidal and wave movements and only experience transient drought seasons. Soil moisture content is inversely proportional to the degree of aeration. Desert soils are therefore, well aerated most of the time (Godoy-Faúndez et al. 2008). Microbial activities in dry soils may be enhanced by increasing the water content, but this should be done in a controlled manner, to avoid water logging, which can inhibit the growth and metabolism of aerobic species. Many hydrocarbonoclastic microorganisms are aerobic, excessively high moisture levels, therefore, may be a rate-limiting factor in waterlogged areas (Wilkinson et al. 2002).

18.7.5 Nutrients

Many extreme environments are characterized by poor organic and inorganic nutrients (Jiang et al. 2012; Speight and El-Gendy 2018c). Most of the indigenous microorganisms in these environments therefore are usually oligotrophs (adapted to low nutrient supply rates). Cold environments (e.g. cold soils) typically do not support significant amount of plant biomass, organic matter is, therefore, deficient offering less favourable growth conditions for microbes (Speight and El-Gendy 2018b). Overall, nutrient stress influences soil microbiota, selecting for organisms adapted to (intermittent or constant) nutrient stress (Dion and Nautiyal 2007; Torsvik and Øvreås 2008). In ecosystems such as polar and high-elevation alpine

soil systems, there is a direct relationship between nutrient levels and moisture content (Dion and Nautiyal 2007). Low nutrient availability is one of the most crucial limitations for microbial physiological and enzymatic processes in desert habitats (Das and Dash 2014). In poor desert soils, microalgae and cyanobacteria, which are major sources of organic materials, are nutrient starved (Dion and Nautiyal 2007). Bacterial survival mechanisms such as the ability for growth at low nutrient concentrations as well as the ability for dormancy to counter starvation have been described (Jannasch 1967; Roszak and Colwell 1987). Another possible survival strategy is a shrinking in cell size via multiple division, thus generating the so-called ultramicrobacteria (Novitsky and Morita 1976, 1977, 1978; Morita 1982). The smaller the bacterial cell, the larger its surface-to-volume ratio, and consequently the greater is its potential for accumulating diluted nutrients from the external environment. Some starving bacteria with a depleted amino acid pool exhibit the so-called stringent response (Neidhardt et al. 1990) which ultimately reduces the protein synthesis rate by inhibiting rRNA synthesis. Other mechanisms for adaptation to low nutrient levels include microbial ability to utilize several substrates as carbon sources simultaneously (Dion and Nautiyal 2007; Eichorst et al. 2007). In areas where fluctuations in nutrient supplies are common, prokaryotes can store nutrients as intracellular polymers (like polysaccharides, poly-β-hydroxybutyrate, polyphosphate). In typical oligotrophic environments, however, particularly cold environments, nutrient supply may be too low to sustain any intracellular storage (Torsvik and Øvreås 2008). There is some research indicating that the organism's affinity for substrates decreases at low temperature due to loss of membrane fluidity that impedes active nutrient transport and the minimum nutrient substrate concentration required for growth increases near the organism's lower temperature limits (Dion and Nautiyal 2007). If liquid water, which is a general necessity for cellular life is present, growth inhibition due to cold temperature conditions may be due to inefficient nutrient uptake, and nutrients invariably become so low to the point where the cell's minimum requirements for sustenance are no longer met (Dion and Nautiyal 2007).

Assuming that proficient microbial degraders are available, unavailability of requisite nutrients may be a rate-limiting factor (Vasudevan and Rajaram 2001). In addition to hydrocarbons, which have been established to serve as carbon sources for some microbial groups, the presence of inorganic nutrients such as nitrogen, phosphorus and potassium is critical because microorganisms require these for incorporation into biomass (Bamforth and Singleton 2005). The supply of these nutrients is however, dependent on certain factors. The nature of the spill or pollution plays a major role; comparison of the biodegradation of hydrocarbons within an oil slick or the biodegradation of soluble hydrocarbons is a typical example (Leahy and Colwell 1990). When considering an oil slick, there is a mass of carbon available for microbial growth within a limited area. In this case, sometimes, diffusion rates are inadequate to provide sufficient nutrients and oxygen to establish optimal C/N and C/P ratios for microbial growth and cellular metabolism (Vasudevan and Rajaram 2001). Toxic effects may also be exerted on microbial consortia by volatile hydrocarbons (Leahy and Colwell 1990). When considering soluble hydrocarbons, nitrogen and phosphorus are probably not limiting since hydrocarbon solubility is

so low as to preclude establishment of an unfavourable C/N or C/P ratio (Atlas 1991).

Petroleum hydrocarbons typically contain low concentrations of inorganic nutrients, and hydrocarbon pollution spikes carbon levels at contaminated sites (Sarkar et al. 2005). Available nutrients may become rapidly depleted during microbial metabolism disrupting the C/N or C/P ratio or both, creating unfavourable conditions for microbial growth (Bamforth and Singleton 2005). Contaminated sites are therefore, commonly supplemented with nutrients such as nitrogen and phosphates using oleophilic fertilizers such as paraffinized urea, octylphosphate, ferric octoate, etc. as well as fish bones, animal meal, biosurfactants and other bulking agents to stimulate the in situ microbial community and therefore, enhance biodegradation (Vasudevan and Rajaram 2001; Van Hamme et al. 2003).

The efficacy of this strategy, however, depends on the characteristics of the contaminated site. For instance, the variable and complex composition of soils as well as other factors such as occurrence of nitrogen reserves and the nitrogen-fixing bacteria may influence the efficacy of any nutrient supplementing intervention (Vasudevan and Rajaram 2001). Nutrient amendment to facilitate hydrocarbon degradation should, however, be done cautiously. For instance, supplementing with high nutrient doses in soils with low moisture content could result in increased ionic strength of the liquid, which may inhibit microbial activity. In temperate soils, the (frequent) freeze-thaw cycles may dynamically redistribute the added nutrients affecting their bioavailability (Margesin 2017). In polar soils, it is prudent to add the necessary nutrients in smaller doses over time rather than all at once. Other concerns include the potential toxicity of these treatments if these are leached into receiving water bodies.

18.7.6 Pressure

High pressure is prevalent in habitats such as the deep sea, groundwater, deep sediments or oilfields (Margesin and Schinner 2001). Barophiles (piezophiles) are microorganisms that require high pressure for growth or thrive under pressure conditions higher than atmospheric pressure (Prieur and Marteinsson 1998). Although barophilic (piezophilic) microorganisms have been isolated and described, their ability to sequester hydrocarbons has not been satisfactorily explored (Grossi et al. 2010; Caumette et al. 2015). Pollutants with densities greater than that of marine waters may sink to the deep benthic zone, where the hydrostatic pressure is notably high (Gallego et al. 2018). A combination of high pressure and low temperatures in the deep ocean affects the metabolism of oil-degrading species, thereby inhibiting microbial degradation (Alexander 1999; Prince and Walters 2016).

The effect of hydrostatic pressure on well-known, conventional oil degraders is worth exploring (Grossi et al. 2010). Hydrostatic pressure seems to impair the metabolism of *Alcanivorax* spp., a typical hydrocarbonoclastic microbe that has been identified in many petroleum-impacted environments but was detected in low

numbers in the Deepwater Horizon (DWH) oil plume. The low *Alcanivorax* abundance in the DWH deep sea plume and sediment was unrelated to HC concentrations, and its role in bioremediation in this case was considered negligible (Mapelli et al. 2017). Buttressing *Alcanivorax* spp.'s oil-degrading potential and ubiquity in oil-polluted environments, and illustrating the effects of high pressure on its growth and proliferation, *Alcanivorax* was abundantly cultivated under atmospheric pressure from oiled beach sands, oil mousses (collected on surface waters) and plume samples (Kostka et al. 2011; Liu Zhanfei and Liu Jiqing 2013; Gutierrez et al. 2013). Also, hydrostatic pressure of 5 MPa and 10 MPa induced substantive reduction in cell replication and inhibited the growth of *Alcanivorax borkumensis*, *A. dieselolei* and *A. jadensis* on dodecane as sole source of carbon, respectively (Scoma et al. 2016a, b; Scoma and Boon 2016).

18.7.7 pH

It has been postulated that the optimum pH range for hydrocarbon mineralization is neutral (Aislabie et al. 2012). Microorganisms are however, able to withstand suboptimal pH although fungal species are more tolerant of acidic conditions, compared to bacteria (Leahy and Colwell 1990; Al-Daher et al. 1998). Studies have shown the effects of pH on the rate and efficiency of petroleum hydrocarbon biodegradation. Improved gasoline biodegradation (almost double the normal rates) in an acidic (pH 4.5) soil was achieved by adjusting the pH to 7.4 (Verstraete et al. 1976). A significant drop in biodegradation rates was however, observed when the pH was further raised to 8.5. Similarly, pH range 5.0–7.8 was assessed to determine the optimum pH for oily sludge mineralization in soil, and 7.8 was observed to be the optimum (Dibble and Bartha 1979). Lower microbial mineralization rates of octadecane and naphthalene at pH 5.0 compared with pH at 6.5 were observed by Hambrick et al. (1980). Octadecane mineralization rates increased further when the pH was raised from 6.5 to 8.0, although naphthalene mineralization rates did not (Hambrick et al. 1980).

Many times, petroleum-impacted environments are not at the optimal pH for bioremediation (Prince 2010). Soil pH is more variable ranging from 2.5 in mine spoils to 11.0 in alkaline deserts, compared to most aquatic ecosystems which have a more steady pH range (Leahy and Colwell 1990). Further, anthropogenic activities may alter pH and thereby impair biodegradability (Margesin and Schinner 2001). Retired gasworks sites, which are usually replete with demolition waste such as concrete and brick, have been used as case studies. The leaching of these wastes increases the pH of the soil, creating unfavourable conditions for microbial metabolism (Bamforth and Singleton 2005). In addition, the oxidation and leaching of coal spoil creates an acidic environment through the release and oxidation of sulphides. The indigenous microorganisms at these sites might not have the capacity to transform PAHs under acidic or alkaline conditions. The pH at these sites may then be adjusted, by the addition of lime, nutrients or fertilizers to facilitate bioremediation (Wilson and Jones 1993; Alexander 1999; Bamforth and Singleton 2005).

Acidophiles have been isolated and described in the literature (Peeples 2014; Speight and El-Gendy 2018a, b, c, d). Many of these are heterotrophs, which are usually resistant to heavy metals and organic compounds, but have demonstrated remarkable potential to replenish acidic environments contaminated by petroleum hydrocarbons (Bamforth and Singleton 2005). Efficient petroleum biodegradation in aquifers with pH of 4.5–5 has been described (Margesin and Schinner 2001). Also hydrocarbonoclastic microorganisms were found in a tropical, acidic forest soil (pH 4–6), 17 years after an extensive oil spill (Amadi et al. 1996).

Twenty-three heavy-metal-tolerant, acidophilic heterotrophic bacteria, isolated from an acidic mine effluent, metabolized a range of aliphatic hydrocarbons (including 5 mM propane-1-ol, acetone, acetaldehyde, propanaldehyde, dodecanoic acid, hexadecanoic acid, dodecane, hexadecane, 1-chlorohexane) as the sole carbon source at pH 3. Stapleton et al. (1998) reported the biodegradation of aromatic hydrocarbons and PAHs in extremely acidic environments. Three soil samples collected from a long-term coal pile storage basin were investigated; the pH value of areas greatly impacted by runoff from the storage basin was 2. The autochthonous microorganisms mineralized around 50% of the supplied naphthalene and toluene to CO_2 and water within 24 weeks, although only about 10–20% mineralization of phenanthrene and anthracene was achieved. 16sRNA sequence analyses indicated the presence of acidophilic bacteria in the soil samples, but a microbial consortium, including eukaryotes, rather than individual acidophiles was suggested to be involved in biodegradation in this acidic environment (Stapleton et al. 1998).

Although many other industrial capabilities and potentials of alkaliphilic and alkalitolerant microorganisms have been described, there is a paucity of data regarding their use in environmental clean-up (Santini et al. 2016). The bioremediation of phenol at pH 10 by alkaliphilic strains such as *Arthrobacter* sp., *Bacillus cereus*, *Citrobacter freundii*, *Micrococcus agilis* and *Pseudomonas putida* biovar B has however, been described (Kanekar et al. 1998). An alkaliphilic and halophilic bacterium *Nocardioides* sp. (pH 9.5–10 and 10% salinity) isolated from Alkali Lake (Oregon) also demonstrated ability to degrade chlorophenol compounds 2, 4-dichlorophenol, 2,4,5-trichlorophenol and 2,4,6-trichlorophenol as the sole carbon source (Maltseva and Oriel 1997).

18.8 Biosurfactants

Biosurfactants are surface-active, amphiphilic molecules that have hydrophobic and hydrophilic domains, synthesized by microorganisms (Ławniczak et al. 2013). They are structurally diverse and are classified based on microbial origin, mode of action, chemical composition and structure, molecular weight and physicochemical properties (Nguyen et al. 2008; Nievas et al. 2008). They possess remarkable capabilities to interact with a versatile range of hydrocarbons as well as the capacity to lower surface and interfacial tension of liquids and form micelles and microemulsions between two different phases (Ławniczak et al. 2013). They are environmentally

friendly, biodegradable and less toxic (compared to their synthetic counterparts) (Karlapudi et al. 2018).

Based on molecular weight, they are classified into low-molecular mass compounds including molecules such as trehalose; lipids, glycolipids, phospholipids, polyol lipids, rhamnolipids and lipopeptides, proteins and surfactins (Smyth et al. 2010). These reduce the surface tension at the air/water interfaces and the interfacial tension at oil/water interfaces. The high molecular weight variants also known as bioemulsifiers (e.g. emulsan, liposan, mannan amphipathic polysaccharides, proteins, lipopolysaccharides, lipoproteins or complex mixtures of lipopeptides, glycolipids, neutral lipids and fatty acids) are amphiphilic and polyphilic polymers that stabilize oil-in-water emulsions (Cameotra and Bollag 2003; Smyth et al. 2010). Although they do not lower the surface tension as effectively as LMW surfactants, they increase the surface area available for bacterial biodegradation (Cameotra and Bollag 2003). Their use as additives to counter the low aqueous solubility of HMW petroleum hydrocarbons and enhance the efficiency of bioremediation has been described (Gan et al. 2009).

Biosurfactants facilitate the transport of hydrophobic contaminants into the aqueous phase through specific interactions resulting in solubilization, thereby increasing their bioavailability, which potentially makes them more susceptible to biodegradation (Maier and Soberón-Chávez 2000). They enhance hydrocarbon biodegradation by two mechanisms (Pacwa-Płociniczak et al. 2011). The first involves increasing substrate availability for microorganisms, while the other involves interaction with the cell surface, which increases the hydrophobicity of the surface, allowing hydrophobic substrates to associate more easily with bacterial cells (Mulligan and Gibbs 2004). By reducing surface and interfacial tensions, biosurfactants increase the surface area of insoluble compounds leading to increased mobility and bioavailability of hydrocarbons (Bordoloi and Konwar, 2009). For LMW biosurfactants, above the critical micelle concentration (CMC), a significant fraction of the hydrophobic contaminant partitions in the surfactant micelle cores. In some cases, this results in a general increase in the bioavailability of contaminants for degrading microbiota. Microorganisms such as *Candida bombicola*, *C. apicola*, *Rhodotorula bogoriensis*, *Pseudozyma* yeasts, *Pseudozyma aphidis*, *Pseudozyma antarctica*, *Pseudozyma rugulosa*, *Alcanivorax borkumensis*, *Mycobacterium* spp., *Nocardia* spp., *Corynebacterium* spp., *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Torulopsis bombicola* have been reported to produce surfactants such as trehalolipids, surfactin, glucolipid, rhamnolipid and sophorolipid (Maier and Soberón-Chávez 2000; Kuyukina et al. 2005; Chevron Cottin and Merlin 2007; Konishi et al. 2007; Kadri et al. 2018).

The capability of biosurfactants and biosurfactant-producing bacterial strains to enhance availability of petroleum hydrocarbons and biodegradation rates has been reported by many authors (Rapp et al. 1979; Rahman et al. 2003; Inakollu et al. 2004; Obayori et al. 2009; Reddy et al. 2010). Hydrocarbonoclastic microorganisms have been demonstrated to synthesize and release biosurfactants which greatly enhance their effectiveness in uptake and sequestration of hydrocarbons (Broderick and Cooney 1982; Singer and Finnerty 1984). Enhanced solubilization

and a concomitant improved mineralization of phenanthrene, pyrene and fluorene by lipopeptide and protein-starch-lipid produced by two *P. aeruginosa* strains have been described (Bordoloi and Konwar 2009). The production and emulsification activities of biosurfactants are influenced by environmental factors such as salinity, pH and temperature, but they have been described to be highly reactive and active at extreme temperatures, pH and salinity (Das et al. 2008; Pacwa-Płociniczak et al. 2011). Hydrocarbon-degrading marine bacteria *Rhodococcus fascians* isolated from the Antarctic produced bioemulsifiers when cultured with *n*-alkanes as sole carbon source (Yakimov et al. 1999). The *R. fascians* strains utilized hexadecane and biphenyl as sole carbon sources at temperatures ranging from 4 to 35 °C, with optimum degradation achieved at 15–20 °C (Yakimov et al. 1999; Margesin and Schinner 2001). Biosurfactant-producing organisms from Tunisian oilfields have also been characterized (Mnif et al. 2011). Psychrophilic strains with high oil-oxidizing and bioemulsifying activities were also described by Chugunov et al. (2000).

A variety of applications for biosurfactants has been described. The molecules could be added externally (influent, spraying or injection) or could be produced on site, which seems especially promising in the case of in situ treatment (Ławniczak et al. 2013). In the latter case, biosurfactants may be produced by bioaugmentation, using metabolically competent strains since autochthonous microorganisms rarely exhibit satisfactory efficiency (Ławniczak et al. 2013). The roles of biosurfactants in biodegradation have been characterized mostly by observing the effects of fractionated preparations (Patowary et al. 2017). However, the successful application of biosurfactants in bioremediation of petroleum pollutants will require precise targeting to the physicochemical conditions of the contaminated areas (Van Hamme et al. 2003). Although the potential of biosurfactants in facilitating bioremediation has been extensively documented, the dynamics of use to replenish polluted extreme habitats are yet to be fully explored.

18.9 Bioremediation of Petroleum Hydrocarbons in Cold Regions

In cold regions such as the Arctic, Alpine, Antarctic, polar and deepwater regions, petroleum hydrocarbon pollution is an important environmental problem (Braddock et al. 1997; Aislabie et al. 1998; Delille et al. 1998; Margesin 2000; Delille and Delille 2000; Lin et al. 2009; Yang et al. 2014). In the cold areas of some countries such as Canada, Russia, the United States (Alaska) and China (Qinghai-Tibet Plateau), petroleum hydrocarbon contamination has been extensively documented (Collins et al. 1993; Margesin and Schinner 1999; Chuvilin et al. 2000; Yang et al. 2009). Resource exploration, transportation, storage and handling of petroleum products with potential for spillage pose significant environmental risks for cold marine and terrestrial ecosystems (Filler et al. 2008). In the Arctic, for example,

crude oil spills from ruptured pipelines represent one of the most significant sources of terrestrial petroleum pollution, followed by shoreline spills from tankers or resupply vessels (Engelhardt 1994). Refined fuel spills are also quite common (Margesin 2017). These are usually caused by infrastructural mishaps, human error during fuel transfer, sabotage or vandalism or natural hazards (Filler et al. 2008). Local physicochemical, geological and biological conditions influence the success of remediation strategies in these regions (Margesin 2017). The unique environmental variables affect the efficiency and rate of bioremediation. Some of these include occurrence of permafrost, water lying above the permafrost, physiology and biochemistry of psychrophiles/psychrotolerant microbes, extreme fluctuations in daily solar radiation levels year round, cold ground and air temperatures as well as annual freezing and thawing of surface layers (Filler et al. 2008).

Petroleum HC contamination in cold regions has potential to be more catastrophic due to the remote locations, limited infrastructure, cold temperature and other environmental constraints related to temperature such as inadequate moisture, low nutrient availability, incidence of competent hydrocarbon-degrading consortia, etc. which pose formidable challenges for remediation (Van Stempvoort and Biggar 2008; Margesin 2017). Hydrocarbon pollutants can persist for several years in these ecosystems because natural attenuation is significantly slower compared to regions with moderate climatic conditions. Bioremediation in cold regions, therefore, may prove to be problematic. For instance, attempts to degrade oil contamination in Arctic marine ice and frozen tundra soil yielded only very limited success (Atlas 1979). Adverse winter temperatures limited the biodegradation of PAHs in estuarine sediment (Shiaris 1989) and of a variety of hydrocarbons in fresh water lakes (Cooney et al. 1985). Gunkel (1967) reported very low hydrocarbon utilization rates at low temperatures. Improved motor oil oxidation rates at 20 °C compared to 5 °C were described by Ludzack and Kinkead (1956), and a similar finding by ZoBell (1969) showed that hydrocarbon degradation was over an order of magnitude faster at 25 °C than at 5 °C. Also, significantly long persistence times for oil in tundra soils were observed by Sexstone and Atlas (1978) and Sexstone et al. (1978). Apparently, hydrocarbon degradation ceases during winter when tundra soils are frozen. In spite of these, however, successful biodegradation of oil in habitats with low temperature has been reported (ZoBell 1973; Cundell and Traxler 1973; Eriksson et al. 2001; Gibb et al. 2001; Hazen et al. 2010; Garneau et al. 2016). Furthermore, it is quite apparent that the microorganisms adapt to the contamination, indicated by the higher microbial activity of certain species post contamination (Whyte et al. 1999). Survival of adapted microorganisms usually depends on their hydration state, their compatible solute content and their ability to switch metabolism to cryoprotectant synthesis (Dion and Nautiyal 2007; Liang et al. 2013).

Cold-adapted autochthonous microbial assemblages capable of rapidly degrading crude oil constituents at subzero or near-zero temperature profiles have been isolated and characterized (Yakimov et al. 2004). Growth at low temperatures requires significant membrane alterations to maintain the fluidity required for nutrient transport across the membrane. Low temperature modifications involve less saturated and less branched membrane fatty acids. Below the minimum growth tem-

perature, the membrane becomes solid, and transmembrane transportation is halted (Dion and Nautiyal 2007). Mechanisms and appendages that facilitate life at these cold temperatures have been investigated (Médigue et al. 2005). Microorganisms may accumulate antifreeze compounds (high salt concentrations, hydrocarbons or amino acids) in the cytoplasm (Margesin et al. 2007; Fuchs et al. 2013; Moreno and Rojo 2014). Bacteria and Archaea have similar adaptive mechanisms, which involve altered membrane composition (cold-adapted lipids) as well as cold-active proteins involved in fundamental cell functions (such as protein synthesis) (Cavicchioli et al. 2000). For psychrophiles, specific cold adaptation implies such drastic changes in cell biochemical composition and makes them unsuited for life outside cold temperatures (Dion and Nautiyal 2007). For instance, the enzymes and ribosomes of cold-adapted microbes become unstable at temperatures 1–2 °C above their 'optimum' temperatures. Accordingly, the psychrophiles have optimum temperatures at or below 15 °C and maximum temperatures below 20 °C. These psychrotrophic organisms can also grow at temperatures close to or even below 0 °C, but their optimum temperature is above 15 °C, and their maximum temperature limit can be as high as 30–40 °C. Cryopegs, which are characteristic features of permafrost regions, are thin films of liquid water, present in permafrost or in permafrost brine lenses. They are generated from layers of unfrozen ground that are perennially cryotic (forming part of the permafrost), but in which freezing is prevented by freeze-point depression due to high concentrations of dissolved substances in the pore water. They may serve as an ecological niche where enzymatically and metabolically active microbes subsist at below freezing temperature (Gilichinsky et al. 2003). They support microbial growth at temperatures as low as −10 °C and metabolic activity at −20 °C and even lower (Bakermans et al. 2002).

The feasibility of bioremediating hydrocarbon-contaminated ecosystems in cold regions has been demonstrated (Margesin and Schinner 2001; Mohn et al. 2001; Yang et al. 2009). A diverse group of bacterial and fungal species capable of not only proliferating under cold conditions but metabolizing hydrocarbons have been identified by numerous studies (Vasco et al. 2011; Kosek et al. 2016). In soil, a broad range of crude oil aliphatics (C_{10}–C_{21}), branched alkanes and a substituted cyclohexane was metabolized by a psychrotrophic *Rhodococcus* sp. at 5 °C (Whyte et al. 1998). Short-chain alkanes (C_{10}–C_{16}) were significantly mineralized better (by a factor of 2–3), compared to long-chain alkanes (C_{28} and C_{32}) at 0 and 5 °C. Long-chain alkanes are less bioavailable at low temperature; factors such as the tendency to form crystals at 0 °C seem to contribute to this, as this increases their persistence and ultimately affects in situ bioremediation in cold climates. Rike et al. (2003) carried out in situ biodegradation of petroleum hydrocarbons in frozen Arctic soil. The study concluded that 0 °C is not the ultimate limit for in situ cold-adapted microbial-mediated biodegradation of petroleum hydrocarbons and that biodegradation can proceed at subzero temperatures during winter in the investigated site (Margesin and Schinner 2001). In Antarctic soils, cold-tolerant species such as *Pseudomonas* spp. and *Sphingomonas* spp. isolated from the soils degraded hydrocarbons such as naphthalene, phenanthrene, fluorene, hexadecane as well as BTEX as sole carbon and energy source (Aislabie et al. 1998, 2000). Using phenanthrene as a model

compound, 53 PAH-metabolizing bacteria were isolated from diesel-contaminated Antarctic soil samples, three of which exhibited a high phenanthrene-degrading capacity (Gran-Scheuch et al. 2017). In Northern soils, microbial populations degraded hydrocarbons at ambient temperatures prevalent during the warmer seasons (Jobson et al. 1972; Westlake et al. 1978).

The technological feasibility of in situ bioremediation of hydrocarbons in cold groundwater systems has been demonstrated by some studies. Bradley and Chapelle (1995) described rapid aerobic toluene mineralization in sediments from a cold (mean groundwater temperature 5 °C) petroleum-contaminated aquifer in Alaska.

18.9.1 Fate of Hydrocarbon Pollutants in Cold Regions

Determining an effective range of suitable remedial strategies for cold regions is a major challenge for environmental managers, engineers and scientists. The degree of success in mitigating the environmental and economic impacts of petroleum pollution in cold regions depends on a variety of factors. The treatment of petroleum hydrocarbon pollution (e.g. oil spill) in cold regions usually involves containment and removal as the first line of action (Kadri et al. 2018). Some possible options include excavation and inland incineration. Excavating and relocating pollutants for off-site treatment are in many cases not viable, because the costs and environmental impacts of bulk extraction may equal or exceed damage caused by the initial pollution (Filler et al. 2008). Similarly, in-ground incineration will not effectively ameliorate oil spill pollution but would rather cause downward migration of pollutants and permafrost degradation through heating. Bioremediation, which is more practical and sustainable, is usually executed after the containment and removal (Yang et al. 2009). Seasonal variations in the rates of hydrocarbon biodegradation under cold temperatures have been observed. In cold regions, hydrocarbonoclastic microorganisms are more abundant during winter than other seasons (Atlas and Bartha 1973; Atlas 1981). For example, higher numbers of hydrocarbon utilizers capable of growth at 5 °C were present in Raritan Bay, N.J., during winter compared to other seasons. Also, rates of hydrocarbon mineralization measured at 5 °C were significantly higher in water samples collected in winter than in summer (Atlas 1981). During summertime, in Arctic surface waters, different structural classes of hydrocarbons were metabolized at similar rates (Horowitz and Atlas 1977). Slower but more extensive biodegradation of petroleum hydrocarbons under cold temperature conditions (0 °C) than at higher temperatures has been demonstrated using a model incubated with estuarine water collected during winter (Walker and Colwell 1974). The better growth observed was attributed to decreased toxicity of hydrocarbons at lower temperatures. Ward and Brock (1976) reported a relationship between seasonal changes in temperature and oxidation of hexadecane with optimum degradation rates at 20–25 °C in the summer. Similarly, crude oil mineralization in soil samples from Louisiana salt marshes varied depending on the season (Jackson and Pardue 1997). During the transition period of spring and fall, the degree of success may be extensively modified, depending on whether or not the contaminated sites

are in close proximity to pollution sources and the occurrence of surface ice or heavy runoff. The characteristics (e.g. solubility and viscosity) of the spilled oil also vary seasonally, and this affects rate and efficiency of biodegradation. Therefore, bioremediation might be needed in certain seasons and not in others. Containment strategy used also depends on the season. Summertime oil spill requires rapid (usually within hours) intervention. In this case, physical removal techniques such as the deployment of a surrounding boom, then vacuum pumping and subsequently, mopping up as much oil as possible using sheets of absorbent materials may be used (Leahy and Colwell 1990).

The influence of co-metabolism makes it difficult to ascertain the direct influence of temperature on bioremediation rates in cold regions. Greater degradation of Metula crude oil at 3 °C than at 22 °C with mixed microbial cultures in beach sand samples was described (Colwell et al. 1978). When 0.1% oil was added, 48% of the added hydrocarbons were degraded at an incubation temperature of 3 °C, compared to only 21% degraded at 22 °C with cultures adapted at the same temperature profiles as the incubation temperature. Under in situ conditions, oil degradation proceeded slowly, but in this study, it was apparent that temperature was not the limiting factor for petroleum degradation in the Antarctic marine ecosystem affected by the Metula spill (Colwell et al. 1978).

Another study assessed crude oil degradation in Arctic marine ice, water and sediment ecosystems. Petroleum hydrocarbons were degraded slowly. Ice immensely inhibited light hydrocarbon losses, and biodegradation of oil on the surface of ice or under sea ice was negligible. It was concluded that petroleum hydrocarbons would remain in cold Arctic ecosystems for protracted periods after oil contamination incidents, although, in these studies, temperature was not specifically elucidated as a major factor limiting hydrocarbon bioremediation except as it related to the occurrence of ice (Atlas and Raymond 1977; Atlas et al. 1978).

18.9.2 Biodegradation of petroleum hydrocarbons in Hot Regions

Thermophilic organisms normally do not thrive at temperatures below 50 °C, whereas the thermotrophs have a lower temperature limit (20–30 °C). At their upper temperature limit, cells become unstable, and irreversible denaturation of proteins and nucleic acids occurs; therefore molecules lose their ability to perform biochemical functions (Price and Sowers 2004; Dion and Nautiyal 2007).

Physiologically and metabolically diverse assortment of thermophilic and hyperthermophilic microorganisms have been identified and characterized from high-temperature, petroleum-contaminated environments (Foght and McFarlane 1999; Orphan et al. 2000; Blanchet et al. 2001). These include sulphate reducers, sulfidogens, fermentative bacteria, manganese and iron reducers, methanogens and acetogens (Davey et al. 1992; Stetter et al. 1993; L'Haridon et al. 1995; Tardy-Jacquenod et al. 1996; Grassia et al. 1996; Greene et al. 1997; Orphan et al. 2000; Magot et al. 2000). Hyperthermophilic Archaea and Bacteria with optimal growth temperatures between 80 °C and 110 °C have been isolated from hot habitats like geothermal and

hydrothermal environments. Thermophiles have been isolated from natural thermal soils such as decomposing litter, volcanic, geothermal and tropical desert soils and from manmade thermal soils such as compost piles and coal refuse piles (Botero et al. 2004). For oil-polluted desert soils, for example, indigenous hydrocarbonoclastic thermophiles such as *Bacillus thermoleovorans*, *Geobacillus thermoleovorans* and *B. stearothermophilus* as well as members of *Anaerolineae*, *Thermotogae*, *Gemmatimonadetes*, *Deferribacteres*, *Spirochaetes* and *Thermoleophilica* have been utilized to restore contaminated hot zones such as in the arid regions (Abed et al. 2006). These microbes were reported to significantly accelerate the rate of in situ bioremediation at these sites. Zeikus et al. (1980) demonstrated microbial methanogenesis at temperatures near 70 °C but below 80 °C in thermal waters, muds and decomposing algal-bacterial mats associated with volcanic activity in Yellowstone National Park. At elevated temperature profiles, bioavailability of petroleum hydrocarbon compounds are significantly improved (Camenzuli and Freidman 2015). High temperatures also reduce viscosity and improve diffusion coefficients, thereby improving mass transfer rate to microbial cells. Also, volatile HC fractions usually evaporate rapidly, leaving more hydrophobic HMW constituents to contend with. This could, however, complicate bioremediation efforts as a tar layer may likely form, which then settles over large areas of coast. This tar layer is highly resistant to biodegradation, and bioremediation in such cases becomes irrelevant. Other remediation strategies like the mechanical removal of tar layers may be explored in such cases (Abed et al. 2006).

Very hot ecosystems such as desert soils are poor in organic matter and water and are usually subjected to excessively high temperature in summer, chilly temperature in winter and extensive light. In spite of these extreme characteristics, desert soils usually accommodate microorganisms including members of actinomycetes, cyanobacteria and other bacteria, fungi, protozoa and phototrophic microalgae. Many of these microbes have the capacity to cope with stress and possess adaptive mechanisms for survival and proliferation. A strain closely related to *Geobacillus pallidus* isolated from a tyrosol-degrading enrichment developed from production water from a high-temperature oilfield in Tunisia utilized crude oil and diesel as carbon sources in the presence of 0–12% NaCl (Chamkha et al. 2008).

Microbes adapted to high temperatures have mechanisms to protect their proteins and nucleic acids from denaturation (Dion and Nautiyal 2007). These strategies involve alterations to the membrane composition and functioning which results in decreased membrane fluidity and as a consequence improved thermostability (Dion and Nautiyal 2007). Biomolecules produced are thermostable, with the capacity to remain biochemically active at temperatures that will otherwise inactivate proteins, lipids and nucleic acids in mesophiles (Rothschild and Mancinelli 2001; Dion and Nautiyal 2007). Some proteins become stable due to alterations in amino acid residues, which confer higher hydrophobicity on them, thereby increasing the stability of subunit interactions (Singleton and Amelunxen 1973; Dion and Nautiyal 2007). The nucleic acids are also thermostabilized, for instance, because of the interactions with histone-like proteins. At high temperature, the membrane fatty acids acquire longer chains, becoming more saturated and branched (Dion and Nautiyal 2007).

18.10 Bioaugmentation

Bioaugmentation involves the addition of specifically formulated microorganisms or an inoculum of microorganisms with known pollutant transformation abilities to a contaminated site to reinforce natural biological processes (Tyagi et al. 2011; Sharma 2012). The development and monitoring of an ideal growth environment in which these selected strains can thrive and function form an integral part of this approach. This intervention is based on the premise that the metabolic capacities of the indigenous microbial community existing in the contaminated site will be enhanced by an exogenous genetic diversity, thus leading to a wider repertoire of productive biodegradation reactions (Leung 2004; Cameotra and Bollag 2003). However, certain limitations to bioaugmentation have been identified. One of the most prominent includes the poor competition/survival of added strains commonly because autochthonous microbial community tends to stifle exotic strains (Maila and Cloete 2004). Selective metabolism of compounds, the tendency for microbes to use up readily degradable substrates (hydrocarbon fractions), probably due to low concentrations and no/poor biodegradability potential of targeted compounds, is another important limitation (Maila and Cloete 2004). Some approaches are available to optimize bioaugmentation potential. The most commonly adapted options for bioaugmentation include the addition of a preadapted pure bacterial strain (or consortium), introduction of genetically engineered microorganisms and the incorporation ('seeding') of biodegradation relevant genes from the augmented strains into a vector to be transferred by conjugation into the autochthonous microbial population (El Fantroussi and Agathos 2005; Tyagi et al. 2011). It is recommended that bioaugmentation be conducted in sites with no indigenous hydrocarbonoclastic microbiota, such as sites contaminated by HMW polyaromatic hydrocarbons (Maila and Cloete 2004).

18.11 Biostimulation

Many microorganisms possess intrinsic capacity to degrade or transform various toxic compounds, but these natural transformation processes are relatively slow (Maier and Gentry 2015). This may be due to factors such as insufficient electron acceptors, low activity of functional microorganisms and inefficient electron transfer, all of which affect the efficiency of bioremediation (Li et al. 2018). In order to achieve desired treatment results, environmental conditions, suitable for microbial growth and activity, must be created (Karigar and Rao 2011).

Biostimulation is a technique developed to achieve optimum conditions for microbial growth within contaminated sites (Nwinyi and Olawore 2017). It also involves stimulating the viable microbial population by adjusting water, air and nutrient supply (Wu et al. 2016; Brown et al. 2017). It involves the introduction of additional nutrients (organic or inorganic), bulking agents such as woodchips, compost and electron donors or acceptors to a contaminated site (Namkoong et al. 2002; Scow and Hicks 2005). Some recent biostimulation approaches include the use of microbial electroremediation cells (Li et al. 2018).

18.12 Bioavailability

Bioavailability, referred to as the bioaccessible fraction by some reports, is the percentage of pollutant that microorganisms can readily access and biodegrade or biotransform (Maier 2000; Ortega-Calvo et al. 2013). It is important because it directly influences the efficiency of bioremediation (Ławniczak et al. 2013; Olaniran et al. 2013). According to experts, poor bioavailability limits the full exploitation of in situ bioremediation protocols (Gogoi et al. 2003; Bamforth and Singleton 2005; Harms et al. 2011). The bioavailability of petroleum hydrocarbons is related to the chemistry of the compounds; molecular structure and weight influence bioavailability. The diffusion rate of the hydrocarbons (in soil or sediment) into microbial cells is influenced by chemical activity, and this will influence the bioaccessible fraction (Ortega-Calvo et al. 2013). Polycyclic aromatic hydrocarbons, for instance, are known to partition into sorbents and NAPLs and also tend to adsorb unto organic particulates, which means the chemical activity gradient expression will be weak, which will affect their uptake and transformation by microorganisms (Ortega-Calvo et al. 2013).

Induction of catabolic gene systems used by microorganisms for biodegradation is a high energy expenditure. The presence and concentrations of contaminants significantly impact the metabolic status and activity of microbial cells. Low contaminant levels mean that these genes will not be induced (Maier 2000). Varying bioavailability of contaminants (in terrestrial environments) may result in either of three scenarios.

The first possibility is that biodegradation will not be prompted because the amount of bioavailable contaminant is inadequate and energy expenditure by microbes is not justified (Maier 2000). In the second case, at low bioavailable concentrations, microorganisms may biodegrade contaminants but in a resting or maintenance state rather than an active, growing state (Maier 2000). In this case, biodegradation will indeed occur, but at a limited rate because the microorganisms are not proliferating. The third possible scenario is ample contaminant levels are available, and biodegradation will proceed at optimal rates (Maier 2000). Over time, microorganisms have evolved strategies to counter poor bioavailability (Dua et al. 2002). One of these strategies is the development of increased cell affinity for hydrophobic surfaces, in the case of hydrocarbons with poor aqueous solubility, this enables the degrading species to attach to the hydrophobic substrate and absorb it directly (Maier 2000). The other is the synthesis of surface-active agents or biosurfactants (already explored above). In addition to improving the solubilization of the compounds, dispersal promotion of microorganisms throughout the polluted matrix can also improve bioavailability (Ortega-Calvo et al. 2013).

18.13 Conclusions

Petroleum hydrocarbons are ubiquitous environmental pollutants, which are introduced into the environment accidentally, due to oversight or deliberately during resource exploration, processing, transport and storage. Other anthropogenic activities such as gasification of fossil fuels and other processes involving the incomplete combustion of organic substances also release petroleum hydrocarbons into the environment. In regions characterized by extreme environmental and climatic conditions, petroleum hydrocarbon contamination represents a significant environmental challenge. Petroleum microbiology is influenced by environmental variables, and unfavourable environmental conditions affect the nature of petroleum pollutants, the extent and efficacy of non-biological removal and microbial metabolic capacities. Microbial growth and degradation abilities and, in fact, overall rates of chemical and enzymatic reactions are strongly influenced by suboptimal environmental conditions and, therefore, determine the success of natural attenuation or bioremediation efforts. Many petroleum compounds are hydrophobic, stable and persistent. They are also potential or proven toxicants to the environment, human health and other life forms. The need for clean-up of contaminated sites is widely acknowledged and has been attempted on varying scales. Output of laboratory and field remediation applications abound in the literature. Numerous conventional remediation schemes/approaches have been in use for quite some time, but bioremediation has proven to be effective and eco-friendly and is publicly accepted. Bioremediation strategies and protocols have been designed, optimized and adopted to replenish petroleum-contaminated terrestrial and aquatic habitats, yielding considerable success. Although studies regarding bioremediation in microaerobic, anoxic, anaerobic, hypersaline and other extreme habitats are fewer compared to those on bioremediation in 'normal' environments, there are studies that have described the feasibility of bioremediation in extreme environments. Microbial consortia and associations as well as microbial responses and adaptations to environmental stress conditions have been and continue to be investigated, enabling us to understand how extreme polluted environments can be restored. Petroleum hydrocarbon bioremediation potential under extreme conditions and relevant studies have been examined in this chapter. Hydrocarbonoclastic extremophiles, their adaptive strategies/responses and oil utilizing capacities have been described by several studies. Some important dynamics, constraints and certain mechanisms of tolerance have been discussed herein. Considering available studies, microbial treatment of extreme petroleum-impacted habitats seems indeed plausible. It is important to further explore extremophiles, identify and characterize those that have oil utilizing potential and optimize bioremediation protocols for their use. More importantly, however, prevention of hydrocarbon pollution in the first instance is critical and should be the priority of all relevant players and stakeholders.

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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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Chapter 20
Oil Spill Removal by Mycoremediation

Rajeev Kumar and Ashpreet Kaur

Abstract Oil spills are always harmful, whether accidental or deliberate as it contains hydrocarbons which are carcinogenic and also cause great damage to marine ecosystem disturbing its food chain and hence a threat to entire marine community. Many chemical and physical methods are used to remove the spilled oil of ocean floor, each having some drawbacks. Bioremediation has a promising future in oil spill removal in which indigenous or exogenous microbes are used to clean the oil spill. These microbes are mostly fungi, bacteria, and yeasts, which are already present in the water. Otherwise not dividing actively, during spillage these proliferate quickly and eat up the hydrocarbons which are essential for their growth producing ultimate end products – carbon dioxide and water. Though not in abundance in marine ecosystem, fungi are found to be better degrader of hydrocarbon than other microbes, and its usage to clean up oil spills is burgeoning. This chapter generally emphasizes on marine fungi, and its role in degrading crude oil components as the oceans is the largest and ultimate receptors of hydrocarbon pollutants.

20.1 Introduction

Petroleum is a mixture of complex hydrocarbons that were formed in ancient times from the prehistoric plant and animals. Hydrocarbons are available in the varied forms: kerogen, asphalt, natural gas, condensates, crude oil, and coal in solid form. Petroleum (crude oil) mainly contains carbon (83–87%) and hydrogen (12–14%) which have a complex hydrocarbon mixture like paraffins, naphthenes, aromatic hydrocarbons, and gaseous hydrocarbons (from CH_4 to C_4H_{10}) (Mukhulyonov et al. 1974). It also contains a small amount of non-hydrocarbons (sulfur, nitrogen, oxygen compounds). Depending upon the majority of hydrocarbons, petroleum is classified as paraffin base, intermediate base, or naphthenic base. It can be classified in four categories – the saturates, the aromatics, the asphaltenes (phenols, fatty acids,

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esters, ketones, and porphyrins), and the resins (pyridines, quinolines, carbazoles, sulfoxides, and amides) (Colwell et al. 1977).

As the demand for petroleum is increasing, so are the chances of disasters. Crude oil finds its way in the marine environment through two principal routes. One route involves human activities related to oil spill extraction, transportation, storage, refining, utilization, or destruction of the platform due to earthquakes or hurricanes. The second route involves natural seepage of crude oil and tar from the bottom of the ocean due to eroding of sedimentary rocks (Hunt 1996). When talking about oil spills, the world's largest oil spill in the Arabian Gulf (or Persian Gulf)/Kuwait can never be ignored. It was a deliberate spill by Iraqi forces to prevent American soldiers from landing by opening the valves offshore, which occurred on Jan 19, 1991, spilling 380–520 million gallons of oil. Deepwater horizon oil spill (BP oil spill or Gulf of Mexico oil spill) being the second on the list (Fig. 20.1).

No two oil spills are the same because of the dissimilarity in oil types, location, and weather conditions involved. Factors influencing degradation of hydrocarbon have been reported (Cooney et al. 1985). Research conducted on microbes shows that they help us in degrading hydrocarbons and get energy by utilizing them (Colwell et al. 1977; Ibe and Ibe 1984; Atlas 1995). Most of the petroleum hydrocarbons are degradable under aerobic conditions by microbes, but a few compounds found in crude oil, like resins, hopanes, polar molecules, and asphaltenes, have nearly unremarked biodegradation rates.

Many microorganisms have the ability to decompose some specified petroleum oil fractions (Bartha et al. 1997). Yuan et al. (2000) suggested introduction of mixed cultures of bacteria and fungi by showing that the hydrocarbon mixture of petroleum cannot be decomposed simultaneously. Also, single cultures of fungi have

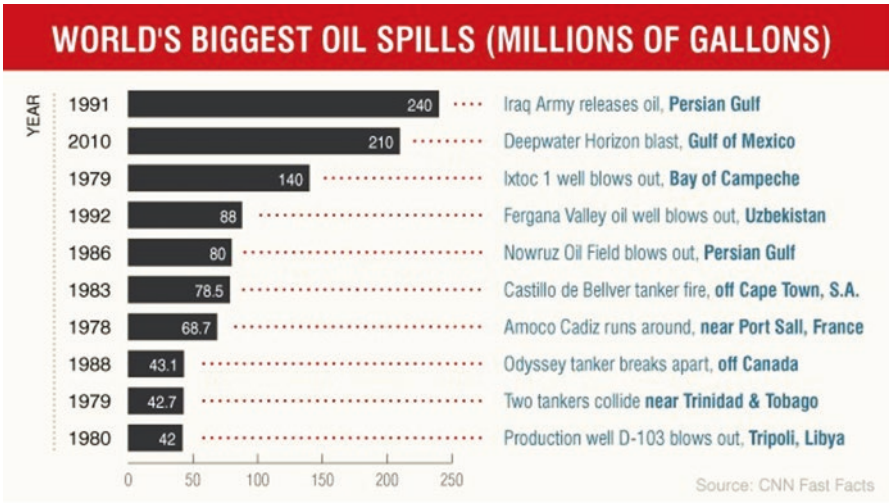

Fig. 20.1 List of ten biggest oil spills in the world. (Source: http://edition.cnn.com/2013/07/13/world/oil-spills-fast-facts/index.html)

been found to be better degraders than mixed cultures (Okerentugba and Ezeronye 2003). Oil is omnipresent and persistent in marine environment (Farrington and McDowell 2004; White et al. 2013), and recently fungi has been taken under study related to hydrocarbon degradation; therefore researchers are interested in the degradation of petroleum by marine fungi recently. The major fungal phyla/subphyla that are involved in the biodegradation of oil are the *Ascomycota*, *Basidiomycota*, and *Mucoromycotina* with specific fungal genera including *Aspergillus*, *Penicillium*, *Cephalosporium*, *Torulopsis*, *Saccharomyces*, *Paecilomyces*, *Candida*, *Gliocladium*, *Yarrowia*, *Pleurotus*, *Geotrichum*, *Talaromyces*, *Cladosporium*, *Pichia*, *Fusarium*, *Alternaria*, *Polyporus*, *Rhizopus*, *Mucor*, and *Rhodotorula* (Harms et al. 2011).

20.2 Sources of Crude Oil and Its Composition

About 1.3 million tons of oil is spilled annually into marine environment from all source. A database of accidental oil spills from tankers, carriers, and barges has been maintained by the International Tanker Owners Pollution Federation (ITOPF) Ltd. since 1967 till date, excluding spills from act of war. Some spills, though large in quantity, needed little or no response due to offshore spillage and hence didn't affect the shoreline. But then there were some spills though less in quantity needed to be responded immediately due to nearness to shoreline. Figure 20.2 shows the percentage of oil entering into the marine environment from different sources (Table 20.1).

A majority of spills are generally from small (<7 tons) incidents, and large oil spill (>700 tons) incidents are few. Approximately 5.73 million tons of oil has been lost due to tanker incidents from 1976 to 2016, but there has been 100-fold reduction in the volume of oil spilt till date. In 2016 it was approximately 6000 tons mainly credited to the large spill in September. Small- and medium-sized spills account for 95% of incidents, but their cause is not known precisely. 40% and 29% occur at loading and discharging, respectively. Whereas information about large oil spills is known with great accuracy, these account for only 5% of incidents. It occurs

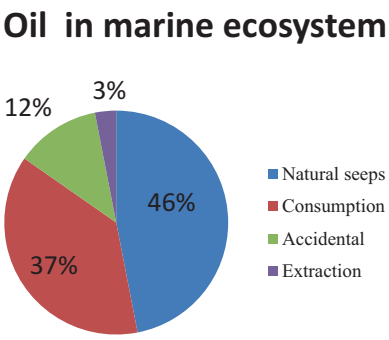

Fig. 20.2 Percentage of oil entering into the marine environment. (Source: National Research Council Report 2003 of US National Academy of Science)

Table 20.1 Major oil spill incidents

Decades	Number of incidents	Quantity of oil spilt	Oil spilt per decade as a % of total spill till date
1970s	788	3,192,000	56%
1980s	454	1,174,000	20%
1990s	358	1,133,000	20%
2000s	181	196,000	5%
2010s	5	39,000	1%

Source: ITOPF (February 2017 report) Oil statistics

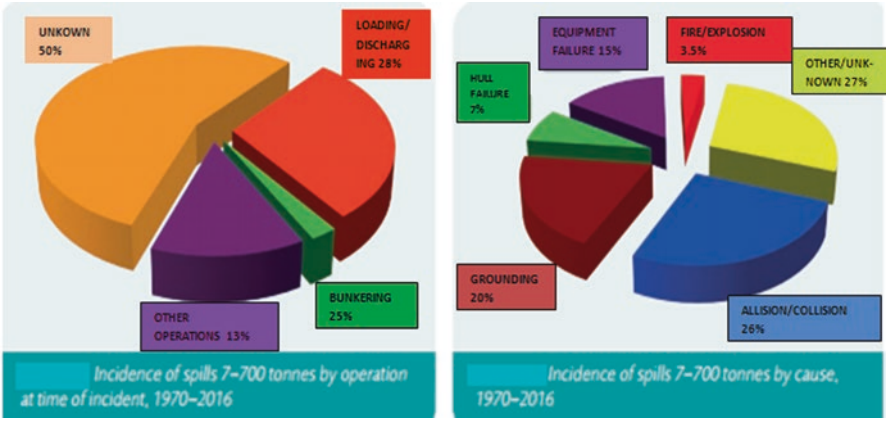

Fig. 20.3 Oil spills of 7–700 tons by operation and cause for the years 1970–2016

due to loading, discharging, at anchor, bunkering, and underway, other and unknown operations.

Figure 20.3 depicts the oil spill chart of 7–700 tons, and Fig. 20.4 depicts the oil spill chart of quantity >700 tons by operation and cause.

Though the composition of petroleum products varies according to the location and the base type (Colwell et al. 1977), petroleum or crude oil can be broadly classified into four chief categories as shown in Table 20.2.

Apart from the above categories, a compound called tarball is produced by weathering of crude oil in marine environment. These are then transported from ocean to the shoreline by sea currents and waves. Tarballs have been found to have high metal concentration and are more persistent than parent crude oil. Shorelines are high on priority for protection due to their sensitivity and difficulty in cleanup. Further research on this sector is going on.

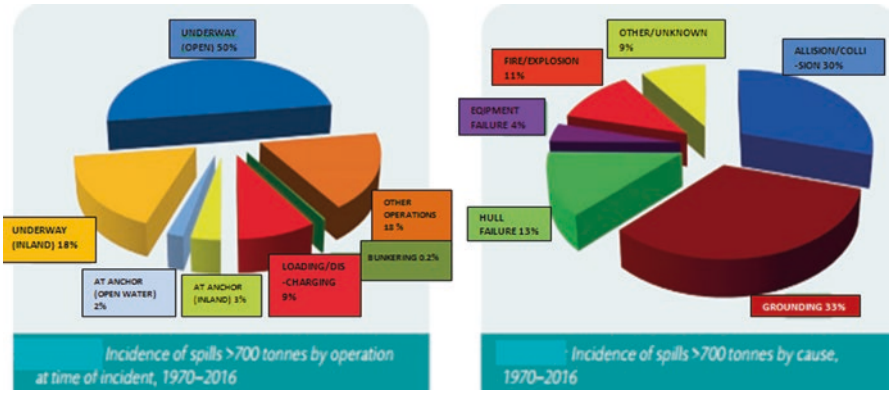

Fig. 20.4 Oil spills of more than 700 tons by operation and cause for the years 1970–2016

20.3 Oil Spill Removal Methods

Present crude oil production is 96.9mb/d as given by the International Energy Agency (IEA), working in the energy, economy, and environmental sector. According to an oil supply forecast made by IEA in March 2017, the global oil demand would exceed the oil supply by 2020, resulting in sharp increase in prices, surpassing the symbolic 100mb/d threshold in 2019, and reaching about 104mb/d by 2022. There will be 45mb/d increase in world oil supply in coming two decades. Figure 20.5 depicts the total global oil demand for 2016, and Fig. 20.6 gives the statistical analysis of the oil demand from 2013 to 2016.

A variety of phenomena are involved in cleaning up oil from water surface. Cleanup methodology depends on factors like oil type, amount of the spill, and environmental conditions, i.e., weather and location. So, method of responses to it can vary. Oil spills can cause great damage to the environment if not cleaned up, and some types of oils are persistent, while others can be degraded relatively quicker. Some oil spills can cause much damage to the environment as it is near to the shoreline as was the *Torrey Canyon 1967* or *Exxon Valdez 1989*, though the amount spilled was less in volume, while other spills are not much of environmental concern. Whatever may be the case, oil spills can be naturally degraded by microorganisms, but it takes much time. So, to augment the rate of degradation, we add some chemicals or exotic species (Fig. 20.7).

Let's have a look at the major oil spill cleanup methods.

Table 20.2 Composition of petroleum products

Parrafins	Naphtenes	Aromatics	Non-hydrocarbons
 Saturated hydrocarbons or alkanes (C_nH_{2n+2})	 Cycloalkanes (C_nH_{2n})	 All compounds containing one or more benzene-like ring (C_nH_{2n+6})	 Present in traces but important component of crude oil
1. N-alkanes, 15–20%, C1–C40, few >C40	1. Cyclopentane, cyclohexane, and their methyl derivatives found in abundance, <C10	1. Resins and asphaltenes are subcategories of aromatics	1. Oxygen, 0–2%, average 0.094%, found in form of phenols, carboxylic acids, and pentacyclic acids (Ourisson et al. 1979)
			2. Sulfur, 0–2%, average 0.6%, present as free sulfur, as hydrogen sulfide, or as organic sulfur compounds
			3. Nitrogen, 0–1%, present basically in quinolines and benzoquinolines (Seifert and Howells 1969)
2. Isoalkanes (branched) < = C10, series of isopropenoids up to C25 and few isoalkanes found	2. C10–C35 usually made by arrangement of 5–6 membered rings	2. These are generally in low amounts (3–30%) as compared to other components	Some metallic elements like iron, vanadium, and nickel are present due to close link between seawater and organic form (petroleum.co.uk)
	3. Mono- and dicycloalkanes, > C10, 50–55% of total cycloalkanes		
	4. Tri, 20% of total, >C10		
	5. Tetra and penta, > C10, 25% of total cycloalkanes		

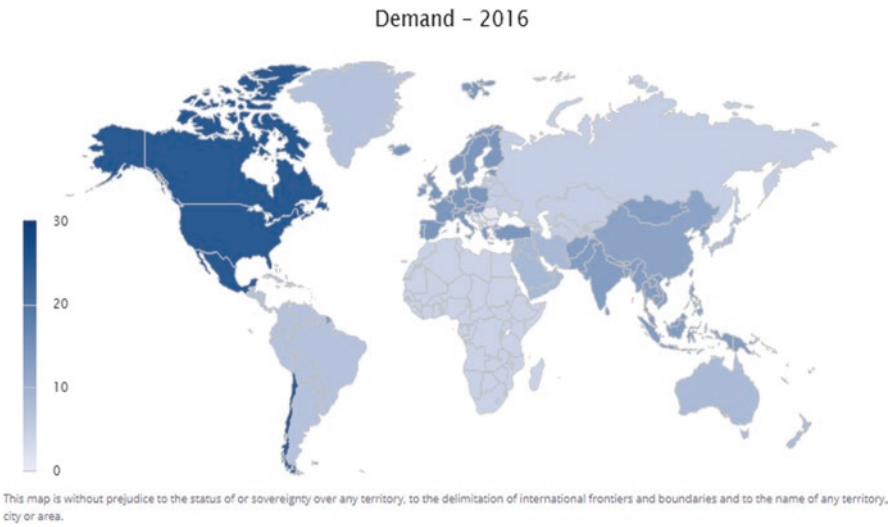

Fig. 20.5 Total world oil demand. (Source: https://www.iea.org/oilmarketreport/omrpublic/maps)

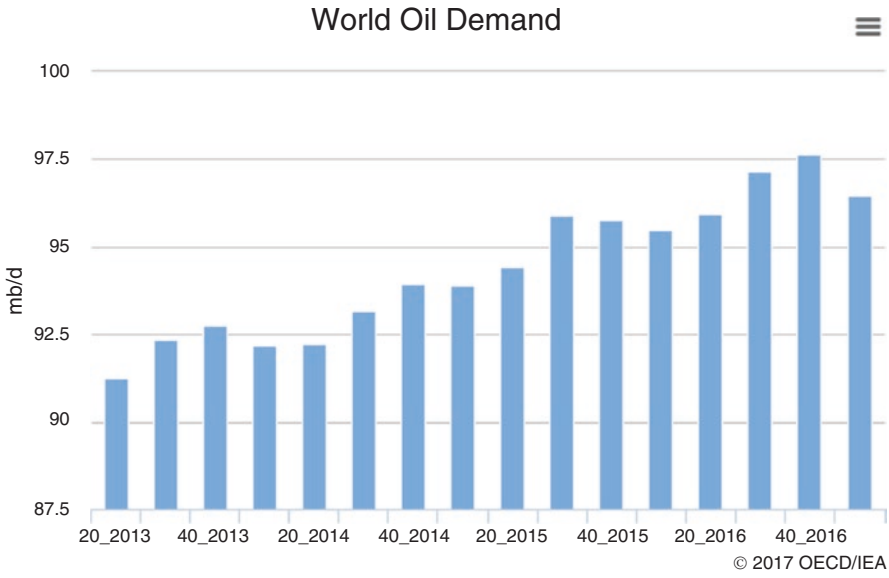

Fig. 20.6 World oil demand from 2013 to 2016. (Source: https://www.iea.org/oilmarketreport/omrpublic)

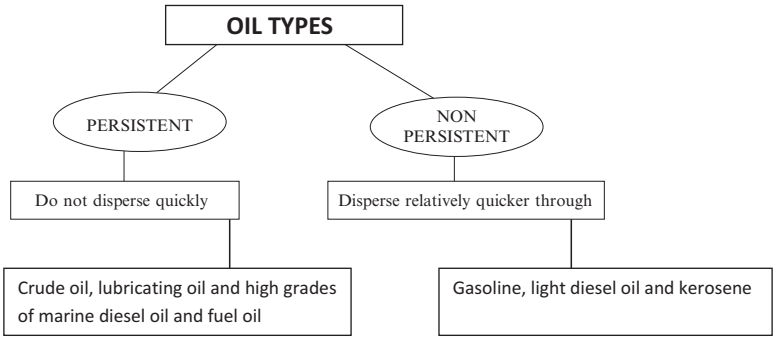

Fig. 20.7 Types of petroleum oil and their uses

20.4 Mechanical Methods

1. Booms – These are the floating barriers made of plastic used to surround and contain the oil; deflect it away from sensitive areas like harbors, mariculture, nature reserves, or coastlines; and concentrate the oil for its easy removal. Different types of booms used are fire booms, hard booms, and sorbent booms, coming in different designs like curtain boom, fence boom, and shore sealing boom. The size and type of material as well as design varies according to the oil type and environmental conditions.
2. Skimmers – Some boats or other devices are installed, which can be combined with booms to remove the oil spilled on the sea surface. It can be towed or self-repelled. Mechanism is to skim off the oil and divert it to a pumping system for storage. It includes "oleophilic skimmers" which have affinity to attract the oil than water, which is later squeezed off to collect the oil. Suction skimmers make use of vacuum pumps to suck out the oil directly from the water. Weir skimmers use gravity to collect oil and store it in underwater tank and physical lifting of oil with mechanical scoops, belts, buckets, or grabs.
3. Sorbents – Insoluble materials used to absorb or adsorb the liquid, generally used to remove the final traces of the oil. These are categorized in *organic* sorbents (peat moss, hay, sawdust, feathers, straw, and other carbon-based products), *inorganic* sorbents (glass wool, sand, clay, volcanic ash (non-water surface), vermiculite, and pumice (water surface)), and *synthetic* sorbents (polyethylene, polyurethane, polypropylene, cross-linked polymers, and rubber materials).
4. In situ burning – It refers to the controlled burning of the spilled oil on the site, contained in the boom and then ignited. It is the fastest, simplest, and effective method for oil spill removal and reduces the need for storage and disposal of collected oil. However, it is limited by some factors like oil thickness, ocean currents, winds, emulsification (Allen and Ferek 1993; Buist et al. 1994; Buist

1995), and water density. The only controversy of this method is its environmental perspective, but, with consideration of air quality (due to burning) and wildlife (due to sinking of residues), it can play a significant role in decreasing the oil quantity on water surface.

20.4.1 Physical and Chemical Methods

1. Photooxidation – Oil reacts with oxygen in the presence of sunlight, breaking into soluble products called tars. Some of the aromatic components (PAHs) also break down and are available for microbes for further process.
2. Evaporation – It depends on the volatility of the hydrocarbons. Oil with greater percentage of light, volatile compound (gasoline, kerosene, diesel) will evaporate faster than the one with heavy compounds. Rough weather, high wind speed, and high temperature promote evaporation.
3. Dispersion – When the oil is broken down into droplets due to ocean currents, turbulence, or winds, these get mixed at the upper level of water surface, and the lighter droplets get suspended in the water, making the droplets easily available for microbial degradation. Sometimes, due to turbulence, water-in-oil emulsion forms which are viscous and persistent and can lead to retardation in the weathering process. It is called emulsification.
4. Dissolution – Some of the water soluble compounds of light hydrocarbons like benzene and toluene get dissolved into the surrounding water.
5. Sedimentation – When most of the lighter oil has weathered or dispersed, some of the particles, close to the coastline, will stick to the suspended sediments and settle down to the ocean floor.
6. Langmuir circulation – Langmuir cells also known as wind rows are formed by wind drive instability. They can create divergence and convergence zones on sea surface which can affect oil thickness and hence weathering rates and cleanup strategies. It also enhances the movement of the slick and vertical dispersion of oil droplets. By pushing the oil droplets downward, it indirectly affects horizontal advection and dispersion that increase the amount of hydrocarbon dissolution into water column (Fig. 20.8).
7. Dispersants – These are surface active agents used to augment the natural dispersion by reducing the surface tension between the oil and water. Surfactant molecule contains an oleophilic part (oil loving) and a hydrophilic part (water loving). When it is sprayed on oil, oleophilic part gets attracted to oil and hydrophilic to water thus causing breaking the emulsion into droplets. But, it poses a serious threat to marine flora and fauna; therefore, its usage remains in doldrums.

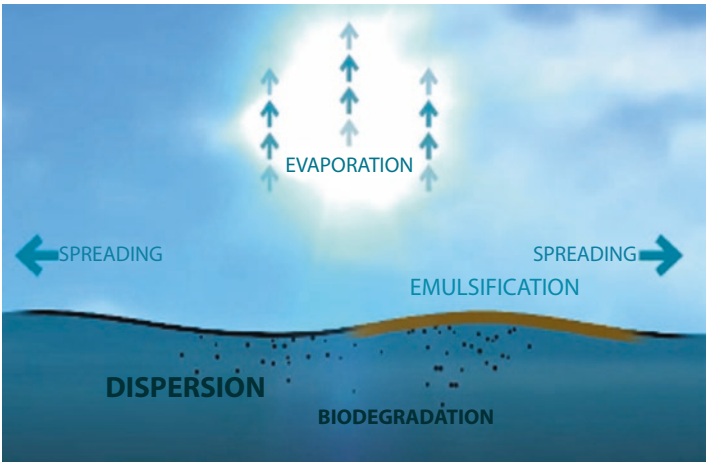

Fig. 20.8 Representation of cleanup methods for oil spills

20.4.2 Biological Methods

It refers to breaking down of complex hydrocarbons into simpler products by microbes like bacteria, yeast, fungi, etc. or addition of fertilizers/microorganisms that increase the rate of natural biodegradation.

1. Bioaugmentation – Also called seeding, addition of microbes isolated from contaminated sites or adding pre-cultured microbes (single strain or consortia) to the site where remediation has to be performed (Leahy and Colwell 1990). It is suited when the native population is low in number or doesn't follow the metabolic routes necessary for the degradation process.
2. Biostimulation – It is the adding up of the limiting nutrients such as phosphorus, nitrogen, and various mineral nutrients to stimulate the growth of native microbes capable of degradation (Atlas 1995; Sarkar et al. 2005; Nikolopoulou and Kalogerakis 2009).
3. Biosurfactants – These amphiphilic, surface-active compounds produced extracellularly or as a part of cell membrane of microbes help in reducing the surface tension between two phases and thus enhancing the solubilization and contaminant removal.
4. Biosparging and bioventing – Addition of air to indigenous oleophilic microbes in saturated and unsaturated zones, respectively, leading to increased biodegradation of contaminants. Usually carried out for groundwater treatment.
5. Bioslurping – Vacuum pumps are used to separate phase petroleum products or contaminants from the vadose zone (for groundwater).
6. Phytoremediation – It is the process in which plants are used to facilitate the restoration of contaminated site. They can directly uptake the petroleum hydrocarbons into their tissues and release enzymes which stimulate the activity of

hydrocarbon-degrading microbes and degradation of contaminants in the rhizosphere due to mycorrhizal fungi and soil microbes (Schnoor et al. 1995; Rhodes 2013).

Some factors limit the above processes like chemical composition and concentration of crude oil, pH, oxygen availability, water activity, temperature, salinity, etc. (Leahy and Colwell 1990).

20.4.2.1 Mycoremediation

Remediation of oil-polluted environment by conventional methods like manual removal, use of chemicals, dispersants, burning, trenching, blasting, removal of sediment, slurry, and others is usually difficult and costly (Michel et al. 2010). The damages from oil-polluted environments are practically irreparable (Porto et al. 2011). The deleterious effects of oil spill incidents, more pandemic in developing countries, have made it more conspicuous to scientists, researchers, and environmentalists that they are finding new ways to speed up the task of oil spill cleanup (UN 2011). The most effective method is to exploit the ability of microorganisms to break down organic pollutants which are their food source or substrate and clean the contaminated sites (Finley et al. 2010). This process is called bioremediation – use of microorganisms, plants, bacteria, fungi, yeasts, or the enzymes secreted by them to return the contaminated site to its original state. In recent years, a new branch of bioremediation called mycoremediation (Singh 2006) has attracted researchers. It refers to the utilization of fungi to degrade or remove toxins from the environment. Fungi are so skilled that they break down many robust, long-chained toxins into simpler and less toxic chemicals (Stamets 2005).

It can also remove heavy metals from the soils. But these beautiful creatures and protectors of environment were recognized very late because they remain hidden underground (Singh 2006).

Fungi are divided in two subgroups: brown rotters (7%) which break the white pulpy cellulose into brown lignin (e.g., *Lentinus ponderosus*, *Gloeophyllum trabeum*, *Serpula lacrymans*, etc.) and white rotter's mycoremediators of toxins held together by hydrocarbon bonds. Enzymes secreted by them include lignin peroxidase, manganese peroxidase, various hydrogen peroxide producing-enzymes, and laccases (Rhodes 2014; Schliephake et al. 2003). Fungi (soil) have been found to be better degraders of petroleum than traditional bioremediation methods including bacteria (Batelle 2000; Ojo 2005). It has also been found to absorb the most harmful pollutants like PAH, PCB, dioxins, pesticides, phenols, chlorophenols, effluents from the paper and pulp mills, dye stuffs, and heavy metals (Atlas and Philp 2005; Singh 2006).

20.5 Morphology and Diversity of Fungi

In marine ecosystem, much research on oil-degrading bacteria is available as it is in abundance, but fungi have not received much attention in bioremediation with regard to its capacity as it is present in lower number than bacteria. Kingdom mycota mainly deals with the study of fungi. Fungi are made up by a structure called hyphae, a branching filament. Many hyphae together form a network called mycelium which makes up the thallus of the fungus. Thallus is the plant body that lacks stems, leaves, roots, and vascular system. Fungi can be unicellular or multicellular, heterotrophic, eukaryotic, nonvascular, and achlorophyllous. These are present everywhere, in air, water, soil, and even in plants and animals. Fungi include mushrooms, yeasts, molds, rusts, smuts, puffballs, truffles, morels, and molds and reproduce by means of spores. These exist as saprobes, mutualists, or parasites based upon the spore case in which spores are produced; fungi are classified into four divisions:

1. *Ascomycota* – (sac fungi) spores are produced in a cup-shaped sac called asci. It is the largest phylum of fungi and can reproduce sexually (plasmogamy, karyogamy, and meiosis) as well as asexually (budding, conidia), e.g., *Aspergillus*, *Claviceps*, and *Neurospora*.
2. *Basidiomycota* – spores are produced in a club-shaped spore called basidium. These are known for the production of large fruit bodies such as the mushrooms, puffballs, brackets, etc. These reproduce sexually (plasmogamy, karyogamy, and meiosis) and asexually (fragmentation), e.g., *Agaricus*, *Ustilago*, and *Puccinia*.
3. *Zygomycota* – spores are produced in round-shaped case called sporangium. They are zygote forming fungi generally found on cheese, bread, and other decaying food and generally reproduce asexually, e.g., *Mucor*, *Rhizopus*, and *Albugo*.
4. *Deuteromycota* – these are known as imperfect fungi as they lack sexual reproduction, and there is little knowledge of their complete life cycles, e.g., *Alternaria*, *Colletotrichum*, and *Trichoderma*.

It is said that more than 600 million years ago, animals and fungi shared a common ancestor. Moreover the widespread of fungi is limited not just on earth but in the entire universe wherever water can be found. Fungi were the first organisms to land on earth around 1.3 billion years ago (Rhodes 2014). At present approximately 5.1 million species of fungus are known to exist (O'Brien et al. 2005) out of which 10,000 are marine fungi (Jones et al. 2015). Though fungus is present in significant diversity on Earth, our ecological knowledge and evolutionary intricacy of fungi are mostly derived from study of cultured fungal isolates from terrestrial environments, but the understanding is expanding as recent molecular methods like environmental DNA, polymerase chain reactions, and clone library approach have aided researchers to investigate environmental diversity. Using DNA sequencing facility at the UHM Hawaii Institute of Marine Biology, researchers have determined some new species of fungus associated with native algae at a depth of 130–500 feet (Wainwright et al. 2017). Pervasiveness of yeast in oceans has been amply documented (Hagler

and Ahearn 1987). As majority of fungal sequences and isolated cultures are from terrestrial environments, it is hypothesized that fungi dwelled on land first. However, all terrestrial life forms must have evolved out of their marine ancestors; therefore, it is now inferred that fungi first developed in oceans and then colonized terrestrial ecosystems (Le-Calvez et al. 2009).

The diversity of fungi mainly depends on availability of nutrients which are generally high in terrestrial environments, high-pressure marine environment at a depth of 1500–4000 m where only a few species can survive, temperature, oxygen, and substrate/host availability (Richards et al. 2005; Bass et al. 2007; Kohlmeyer and Kohlmeyer 1979). It is known that obligate marine fungi grow and sporulate exclusively in marine environment, whereas facultative marine fungi grow in terrestrial or freshwater habitats but can also grow in marine environment. It was asserted earlier that addition of new species is bleak in future and there are less than 500 marine fungi (Kohlmeyer and Kohlmeyer 1979) but a dramatic rise in the species number has been seen since then.

Marine fungi breed on a wide variety of substrata like wood and sediments, intermingled with algae and lichens, coral skeleton, calcareous material, shell rocks, guts of marine arthropods, and fishes; no doubt why it is difficult to study about marine fungi (Hawksworth and Rossman 1997; Kevin et al. 1998). Earlier it was found that fungi are present mainly in the littoral regions of euphotic zone, but now it has been found in deep-sea habitats, pelagic waters, coastal regions, hydrothermal vents, anoxic habitats, and ice-cold regions (Manohar and Raghukumar 2013). Fungi have been isolated even from depth of 10,897 m in the Mariana Trench in the Pacific Ocean (Takami et al. 1997). Sequences of fungi have even been reported in aphotic zone at 250–3000 m in the Antarctic polar front (Lopez-Garcia et al. 2001). Bartha et al. (1997) listed 14 genera of aquatic fungi; Davies and Westlake (1979) identified 60 fungal strains growing on some hydrocarbons. But still a major chunk of the fungi present in oceans have found their way from terrestrial ecosystem by winds or runoffs (Richards et al. 2012). Fungi are suited to PAH degradation relative to bacteria because they can degrade high molecular weight compounds, while bacteria degrades only small molecular weight compounds and can function in very low oxygen level conditions which is specific of PAH-contaminated zones (Peng et al. 2008; Fernández-Luqueño et al. 2010).

The chief fungal group present in marine environment is that of ascomycetes, basidiomycetes, and chytrids and no glomeromycetes or zygomycetes. Yeast is usually the dominant form in deep-sea fungal community (Bass et al. 2007). The major fungal phyla/subphyla involved in the biodegradation of oil include the *Ascomycota*, *Basidiomycota*, and *Mucoromycotina*, with specific fungal genera including *Aspergillus*, *Candida*, *Cephalosporium*, *Penicillium*, *Torulopsis*, *Saccharomyces*, *Paecilomyces*, *Gliocladium*, *Yarrowia*, *Pleurotus*, *Pichia*, *Geotrichum*, *Talaromyces*, *Cladosporium*, *Mucor*, *Fusarium*, *Alternaria*, *Polyporus*, *Rhizopus*, *Rhodotorula*, *Aureobasidium*, *Hansenula*, *Rhodosporium*, *Trichosporon*, *Cunninghamella*, *Cyclothyrium*, *Mortierella*, *Psilocybe*, *Yarrowia*, *Beauveria*, *Verticillium*, *Drechslera*, *Geotrichum*, *Phialaphora*, and *Trysanophara* (Ahearn and Meyers 1971; Cerniglia and Perry 1973; Walker and Corwell 1974; Le petit et al. 1970;

Al-Nasrawi 2012; Da Silva et al. 2003; Hassanshahian et al. 2012; Passarini et al. 2011; Walker et al. 1975; Fedorak et al. 1984; Bossert and Bartha 1984). Over 1000 yeast species have been isolated from samples collected from fresh, estuarine, and marine waters of Southern Florida and adjacent areas, and they showed a definite distribution pattern; some confined to fresh water or seawater (*Hansenula*, *Torulopsis*, *Pichia*, *Saccharomyces*, etc.), and some spread widely in all habitats (oxidative sp. of *Candida*, *Cryptococcus*, and *Rhodotorula*) (Ahearn et al. 1968).

20.6 Mechanism

Generally bacteria are considered to be the primary degrader of hydrocarbon, but no single species is found to be the ultimate degrader, and hence a consortium is always preferred. Initial steps in biodegradation of hydrocarbons involve oxidation of substrate by oxygenases for which molecular oxygen is required.

Biodegradation of n-alkanes occurs readily. In marine environment fungi utilize short-chained n-alkanes (C_8–C_{18}). Like other organisms, the basic route of metabolism in fungus is monoterminal oxidation to corresponding alcohols, aldehydes, and fatty acids. The fatty acids are further oxidized to acetate and shorter fatty acid through beta oxidation or may be incorporated into cellular lipids (Singer and Finnerty 1984). Highly branched alkanes are known to follow omega oxidation with formation of dicarboxylic acids. Degradation of cycloalkanes is comparatively difficult. Generally a formation of aromatic intermediate occurs followed by cleavage of the aromatic ring structure. Aromatic hydrocarbon rings are hydroxylated to diols which forms cleaved rings with the formation of catechols which are further degraded to intermediates of dicarboxylic acid cycles. Fungi generally form trans-diols (Atlas 1981, 1995).

Some fungi are found to oxidize alkane entirely by subterminal oxidation to secondary alcohols and then to ketones (Rehm and Reiff 1981). Alkenes are oxidized at sites of unsaturation or terminally. Branched chain hydrocarbons like pristane are oxidized or cooxidized by certain yeasts (Hagihara et al. 1977).

Fungi oxidize PAHs via cytochrome P450 monooxygenase and epoxide hydrolase reactions to trans-dihydrodiols. Fungi also form glucuronide and sulfate conjugates of phenolic aromatic hydrocarbon. 1-Naphthyl glucuronic acid and 1-naphthyl sulfate were major soluble metabolites formed by fungal metabolism of naphthalene. *Cunninghamella elegans* was found to oxidize anthracene to trans-1,2-dihydroxy-1,2-dihydroanthracene and l-anthryl sulfate. The reactions were found to be monooxygenase-catalyzed (Cerniglia et al. 1982). Phenanthrene is oxidized by *C. elegans* at 1,2 and 3,4 position to form phenanthrene trans-1,2- and trans-3,4-dihydrodiols (Cerniglia and Yang 1984). Some major PAHs oxidized by fungal species are listed by CE Cerniglia in journal of *Industrial Microbiology and Biotechnology* (1997) – fungal metabolism of PAH past, present, and future applications in bioremediation (Fig. 20.9).

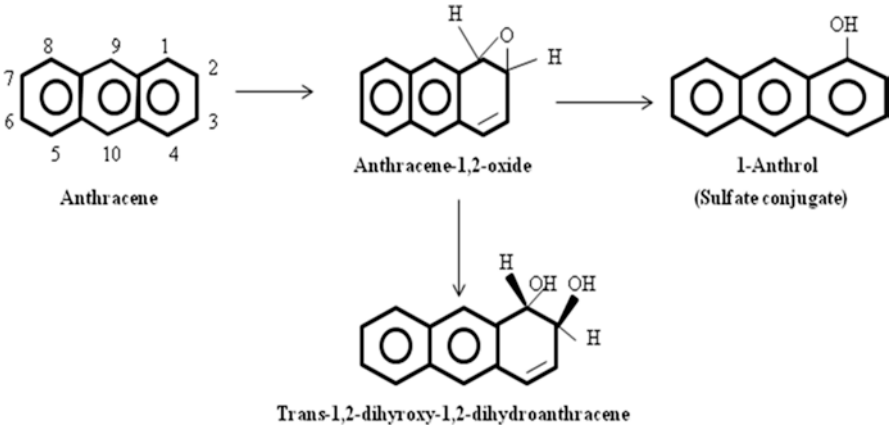

Fig. 20.9 Pathway for fungal oxidation of anthracene by *C. elegans*

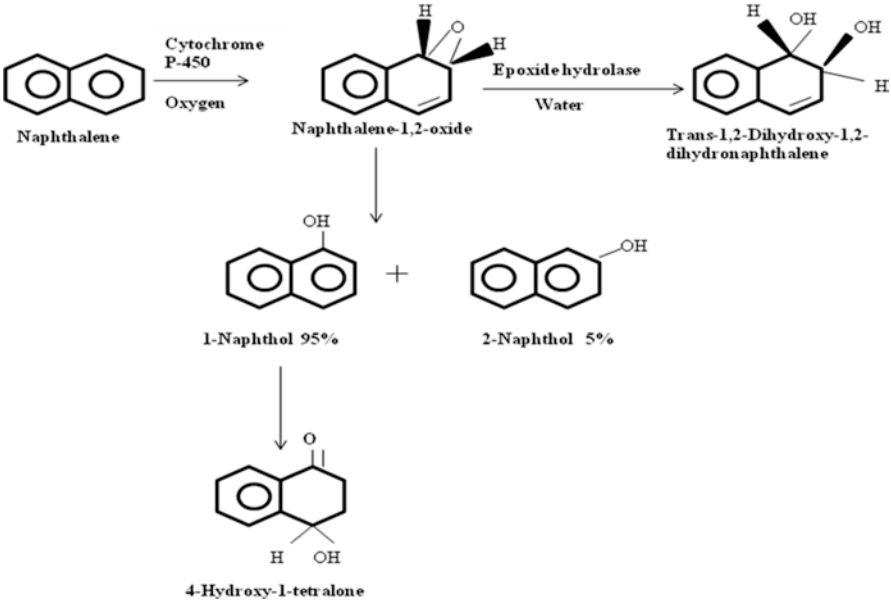

Fig. 20.10 Pathway for fungal oxidation of naphthalene by *C. elegans*

C. elegans oxidizes naphthalene to naphthalene-1,2-oxide which is unstable and undergoes further reactions like (1) rearrangement to form 1-naphthol (major) and 2-naphthol (minor) and (2) enzymatic hydration catalyzed by epoxide hydrolase to form trans-dihydroxy-1,2-dihydronaphthalene (Cerniglia and Gibson 1977, Cerniglia and Gibson 1978; Cerniglia et al. 1983) (Fig. 20.10).

C. elegans, *Saccharomyces cerevisiae*, *Neurospora crassa*, *C. bainieri*, *Aspergillus ochraceus*, and various yeast strains have shown oxidation of benzo[a]pyrene (Cerniglia et al. 1983). The filamentous fungus *C. elegans* oxidized benzo[a]pyrene to trans-9,10-dihydroxy-9,10-dihydrobenzo[a]pyrene, trans-7-8-dihydroxy-7,8-dihydrobenzo[a]pyrene, benzo[a]pyrene-1,6- and 3,6-quinone, and 3- and 9-hydroxybenzo[a]pyrene and oxidized *benz[a]anthracene* to form trans-8,9-dihydroxy-8,9-dihydrobenz[a]anthracene, trans-10,11-dihydroxy-l0,ll-dihydrobenz[a]anthracene, and a trace amount of trans-3,4-dihydroxy-3,4-dihydrobenz[a]anthracene (Cerniglia and Gibson 1979; Dodge and Gibson 1980; Cerniglia et al. 1980). PAHs are generally difficult to be degraded due to high molecular weight, but ligninolytic fungi degrade most of these by oxidizing them and producing a nonspecific enzymatic extracellular complex which is generally used for lignin depolymerization. These lignin-degrading enzymes comprise of lignin peroxidase (LiP), manganese peroxidases (MnP), and laccase. *Aspergillus sclerotiorum* was found to degrade 99.7% pyrene and 76.6% benzo[a]pyrene, and *Mucor racemosus* was found to degrade more than 50% of benzo[a]pyrene when subjected to metabolism evaluation using HPLC-DAD-MS technique. Pyrene and benzo[a]pyrene were converted to more water soluble and less toxic metabolites, pyrenesulfate, and benzo[a]pyrenesulfate, respectively, which can be used as a source of carbon and energy by other microorganisms. Mechanism of hydroxylation is mediated by cytochrome P450 monooxygenase followed by conjugation with sulfate ions. Formation of conjugate is believed as a method of detoxification (Passarini et al. 2011). Asphaltenes are also considered one of the recalcitrant components in the oil and form tarballs when spilled in water and settle with the sediments thus polluting the coasts. About 71% reduction in the tarball content was brought about by thraustochytrids (Raikar et al. 2001). *Cyclothyrium* sp. isolated in Brazil from industrially polluted estuarine sediment was studied under UV spectral analysis and proton nuclear magnetic resonance spectrometry. It changed biphenyl to 4-hydroxybiphenyl and anthracene to anthracene trans-1,2-dihydrodiol and metabolized 90% of phenanthrene to phenanthrene trans-9,10-dihydrodiol as a major metabolite and 2-hydroxy-7-methoxyphenanthrene as a novel metabolite (Da Silva et al. 2004) (Fig. 20.11).

P. ostreatus oxidizes phenanthrene to trans-9,10-dihydroxy-9,10-dihydrophenanthrene by incorporating one atom of $^{18}O_2$ in it, and the inhibitor of P450 monooxygenase reduces the formation of this product. So it can be known from these results that cytochrome P450 monooxygenase is involved in an initial oxidation of phenanthrene. Laccase may be involved during the pathway of PAH products at a later stage.

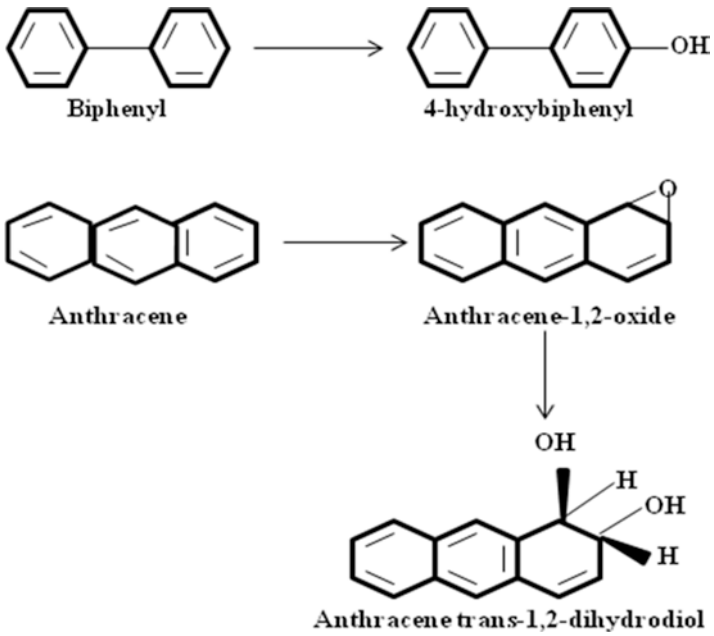

Fig. 20.11 Proposed pathway for metabolism of biphenyl and anthracene by *Cyclothyrium* sp.

20.7 Status of Mycoremediation: A Case Study on BP Oil Spill (2010)

The Deepwater Horizon disaster was the biggest marine oil spill that the USA has seen. On April 10, 2010, an explosion on the Deepwater Horizon spilled approximate 5 m barrels of oil in the Gulf of Mexico and resulted to the death of 11 workers on board. Oil spill affected the states of Texas, Mississippi, Alabama, Florida, and Louisiana. About 1.8 million gallons of dispersant was used to clean up the oil spill.

It has been widely promoted by mycologist-turned-environmental guru, "Paul Stamets," that mushrooms can break down the lighter hydrocarbons and the higher hydrocarbons to lighter hydrocarbons present in petroleum and turn them back into inert matter. He believes that fungi can clean up everything from oil spills to nuclear meltdowns. His book *Mycelium Running: How Mushrooms Can Help Save the World* describes methods for mycoremediation. On hearing about the BP oil spill, he regretted and believed that he can offer a practical solution to the problems by the help of his best friends – *mushrooms*. Stamets calls fungi the "interface organisms between life and death" as they focus on breaking down the indigestible substances into smaller particles which other living things can use as nutrients. It is this aptitude to digest complex organic compounds that makes fungi so promising for cleaning up oil.

A revolution in the field of mycoremediation has been brought by the aptitude of the oyster mushroom *Pleurotus ostreatus* to metabolize several pollutant PAHs. *It* has shown an extraordinary resistance to salty conditions and is capable of growing and reproducing in seawater. This has led to stirring opportunities in the mycoreme-

diation of marine oil spills. The public and government are showing great curiosity in using this technology to deal with polluted environments in the wake of the Gulf of Mexico oil spill.

Paul comes up with an unique solution, experimental but not yet proven, to clean BP the oil spill – MycoBooms – straw is colonized with oyster mushroom (*Pleurotus ostreatus*) mycelium encased in hemp tubes and is used to suck up oil from surrounding water. Absorbed oil is then broken down by the mycelium present inside the straw, thus, starting the decomposition of oil process, reducing the complex hydrocarbons into simpler, more unstable forms. As the mycelium ages, nonpathogenic bacterial community begins to dwell which breaks down the oil in their own ways. Below images show the inoculation and absorption of oil by fungi (Plate 20.1).

Straw is inoculated with fungal mycelium which is absorbing oil from water

Further absorption of oil from water occurs by straw inoculated with mycelium

Oil is being added to straw inoculated with mycelium

Oyster mushroom starts to grow in the oil soaked straw

Mycoboom floating on salt water

Mushrooms grows from the end of a mycoboom

Plate 20.1 Absorption of petroleum by mushroom

20.8 Conclusion

Oceans are considered as huge reservoirs of biodiversity having numerous microbial communities playing an important role in nutrient regeneration cycle as decomposers of dead and decaying organic matter, some founded and worked upon, and some yet to be revealed. Nature has its own ways to replenish if kept untouched. But a constant disturbance by man has led the natural recovery of environment to degrade. Still, nature has many things to offer. Hence, marine-derived fungi can be called as source of enzymes of human interest. Oil spill accidents cause severe physical, chemical, and biological hazards in local marine environment from death of flora and fauna to economic losses in tourism and marine resource industries. Most of the marine fungal specie can be helpful in degrading the spilled oil by converting the hydrocarbons into more water soluble and less toxic to marine environment. In most of the fungal genera, the primary route of metabolism is the monoterminal oxidation to corresponding alcohol, aldehyde, and fatty acid. Microbial degradation of oil spill depends on numerous factors like nature of oil, temperature, nutrients present, composition of oil, and ambient and seasonal environmental conditions. Supplementing the spilled site with microbes to enhance biodegradation or adding oleophilic fertilizers modifies the environment and thus can speed up the biodegradation process. There are certainly other methods to treat the oil spills, but the most effective method is the use of microbes. Bioremediation is gaining much popularity recently and can be further searched upon for more benefits.

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Chapter 21
Treatment of Oily Wastewater Using Hydrogels

Zizi Abdeen

Abstract This chapter discusses the application of hydrogels in wastewater treatment. Oily wastewater is produced in large quantities from activities and processes in the petroleum industry. Moreover, the draining of these effluents pollutes the environment and diminishes the yield of oil and water. Oily wastewater is associated with important threats to air, water, soil, and humans due to the dangerous nature of its oil contents. Hydrogels have been used to treat oily wastewater by an adsorption method. This chapter discusses the monitoring and handling of water remediation in the petroleum industry using hydrogels, such as polyvinyl alcohol (PVA) cross-linked hydrogels, PVA foam, and chitosan-based polyacrylamide hydrogels. The capacity of these hydrogels to adsorb and trap crude oil was determined by gravimetric methods under the best possible conditions. The treatment of oily wastewater can be enhanced at a low cost by trapping crude oil in an open marine environment.

21.1 Introduction

The treatment of oily wastewater is a major issue for the petroleum industry. Refinement, storage, and transportation in petroleum and petrochemical production processes produce large quantities of oily wastewater (Ahmed et al. 2007; Machı'n-Ramı'rez et al. 2008). Pollution from oily wastewater has some obvious impacts, including contamination of drinking water and other water resources, including groundwater and aquatic habitats; negative effects on human health; atmospheric pollution; negative effects on agricultural production; destruction of the natural landscape; and safety issues that arise from the use of oil burners (Poulopoulos et al. 2005).

Aliphatic and aromatic petroleum hydrocarbons with different concentrations are present in wastewater from refineries. The aromatic fraction is not easily degraded by

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common treatments and is quite toxic. Thus, there is a need to develop novel techniques to eliminate or reduce these pollutants as much as possible. Petroleum hydrocarbon biodegradation is a difficult process that depends on the amount and nature of the hydrocarbons present, such as saturates, aromatics, asphaltenes, and polar compounds (Colwell et al. 1977). Polycyclic aromatic hydrocarbons (PAHs) (Moustafa 2004) are a small component of crude oils, but they are common environmental pollutants. PAHs are generated from oil and many pyrolysis processes, causing concern because of their potentially harmful effects on human health (Baum 1978). A number of treatment methods can be used to reduce or eliminate the unfavorable effects of oily wastewater. Some examples of these treatments include electrochemical treatments, membrane filtration, biologic media, adsorption, flotation and coagulation of chemicals, ultrasound-dispersed nanoscale zero-valent iron particles, titanium dioxide, ultraviolet vacuums, natural minerals, and hybrid technologies.

Electrochemical methods cause instability in oil emulsions in wastewater from an electrical current. Electrocoagulation and electroflotation methods are also commonly used in oily wastewater treatment. Membrane filtrations physically separate liquid content from a suspension using a membrane and the application of pressure. Commonly used membranes include ultrafiltration and microfiltration membranes, which are made of ceramic and polymeric materials. Additionally, reverse osmosis membranes may be used in oily wastewater treatment. Biological treatment includes the use of microorganisms that produce the lipase enzyme, which destroy the biodegradable organic substances in oily wastewater (Jamaly et al. 2015). Also, a bioremediation method may be used to convert chemical compounds into energy, cell mass, and biological waste products via living organisms, mainly microorganisms (Rahman et al. 2002).

Because the adsorption process is one of the most effective, efficient, and low-cost methods of oily wastewater treatment, it is broadly used in wastewater systems (Vieira and Beppu 2006). In nearly all cases, the adsorbents have very large internal pores to provide enough surface area for adsorption. However, diffusion restrictions within the particles can reduce the adsorption rate and the available capability (Liao and Chen 2002). Hence, researchers are investigating novel adsorbents with lower diffusion resistance, large adsorption capacity, and environmental friendliness to eliminate crude oil in aqueous solutions.

Natural materials have been used for the adsorption of waste oil, including peat (Solisio et al. 2002), organic bentonite (Panpanit and Visvanathan 2001), and activated carbon (Inagaki et al. 2002). Activated carbon has low efficiency and high cost for the treatment the oily wastewater (Ibrahim et al. 2010). Thus, lower-cost options were investigated by researchers in recent years (Moazed and Viraraghavan 2005). Polymeric hydrogels are hydrophilic homopolymers or copolymers with structures of a three-dimensional crosslinked network (Lee and Huang 2008; Zheng et al. 2007). Because of their superior hydrophilicity properties, they can swell rapidly in aquatic media, which helps to reduce the time to attain adsorption equilibrium (Kaşgöz et al. 2008). Different functional groups have been used to prepare polymeric hydrogels, such as carboxylic acid, amine, hydroxyl, amidoxime, and sulfonic acid groups. When these groups are attached onto polymeric networks, they can be adapted easily

for a particular application. Polymer hydrogels have a higher adsorption rate and adsorption capacity, which gives them many advantages as novel, responsive, and high-capacity adsorbent materials in an adsorption process (Zheng and Wang 2010).

Another advance in polymer hydrogels is the incorporation of environmentally friendly biodegradable polysaccharides, such as starch (Chang et al. 2008) cellulose (Demitri et al. 2008) and chitosan (Abdeen 2005, 2011; Abdeen et al. 2013; Abdeen and Somaia 2014; Alsabagh et al. 2014), inside the polymer hydrogel. Polyvinyl alcohol (PVA) and polyhydroxyethylmetacrylate are common hydrophilic polymers used for hydrogel synthesis (Abdeen 2005, 2011, 2016; AL-Sabagh and Abdeen 2010; Abdeen et al. 2015). PVA is a low-cost polymer that has attractive properties, such as water solubility, biocompatibility, and biodegradability (Matsumura et al. 1993). However, PVA with high hydrophilicity can prevent the uptake of hydrophobic contaminants if the matrix porosity is not high enough (Cunningham et al. 2004). Modification procedures for PVA use a crosslinker in a physical or chemical method (Abdeen 2005, 2011; AL-Sabagh and Abdeen 2010) to diminish its hydrophilicity (Abdeen 2011; AL-Sabagh and Abdeen 2010). Limited research is available on the preparation and use of PVA foam for the degradation of oil. Therefore, it may be possible to design a better PVA foam carrier for oil and advance a new technology for the treatment of wastewater.

21.2 Treatments of Wastewater by Hydrogels

21.2.1 Hydrogel Preparations, Structures, and Properties

Over the years, researchers have defined hydrogels in different ways. Most commonly, a hydrogel is defined as a cross-linked polymeric network that swells in water and is obtained by the simple reaction of one or more monomers. Hydrogels are also known to keep a large amount of water inside their structures; however, they do not dissolve in water. Hydrogels have received considerable attention over the past 50 years as a result of their great potential for a wide range of applications (Li et al. 2013). They have a great degree of flexibility and are extremely similar to natural tissue because of their bulky water content. The hydrophilic functional groups attached to the polymeric backbone give hydrogels the ability to absorb water, whereas the cross-linking between their networks chains provides resistance to dissolution.

Several natural and synthetic materials fit the definition of a hydrogel (Enas 2015). Throughout the past two decades, natural hydrogels were steadily replaced by synthetic hydrogels, which have long service lives, high water-absorption capacity, and high-strength gels. Synthetic polymers usually have well-defined structures that can be customized while being degradable and functionalized. In addition, hydrogels can be prepared from purely synthetic components and are stable in a variety of conditions and temperature ranges (Enas 2015).

Recently, hydrogels have been defined as two- or multi-component systems consisting of polymer chains with a three-dimensional network and water that occupies

the space between macromolecules. The properties of the polymer depend on the nature and density of the network joints. These structures hold diverse amounts of water in an equilibrium, usually in the swollen state. The mass fraction of the polymer is less than the mass fraction of water in a hydrogel. In practice, synthetic polymers that water-soluble in non-crosslinked forms are used to attain high swelling degrees (Enas 2015).

Natural polymers that form hydrogels include proteins (e.g, collagen, gelatin) and polysaccharides (e.g., starch, alginate, agarose). Hydrogels formed from synthetic polymers are conventionally prepared using methods of chemical polymerization. Hydrogels are polymer networks that possess hydrophilic properties and are prepared based on hydrophilic monomers. Sometimes, in hydrogel preparation, hydrophobic monomers are used to regulate the properties for specific applications (Enas 2015).

A hydrogel's cross-linked hydrophilic polymeric network creates a flexible structure. Therefore, any method that can be used to produce a cross-linked polymer can also be used to create a hydrogel. Commonly, copolymerization/crosslinking free-radical polymerization methods are used to create hydrogels by crosslinking the hydrophilic monomers with multifunctional cross-linkers. Both natural and synthetic water-soluble linear polymers can be cross-linked in a number of methods to form hydrogels (Enas 2015), as follows:

1. Polymer chains are linked by a chemical reaction.
2. The main-chain free radicals are generated using ionizing radiation that can recombine as cross-link junctions.
3. Entanglements, electrostatics, and crystallite formation are examples of physical interactions.

Several of the different polymerization methods can be used to form gels, as well as for the polymerization of bulk, solutions, and suspensions. Generally, the monomer, initiator, and cross-linker are the three basic parts needed to prepare a hydrogel. Diluents, such as water or another aqueous solutions, can be used to control the polymerization heat and the required properties of hydrogels. Finally, the hydrogel is washed to eliminate unwanted products, such as non-reacted monomers, initiators, and cross-linkers that remain from the process of preparation (Fig. 21.1).

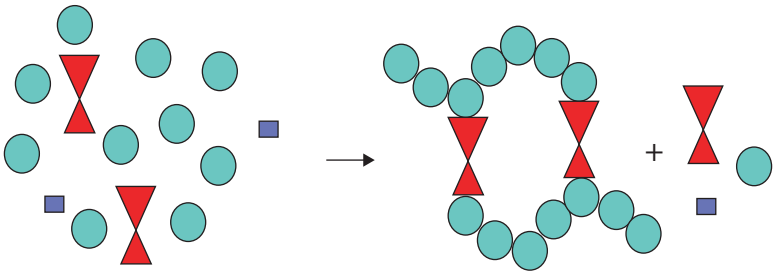

Fig. 21.1 Schematic diagram of hydrogel preparation

21.2.2 Preparation of PVA Hydrogel Foam

Researchers have prepared PVA and PVA foam hydrogels by crosslinking methods using a cross-linker such as epichlorohydrin (Abdeen and Moustafa 2016). Based on PVA and calcium carbonate, PVA foam was prepared and foamed by adding hydrochloric acid, which produced a macroporous carrier (Abd El-Hady and Abd El-Rehim 2004). The chemical crosslinking agent, epichlorohydrin, was used to form structures with a network to improve the stability of the foam. The attractive properties of PVA foam crosslinked as an oil carrier were found in further investigations. For comparison, a foam carrier of crosslinked PVA prepared and used for the adsorbent matrix preparation. The capacity and efficiency of each PVA and its foam matrixes were crosslinked using epichlorohydrin to adsorb oil from water under diverse conditions (AL-Sabagh and Abdeen 2010). This data on the treatment of waste effluents of oil will be helpful in future applications.

21.2.3 Preparation of Chitosan Hydrogel

A linear polysaccharide, such as chitosan, can be produced from the partial substitution of the N-acetyl groups of chitin in the presence of an alkaline solution (Torres et al. 2006). The deacetylation chitin (chitosan) (Abdeen 2005), which is a natural polysaccharide, consists of repeated glucosamine units resulting from the deacetylation of chitin found in insects and crustacean exoskeletons (Abdeen 2005). When chitosan is used in its natural form, it has a tendency to agglomerate forming gels (Amit and Mika 2009) as well as other adsorption process difficulties, such as solubility in acidic media, which prevents it from being recycled. Furthermore, its small internal surface area limits access to covered sites of adsorption (amino groups), so the maximum capacity and adsorption process speed decrease. To minimize these problems, chemical modification of the polymer uses substances that increase the characterization of chitosan as an adsorbent, such as reticulating agents (Barros et al. 2006).

Chitosan hydrogel is prepared by carefully mixing a prepared chitosan solution of 1% acetic acid with a certain concentration percentage of a cross-linker (epichlorohydrin or glutaraldehyde) (Abdeen 2005, 2011, 2016; Abdeen et al. 2013; Abdeen and Somaia 2014). Chitosan has been prepared by decalcification of the chitinous material (shrimp shells) in 1.0 M HCl (3.0% w/v) at room temperature with constant stirring for 1.5 h. The decalcified product was then filtrated, washed, dried, and deproteinized at 50 °C with 4% NaOH solution with constant stirring for 5 h. The product was filtered and washed using deionized distilled water until the pH reached 7. After that, it was dehydrated twice using methanol and once using acetone, and finally dried. The odor was removed by adding dried chitin to a boiling 0.1% solution of potassium permanganate and to a 15% solution of oxalic acid to remove the color. The chitin product was filtered, washed with distilled water, and then dried.

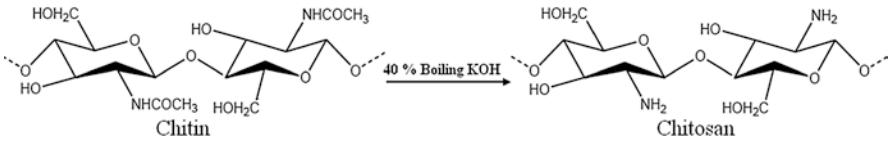

Fig. 21.2 The deactylation of chitin to prepare the chitosan

To prepare the chitosan, the dried chitin was added to a three-necked flask containing 40% (w/v) KOH solution and refluxed at 135–140 °C under a nitrogen atmosphere for 2 h (Abdeen 2005, 2011). The chitosan (deacetylated chitin) was filtered, washed using distilled water, and then dried (Fig. 21.2).

Chitin and chitosan derivatives have attracted a great deal of interest as effectual biosorbents because of their low cost and bulky functional groups of amino and hydroxyl, which have considerable adsorption potential to eliminate different pollutants from water (Amit and Mika 2009). They are widely used because of their characteristics of nontoxicity, abundance in nature, biocompatibility, and ability to biodegrade (Boddu et al. 2008; da Silva Grem et al. 2013).

21.2.4 Chitosan-g-P (AAm) Hydrogel Preparation Via Grafting by Gamma Irradiation

Chitosan is an interesting example of a prospective natural polymer. It has advantages in terms of flocculation efficacy and a reduction in the lifecycle of environmental issues related to synthetic flocculants. However, because most polysaccharides have restricted charges and flocculation for many applications, with a required density of molar charge above 50%, methods to increase the charges of polysaccharides are being investigated. To facilitate the process of adsorption, a more efficient and environmentally friendly adsorbent must be developed to eliminate oil in aqueous solutions (Peniche et al. 2003; Sokker et al. 2011; Aly 2017).

Among the different modification methods for grafting chitosan, the most promising method uses the plentiful groups of amino and hydrogels in the backbone of chitosan in a reaction with vinyl monomers in mild conditions. To date, much research was carried out on the grafting copolymerization of chitosan with vinyl monomers (Navarro and Tatsumi 2001). Generally, the grafted copolymers have the major properties of both initial reactants. Usually, they are able to biodegrade to some degree due to the presence of the polysaccharide backbone. They are reasonably stable in the presence of shearing conditions, which has been attributed to the flexible attachment of synthetic polymers onto the backbones of rigid polysaccharides. When a flexible polyacrylamide is grafted onto the backbone of a rigid polysaccharide, the probability of the flocculant moving toward the contaminant particles increases, and consequently the polysaccharide's flocculant ability increases (Al-Karawia et al. 2011). Acrylamide with certain concentration percentages can be mixed carefully with a

solution of chitosan containing 1% acetic acid in glass vessels, deoxygenated using nitrogen bubbled for a period of time, and irradiated with 60Co-ray at different irradiation doses. The grafted copolymer was dried and then ground to a 300-m mesh.

21.3 Hydrogels as Adsorbent Materials for Oil

The removal of oils and organic contaminants from wastewater has received much attention from academic researchers and commercial ventures, owing to the need for industrial cleaning products for oily wastewater, oil/chemical spills, and leaks. Because of the increasing rate and capacity of polymer hydrogel adsorption (Liao and Chen 2002), they could serve as a new type of adsorbent materials with rapid responsiveness and higher capacity (Zheng and Wang 2010; Bulut et al. 2009). They have a crosslinked three-dimensional network of hydrophilic homopolymers or copolymers (Lee and Huang 2008; Zheng et al. 2007) that could swell rapidly in an aquatic solution, thus decreasing the time to attain equilibrium of adsorption (Kaşgöz et al. 2008).

The hydrogels of each PVA and its foam could be superlative adsorbent materials for eliminating crude oil from oily wastewater; in addition, they can be used to examine the adsorption performance of oil. Once a sample of hydrogel is sited in synthetic oily wastewater, the processes of adsorption and degradation start on the hydrogel's surface and in a mixture of the water/oil, in that order. The oil adsorption performance onto the prepared hydrogels, the percentage of the degradation process using different dosages of hydrogel, and equilibrium times were reported elsewhere (Abdeen and Moustafa 2016).

The adsorption capacity of PVA and its foam for crude oil has been calculated by the degradation percentage: 59.2% and 69.5%, respectively (Abdeen and Moustafa 2016). The superior adsorption capability for oil shown by the PVA foam could be due to its structure, which is more porous than the PVA gel. Therefore, the different adsorption capacities observed among these gels were attributed to the number of binding sites that were available to eliminate the oil at a particular initial concentration, which increases the dose of the gel (Abdeen and Moustafa 2016). Therefore, this adsorption increase could be related to a greater number of hydrogel particles on the surface area and more available surface sites for adsorption (Ferrus and Pages 1977). Also, at a somewhat low dose of PVA foam, the results showed that the percentage of degradation was high compared with the PVA gel. At a high dose of PVA foam, the removal efficiency decreased (Abdeen and Moustafa 2016). This may indicate a grouping of factors, such as an elevated molecular mass or higher charge density, contribute to the somewhat high PVA hydrogel adsorption activity. However, polymers with oppositely charged particles have attraction between them, which might result in a higher charge density polymer, due to the high number of charges per polymer molecule (Abdeen and Moustafa 2016). It is clear from the SEM micrograph that the PVA foam has more pores than PVA, and of a larger size. As a result,

PVA foam has a large surface area that provides extra sites for crude oil adsorption in larger amounts than PVA, which has a lesser adsorption efficiency than PVA foam.

Earlier studies have shown that the economic quantity of hydrogels is nearly 1.0 g/L in solutions for use in the treatment of contaminated oily wastewater of an oil concentration of 5.0 g/L. Also, the ability of oil adsorption has been studied at an initial concentration of 5.0 g/L using hydrogel adsorbents of 1.0 g/L at a pH range of 2.0 to 9.5 after 3 hrs. The results have showed that the degradation percentage of crude oil was higher using PVA and its foam as adsorbents (71.52% and 80.82%, respectively) at pH 3; the adsorption rates were 74.05% and 81.80% at pH 9 and 58.3% and 67.6% at pH 7, respectively. Therefore, it could be deduced that more acidic conditions aggravated oil formation (Peniche et al. 2003; Aly 2017), whereas hydrogels of PVA and PVA foam promoted a physicochemical effect that actually helped to demulsify and increase the size of droplets to improve the oil adsorption. Furthermore, the alkali state operates as a catalyst for the reaction of each molecule of oil and the hydroxyl groups of hydrogels that are present inside the adsorption site.

The effect of contact time on oil adsorption efficiency onto hydrogels was also investigated. The oil adsorption efficiency increased with time until it reached an almost constant value after which almost no additional oil was removed from the solutions. At the point of equilibrium, the amount of oil desorbed from the preparing hydrogel was in a dynamic equilibrium state with the oil amount that could be adsorbed on the hydrogel. The results showed that the adsorption of oil was faster at the initial step of the contact time, after which it became slower closer to the equilibrium. This may be due to the fact that more vacant surface sites were available during the initial step for adsorption; after a period of time, it was difficult for the remaining vacant surface to be occupied because of repulsive forces between the molecules of the solute on the solid and bulk phases (Abdeen and Moustafa 2016).

The higher adsorption capacity of the PVA foam (shown in Fig. 21.3) than that of PVA hydrogels could be related to the microstructure of the PVA foam, which incorporates more regular pores with larger sizes, higher surface areas, higher degrees of adsorption inside their structures, and higher porosity than that of PVA. Higher adsorption capacities of PVA and PVA foam gels result in an effective matrix for the adsorption of oil under similar conditions, such as the weight of hydrogel, degradation time, pH, and temperature. Thus, an adsorption with high capacity was shown for the degradation percentage of oil (Abdeen and Moustafa 2016). Therefore, the hydrogels' capabilities to adsorb oil improve the degradation of oil. The obtained data from petroleum hydrocarbon degradation via adsorption of oil by immobilization on the hydrogel matrix suggests that the degradation rate is higher when using a PVA foam adsorbent than when using PVA. This may be attributed to the varied surface area of each adsorbent, assuming that this is responsible for the capacity of adsorption and rates of degradation obtained using the hydrogel samples. The degradation rate was noted to be much higher when using PVA foam than in the matrices of PVA; this could be related to the increased crude oil diffusion into the hydrogel pores as a result of increasing their number and size compared with that of PVA (Abdeen and Moustafa 2016).

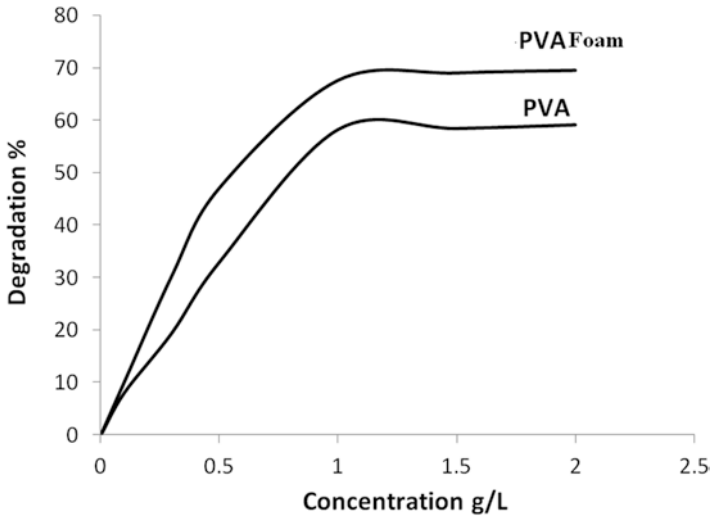

Fig. 21.3 Degradation percentage of oil in wastewater using hydrogels with different concentrations for 3 h at pH 7. (Abdeen and Moustafa 2016)

21.4 Oily Wastewater Treatment Using an Oil Adsorption Method

Crude oil of certain concentrations was placed in a series of water volumes. Different doses of the preparing gel were added to the suspension. In addition, the time of contact and pH were adjusted to obtain the ideal condition for removing crude oil from an aqueous solution. The experiments were done at a temperature of 30 ± 1 °C. The mixtures were excited using a mechanical shaker. After removing the samples, the degradation percentage of oil was calculated by removing the solvent using a rotary evaporator, and the residue of oil was weighed (Abdeen 2016; Abdeen and Moustafa 2016).

21.5 Physical and Chemical Properties

A basic understanding of hydrogel properties has not been enough to create a reasonable design for a new system of gels. These designs must identify how the solute molecules interact with the gel, particularly how they segment between the phase of the gel and the phase of the surrounding liquid. Segmentation depends on two main factors: size exception and molecular attraction/repulsion (Naziha et al. 2015).

The hydrogel swells with cross-linked polymer networks in an aqueous media. The absorbing solution acts as a selective filter to permit the free diffusion of an amount of solute molecules, while the polymer network acts as a matrix to retain the

liquid inside. The gel could be absorbed from 10% to 20% (qualitative lower limit) up to thousands of times its dry weight after immersion in water. In a hydrogel, the water characteristics can be determined the nutrients' overall penetration into the hydrogel and cellular products out of the gel. When dry hydrogels start to absorb water, the first water molecules entering the hydrogel matrix will hydrate the mainly polar and hydrophilic groups, forming primary-bound water. The same occurs as the polar groups are hydrated; the network swells and exposed hydrophobic groups, which also interact with molecules of water, form hydrophobically-bound water or secondary-bound water. Primary- and secondary-bound water frequently combine and then are described as the total bound water.

When the polar and hydrophobic sites interact with bound water molecules, the additional water will be imbibed by the network because the network chains have an osmotic driving force toward infinite dilution. This further swelling is resisted by the chemical or physical crosslinking, leading to an elastic network with retraction force. The use of Differential scanning calorimetry was based only on the fact that free water could be frozen, and therefore on assuming that the measurement of the endothermic temperature on warming the frozen gel represented the free water melting; this value produced a free water amount in the hydrogel sample that can be tested. After that, the bound water can be obtained by calculating the difference between the total water content measured in the hydrogel test specimen and the free water content (Naziha et al. 2015). The degree of swelling is measured by pre-weighing the dry sample and submerging it in distilled water for a known time period. The excess surface water excess is removed using absorbent paper, and the swollen sample is then weighed. The preceding steps are repeated until there is no increase in the swollen sample weight. The following equation is using to calculate the degree of swelling (DS):

$$\mathrm{DS} = \left(m - m'\right) / m' \left(1\right),$$

Here, the swollen and dry samples weights are represented by *m* and *m'*, respectively (Ferrus and Pages 1977). The PVA and PVA foam carriers have swelling behaviors (Abdeen and Moustafa 2016) and were tested at pH 7 and 30 °C (Fig. 21.4). The rates of equilibrium swelling versus the ratios of the cross-linker relationship are represented in Fig. 21.3, which shows that the water uptake of the PVA matrix decreases with an increasing crosslinker ratio over the definite ratio. A significant crosslinking density occurs with an increase in the ratio of crosslinker to achieve the best swelling properties (9.1); after that, this property will decrease (from 9.1 to 6.2) with an increasing density of crosslinking (Abdeen 2011; Abdeen and Moustafa 2016; AL-Sabagh and Abdeen 2010). The hydrogel sample's water uptake increases with time, but the swelling properties of PVA are higher than that of PVA foam; this is may be due to an increase in the crosslinking density of PVA foam, leading to a decrease in the water uptake. It also may be attributed to the presence of a large number of large-size pores in the hydrogel of PVA foam, which might decrease the retention of water. Moreover, the oxygen in $CaCO_3$ could create a physical bond

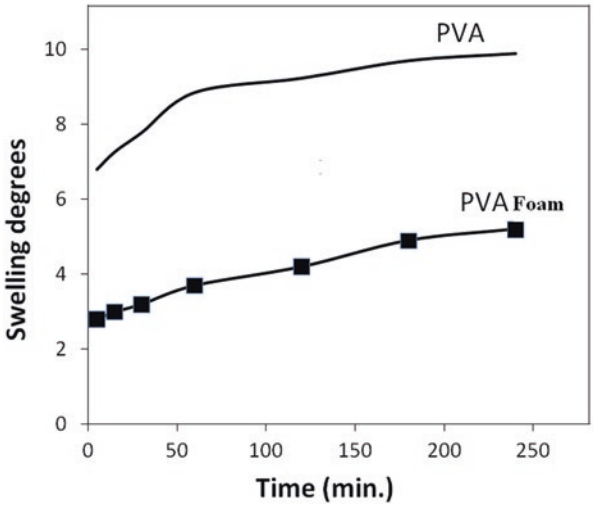

Fig. 21.4 Swelling degrees of PVA foam and PVA hydrogels over a period of time at pH 7. (Abdeen and Moustafa 2016)

between it and the hydroxyl group of PVA, resulting in a decrease of water molecule penetration into the hydrogel network (Abdeen and Moustafa 2016).

21.6 Structural Properties

Fourier transform infrared spectroscopy has been used to verify that the formation of hydrogels occurred. Fig. 21.5 shows the infrared spectra for hydrogel samples of PVA and its foam (Shimadzu 8001, Japan). In Fig. 21.5 of the PVA and its foam (Abdeen and Moustafa 2016), at 3440–3100 cm^{-1} a broad peak exposed the hydroxyl groups, with stretching vibrations from 2940 to 2900 cm^{-1} resulting from the C-H band. In addition, the PVA spectrum has a hydroxyl group with a broad bonded peak at approximately 3263 cm^{-1}; however, it becomes less broad;y condensed and moves to 3313 cm^{-1} in the PVA foam infrared spectrum. Because of intramolecular and intermolecular hydrogen bonding among PVA hydroxyl and other molecule groups, the hydroxyl group band moved to lesser frequencies as shown in the IR spectrum of PVA. The peaks at 1419, 871, and 713 cm^{-1} are related to the calcium oxide stretching and the bending vibration of calcium carbonates (Abdeen and Moustafa 2016). From this result, it is clear that the network structures are forming on the PVA chains with definite numbers of hydroxyl groups. Consequently, as crosslinked network structures formed, a strong band of the ether group formed in PVA and PVA foam at 1090 cm^{-1}. In the PVA foam spectra, these bands are weaker than in PVA because of the increased crosslinking in hydrogels. The band of C-O

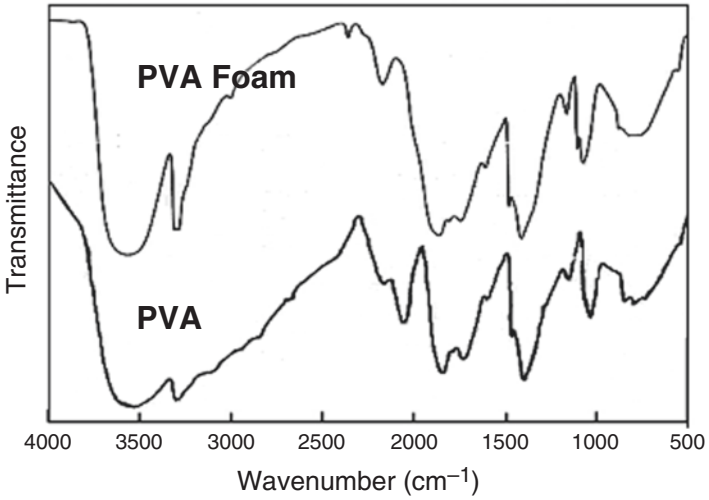

Fig. 21.5 Fourier transform infrared spectra of PVA foam and PVA hydrogels

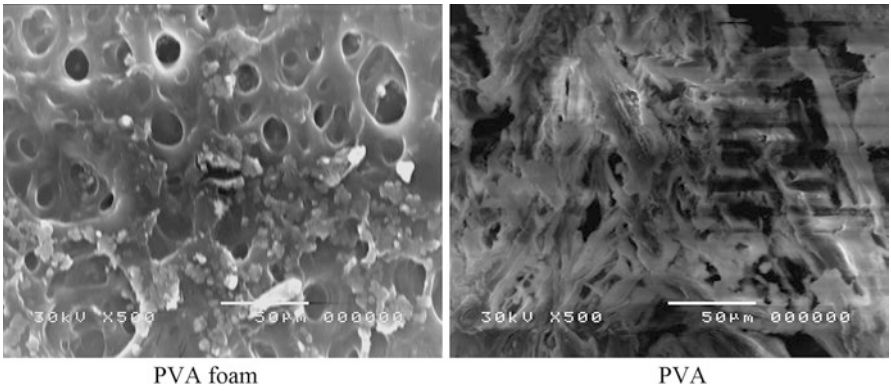

Fig. 21.6 SEM photographs of PVA foam and PVA hydrogels (Abdeen and Moustafa 2016)

stretching for the secondary alcohol group is shown at 1095 cm^{-1} (Abdeen and Moustafa 2016; Abdeen 2011).

Various microscopic examinations of hydrogels have been performed. They include qualitative and quantitative investigations, from simple morphological examinations of materials to very complex biocompatibility studies. The surface morphology can be examined by using topographic microscopy techniques. With increasing powers of magnification, these techniques include optical microscopes, stereo microscopes, scanning and transmission electron microscopes, tunneling microscopes, and atomic force microscopes. For example, Fig. 21.6 (Abdeen and Moustafa 2016) shows electron scanning microscopy that was used to assess the surface morphology of hydrogels before and after the adsorption of oil. It used gold

with a thin layer to coat the sample (around 110A) before evaluating. The surface morphology of the PVA foam hydrogel is wholly porous with a non-smooth surface, whereas the PVA hydrogel has a smaller amount of pores. Thus, a perfect carrier hydrogel should have a structure with more pores to facilitate the spread of solutes (Abdeen and Moustafa 2016).

21.7 Conclusion and Future Aspects

The effective treatment of oily wastewater runoff is required to improve environmental conditions and quality of life. Thus, the need for a novel, efficient approach is urgent. Because of their uptake capability for crude oil , hydrogels can act as an adsorbent for oily wastewater in different environments, including rivers and seas. As can be seen from the results presented in this chapter, these hydrogels should be applied for the elimination of crude oil in any oily wastewater.

Future studies on oily wastewater treatment technologies should focus on the following areas:

1. A new combined process that maximizes the advantages of existing methods while avoiding their weaknesses
2. Methods to increase oily wastewater treatment efficiency and diminish processing costs by providing a solid theoretical foundation through the in-depth study of oily wastewater degradation mechanisms
3. Environmentally friendly research approaches, including technology for supercritical water oxidation that avoids secondary pollution, provides superior wastewater treatment rates, and utilizes a relatively simple device with automatic controls

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Chapter 22
Novel and Cost-Effective Technologies for Hydrocarbon Bioremediation

Rajeev Kumar and Pooja Yadav

Abstract Hydrocarbon contamination of soil and water is increasing day by day around the world. Remediation of these contaminated sites using microorganisms (bioremediation) is the most efficient and environment-friendly method. Bioremediation is executed either on the site of contamination known as in situ or off the site of contamination known as ex situ. Among these two, ex situ bioremediation technologies are more expensive because the cost of excavation gets added up. But, on the other hand, installation of the equipments required for in situ technologies is also of major concern. So, it becomes very important to find the correct technology for bioremediation of a particular site to get the desired results. In this chapter various cost-effective technologies are discussed such as land farming, phytoremediation, bioreactors, biopiles, etc. Also, the two techniques, bioaugmentation and biostimulation, for enhanced bioremediation are discussed. Principles, advantages, and disadvantages of the techniques are described.

22.1 Introduction

One of the main problematic issues for the environment today is hydrocarbon pollution (Das and Chandran 2011). Introduction of hydrocarbon into the environment takes place either because of accidental reasons or human activities resulting in polluting the environment (Singh and Chandra 2014). At present, the accepted traditional methods are excessively expensive when the amounts of contaminants are large and chemical treatments are successful for the degradation of petroleum products, but they fail to keep up with the needed properties; also, often a lot of hazardous compounds are formed by them involving a great level of risk (Jain et al. 2011). Physical and chemical methods result in a lot of negative consequences because of which the bioremediation technology is adapted on a large scale and regarded as one of the best technologies for treating the environment polluted by hydrocarbon (Singh

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and Chandra 2014). Bioremediation is defined as the use of microorganisms to degrade, break down, transform, and/or essentially remove pollutants from soil and water. Bioremediation method depends upon bacteria, fungi, and plants to remediate pollutants when the organisms perform their regular functions/activities. Metabolic functions of these organisms utilize the chemical pollutants as a source of energy, leaving behind nontoxic compounds. Basic requirements for bioremediation are (1) contaminant, (2) electron acceptor, and (3) microbes that can degrade a certain pollutant. Usually, a pollutant gets degraded in a short span of time if it occurs naturally in the environment or chemically resembles a compound that occurs naturally. It is because microbes that can degrade the compound are likely to have already evolved (State of Mississippi, Department of Environmental Quality, 1998). Petroleum hydrocarbons are an example of chemicals that occur naturally; and hence, microbes which are able to degrade them are already present in the environment (MSU). Thus, bioremediation techniques are efficient because of their efficiency, safety for long-term use, expenses, and simplicity to monitor (Jain et al. 2011).

22.2 Technology for Bioremediation of Contaminated Soil and Water by Hydrocarbons

When the contaminated soil is removed from the subsurface for its treatment, then this technique is known as ex situ bioremediation. While in the case of in situ remediation technologies, the contaminated soil is treated at its native place without translocation. Several thermal, chemical, and physical remediation practices have been unsuccessful from getting rid of the pollution. It is because those techniques result in shifting of contaminant from its original phase to another such as creating air pollution. Bioremediation degrades contaminants and therefore is a worthwhile and environment-friendly method that results in being economically profitable as well (Iranzo et al. 2001). Ex situ and in situ methods have specific costs and advantages associated with them.

22.3 Ex Situ Bioremediation Technologies

Bioremediation technology which is ex situ requires the contaminated portion to be excavated and transferred to a place where it can then be treated. Ex situ technology makes it possible to have more control on environmental conditions, enabling increase in biodegradation rate (Aislabie et al. 2006). Because of the ability to intermix sample that is contaminated, the treatment is generally more uniform and takes less time than in situ treatment techniques. However, ex situ technologies are more expensive because of the extra procedures associated with it such as excavation, site preparation, and operation. Excavation enhances introduction to contaminants. Site

preparation mostly requires setting up a liner system in the area of treatment to prevent contaminants from moving into the subsurface and surface water runoff control systems to prevent off-site transport. Other requirements, such as measures to maintain suitable environmental conditions, include application of moisture and nutrients or aeration via blower systems or mechanical agitation (Brown et al. 2017). The major advantage of ex situ bioremediation technology is that it does not need any large exploratory assessment of polluted site before remediation; this makes the primary stage cost-effective. By excavation of polluted soil, the effect of soil porosity on transportation process gets reduced. Ex situ bioremediation technologies cannot be used for areas that are beneath buildings, inner city, and sites where work is going on (Philp and Atlas 2005). But, on the other note, excavation technique of ex situ bioremediation is likely to interrupt soil structure; as a result, polluted site and sites around it go through more disturbances. Ordinary to considerable techniques required for any ex situ bioremediation technology shows that more manpower and cost are required to construct any of the technology. Mostly, this technology demands large area for operating. Usually, ex situ bioremediation technologies are faster, simpler to govern, and utilized to deal with a large number of pollutants (Prokop et al. 2000). These technologies include land farming, biopiles, and windrows that are discussed in detail below (Brown et al. 2017).

22.3.1 Land Farming

Land farming is considered the easiest bioremediation technology because of its low price and minimum equipment need to operate. The title "Land Farming" explains a process in which hydrocarbon-contaminated soils are laid out with a height of 0.3–1.0 m, addition of nutrients is done, and soils are mixed regularly (Krysta et al. 2008). Mostly, it is considered as ex situ bioremediation technique, whereas at times, it is considered as in situ bioremediation technique. This discussion is because of the site of treatment. Depth of pollutant is an important factor in considering the site for the treatment of contaminated soil. In this technique, common thing is that the contaminated soil is usually excavated and/or tilled, but the site for its treatment actually decides the kind of bioremediation, whether in situ or ex situ. Commonly, it is considered as an ex situ bioremediation technique. It has been said that whenever pollutant lies less than 1 m in height below the ground surface, bioremediation can be done without excavation, whereas pollutant lying more than 1.7 m in height below the ground surface, then it has to be translocated to surface for efficient bioremediation (Nikolopoulou et al. 2013). Adding nutrients (potassium, phosphorous, and nitrogen), irrigation, and tillage are the main procedures. It was reviewed that tillage and irrigation without inclusion of nutrients in soil with suitable biological activity enhanced heterotrophic and diesel-degrading bacterial counts which helps in improving the rate of bioremediation. In land farming technology, an important role is played by aeration in removing the contaminant specifically in regions having cold climate. Usage of land farming is generally done for

remediation of polyaromatic hydrocarbon-contaminated area (Silva-Castro et al. 2012; Cerqueira et al. 2014). Biodegradation and weathering are the two remediation mechanisms included in hydrocarbon-contaminant removal. Land farming has regularly been doing well in warmer southern climates (McCarthy et al. 2004). For example, in a 12-month-long experimental period of an Australian land farm, TPH levels were repaired from 4644 ppm to <100 ppm (Krysta et al. 2008). The building of an appropriate land farming design with an impermeable liner curtails pollutant leaching into surrounding sites while bioremediation procedure takes place (Da Silva et al. 2012). Land farming bioremediation technology is very easy to construct and execute; it needs less capital and can be utilized for the treatment of large amount of contaminated soil with minimum impact on the environment and minimal utilization of energy (Maila and Colete 2004). However, the simplest technique (Fig. 22.1),

The application and productiveness of this procedure consists of:

- Large portion of space.
- Suitable conditions for bioremediation of the hydrocarbon pollutants (e.g., temperature, moisture) are uncontrollable, because of which the time of bioremediation increases.
- Biodegradation of inorganic pollutants does not take place.
- Pretreatment of volatile contaminants must be done to avoid air pollution.
- While tilling, regulation of dust particles in the environment should be taken care of.
- Construction of runoff facility for collection must be done.

The above limitations make land farming technology for bioremediation long drawn out than the other ex situ bioremediation technologies. This technology is

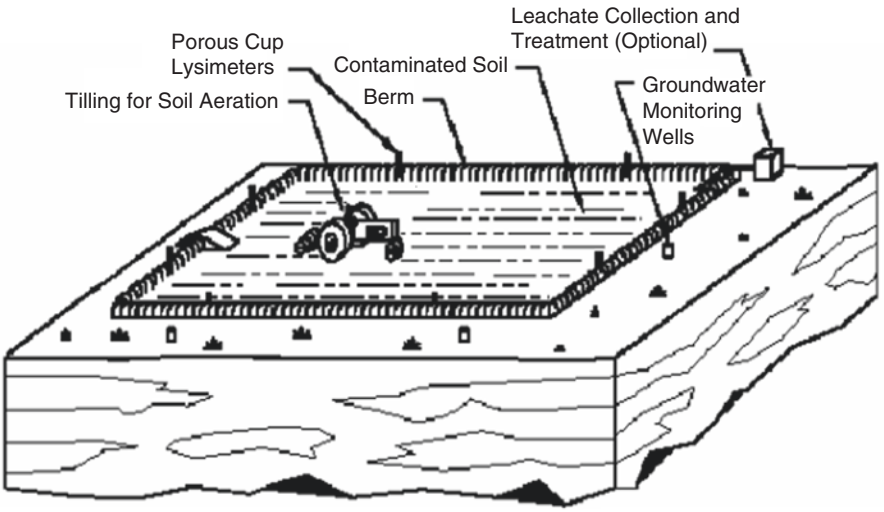

Fig. 22.1 Land farming technique. (Source: United States Environment Protection Agency 2004)

used on a large scale and has been applied to different kinds of wastes, largely for degrading oily sludge and various petroleum refinery wastes (Azubuike et al. 2016).

22.3.2 Biopiles

Biopiles are piles of the contaminated soil mixed with water and nutrients, and the microbial communities are stimulated via aeration. Microbial activity is inversely proportional to the concentration of heavy fraction hydrocarbon (HFH). Biopiles are focused at decreasing the hydrocarbon concentration which is present in polluted soil through the process of biodegradation. Biopile technology is used most commonly to remediate petroleum hydrocarbons, especially soils rich in sandy granules (Iturbe and López 2015). The components of this technology are aeration, irrigation, nutrient and leachate collection systems, and a treatment bed. The useful functions and cost-effectiveness of this technology make it more frequently used, which facilitates efficient biodegradation in the situation that nutrient, temperature, and aeration are properly governed (Whelan et al. 2015).This technology works effectively in remediating pollutants in extreme environmental conditions such as very cold climate (Dias et al. 2015; Gomez and Sartaj 2014; Whelan et al. 2015). The time for treating the polluted site can be decreased because of the adjustability of this technology because the thermal system can be combined with biopile design to make microbial activities and availability of the contaminate rise. Hence, the rate of biodegradation gets increased (Aislabie et al. 2006) (Fig. 22.2).

Moreover, biopile can be injected with the heated warm air which helps in facilitating intensified bioremediation. The study of Sanscartier et al. (2009) stated that biopiles that are humid in nature have very low concentration of final total petro-

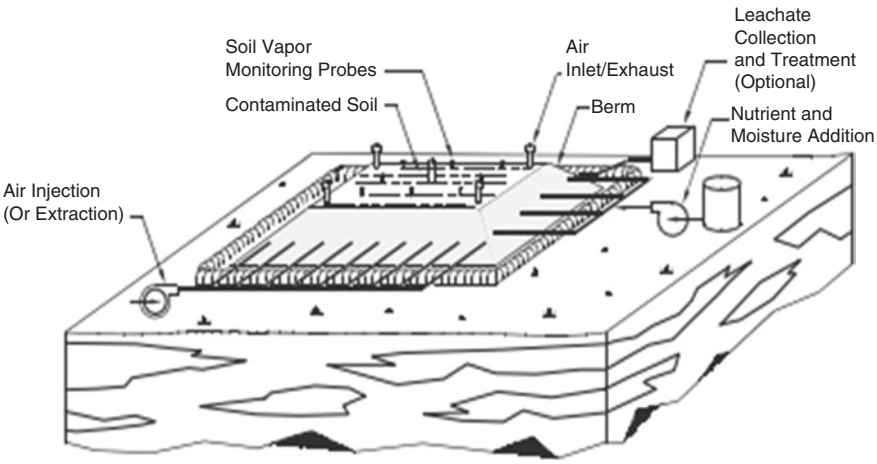

Fig. 22.2 Biopile system. (Source: United States Environment Protection Agency 2004)

leum hydrocarbon compared to biopiles that are heated and are passive in nature because the conditions such as optimal moisture content, decreased leaching, and minimum volatilization of less degradable pollutants prevail. Also, it was reported that because of biopiles, a large volume of contaminated soil can be remediated in a restricted amount of space. The format of biopile arrangement can comfortably be scaled up to an experimental setup to achieve identical performance obtained while performing laboratory studies (Chemlal et al. 2013). To have considerable efficiency of biopile sieving and aeration of polluted soil is important before processing (Delille et al. 2008). Bulking agents like straw, saw dust, bark, and other organic matters have been included to amplify remediation process in a biopile setup (Rodríguez-Rodríguez et al. 2010). However, biopile technology saves space as compared to other ex situ bioremediation techniques, consisting of land farming, cost of maintenance and procedure, and lack of power supply in remote areas, which otherwise enables constant allocation of air in polluted piles of soil though air pumps are some limitations of the biopiles. Moreover, large amount of heating of air can make the soil dry which undergoes bioremediation, which further results in restraining microbial activities, and volatilization is promoted instead of biodegradation (Sanscartier et al. 2009).

22.3.3 Windrow

A defined or shaped pile is made out of the polluted soil, and that pile gets aeration by regular turning in a defined time period of the windrows by specific machinery which can be self-propelled or mounted excavator. As one of the ex situ bioremediation technologies, windrows depend upon periodic turning of piled contaminated soil to increase remediation by enhancing degradation activities of indigenous and transient hydrocarbonoclastic bacteria that are present in polluted soil. The turning of polluted soil in defined time interval, accompanied by the adding up of water, results in an enhanced aeration, ordered distribution of contaminants, and degrading actions of microbes and nutrients. Hence, rate of bioremediation gets enhanced, which is achieved through assimilation, biotransformation, and mineralization (Barr 2002). Comparison was made between windrow technology and biopile technology, and in the results, an enhanced hydrocarbon removal rate was observed. However, the enhanced efficiency of the hydrocarbon removal by the windrow technology was due to the soil type that was said to be powdery in nature (Coulon et al. 2010). Windrow is usually the most cost-effective method out of all the ex situ bioremediation technologies. However, windrow technology cannot be regarded as the best technology to be used for bioremediation of contaminated soil with volatiles because of being related to the periodic treatment. Utilization of windrow technology has been involved with the release of methane because the anaerobic zone id developed within piled contaminated soil, which generally occurs after the decreased aeration (Hobson et al. 2005).

22.3.4 Bioreactor

Bioreactor is a container in which raw materials are transformed into specific products followed by series of biological reactions. Various modes of operating bioreactor are as follows: batch, fed-batch, sequencing batch, continuous, and multistage. Operating mode is chosen depending majorly upon the expenditure cost and the economy of the market. Contaminated soils and groundwater are treated in a defined and controlled manner with the help of bioreactor technology. Two types of bioreactors are compost-based reactors and slurry-based reactors. Compost-based reactors are a controlled in-vessel biological approach by which biodegradable hazardous materials are converted to non-harmful and stable byproducts by the usage of microorganisms under raised temperature. These bioreactors are of two common types: plug flow reactors (vertical and horizontal) and agitated-bed reactors. The raised temperature, typically 120–160 degree Fahrenheit, is because of the released heat by microorganisms during the degradation of the organic matter. Aerobic composting causes degradation of the sewage sludge, whereas anaerobic processes usually facilitate treatment of hazardous waste. This method of composting polluted soil makes use of a bulking agent, because of which porosity of the media gets increased. The use of conveyors must be done for the transportation of material in compost-based reactor system. Efficiency depends on moisture content of contaminated soil, pH, oxygen content, temperature, and C:N ratio (Cookson et al. 1995, EPA). Slurry-phase biological treatment is carried out in a slurry-based reactor for treating a mixture of water and excavated polluted soil. Polluted soil and water are intermixed in a concentration, determined by contaminants' proportion in soils, rate of biodegradation, and physical nature of the soils. In case of prewashed soils, the contaminated fine particles and washed water are treated in the reactor. Between 5% and 40% solids (by weight) are contained in slurry which depends on the nature of the biological reactor. The soil is mixed with nutrients and oxygen in the reactor (Fig. 22.3).

Any of the three among acids, microorganisms, and alkali is added as per the requirements of the treatment. On completion of biodegradation, dewatering of soil slurry takes place (Cookson et al. 1995; EPA; Alexander 1994). Conditions in a bioreactor reinforce natural process of cells by resembling and controlling their natural environment to maintain regular conditions for growth. Contaminated samples can either be dry matter or slurry for remediation in a bioreactor; in any of the two cases, bioreactor used for remediation of contaminated soil has various pros compared to any other ex situ bioremediation technologies. Superior control on parameters of bioprocesses such as temperature, pH, agitation, aeration rates, etc. is one of the main advantages of bioreactor-based bioremediation. Because of the capability to maintain and regulate parameters of the process in a bioreactor, biological reactions within the vessel can be increased to efficiently decrease the time of bioremediation. Majorly, controlled bioaugmentation, nutrient addition, increased bioavailability of pollutant, and mass transfer are some of the limiting factors of bioremediation process, and they can be maintained in a bioreactor which makes

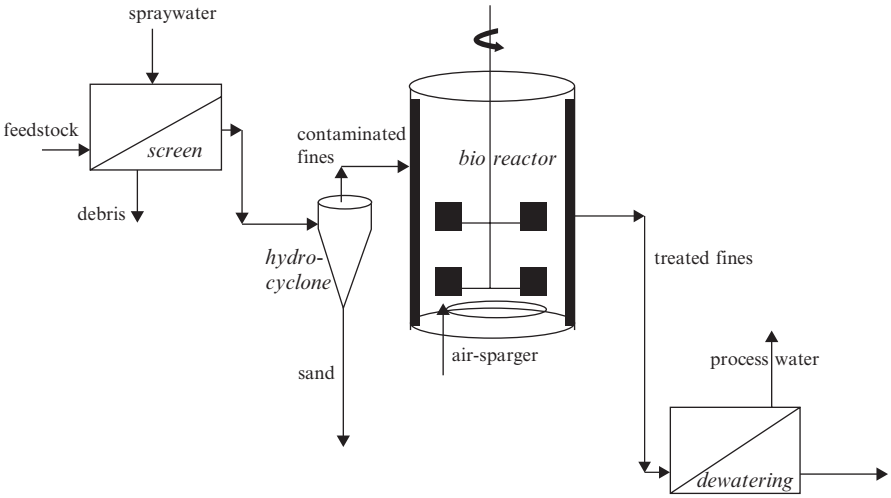

Fig. 22.3 Typical slurry bioreactor. (Source: Kleinjntnens and Luyben 2000)

bioreactor-based bioremediation more efficient than other technologies. Bioreactors are used to remediate soil/water contaminated with hydrocarbons. For bioremediation, the use of various bioreactors has resulted in removing pollutants in a wide range. The bioreactor designs are flexible in nature as they allow maximum biodegradation while reducing abiotic losses (Mohan et al. 2004). Long- or short-term working of bioreactor carrying crude oil-contaminated soil slurry allows tracking of differences in dynamics of microbial population which simplifies characterization of core bacterial communities indulged in process of bioremediation (Chikere et al. 2012; Zangi-Kotler et al. 2015). Also, because bioreactor is a system which is enclosed, genetically modified microorganism (GEM) can be used for bioaugmentation after which the destruction of organism can be done before returning the treated soil for the purpose of landfilling. This process restricts foreign gene to move into the environment after bioremediation. For the most effective use of bioremediation process, understanding of microbiological processes holds a really important position (Piskonen et al. 2005). Moreover, full-scale practice of bioreactor-based bioremediation is not commonly done because of a few reasons such as the following: bioreactor is an ex situ technique; therefore, contaminated soil or other substances that needs to be remediated can be too much in volume, requiring increased manpower, cost, and safety requirements for transferring contaminant to the site of the treatment, hence making this technology cost-ineffective (Philp and Atlas 2005). Also, because of the number of parameters of bioprocesses, a parameter which is not regularly controlled or properly maintained can become a limiting factor, which decreases microbial activities and thus decreases effectiveness of bioreactor-based bioremediation. Moreover, different pollutants react differently to different bioreactors; availability of the most appropriate design is of utmost significance. Above all, bioreactor suitable for a bioremediation is capitally intensive (Azubuike et al. 2016).

22.4 In Situ Bioremediation Technologies

In situ bioremediation technologies treat contaminants at the site of pollution. It does not demand any excavation; therefore, almost no disturbance is associated to the site soil structure. According to Brown and Crosbie (1994), the most notable petroleum hydrocarbon pollutant load is contained by unsaturated and remaining saturated soils. Such soils result in being a continuous source of groundwater pollution, if they are left untreated. When excavation of soil is prohibitively expensive or difficult, as might be the case with deep subsurface contamination (such as leaking underground storage tanks) or sites that are in close proximity to structures, in situ bioremediation strategies are likely a more effective strategy for remediation. An important primary step for in situ bioremediation is the assessment of conditions of the site to be remediated, the bioavailability of the pollutants, and the evaluation of limiting components that needs to be modified during the process of remediation (Menendez et al. 2007). In situ remediation includes technologies such as natural attenuation, bioventing, biosparging, bioslurping, and phytoremediation. Because of the lack of cost associated with excavation and transportation, in situ bioremediation technology is less expensive. However, this remediation technology is less controllable and less effective in nature (Koning et al. 2000). For achieving a successful in situ bioremediation of the contaminated site, significant environmental conditions, such as the electron acceptor, moisture content, availability of nutrients, pH, and temperature, should be suitably present (Philp and Atlas 2005). Ex situ bioremediation technology is not affected by the soil porosity, whereas it plays a very important role in remediation of contaminated site by in situ bioremediation technologies (Azubuike et al. 2016).

22.4.1 Natural Attenuation

To clean contaminated soil or polluted groundwater, natural processes are used, and this method is known as natural attenuation. According to the USEPA (1995), natural attenuation is "the use of natural processes to contain the spread of the contamination and reduce the concentration and amount of pollutants at contaminated sites." It is also termed as intrinsic remediation, bioattenuation, and intrinsic bioremediation. In this, the pollutants are left at the site, and then to clean up the site, naturally occurring processes are also left at the same place. In the natural processes, biological degradation, volatilization, dispersion, dilution, radioactive decay, and sorption of the pollutant onto the organic matter and clay minerals in the soil takes place. Most significantly, it remediates the aquifer when the polluting source is removed or even when the source is still present and after removal of hot spots as well. Although natural attenuation can be applied to a lot of sites, it is hardly applied as a single remediation process because it is a non-engineered biodegradation process, making it time-consuming (Mulligan 2001). For preventing risk to the environment, long-term monitoring becomes really essential in this technique (Catherine et al. 2004).

The technology depends on both microbial aerobic and anaerobic processes for bioremediating hydrocarbon contaminants. Natural attenuation costs less if compared to other in situ techniques because no external force is required for it. But the process should be regularly be monitored to make sure that bioremediation is taking place. According to the United States National Research Council (US NRC), three things that are important for natural attenuation are as follows: conformation of pollutant loss from the remediated site, substantiation established from laboratory analyses that isolated microorganisms from polluted sites which have inherent capability to degrade or convert pollutants of the polluted site, and confirmation of understanding of biodegradation capabilities at the site (Philp and Atlas 2005). In accordance with these criteria, isolated bacteria which degrade hydrocarbon from refinery oil-contaminated soil and presented the biodegradation capabilities of those isolated bacteria by making them grow on a medium of mineral salt with saturated and unsaturated hydrocarbon substrates as the only source of carbon and by their potentials to decrease concentration of hydrocarbons. Moreover, biodegradation is indicated as the major process for removing contaminant during natural attenuation. The main limitation of natural attenuation is that it takes a longer period of time to reach a particular level of contaminant concentration because of the absence of an external force to accelerate the process. Therefore, before application of natural attenuation, risk assessment is needed to make sure that the time taken for bioremediation is less than the time set forth for contaminant to reach exposure point corresponding to the nearest populations. Furthermore, it was investigated that natural attenuation does not sufficiently remove polyaromatic hydrocarbon and reduction in contaminated soil eco-toxicity (García-Delgado et al. 2015).

22.4.2 Phytoremediation

Phytoremediation is using living green plants directly for in situ biodegradation of pollutants in soils, surface water, and groundwater (UNEP). Ex situ bioremediation treatment is prohibited by huge amount of contaminated soil because of economic factors, and therefore, comparative less expensive in situ bioremediation technique such as phytoremediation is used (Kamath et al. 2004). There are a lot of advantages associated with phytoremediation. They are cost-effectiveness, aesthetic benefits, and long-term pertinence. Also, phytoremediation can be used as a refining in situ technology that helps in minimizing land irritation and excludes transportation and liability expenses required in case of ex situ technologies and disposal. Processes such as physical, chemical, and biological are used by phytoremediation for removing, degrading, converting, or stabilizing pollutants present in soil and water. For selecting a plant to be used as a phytoremediator, certain parameters are examined such as root system of the plant, which is fibrous root plant or tap root plant depending on contaminant depth; biomass of the plant above the ground, which should be absent for consumption by the animals; contaminant toxicity to the plant; survival and adaptability of the plant in existing conditions; growth rate; and time needed to reach the required level of degradation of contaminants. Moreover, resistance of

plant toward diseases and pests is equally important (Lee 2013). Plants can be used in a lot of ways for remediating pollution. Plants behave as filters to eliminate contaminants from soil or water; plants break down organic contaminants or suppress inorganic pollutants. For a successful process of phytoremediation at any polluted site, all the mechanisms work simultaneously. Phytoremediation is a cost-effective technology which is operated by solar energy and results in a natural cleaning of the environment; all together, sites with low levels of hydrocarbon contamination are significantly benefitted by this technique. In fact, phytoremediation is useful for the treatment of a large variation of pollutants. Several natural processes are put to use by phytoremediation technique for the cleanup of contaminants present in soil/water. Removal of pollutants is encouraged by various mechanisms of a plant; it includes uptake of contaminants then their concentration, transformation, and stabilization and also rhizosphere degradation; here, the bacterial growth is facilitated by the plant beneath the ground where contaminants break down. Phytoremediation can be applied in both ways, either in situ or ex situ. It is responsive to a large number of pollutants. Soil disturbance is decreased by the application of technique on site, and chances of spreading the pollutant also reduce, waste for land filling gets decreased significantly, and it is cost-effective in nature. Moreover, it abolishes the use of equipments and is a simple technique that does not require maintenance or any specialized labor and has high aesthetical value (Etim 2012) (Fig. 22.4).

The six types of methods used in phytoremediation are:

- Phytodegradation: pollutants are taken up, stored, and degraded in the tissue of the plant.
- Phytostimulation or rhizodegradation: for degrading the pollutant, accessible soil microbes and plant use their rhizospheric associations.
- Phytovolatilization: plants take up the pollutants, convert them into nontoxic volatile material, and release it into the atmosphere.
- Phytoextraction: plants transport pollutants from the contaminated site through roots and accumulates them in the shoots.
- Rhizofiltration: roots of the plants are used to decontaminate soil/water via absorption or adsorption.
- Phytostabilization: bioavailability of the pollutant gets reduced because contaminants get bound by the plants.

22.4.3 Bioventing

Bioventing is a process in which oxygen is provided to the microorganisms present in the soil to stimulate the natural biodegradation of the pollutants present in the soil. To supply only enough amount of oxygen, low air flow rates are used which encourages the microbial activity in unsaturated zone. Generally, oxygen is provided through direct air injection into remaining pollution in the contaminated soil. Furthermore, compounds that are volatile in nature are degraded as vapors moving at a slow pace through soils which are biologically active (US Air Force Center for

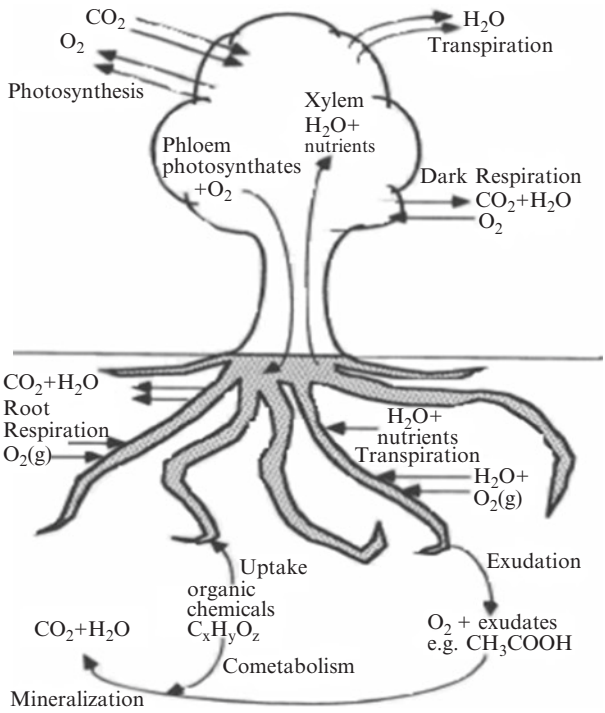

Fig. 22.4 Illustration of phytoremediation. (Source: Schnoor 2004)

Environmental Excellence) (Hinchee 1993). Factors limiting the applicability and effectiveness of the technique are as follows: (1) permeability of the soil, low permeability of soil reduces bioventing; (2) air around structure, air should not be present near the area of concern because its presence builds vapors in the influence radius of wells injecting air; (3) supervision of the gases released at the surface of the soil is needed; (4) aerobic biodegradation is generally not successful for a lot of chlorinated compounds; and (5) soil moisture content, if low (due to bioventing), it hinders biodegradation (Office of Research and Development, EPA) (Fig. 22.5).

Large variety of petrochemical compounds gets reduced due to bioventing, including fuel oil, bitumen, and gasoline. These hydrocarbons together are measured as total petroleum hydrocarbons (TPHs). Moreover, usually within 1 year, bioventing significantly reduces these compounds below detection level into a subset called as BTEX (benzene, toluene, ethylbenzene, and xylenes). Among all the petroleum hydrocarbons, BTEX compounds are most soluble, mobile, and toxic in nature (US EPA 1995a). Bioventing requires regular monitoring, where system performance is assessed by supervisors, air flow is adjusted which refines air environment, determination of the correction situation is done for an operation to conclude, and then the results are evaluated. Detailed and careful account of degradation progress may result in decreased expenses (Hellekson 1999). Nutrients and moisture are added as modification to stimulate bioremediation to obtain the result as microbial transfor-

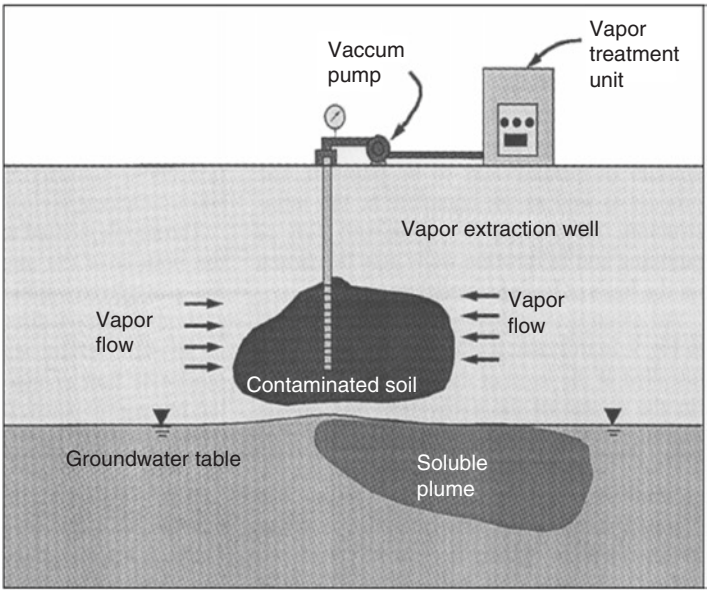

Fig. 22.5 Illustration of bioventing system. (Source: Held and Dörr 2000)

mation of contaminants into a nontoxic form (Philp and Atlas 2005). Bioventing is especially used for restoration of the sites contaminated with light splatter of petroleum hydrocarbons (Höhener and Ponsin 2014). High rate of airflow is of no use to enhance biodegradation rate or for significant biotransformation of the contaminants. This is because for the demand of oxygen, the saturation of air by different air injecting rates takes place early in the subsurface during biodegradation. However, low air injection rate causes a considerable rise in biodegradation of the pollutant. Therefore, it shows that in bioventing, air injection rate is one of the primary factors for dispersal of contaminants, redistribution, and surface loss. Frutos et al. (2010) reported the significance of bioventing technology for remediating soil polluted by phenanthrene and observed pollutant removal of more than 93% after a time period of 7 months. In removing diesel impurity from clayey soil, airflow intensities and intervals derived significant difference, which shows that air injection for a longer interval of time and low rate of air injection can be cost-effective for bioventing in diesel-contaminated clayey soil (Thomé et al. 2014). Apparently, airflow rates and air intervals are the basic factors for bioventing, but the effectiveness of bioremediation based on bioventing depends upon the total number of air injection points, which makes distribution of the air uniform. Regardless of the fact that aeration of vadose zone is promoted by the technique of bioventing, the process of anaerobic bioremediation can be done specifically for the remediation of vadose zone contaminated by chlorinated compounds (recalcitrant under aerobic conditions). For the latter, mixture of nitrogen and low concentration of oxygen and hydrocarbon can be used instead of air/oxygen for chlorinated vapors to get reduced, where hydrogen plays the part of an electron

donor (Mihopoulos et al. 2000, 2002; Shah et al. 2001). Input of pure oxygen can result in presence of higher proportion of oxygen as compared to air injection, in a low permeability soil. Moreover, for increasing the rate of biodegradation in case of recalcitrant compound, for their partial oxidation, ozonation can be used (Philp and Atlas 2005). In in situ bioremediation techniques, attaining same results as that of laboratory studies cannot always be achievable because of different environmental conditions and various characteristics of the vadose zone in which air is injected; therefore, in this technique of bioventing, time taken can be increased. Because of greater rate of airflow, volatile organic compounds get transferred to the soil vapor phase, for which off-gas treatments of the obtained gases are done before releasing them into the atmosphere (Burgess et al. 2001). By combining bioventing technique and biotrickling filter technique to decrease both pollutant and outlet gas emission levels, particularly this issue can be solved. Therefore, decreasing the extended amount of time period related to bioventing (Magalhães et al. 2009).

22.4.4 Biosparging

In biosparging, the atmospheric air is injected into the aquifer or saturated zone to increase the biological activity of the microorganism. The in situ bioremediation technique is used for both saturated and unsaturated zones. This technology was formed to decrease the utilization of energy. Small channels for the air to move to the unsaturated zone of soil are formed by injecting air into aquifers. Air is pulsated into the soil for forming the required number of branches in these channels. Because of biosparging, volatile pollutants get transferred to the unsaturated soil zone; as a result, to extract the volatile vapors, soil vapor extraction is generally used, and then they are treated at the surface (Held and Dörr 2000). When the sparge points are located beneath the pollution zone, the biosparging becomes effective because air has a tendency to flow in upward direction. This flow of air in upward direction forms an influence cone, and the amount of air pressure during the injection decides the degree of branching and angle of the cone. For each sparge point, the pilot test determines the degree of influence. To measure the zone of influence for the sparge point, monitoring wells are installed surrounding the point, and then level of groundwater and dissolved oxygen content are determined. For removing pollutants effectively from the soil using in situ technique of biosparging, the soil should be proportionately homogeneous throughout the polluted zone (Held and Dörr 2000).

22.4.5 Bioslurping

In bioslurping technique, anaerobic biodegradation of hydrocarbon contaminants takes place, while elements of bioventing and vacuum-enhanced pumping are combined for extraction of free product from the groundwater and soil (CPEO 1998;

FRTR 1999). Removal of free product done by vacuum recovery along with groundwater and vapor extraction results in eliminating highly volatile vapors from the vadose zone; in both the vadose zone and the capillary fringe, biodegradation is stimulated by bioventing (GWRTAC 1996; Midwest Research Institute 1998; Yen et al. 2003). Slurp tube is well installed in bioslurping system whose length can be adjusted according to the requirement. After being attached to a vacuum pump, the slurp tube is moved down inside the light nonaqueous phase liquid (LNAPL) layer for removing free products and some quantity of groundwater as well. Process of vapor extraction takes place when the slurp tube starts extraction vapors because pumping results in decreased levels of LNAPL layers. The mixture of product and groundwater, liquid in nature, is withdrawn from the slurp tube and then moved to a water/oil separator and the vapors to a liquid/vapor separator (GWRTAC 1996; Cresap 1999). Moreover, the aeration of the unsaturated zone takes place because of the process of extraction taking place through slurp tube; the rate of aerobic degradation and amount of oxygen get enhanced because of this (GWRTAC 1996; FRTR 1999; RAAG 2000). Bioslurping, specifically used for addressing floating light nonaqueous phase liquid layers, is commonly utilized for the sites having overloaded compounds having size of grains between fine and medium; also, it is eminently utilized for the sites having material of grain size between medium and coarse (GWRTAC 1996). Sites having shallow groundwater or groundwater below 30 m can also make an efficient use of the technique (Midwest Research Institute 1998; Cresap 1999; Yen et al. 2003). Some important factors regarding the procedure of bioslurping technology are:

- By extracting the free product, bioslurping enhances remediation process (Midwest Research Institute 1998; FRTR 1999; Yen et al. 2003).
- Aquifer smearing gets reduced by this technique. In an aquifer, LNAPL travels deeper into the aquifer when water table is lowered. Because of smearing the vertical height of pollutant is increased as a result of the pollutant interaction with saturated soils (GWRTAC 1996; Cresap 1999).
- Natural in situ bioremediation process of the vadose zone soil gets increased by bioslurping (Midwest Research Institute 1998).
- Excess of moisture content in soil decreases the air permeability of it and reduces its capability of transferring oxygen; microbial activity is restrained when moisture content is very less.
- Bioremediation process becomes slow in speed at the sites having low temperature.
- Before discharging, treatment of extracted groundwater and emissions from the bioslurper is needed (CPEO 1998).

While comparing to other bioremediation systems, bioslurping is regarded as the most cost-effective technique. The cost associated with the method gets decreased because bioslurping extracts soil-gas even at the concentrations that are below regulatory limits resulting in decreasing the cost associated with storage and disposal (GWRTAC 1996; FRTR 1999; Cresap 1999).

22.5 Bioaugmentation

Bioaugmentation is a technology that enhances the biodegrading potential of polluted sites by introducing single strains or an association of microorganisms having required catalytic abilities. Furthermore, genetically engineered microorganisms (GEM) showcasing increased degrading abilities comprise a large variety of aromatic hydrocarbons also having potential for soil bioaugmentation. Bioaugmentation approach is assumed to be applied after the failure of biostimulation and bioattenuation techniques (Vogel 1996; Iwamoto and Nasu 2001; El Fantroussi and Agathos 2005). According to Forsyth et al. (1995), for application of bioaugmentation, soils should (1) have low or non-detectable amount of microbes that can degrade pollutants, (2) have compounds needing bioremediation by multiple processes, and (3) be where which expenses of non-biological methods supersede expenses of bioaugmentation. Furthermore, microorganisms should be introduced in the soil where areas contaminated by materials need long period of time for adjustment. Bioaugmentation of soils contaminated by aromatic hydrocarbons was reviewed, and the technology was proven beneficial in remediation of sites polluted by aromatic hydrocarbons but also suffer from a lot of problem to the environment. Most of the problems were faced by the strains that need survival in the soil. After soil inoculation, reduction in the amount of exogenous microorganisms was seen. Researches show that the ability of bioaugmentation gets affected by both biotic and abiotic factors (Cho et al. 2000; Bento et al. 2005; Wolski et al. 2006). Abiotic factors such as temperature, moisture, pH, and organic matter play an important role. Also, aeration, nutrient content, and type of soil affect the effectiveness of bioaugmentation (Agnieszka and Zofia Piotrowska-Seget 2010). It can be concluded that the microbial populations present at the site might not be able to remediate various kinds of significant compounds that exist in products such as petroleum. Factors for deciding the usage of bioaugmentation are when the hydrocarbons for bioremediation of the contaminated site are less in amount, when the time period taken for remediation is the basic factor, and when seeding decreases the lag period for initiating remediation (Forsyth et al. 1995). Bioaugmentation can be efficient at the site if the seed microorganisms are able to degrade major amount of petroleum hydrocarbons, when genetic stability and viability while storing are maintained, sustaining in foreign and unfavorable environmental conditions, efficiently surviving along with the microorganisms already present at the site, and sliding through the sedimentary pores to the pollutants. In bioremediation, study of microbes makes the choice of microorganisms simplified that have the ability to degrade and produce compounds that can be implemented for biotechnology in the oil/petrochemical industries. Effectiveness of bioaugmentation technology relies upon the utilization of microbial strains as inoculums or consortia of microbes that have already been adjusted at the polluted site. Microorganisms in inoculum, foreign in nature and are successfully put in an application, have their effectiveness dependent upon the capability to be a competitor with native microorganisms, predators, and several abiotic components. Factors influencing the rapid growth of microorganisms utilized for

the process of bioaugmentation are the chemical structure and concentration of contaminants, the amount of the pollutant available to the microorganisms, the proportion and nature of community of microorganisms, and the kind of physical environment present. All the above factors are considered before selecting microorganisms to carry out the process. This technique of bioaugmentation is basically an implementation of microorganisms that are isolated from a polluted site or attentively chosen and modified genetically to carry out degradation of sites polluted by petroleum hydrocarbons after assuming or confirming that native microorganisms present at the contaminated site are unable to degrade the petroleum hydrocarbons.

22.6 Biostimulation

For increasing the number of native microorganisms, nutrients are added externally to a contaminated site, and this process is known as biostimulation. Hydrocarbon pollution at a site creates a speedy decrease in the available amount of main inorganic nutrients, such as nitrogen and phosphorous. Therefore, to degrade hydrocarbons, nutrient supplementing usually emphasizes on introduction of nitrogen and phosphorous in any of the organic or inorganic form, usually calculated from C/N ratios (Sarkar et al. 2005; Sang-Hwan et al. 2007). The aim of this technique is to enhance the actions of native microbes which are responsible for degrading hydrocarbon contaminant from the affected soil site; it is generally used for the treatment of oil-contaminated soil. Fertilization, a method for enrichment of nutrients, is a bioremediation technique where fertilizers containing phosphorus and nitrogen that are used for crops during farming are added to polluted soil site for stimulating the growth and actions of native microbes which degrade the hydrocarbon contaminants (Thieman and Palladino 2009). Plenty of crucial elements like phosphorous, oxygen, nitrogen, carbon, and hydrogen are needed by microbes to build macromolecules; these microorganisms are provided with essential elements by the introduction of fertilizer to replicate and flourish. At times, products like wood chips, straw, and manure give microorganisms the needed source of fertilizer containing carbon. Biostimulation process works by the introduction of more nutrients that enables microorganisms to reproduce and enhance in number and growth, therefore enhancing the biodegradation (Thieman and Palladino 2009). Added inorganic nutrients behave as a fertilizer for stimulating biodegradation by native microbes at few sites; but in other cases, it is an intended stimulation of native bacteria that degrades xenobiotic compounds by making use of electron acceptors, addition of nutrients, electron donors, or water (Widada et al. 2002). Better results are obtained when inorganic nutrients are used in a combination rather than using one nutrient at a time (Sutherland et al. 2000). Experiments done by Liebeg and Cutright (1999) in lab showcased that for stimulating the activities of native microorganisms, macronutrients at a low level and micronutrients at a high level were needed. Solution of 75% sulfur, 3% nitrogen, and 11% phosphorus resulted in the largest amount of stimulation. Adding carbon as a source of nutrient at polluted site increases the rate of degradation because it is

responsible for biodegradation of the contaminant by stimulating the microbe action. Adding carbon in form of pyruvate encourages the growth of microorganisms and increases the rate of degrading polycyclic aromatic hydrocarbons (Lee et al. 2003). Use of composting as a bioremediation technique is a form of biostimulation. Composting depends upon mixing of main components of compost and polluted soil, so that when the compost is developed and matured, the contaminants get degraded by the active bacteria present in the mixture (Semple et al. 2001). Spent mushroom compost and mushroom compost are used for the degradation of organic pollutants at the affected sites (Eggen 1999; Trejo-Hernandez et al. 2001). Spent mushroom compost increases the degrading efficiency of polycyclic aromatic hydrocarbons by 82%. Spent mushroom compost when added to affected site decreases the toxicity, added enzymes and nutrients of the microorganisms responsible for degrading polycyclic aromatic hydrocarbons (Lau et al. 2003). Organic wastes like banana peel, spent mushroom compost, and brewery spent grain increase the degradation of used lubricating oil up to 90% loss within a period of 3 months (Abioye et al. 2009, 2010). Also, melon shell has an ability to stimulate degradation of crude oil in polluted soil by 75% within a period of 28 days (Abioye et al. 2009). Nature of the polluted soil results in these nutrients becoming limited or not; thus, adding of nutrients is important for enhancing biodegradation (Kim et al. 2005). In sub-Antarctic intertidal sediments for a period of 1 year, efficacy of fertilizers for crude oil remediation were evaluated by Pelletier et al. (2004), and it was observed that chemical, microbial, and toxicological parameters indicated the efficiency of several fertilizers in a pristine environment. Noticed that commercial oleophilic fertilizers having nitrogen and phosphorus when added to site polluted by hydrocarbons enhanced the population of microbes that degrades hydrocarbon and the total petroleum hydrocarbon degradation, and a loss of 77–95% of alkanes and 80% of polycyclic aromatic hydrocarbons in polluted soil within a period of 180 days was reported. Another study reports that, in the presence of poultry manure as organic fertilizer, the degradation of polluted soil enhanced, but the extent of degradation was affected by incorporating a different substrate of carbon (Okolo et al. 2005). But, degradation activity is hindered by extra amount of nutrient concentration, and reports by various authors show that high levels of nitrogen, phosphorous, and potassium have a negative impact on the degradation of hydrocarbons (Oudot et al. 1998) and specifically on the aromatic hydrocarbons (Carmichael and Pfaender 1997).

22.7 Future Aspects

Remediation of water and soil contaminated by hydrocarbon is necessary to live a healthy life. Bioremediation of hydrocarbon-contaminated soil and water is better and an environment-friendly technique if it is effectively monitored and then applied. Bioremediation technology has an edge over other treatment techniques because of several reasons. Despite this advantage, the capability of the technology

still needs to be fully explored and put to use for restoring the surrounding environment. It might be because of a reason that the techniques take much more time to completely remediate the contaminated site than other methods of treatments that are available. Though, to overcome this limitation, addition of required nutrients and microbes having the ability to degrade hydrocarbon contamination can be done. Research and development in the upcoming future needs to emphasize on using nutrients that are low-priced, environment-friendly, and largely available and also have the ability to enhance the activity of microorganism for the treatment of hydrocarbons.

22.8 Conclusion

Bioremediation itself is the most novel and cost-effective technology as compared to the other available techniques for hydrocarbon remediation. For its efficient and feasible use, correct site selection is the primary step that needs to take place. Ex situ bioremediation technologies are largely expensive than the in situ technologies because of the additional expenses related to excavation. The monetary value of installation of equipments on the site of remediation and measures taken for controlling subsurface add on to the difficulties and expenses for technologies of in situ bioremediation. Apparently, the amount of money required for bioremediation should not be the only criteria for selecting a technology; a lot of characteristics such as soil type, pollution depth, relative site location, etc. need to be analyzed before deciding the most novel and cost-effective bioremediation technology for particular hydrocarbon contamination.

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Chapter 23
Role of Filamentous Fungi to Remove Petroleum Hydrocarbons from the Environment

Ihsan Flayyih Hasan and AI-Jawhari

Abstract Excessive use of petroleum hydrocarbons is causing many problems in the ecosystem. Practically speaking, injudicious use and inappropriate discharge of all forms of hydrocarbons compounds are harmful for the ecosystem. On the other hand, hydrocarbon components like polyaromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) and their biodegradation products are known for their carcinogenic behavior. The reason of persistence of carbon-based compounds (petroleum hydrocarbons) for a long time in the ecosystem depends on many factors such as the physical factors, type of soil, type of microbes in that particular environment, water and sediment of that area, and above all the chemical nature of the petroleum hydrocarbon. The degradation rate of any hydrocarbon product depends upon the chemical nature of the compound, influence of physical factors (here temperature plays a significant role), and accessibility of hydrocarbon as carbon source for microbes, especially the extracellular enzymes secreted by the microbes. The hydrocarbon compounds released in the soil sediments are easy to degrade compared to the aquatic system; since the diversity of microbes in soil and sediment is more, therefore, released hydrocarbon compounds are easily degraded into simple and nontoxic components. Filamentous fungi are a very important biodegrader, owing to their greater biomass compared to bacterial cell. The fungi have more surface area for biosorption and enzyme secretion for efficient biodegradation of petroleum hydrocarbons. In addition to fungi, other organisms such as bacteria and algae have also been employed as an efficient hydrocarbon biodegrader. The main problem with petroleum hydrocarbon biodegradation is that owing to the recalcitrant nature of petrochemicals, the process is complicated, and it also takes a long time for mineralization. Environmental factors also determine the fate of petroleum hydrocarbons in aquatic and terrestrial ecosystem and also rely on several climatic conditions such as temperature, light, aerobic and anaerobic conditions, pH, wind, availability of nitrogen compounds, presence of humic acids, and salinity. There are several methods and approaches used all over the globe to remove or biodegrade the unwanted hydrocarbons using physical and chemical means, but these approaches are not efficient, and moreover

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they are not cost-effective. The use of biological means by applying potential microbes for bioremediation is an efficient, eco-friendly, and cost-effective tactic without addition of any unwanted load on the environment.

23.1 Introduction

Since last many decades, after the large-scale industrialization and accidental petrochemical product discharges, huge quantities of petroleum products polluted the aquatic and terrestrial ecosystem, and this spill resulted in enormous side effects on the quality of the soil, sediments, and water in affected parts of the world. Petroleum products are also significant sources of environmental contaminants on the beaches and near oil exploration and dump sites. Man can avoid such pollution, but most notably the aquatic flora and fauna have to suffer from this petrochemical-based pollution (Bobra et al. 1983); excessive discharge of hydrocarbon products on land also influences the plants and animals.

The soil fungi possess unique hydrocarbon decomposing capabilities to deal with a variety of innately occurring chemical components that perform as possible carbon source. The petroleum hydrocarbon contaminants bear comparable structure of molecules which enable efficient fungal strains to act upon them, and they degrade it using biosorption or enzyme excretion phenomenon. When a particular area is polluted, the capability to interact with carbon-based pollutants and convert them into less toxic or nontoxic substances and also obtain energy from that is selected for further studies; this efficient fungal strain may be further developed using genetic engineering techniques, or it may be mutated to perform much better under adverse climatic conditions (Fernandez Luqueno et al. 2010). Additionally, in another study conducted by Peng et al. (2008), they reported that the genes responsible for polyaromatic hydrocarbon biodegradation are located on the genome as many homologous loci. This genome provides chances of rearrangement of genes and also possibilities for mutation, which may enhance or decrease a particular trait in an organism. Additionally, the microbiome owing to their small genome has the potential to develop genetic and phenotypic variety to overcome the various biological and nonbiological stresses (Fernandez-Luqueno et al. 2010). In another interesting study conducted by Grishkan et al. (2003), under environmentally stressful condition, in some soil fungi, sexual reproduction was observed to increase. This swing or change in sexual reproduction also enhances the genetic diversity in soil fungi, and it can be ascertained at different stages.

In an aquatic ecological unit, fungi play a significant role in removing dangerous and perilous compounds. On the other hand, the soil and sediment particulate matter polluted with raw petroleum oil is one of the preferential ecological niches of fungi; here the fungi utilize the crude oil and use that carbon as an energy source and bioremediate the petroleum products (Al-Nasrawi 2012).

The chemical composition of petrochemical oil varies from sources to sources; therefore it is not possible to write here a precise chemical composition of crude oil in general. Commonly we can say that most of the petrochemical oil contains at

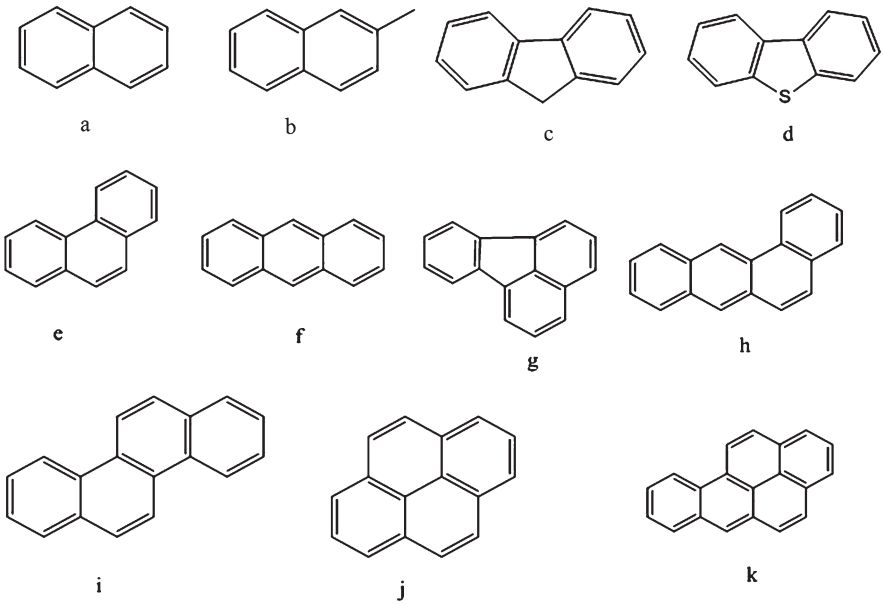

Fig. 23.1 Structure of commonly available PAHs
Structure of representation PAHs. (a) Naphthalene, (b) 2-methylnaphthalene, (c) fluorene, (d) dibenzothiophene, (e) phenanthrene, (f) anthracene, (g) fluroanthene, (h) benz[a]anthracene, (i) chrysene, (j) pyrene, (k) benzo[a]pyrene

least the following chemical groups: alkenes, alkynes, cycloalkanes, aromatics, and polyaromatics (Peterson 1994). However, the crude oil also contains some additional water, nitrogen, and sulfur-containing chemical compounds, besides small amount of metals like nickel and vanadium. The various chemical components of crude oil present in any environment could be measured by numerous modern techniques including silica gel chromatography (Atlas 1981) and solid phase microextraction (SPME) (Gomes et al. 2009).

GC/MS has also been employed, though this technique is time-consuming to realize the precise chemical composition of any petrochemical mixture (Brown et al. 1999). Besides bearing normal and less toxic components in crude oil, there are several chemical compounds which are of special interest owing to their carcinogenic properties, and these chemical components are the polyaromatic hydrocarbons (PAHs). The most commonly available PAHs are shown in Fig. 23.1 (Harayama 1997).

23.2 Petroleum Hydrocarbons and Environmental Toxicity

The environmental toxicity of petroleum hydrocarbon products depends on the chemical and physical properties of the carbon compounds. The fact is that the water-soluble petrochemical fraction is very harmful fraction for the environment,

since this water-soluble part is directly and easily available for uptake or adsorption by micro- and macroorganisms. The amount and concentration of any petrochemical product absorption depend upon the contact duration, chemical nature of petrochemical products, and type of organisms (surface type) (Peterson 1994). It has been observed that water-soluble fractions of the aromatics and polyaromatics of petrochemicals were reported to be toxic in nature and sometimes also act as carcinogenic agent (Keith and Telliard 1979). Specifically the polyaromatic hydrocarbon substances having four or five ring structures are notorious for their carcinogenic nature (Cerniglia 1992). But on the other hand, the nonaromatic petrochemical compounds are not supposed to be very toxic; this is because of their simple chemical structures (Peterson 1994). It has been reported that hydrocarbons having hydrophobic nature are quite harmful for common soil and water microbes such as bacteria, fungi, and algae, since these hydrophobic hydrocarbons got accumulated in the membrane structures and, therefore, result in membrane integrity loss (Sikkema et al. 1995). There are more fractions of petrochemical products which can lead to the noxious effects, and the poisonous aspects of polyaromatic hydrocarbons are studied in detail. Generally the polyaromatic hydrocarbons are considered to be carcinogens, since they lead to mutation in nucleic acids. The most common and hazardous polyaromatic hydrocarbons are benz(a)anthracene, dibenz(a,h) anthracene, and benzo(a)pyrene, because these hydrocarbon compounds seem to be the lesser bioavailable polyaromatic hydrocarbons. Generally, the 3% solubility results show low bioavailability. Therefore, owing to less solubility, it should be included in the risk evaluation (Shor et al. 2004). In studies related to toxic compounds, it is also imperative to take into account the probable noxious metabolites of polyaromatic hydrocarbons. The initial step in the biodegradation of polyaromatic hydrocarbons in eukaryotic organisms is establishment of trans-dihydrodiol compound. These trans-dihydrodiol compounds are in equal amount with trans-diol epoxide compounds (Flowers et al. 1997). During the course of reaction, dihydrodiol compounds are converted into catechols, which finally autooxidize to quinone compounds (McCoull et al. 1999). The lethal metabolic compounds add some additional molecules on DNA strand, which leads to the damage of DNA (Stowers and Anderson 1985). In case of animals, these additional molecules displayed very slow turnover, which leads to a buildup of these additional molecules or adducts. As a result, quinones and trans-diol epoxides are the reactive species formed, may lead to DNA scission, which prove lethal to organism.

23.3 Toxicity of Polyaromatic Hydrocarbons (PAHs)

PAHs are quite insoluble in water and are, therefore, difficult to biodegrade (Fellenberg 1990). Figure 23.2 shows the molecular structure of polyaromatic hydrocarbons (PAHs). These compounds cause many problems in the environment; some of PAHs also acts as carcinogenesis in the human body (Curfs et al. 2003).

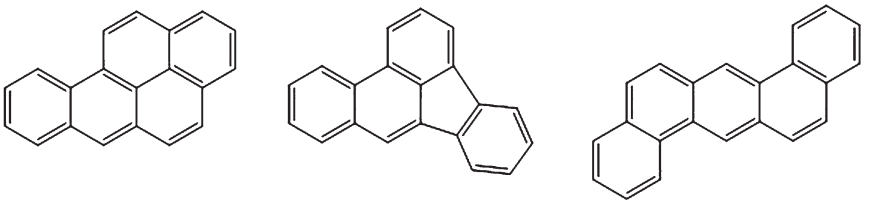

Fig. 23.2 Structure of various polycyclic aromatic hydrocarbons (PAHs)

There are several literatures that indicate the potential of microorganisms and plants to degree PAHs to regular cell metabolites (Trenk and Sandermann 1978, Trust et al. 1995; Bisht et al. 2014a,b; Alegbeleye et al. 2017). Also Al-Jawhari (2016) reported that fungal strains of *Aspergillus niger* and *Penicillium funiculosum* can effectively degrade anthracene to other compounds.

23.3.1 Fate of PAHs in the Environment

When PAHs and petroleum hydrocarbons reach the water or any aquatic phase, a very fast partitioning between the water, air, and sediment parts of the environment takes place (Knap 1982). The insoluble fraction forms a layer of 0.01 to 3.0 mm thickness on the water layer (Lichtenthaler et al. 1989). During the first few hours, some parts of PAHs evaporate, and other parts are absorbed in the sediment. When the hydrocarbon products are concentrated, at that time adequate nonaqueous phase liquids (NAPLs) can be materialized. The remaining hydrocarbons are present in the aqueous layer or as a thin film on the aquatic water surface. The lighter fractions of the petro-hydrocarbons are removed within 24 h by the process of evaporation. Studies show that the amount of oil that evaporates strongly depends on the type of oil. The evaporation of alkanes is possible until an 18 carbon chain (Knap 1982). The mass loss due to evaporation can range from 0.1% for heavier oils to 17.3% for lighter oils (Wang et al. 1998). Evaporation of lighter fractions stimulates biodegradation, because the lighter fractions are more toxic to degrading bacteria (Delille et al. 1998). After the partitioning the degradation starts in the different compartments.

23.4 Bioaccumulation

Polyaromatic hydrocarbons are recognized for their bioaccumulation in the living beings. PAHs accumulate in the cells and tissues of animals rich in lipids, and these organic compounds are especially observed in the fish liver and in the pancreas of other organisms. More hydrophilic PAHs are taken up by aquatic organisms from

the well-oxygenated water. The other uptake pathway for the more hydrophobic PAHs is by food and sediment. The food uptake pathway implies a buildup of polyaromatic hydrocarbons in the food chain. This accumulation of PAHs in food chain is of very interest for scientists, since the humans consume fish and other animals and also they are on the higher end of the food chain. In case of animals, the PAH uptake recorded a variation, and the uptake depends upon the season (Meador et al. 1995). The uptake of PAHs in plants has also been examined, and it was found that amount of PAH in plant parts is due to the result of a partitioning of PAH molecules between the two phases, the gas phase and absorbed phase. Owing to the seasonal variation, a great dependence of this gas phase and absorbed phase partitioning on temperature change has been observed (Simonich and Hites 1994).

23.5 Sorption in Sediments and Soil

Sediment absorption is an important aspect for degradation of hydrocarbons, because absorption makes the hydrocarbons in general less available for the process of degradation. Uptake and bioremediation of hydrocarbons by microorganisms were shown to be much slower from the sediment region compared to the situation, when the hydrocarbons are present in a solved state (Pignatello and Xing 1996). The absorption of carbon-based compounds depends on many factors. First of all, the composition of sediments is an important factor. Further the presence of other organic substances in the soil can have an influence. At last also the environmental condition as, for example, pH, salinity, and water temperature is very important in absorption. pH was found to have a minor influence, with a 6–9% reduction in absorption with an upsurge of one pH unit in the pH 6.5–8.5 domain (Meyers and Quinn 1973).

23.6 Role of Filamentous Fungi in Degradation Petroleum Hydrocarbons

Fungi are known to degrade hydrocarbons. They use another mechanism for degradation of hydrocarbons than bacteria, and they are, therefore, may be able to degrade the hydrocarbon compound left by the normally faster degrading bacteria. This can be useful for the five-ring PAHs, which are only poorly degraded by bacteria. Fungi secrete extracellular oxidizing enzymes for degradation of lignin (Field et al. 1992). These enzymes are able to make reactive peroxide from oxygen (Barr and Aust 1994), especially the white rot fungal strains are able to break down lignin. Lignin is a complex random molecule containing a lot of aromatic groups. For example, the fungal strain *Phanerochaete chrysosporium* could degrade PAHs (Bumpus et al. 1985; Bumpus 1989). Fungal species can produce a series of degradation-related

enzymes extracellularly, such as lignin peroxidase (LiP), manganese-dependent peroxidase (MnP), laccase, and tyrosinase, which could help to improve the bioavailability of polyaromatic hydrocarbons in the ecosystem (Gianfreda and Rao 2004). Besides, because of the complex structure of lignin, ligninolytic fungi usually produce very low substrate-specific extracellular enzymes and make them more effective for biotransformation of different compounds (Haritash and Kaushik 2009). Soil fungi can manufacture ligninolytic enzymes which have also been found to degrade different PAHs under microaerophilic or very less oxygen tension situations (Silva and Barbosa 2009). Additionally, Clemente et al. (2001) observed that the degree of remediation has some correlation with types of extracellular enzymes production. For example, the strain which could remove 69% of naphthalene showed MnP activity. Another strain which exhibits 17% of removal could produce LiP and laccase. However, data referring to the involvement of nonwhite rot fungi involved in PAH bioconversion is little known. Capotorti et al. (2004) demonstrated that a nonwhite rot fungi *Aspergillus terreus* isolated from a polyaromatic hydrocarbon-contaminated soil can metabolize BaP and pyrene to pyrenyl sulfate and benzo[a]pyrenylsulfate, correspondingly, which suggested a mechanism of the hydroxylation by cytochrome P-450 monooxygenases trailed by conjugation with SO_4 ions. Veignie et al. (2004) suggested a theoretical unconventional metabolic pathway involved in metabolism of BaP by fungi *Fusarium solani*. This they have done by analysis on the enzyme mechanisms which are involved in the consecutive process in degradation of BaP. In another study conducted by Naranjo et al. (2007), the authors isolated many autochthonous fungal strains which could use extra heavy crude oil (EHCO) and polyaromatic hydrocarbons as solitary source of carbon and energy from extra heavy crude oil-contaminated soils. These fungal strains were identified as *Trichoderma*, *Pestalotiopsis*, *Neosartorya*, *Phoma*, *Fusarium*, *Paecilomyces*, *Penicillium*, *Aspergillus*, *Cladosporium*, and *Pseudallescheria*. In one study, AI-Jawhari (2016) refers that *A. niger* and *P. funiculosum* degraded anthracene to other compounds. It was also observed that the dry weight of fungal mycelial of *Penicillium funiculosum* was more than the control; on the other hand, the fungal mycelia dry weight of pure culture of *Aspergillus niger* was less than the control. Results on biochemical and phenotypic studies of fungus also disclosed the capability of the non-WRF filamentous fungal strains to biosynthesize the extracellular oxidative enzyme and proposed that there might have a connection between the lignin degradation system (LDS) and extra heavy crude oil bioremediation. Fungal strains are capable to break down lignin which makes them also possible candidates for PAH degradation. The degrading enzymes lignin peroxidase and manganese peroxidase have shown to be able to degrade some model lignin compounds. Peroxidase has showed to be involved in degradation of PAH to quinones (Hammel et al. 1986). Fungi do not degrade the hydrocarbons completely to CO_2 as bacteria often do. In the highest conversion of hydrocarbons shown, only 19% was converted (Field et al. 1992). Instead they form a range of degradation products which are solved in the aqueous phase or become bound to organic fraction in the soil. For benzo[a]pyrene it was found that nearly all degradation product was bound to the compost fraction used in that experiment. The degradation rate of benzo[a]

pyrene was found to be double of the degradation rate in the culture without the fungi. After a month the degradation stopped, which is suggested to be due to nutrient limitation (McFarland et al. 1992). Also AI-Jawhari (2015) found that the maximum crude oil bioremediation was 95% by the pure culture of *A. niger*, after 28 days treatment. But the maximum bioremediation percentage of crude oil with mixed cultures of *Aspergillus niger* and *Aspergillus fumigatus* was 90%, and the lowest bioremediation rate of crude oil was observed in consortium of four fungal strains. The presence of fungi and bacteria in one culture leads to an increase in degradation of crude oil. The culture showed a breakdown rate which was much more than the total amount of degradation in a bacterial and a fungal culture separately. Bacteria, which have shown no growth on benzo[a]pyrene, are able to show growth in presence of fungi; it means that fungi and bacteria supported each other. A degradation rate of 27% in 56 days was observed in case of benzo[a]pyrene and 19% in case of dibenz[a,h]anthracene using the consortium culture. Biodegradation mechanisms of bacteria and fungi show a remarkable difference with cis-trans hydroxylation for fungi and cis-cis hydroxylation for bacteria (Cerniglia 1992).

Limited work has been carried out on the biotransformation ability of yeast on PAHs. Romero et al. (1998) have studied on phenanthrene-degrading yeast *Rhodotorula glutinis* isolated from a polluted water stream and observed that its degradative rate was almost equal to that of another phenanthrene-utilizing bacteria *Pseudomonas aeruginosa* which was isolated from the same habitant. Hesham et al. (2009) reported one yeast strain *Candida viswanathii* had ability of biodegrading a blend of high and low molecular weight polyaromatic hydrocarbons with a biodegradation efficacy of 55.53% for BaP, 60.7% for pyrene, 77.2% for phenanthrene, and 89.7% for naphthalene after 10 d of incubation period.

23.7 Metabolic Pathway of PAHs

For metabolic pathways of PAHs, both fungal and bacterial strains are able to metabolize a varied diversity of PAHs; however, their respective principle pathways are different (Fig. 23.3).

Knowledge about the mechanisms on fungal degradation of PAHs is less than that of the bacteria. In general, PAHs can be degraded to metabolites of phenolic compounds by fungi through a co-metabolic progression (Cerniglia 1993; Harayama 1997), and in this process, minimum two ways or mechanisms are implicated. One method is based on the cytochrome P-450 system, which can catalyze the oxidation of PAHs into arene oxides and initialize the PAH metabolism. The other is the nonspecific oxidation reaction carried out by the extracellular enzymatic system (Hammel 1995; Bogan and Lamar 1996), which can lead to the production of hydroxylated aromatic compounds and quinones (Muncnerova and Augustin 1994).

Extracellular enzymes are the powerful biocatalysts which are effectively involved in biotransformation of carbon-based contaminants and are able to comprehensively reframe the arrangement, toxicological properties, and structure of

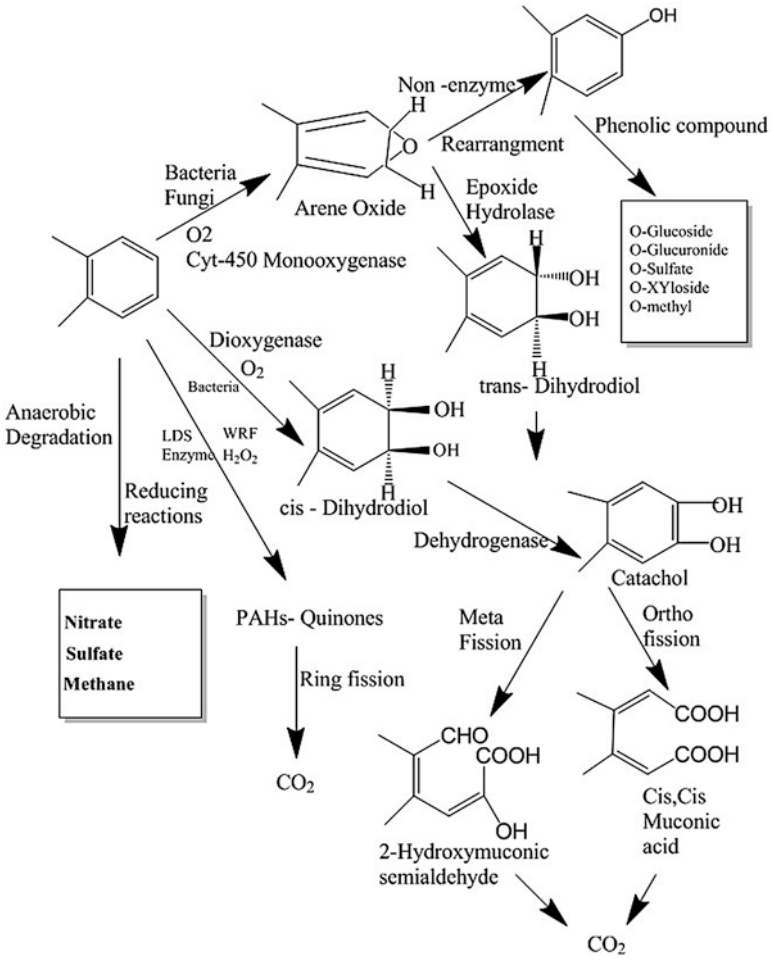

Fig. 23.3 Biodegradation of the aromatic rings by ortho- or meta-cleavage forms by microbial means. (Wu 2010)

pollutants or may entirely mineralize the carbon-based molecules into harmless and mild inorganic final products. The degradative efficiency by enzymatic systems depends on the biodegradability, molecular structure of substrates, and interaction between the enzymes and the substrates (Gianfreda and Rao 2004). In general, fungi possess very powerful extracellular enzymatic systems, and these enzymes from fungi include a great variety of oxidoreductases and hydrolases. Both of these enzyme types might exhibit a biodegradative ability and convert the substrates into the simple forms or partly break down compounds or may transform into oxidized products which can be simply without any difficulty taken up by the cells. In another interesting study conducted by Meulenberg et al. (1997), they have conveyed that the partial oxidation of polyaromatic hydrocarbons by extracellular enzymatic sys-

tem results into the products which are water soluble and also having augmented polarity results in a better biodegradation. So the extracellular enzymes from fungal strains play a very significant role in biodegradation process.

23.8 Fungal Remediation of Petroleum Hydrocarbons in Soils

Generally there are four pathways for the bioremediation of PAH-contaminated soils, but it depends upon the soil status, structure, its organic matter content, compactness, and type of microbiome present in that soil (Mahro et al. 1994). For effective bioremediation process to occur, soil bacterial and fungal strains are required to excrete the extracellular enzymes. These extracellular enzymes were produced in response to the existence of contaminants in a particular area, since the microbes use that organic contaminant as carbon source and energy. For amelioration of PAH soils, the use of compost and related material has been reported to have great potential, since in the compost presence of various microbes accelerates the process of bioremediation. Another way to accomplish actually could be in situ restitution by employing plants to carry out bioremediation process in rhizosphere or rhizoplane. Therefore, the process of phytoremediation is of special attention as it may act as a secondary improving tactic for polyaromatic hydrocarbon-polluted soil, which has been earlier remedied by the process of land farming or by polyromantic hydrocarbon-degrading microbes. Moreover, the growth of plants may be augmented using plant growth-enhancing microbes (Bisht et al. 2014a). One can also go for another different style or attempt; it could be alternate or unconventional to a microbial call or plant part, here the application of extracellular enzymes, such as protease, oxidoreductases, amylases of fungal origin with known capabilities to transmute or even to decompose polyaromatic hydrocarbons effectually (Andreoni and Gianfreda 2010).

23.9 Biodegradation of PAHs: Factors Involved

There are several factors which are involved directly or indirectly influencing the biodegradation of PAHs in soil. Biodegradation by fungal strains relies on the PAH properties and type of soils. Attachment or covalent binding of polyaromatic hydrocarbons to organic content of soil, such as minerals of clay and size of clay particle, influences the availability, uptake, and transport by efficient fungal strains, which ultimately result in effective mycoremediation in the nature. There are numerous means and procedures, such as cation and ion exchange, chemical binding, covalent bonding, hydrogen bonding, and exchange of ligands, and the most common van der Waals forces might be intricate in the adsorption process, though the above said

mechanisms are not yet fully realized (Margesin et al. 2000; Khashayar and Mahasa 2010). Certain other environmental constraints or considerations such as soil moisture content, soil pH, soil redox potential, status of soil oxygen, temperature, and soil nutrient status also impact the rate of PAH remediation. It has been observed that highest rate of bioremediation happened when environmental conditions were in favor of microorganisms involved. It is imperative to understand the features and characters of the polluted site before starting the process of bioremediation treatment. The essential and fundamental information such as type of microbes available in soil, or type or population density of microbes to be applied in soil, residual PAH concentration, and the bioremediation potential of microbes and natural factors such as soil type, soil pH, and temperature of the location, etc. are some of the important points which are to be kept in mind for effective restitution process (El-Tarabily 2002).

23.10 Conclusion

Filamentous fungi are very vital, imperative, and significant in terrestrial and aquatic ecosystem beside other microorganisms, e.g., algal cells and bacterial algae to degrade several pollutants including crude oil and utilize them as nutrient sources. They may also metabolize these pollutants to substrates with low harmful effect on the environment. In this chapter, the data presented has enriched our acquaintance and understanding of PAHs and behavior of fungal strains in polluted locations. Moreover, in addition to the process or means used by these fungal strains to remove or bioremediate the pollutants in the ecosystem, we can employ these potential fungi for bioremediation purpose in the future too. Contaminated soil and water regeneration by the potential culture of filamentous fungi such as *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus versicolor*, *Penicillium funiculosum*, and *Rhizoctonia solani* have been assuring as these can mitigate and diminish the hydrocarbon pollution to the satisfactory or normal levels for reclaim of terrestrial water within short span.

23.11 Future Aspects

We need now to study about the role of filamentous fungal strains in bioremediation of petroleum hydrocarbons from ecosystem; also we need to know if there is any stage of fungal growth or life cycle which is required to degrade these organic hydrocarbon compounds. Also investigation of the metabolic pathways of polyaromatic hydrocarbons by selected fungi and evaluation of the degradation-related enzymes production during the degradation process are imperative. However, it will more helpful in future to use fungi having cloned gene or genetically engineered fungal strains to produce extracellular enzymes responsible to efficient degradation of hydrocarbon pollutants. Another interesting aspect of bioremediation research

would be to enhance the accessibility and convenience of hydrocarbon pollutants in soil and water potential microbiome; if this is achieved and applied successfully under field conditions, then the technique will certainly improve the bioremediation efficacy. Novel practices and means for encouraging transport of hydrocarbon pollutants to the microorganisms might include fracturing of the subsurface matrix using high pressure and pollutant solubilization by applying heat (using hot water or steam or maybe hot air; if required, surfactants may be used). Innovation and invention of upgraded techniques for microbial dispersion might also increase the contact of microorganisms with the hydrocarbon contaminants and will lead to more successful bioremediation.

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Chapter 24
Petroleum Biodegrading Property of Microbial Consortia from a Contaminated Site

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Abstract Hydrocarbons enter into the environment mainly via waste disposal, accidental spillage and leakage tankers. Hydrocarbon toxicity is caused by low-boiling-point aromatic hydrocarbons including benzene, toluene, xylene, naphthalene etc., and the toxicity is spread in almost all compartments of the environment, e.g. plants by damaging protoplast, animals by causing various lung diseases and cancers and atmosphere by decomposing the ozone layer, and it can be prevented also by using hydrocarbon-degrading microorganisms. In this investigation, at first six hydrocarbon-degrading bacterial strains were isolated from oil-contaminated soil near a petrol pump of petrochemical industries at Budge Budge, Kolkata. Their hydrocarbon-degrading ability was checked by their ability to grow in mineral salt-based Bushnell Haas medium supplemented with petrol as a sole carbon source. Along with gram characterization and different biochemical tests, their hydrocarbon tolerance level was measured, which was found to be up to 8% for isolates 1, 3 and 4. The isolates were also examined for the activity of three different hydrocarbon-degrading enzymes, namely, laccase, tyrosinase and catechol oxidase. It was found that isolate 3 had the highest activity for these enzymes and it could also degrade the petroleum to produce CO_2 within 7 days. The isolate also had metal-adhering ability by forming biofilm on it. From the colony morphology and microscopic analysis, it was presumed that isolate 3 was an actinobacteria, which was finally proved by their ability to grow in selective medium – starch casein agar – and 16S rDNA sequencing, and it was found that isolate 3 was *Streptomyces bacillaris* S4BW2. So these isolates seemed to have potential for bioremediation of hydrocarbon pollution and, thus, can serve as a potential tool to degrade petroleum waste.

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24.1 Introduction

Hydrocarbon-degrading organisms are ubiquitously distributed in the environment, comprising less than 1% of the total microbial community in terrestrial ecosystem. But under the influence of oil and hydrocarbon pollutants, the population of hydrocarbon-degrading microbes rises to 10% of the community. The physical, chemical and biological factors that govern the rate of hydrocarbon degradation by bacterial community include composition, state and concentration of oils and hydrocarbons in soil, absorptivity of soil particles, dispersion- and emulsification-enhancing rates of water content of soil, temperature, pressure, nutrient availability, salinity, pH and moisture content in soil (Leahy and Colwell 1990). In the eubacterial community, heterotrophs, polycyclic aromatic hydrocarbon (PAH) degraders and TPH degraders are persistent (Viñas et al. 2005).

Soluble fractions of oil, comprising of low-boiling-point aromatic hydrocarbons like benzene, toluene, xylene, naphthalene, phenanthrene, etc. are the main sources of oil toxicity (Islam et al. 2013). Microbes play a major role in detoxification of toxic hydrocarbons to relatively non-toxic varieties, like carbon dioxide, water and biomass. Otherwise xenobiotic compounds exhibit carcinogenic, mutagenic and teratogenic effects on human population, which make bioremediation of pollutants a bare necessity in modern era, where microbes mineralize, immobilize or transform toxic hydrocarbons to non-toxic products in a cost-effective, less time-consuming and efficient manner.

Petroleum gets into the environment due to events such as accidental spills, waste disposal, tankers leakage and losses during transportation or shortage (Okoh 2006). The petro-fuel is a complicated mixture of normal, branched and cyclic alkenes and aromatic compounds, which is obtained from the middle distillate fraction in the course of petroleum separation. The lethal effects of crude petro-oils and refined petro-oils on flora, faunas, humans and the environment are horrible (Elliot 1997).

Hydrocarbons in soil are initially attacked by oxygenases produced by soil microbiota, using oxygen as the second substrate. Degradation by peripheral pathways leads to the production of C2 compounds that enter the citric acid cycle to form C6 and C4 TCA cycle intermediates (Adams et al. 2015). Carbon dioxide is produced as a byproduct along with hydrogen peroxide as a reaction intermediate, utilizing ammonium phosphate, sulphate and ferrous ions. Thus organisms increase in cell biomass using hydrocarbon as its sole carbon source.

The bioremediation process depends on the hydrocarbon-degrading ability of microbes, which is present naturally and are highly efficient due to their simplicity and cost-effectiveness. Bioremediation is a process of using microorganisms to degrade toxic chemicals into organic chemicals, leading ultimately to the formation of carbon dioxide, water and biomass (Dua et al. 2002).

This study was undertaken to gain insight into the concept of bioremediation by the isolation of bacteria growing in oil-contaminated soils near petrol pumps of petrochemical industries in Kolkata which can degrade petroleum products. The experiment was further progressed to study the morphological and Gram character-

ization of the isolated organisms; biochemical testing such as IMVIC, catalase and oxidase tests; determination of sensitivity assay of the isolated organisms to different concentrations of petrol and kerosene; determination of the presence of hydrocarbon-degrading enzymes like laccase, monophenol oxidase like tyrosinase and polyphenol oxidase like catechol oxidase; determination and estimation of the amount of CO_2 liberated by the organisms during degradation of petroleum and in the absence of petroleum; identification of the nature of the most efficient organism by growing it in a selective media; and finally phylogenetic analysis of the most efficient organism by performing 16S rDNA sequencing.

24.2 Materials and Methods

24.2.1 Collection and Characterization of the Soil Sample

Soil samples were collected from oil-contaminated sites near a petrol pump of petrochemical industries at Budge Budge, Kolkata. The samples were collected in sterile containers at a 5 cm depth from the surface of soil to avoid contamination. Collected samples were transferred to the laboratory under sterile condition for physical and chemical characterization like pH and water conductivity.

24.2.2 Isolation of Petroleum-Degrading Bacteria

10 gm of soil sample was diluted in 90 ml of PBS, and dilutions of the sample (10^{-3}, 10^{-4} and 10^{-5}) were prepared. The diluted samples were spread on sterile Bushnell Haas agar plates. The medium (composition – $MgSO_4.7H_2O$[0.2 g/l], K_2HPO_4[1.0 g/l], KH_2PO_4[1.0 g/l], $FeCl_3$[0.05 g/l], NH_4NO_3[1.0 g/l], $CaCl_2$[0.02 g/l], pH -7.2) contains all the nutrients except a carbon source (Bushnell and Haas 1941). So the plates were supplemented with 1% filter-sterilized petrol and incubated at 37 °C for 2–3 weeks except the control plate. After incubation, the bacterial colonies grown on BH plates were characterized morphologically and biochemically.

24.2.3 Determination of Tolerance Level to Different Petroleum Concentrations

Different concentrations of petrol and kerosene (1%, 2%, 4% and 8%) containing BH agar plates were inoculated with the isolated cultures and finally incubated at 37 °C for 7 days. Tolerance to petrol and kerosene was observed by the ability of organisms to grow on BH agar plates containing the respective concentration of the hydrocarbons.

24.2.4 Determination of Activity of Different Hydrocarbon-Degrading Enzymes in the Isolates

24.2.4.1 Laccase Assay

Laccase can catalyse the ring cleavage in aromatic compounds. So to assay laccase activity, sterile nutrient agar plates supplemented with 0.02% bromophenol blue were inoculated with the isolates and a control organism which is not petroleum degrading. The plates were incubated at 37 °C for 24 h. The change in the intensity of the blue colour of the plate due to the destruction of the ring structure of bromophenol blue around the growth of the organism was observed only around test isolates but not around the control organism.

24.2.4.2 Monophenol Oxidase or Tyrosinase Assay

Tyrosinase catalyses the destruction of resonance structure of aromatic compounds by hydroxylation. To assay tyrosinase, sterile nutrient broth-containing test tubes, each of which contains 0.1 mM tyrosine, were inoculated with the isolates separately. A control set was maintained for each isolate where the tyrosine was replaced by sterile water. Tyrosinase would be converted to its o-quinone derivative via dihydroxyphenylalanine, when subjected to tyrosinase, which cannot absorb light of 280 nm like tyrosinase. So tyrosinase activity can be estimated by the decrease in OD value at 280 nm in the test tube compared to the control set. To avoid the interference of 280 nm light absorbance of extracellular proteins, 0.1(N) NaOH was used to degrade and coagulate the proteins, which can be separated by centrifugation before taking the optical density readings. Both test and control tubes were incubated at 37 °C, and OD readings were taken at day 0, day 1 and day 3.

24.2.4.3 Polyphenol Oxidase or Catechol Oxidase Assay

Catechol oxidase catalyses the oxidation of ortho-diphenols into ortho-quinones with the reduction of molecular oxygen to water. To assay catechol oxidase, sterile nutrient broth supplemented with 0.1 mM catechol was prepared, into which the isolates were inoculated separately. A control set was maintained for each isolate where the catechol was substituted by sterile water. Catechol or 1,2-dihydroxybenzene, like other polyphenols, can form adduct with Folin-Ciocalteu, which can be stabilized by the addition of Na_2CO_3 solution, and this adduct can absorb light at 765 nm. But quinone derivatives of catechol, produced after catechol oxidase reaction, cannot form this adduct. So the catechol oxidase activity can be estimated by the decrease in OD value at 765 nm in the test set compared to the control set. Both test and control set tubes were incubated at 37 °C, and OD readings were taken in day 0, day 1 and day 3.

24.2.4.4 Estimation of CO_2 Evolution

Microbial metabolism of petroleum products generates C2, C4 and C6 TCA intermediates, which is finally converted to CO_2 and biomass. Thus incubating hydrocarbon-degrading bacteria in a medium supplemented with hydrocarbon may produce CO_2 as a byproduct. So the measurement of CO_2 liberated due to petroleum degradation, by observing the change in pH of CO_2 . trap like NaOH, can be a very important tool to determine the rate of petroleum degradation. This test has been carried out for isolates '1' and '3' only, due to their efficient results in the previous experiments. Two BH agar medium-containing flasks, supplemented with petrol, were then separately inoculated by isolates '1' and '3'. In the control set, no petrol was added. Small tubes containing freshly prepared 0.1(N) NaOH were then hanged to trap the evolved CO_2 inside the flasks, whose mouths were sealed with a rubber cork and incubated at 37 °C. After a week, when the microbial colonies start to develop, the pH of residual NaOH was determined by titrating it against 1(N) HCl.

24.2.4.5 Study on the Ability of Isolate '3' to Colonize on Metallic Surface

Most often it is found that many of the hydrocarbon-degrading bacteria form biofilm on the metallic surface of the aeroplane fuel tank and thus damage it. So the ability to colonize on the metallic surface was studied. This study was done only for isolate '3' as this isolate had showed the highest laccase, tyrosinase and catechol oxidase activity. In this study, at first the metallic fragments were sterilized and then inoculated with the isolate and placed in a moist chamber. After placing, they were incubated at 37 °C for a week. A control set was also maintained were a metallic fragment without inoculation was incubated in a moist chamber. After incubation the test metallic fragment was observed via phase-contrast microscopy for the presence of extracellular capsule-like material on the surface of the microbial cells, which is normally found on the surface of the biofilm. Along with phase-contrast microscopic observation, they were also checked for viability by inoculating them into a starch casein agar plate.

24.2.4.6 Identification of Isolate '3'

Due to having the highest activity in different petroleum-degrading enzymes and metal-adhering ability, isolate '3' was identified. Identification of isolate '3' was done by three approaches – morphological characterization, their ability to grow on specific selective medium and lastly by 16S rDNA analysis. The microscopic analysis and colony morphology (powdery colonies) of isolate '3' showed a similar characteristic to that of the genus *Actinomycetes*. To confirm its nature, it was inoculated in an *Actinomycetes* selective medium – starch casein agar (composition: soluble starch[10 g/l], casein[0.3 g/l], KNO_3[2.0 g/l], NaCl[2.0 g/l], K_2HPO_4[2.0 g/l],

$CaCO_3$[0.02 g/l], $MgSO_4.7H_2O$[0.05 g/l], $FeSO_4.7H_2O$[0.01 g/l], Agar[20 g/l], 50% sea water, nystatin[50 mg/l], benzyl penicillin[0.88 mg/l], pH-7) – and incubated at 37 °C, and the presence of growth was noted. Then for 16S rRNA sequencing of 'isolate 3', genomic DNA of the isolate was extracted and for analysis of the gene of 16s rRNA was amplified via polymerase chain reaction. The PCR reaction was carried out using the following primer set: (1) forward primer (5′-AGAGTTTGATCMTGGCTCAG-3′) and (2) reverse primer (5′-CGGCTACCTTGTTACGACTT-3′).

The PCR fragments were then analysed by 1.5% agarose gel electrophoresis using 100 bp DNA ladder and then visualized in gel documentary system. After this amplification of the 16S rDNA was sequenced, and it is compared to 16S rDNA sequences available in the nucleotide databases of the GenBank using the basic local alignment search tool (BLAST) at the National Center for Biotechnology Information (NCBI), to determine the approximate phylogenetic position and percent homology scores to identify the isolate. And after comparing with other strains, a phylogenetic tree of isolate '3'was also prepared using Clustal Omega software.

24.3 Results

24.3.1 Physical and Chemical Characterization of the Soil Sample

The physical and chemical characteristics of the sample are given below (Table 24.1).

24.3.2 Isolation of Petroleum-Degrading Bacteria

Six different types of colonies were obtained from the BH agar plates containing 1% petrol; these colonies were absent in the control plate that lacks the petrol. The isolated colonies were designated as isolate 1 to isolate 6 (Fig. 24.1).

Table 24.1 Physical and chemical characteristics of the collected sample

Colour	Brownish black colour
Texture	Grainy to smooth texture with certain gravels that were removed prior to analysis
Odour	An odour of petroleum emanates from the sample
pH	6.2
Electrical conductivity	177.15 µsiemens

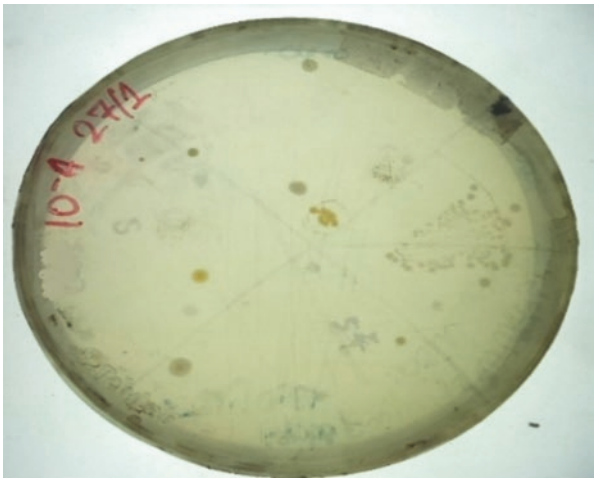

Fig. 24.1 The BH plate showing the colonies of the isolates obtained on inoculating 10^{-4} dilution of soil suspension

Table 24.2 Morphological and biochemical characterization of the isolates

Characterization of the isolates	Designation of isolates					
	1	2	3	4	5	6
Morphology	Smooth, medium, whitish round	Small, pale yellow, smooth, irregularly shaped	Whitish, powdery, rough, round colonies	Yellowish, big, smooth, round colonies	Whitish, smooth, small irregularly shaped	Reddish, tiny, smooth, round
Gram character	G(+)	G(−)	G(+)	G(+)	G(−)	G(−)
Indole test	−	−	−	−	−	−
Methyl red	−	−	−	−	+	−
Voges-Proskauer	+	+	+	+	−	+
Citrate utilization	+	+	+	+	−	−
Catalase	+	+	+	+	+	+
Oxidase	−	+	+	−	+	+

'+' denotes positive result, whereas '–'denotes negative of result

24.3.3 Morphological and Biochemical Characterization of the Sample

The morphological and biochemical characterization of the isolates are provided in Table 24.2.

Table 24.3 Sensitivity profile of the isolates to different concentrations of petrol and kerosene

Designation of isolates	Concentration of petrol				Concentration of kerosene			
	1%	2%	4%	8%	1	2	4	8
1	+	+	+	+	+	+	+	+
2	+	+	+	–	+	+	+	+
3	+	+	+	+	+	+	+	+
4	+	+	+	+	+	+	+	+
5	+	+	+	–	+	+	+	–
6	+	+	–	–	+	+	+	–

'+' denotes presence of growth, whereas '–' denotes absence of growth

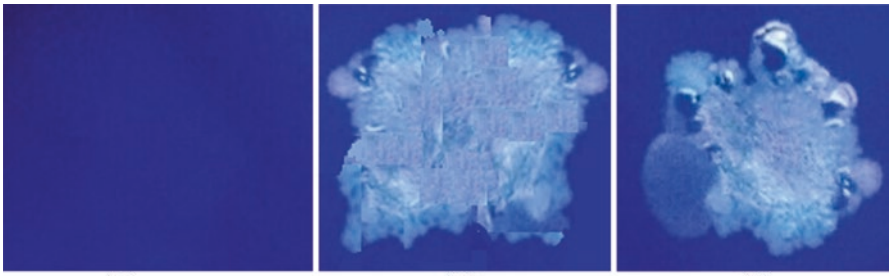

Fig. 24.2 Observation of the laccase assay where isolates 1 and 3 show discolouration, while other isolates did not show any change. (**a**) Original colour of the plate. (**b**) Discolouration done by isolate 1. (**c**) Discolouration done by isolate 3

24.3.4 Determination of Tolerance Level to Different Petrol and Kerosene Concentrations

The tolerance level of the isolates to different concentrations of petrol and kerosene was observed and provided in Table 24.3.

24.3.5 Activity of Different Hydrocarbon-Degrading Enzymes in the Isolates

24.3.5.1 Laccase Assay

Due to the laccase activity, discolouration was observed around colonies of isolate 1 and isolate 3 only, but the discolouration was not observed in case of other isolates (Fig. 24.2).

Table 24.4 The changes in O.D. values at 280 nm and tyrosinase activity of the isolates

Designation of isolates	O.D. values at 280 nm			Tyrosinase activity (O.D./day)
	Day 0	Day 1	Day 3	
1	0.293	0.233	0.128	0.055
2	0.295	0.160	0.087	0.069
3	0.315	0.235	0.005	0.103
4	0.190	0.143	0.047	0.047
5	0.115	0.074	0.020	0.032
6	0.183	0.140	0.102	0.027

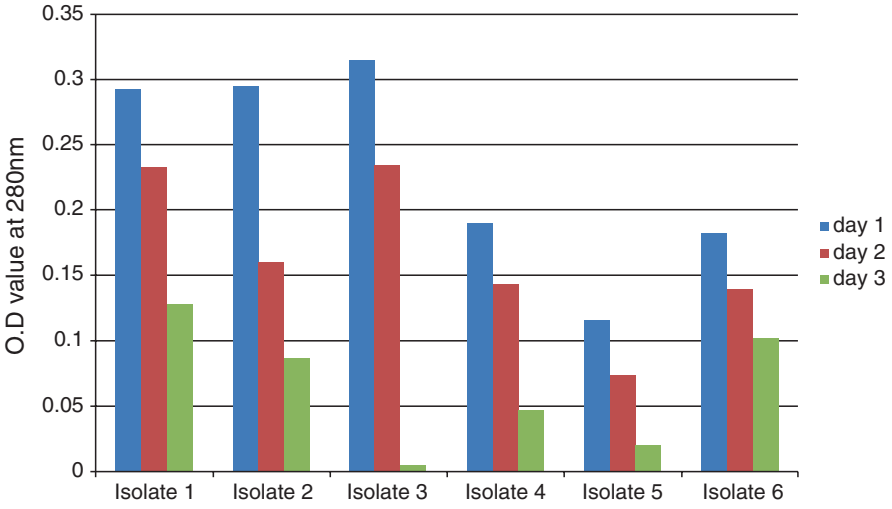

Fig. 24.3 The graphical representation of the changes in O.D. values in the reaction medium due to the tyrosinase activity of different isolates. Y-axis denotes the O.D. values of the test culture solutions at 280 nm, and the X-axis denotes the test isolates

24.3.5.2 Tyrosinase Assay

In the span of 3 days, the changes in O.D. values at 280 nm were taken into account for the six isolates. It is noted that the O.D. values decreased subsequently owing to the fact that tyrosine's concentration decreased. The changes in O.D. values at 280 nm are tabulated in Table 24.4 (Fig. 24.3).

24.3.5.3 Catechol Oxidase Assay

In the span of 3 days, the change in O.D. values of the samples was observed at 765 nm for the six isolates. It is noted that the O.D. values were decreased subsequently due to the catechol oxidase activity of the isolates, which is responsible for

Table 24.5 The O.D. values of the isolates for catechol oxidase and catechol oxidase activity at 765 nm

Designation of isolates	O.D. values at 765 nm			Catechol oxidase activity (O.D./day)
	Day 0	Day 1	Day 2	
1	0.582	0.475	0.376	0.103
2	0.622	0.484	0.352	0.135
3	0.653	0.432	0.325	0.164
4	0.651	0.512	0.407	0.122
5	0.619	0.547	0.439	0.090
6	0.540	0.444	0.386	0.077

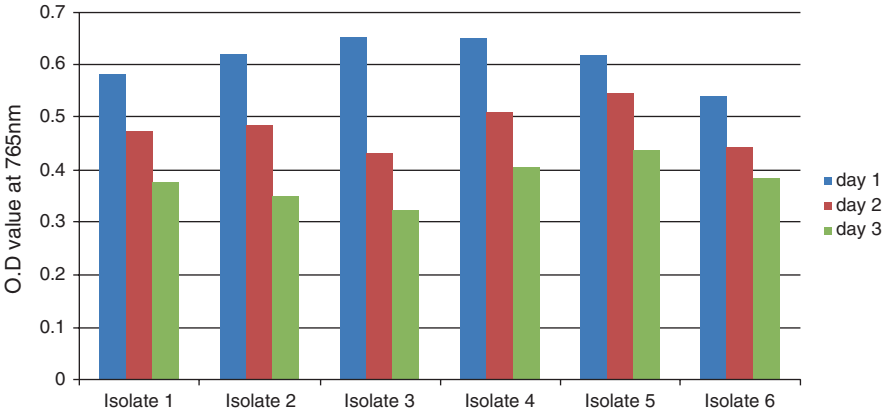

Fig. 24.4 The graphical representation of the changes in the O.D. at 765 nm values in the reaction medium of catechol oxidase assay due to the catechol oxidase activity of different isolates. Y-axis represents the O.D. values of the test culture solutions at 765 nm and X-axis represents the test isolates

the reduction in the concentration of the catechol, which reduces the amount of the catechol Folin-Ciocalteu adduct. The changes in the O.D. values at 765 nm were tabulated in Table 24.5 (Fig. 24.4).

24.3.6 Estimation of CO_2 Evolution

The results of the CO_2 evolution assay were provided in Table 24.6.

The amount of HCl utilized is inversely proportional to the amount of CO_2 produced. Thus isolate '1' produced more CO_2 than isolate '3', and it can be calculated that isolates '1' and '3' have produced 4 ml and 7 ml of CO_2, respectively, in comparison with the control set in the course of incubation period (Fig. 24.5).

Table 24.6 The amount of CO_2 evolved for isolates 1 and 3

Culture	Vol of 1(N) HCl utilized (ml)	Amount of CO_2 evolved (ml)
Control (3)	12.5	–
Test culture 1	9.1	7
Test culture 3	10.75	4

Fig. 24.5 Reaction setup of CO_2 evolution assay showing the hanging tube containing 0.1(N) NaOH

24.3.7 Metallic Surface Colonizing Study

After the incubation, formation of biofilm was observed on the surface of the metallic fragment. When it was observed under phase-contrast microscopy, white halos were found on the surface of the microorganism, which correspond to extracellular capsule-like material. And when this biofilm was tested for its viability, it was found that the organism remained viable and can grow on starch casein agar plate (Fig. 24.6).

24.3.8 Identification of Isolate '3'

When isolate '3' was tested for its ability to grow in starch casein agar plate, growth was observed on the agar plate. PCR amplification using the above-mentioned primers for the 16S rDNA was also observed, and the amplification gave a band of less than 1500 bp DNA fragment. From the sequencing of the rDNA and its alignment in BLAST programme, it was identified as *Streptomyces bacillaris* S4BW2, and it shows 100% similarity in the alignment (Figs. 24.7 and 24.8) (Table 24.7).

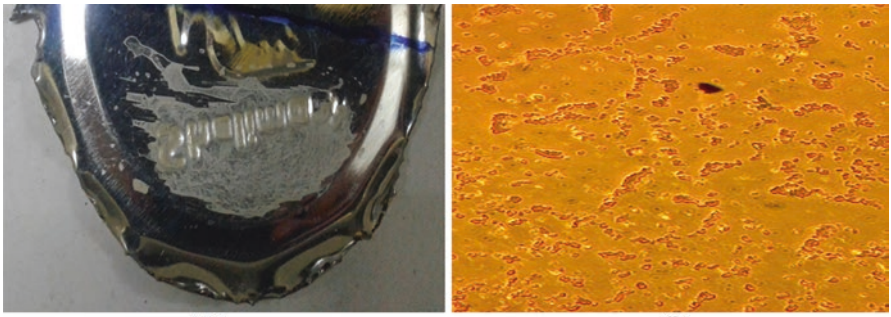

Fig. 24.6 (**a**) Formation of biofilm on the surface of metallic fragment by the formation of extracellular polysaccharides, like capsules, after 7 days of incubation. (**b**) Halos surrounding the organisms produced after 7 days of incubation, which correspond to the presence of capsules required for biofilm formation on the surface of metals

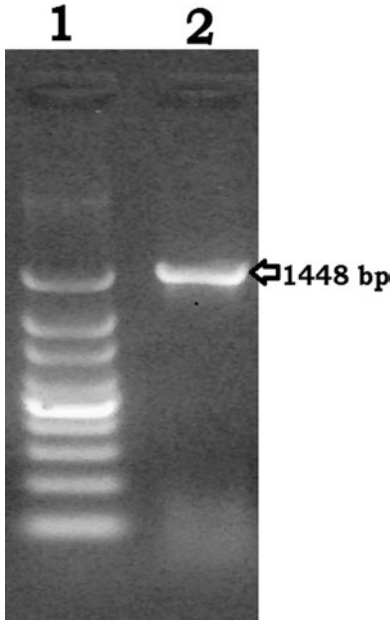

Fig. 24.7 0.8% Agarose gel showing single 1.5 kb of *16S rDNA* amplicon. Lane 1, 100 bp; DNA ladder; lane 2, *16S rDNA* amplicon

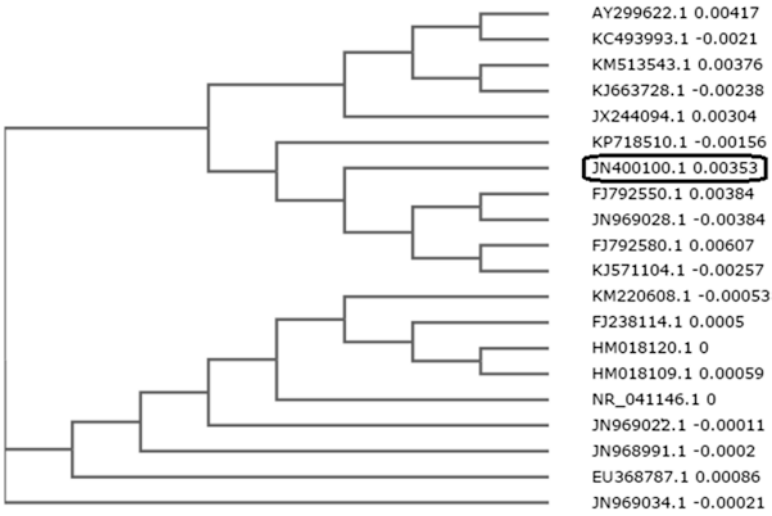

Fig. 24.8 Phylogenetic tree of isolate '3' obtained after analysing the BLAST result

Table 24.7 BLAST result of 16S rDNA gene sequence of isolate 3

Designation of the isolate	BLAST match	Strain	rDNA	Accession no.	Identity
Isolate 3	*Streptomyces bacillaris*	S4BW2	1048 bp	JN400100.1	100%

24.4 Discussion and Conclusion

The samples were collected from oil-contaminated sites near a petrol pump of petrochemical industries, and it was transferred to the laboratory in sterile condition for analysis. Physical and chemical characterization of the sample was performed followed by isolation of petroleum-degrading bacteria from the sample. In order to isolate a mineral-based media, Bushnell Hass (BH) medium was used which lack any carbon source (Jyothi et al. 2012), and this medium was supplemented with petrol as the sole carbon source. Thus only six types of colonies were obtained which grew on those plates, thereby indicating their ability to degrade petroleum (Varjani et al. 2013). Characterization of the six isolates was performed which included gram characterization (Subathra et al. 2013) and biochemical tests such as IMViC, oxidase test and catalase tests (Karigar and Rao 2011). Sensitivity assay of different concentrations of petrol and kerosene was performed in which isolates 1

and 3 showed dense growth at maximum concentration of petroleum and kerosene. Various hydrocarbon-degrading enzyme assays were also performed such as laccase assay (Haritash and Kaushik 2009) and tyrosinase assay (Panda et al. 2013), and isolate 3 was shown to be the most efficient in all of these enzyme assays mentioned above. Quantitative analysis of CO_2 was performed in the presence and absence of petroleum and isolate 1 produced more CO_2 than isolate 3. Since the isolated petroleum-degrading microorganisms are able to colonize on metal surfaces by producing biofilm, it can reduce the fuel consumptions of the automobiles by surviving on the metal surface of petroleum containers contaminated with isolate 3 or similar organism. Isolate 3 was grown on a selective media, i.e. starch casein agar, in order to detect the nature of it. This medium is specific for *Actinomycetes* sp., and growing isolate 3 in this medium showed positive result. As isolate 3 showed the most efficient results 16S rRNA analysis was performed, and the result is *Streptomyces bacillaris*.

Review of literature reveals that individually extensive work has been done in isolating petrol-degrading bacterial isolates from oil-polluted soil. In the study carried out by Jyothi et al. (2012), seven petroleum-degrading strains were isolated from three soil samples near petrol and diesel pumps in Hussain Sagar Lake, Hyderabad, and these bacterial strains showed 99% similarity in 16S rRNA sequence with *Staphylococcus aureus*, *Lactobacillus acidophilus*, *Neisseria flavescens*, *Corynebacterium xerosis*, *Bacillus megaterium*, *Micrococcus luteus* and *Bacillus cereus*, respectively.

In the study carried out by Panda et al. (2013), from an old oil jetty of Paradip Port, Orissa, India, petroleum-polluted soil, marine water and soil sediment samples were collected. The amount of petro-hydrocarbon biodegradation was measured by gravimetric assay technique after a gap of every 5 days. From these soil sediments, one effective Gram-negative bacterium was isolated and further selected on the basis of petro-hydrocarbon remediation efficacy, which was later identified as *Pseudomonas* sp. This *Pseudomonas* sp. demonstrated diesel oil degradation up to 49.93% in 20 d against 0.5% of diesel in 100 ml BHM.

In another study conducted by Subathra et al. (2013), three efficient petro-oil-degrading bacterial isolates *Bacillus subtilis* I1, *Pseudomonas aeruginosa* I5 and *Pseudomonas putida* I8 were isolated from soil sediment samples from areas surrounding the Ennore creek. The measureable study of oil degradation was carried out using gravimetric technique, and a maximum degradation rate of 55% was observed in isolate *Pseudomonas aeruginosa* I5.

In another interesting study carried out by Islam et al. (2013), petroleum biodegrading bacterial isolates were isolated from various oil-polluted water and soil samples. Nine bacterial strains were isolated which were capable of biodegrading diesel, petrol and kerosene having variable tolerance capacities. On the basis of morphological and biochemical tests, the bacterial isolates belonged to the following genera, e.g. *Streptococcus*, *Staphylococcus*, *Corynebacterium*, *Micrococcus*, *Bacillus* and *Klebsiella*. It was observed that isolate *Staphylococcus* spp. tolerated as high as 7% petroleum concentration as compared to other isolates. Other isolates also exhibited various concentrations of petroleum tolerance. In a nutshell we can

say that all of the isolates were able to biodegrade petro-products completely within 7 days and generated CO_2, with exceptions of *Klebsiella* sp. and *Bacillus* sp. These two isolates carried out complete biodegradation of kerosene in 15 days. On the basis of the above-mentioned results, we can say that these isolates could be employed in remediation of petroleum-polluted environment.

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Chapter 25
The Role of Microbes Toward Biodegradation of Hydrocarbons

Varsha Dogra, Rajeev Kumar, Sandeep Kumar, and Gurpreet Kaur

Abstract Environment contamination by hydrocarbons (HC) has caused lots of implications and the use of HCs has been increasing over the years due to their several applications in different industries. HCs are compounds composed of hydrogen and carbon; it is described as an enormous contaminant with carcinogenic, mutagenic, and toxicity potential for the flora and fauna. HCs are very difficult to get rid of the environment as they are difficult to degrade. Accidental release of the petroleum products leads to the degradation of the environment. Oil spills in the ocean, crude oil-carrying pipeline leakages, production of by-products, and HC refining lead to environment pollution which is causing loss of biodiversity. The search for the natural methods for the degradation of HCs and their by-products has increased with the advancement of technologies as they are creating lots of environmental problems. In this book chapter, we have tried to sum up all the environment-friendly remediation methods for the removal of HCs such as phytoremediation, rhizoremediation, bioaugmentation, and bioremediation by enzymes, algae, bacteria, fungi, microbial consortium, and protozoans.

25.1 Introduction

HC compounds consist of hydrogen and carbon in their structure, these compounds have become one of the major environmental problems worldwide. The environment is getting degraded with increased use of the HC in the petroleum industries

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and because of their accidental release into the environment. Some HCs are mutagenic, carcinogenic, and neurotoxin to flora and fauna. Accidental oil spills and pipeline leakage during the transportation, refining, and storage of petroleum and its derivatives lead to the widespread damage to the biodiversity. These pollutants get accumulated into the tissues of marine flora and fauna which may lead to the death or genetic changes, further causing biomagnification by entering into the food chain.

HCs lack functional groups, are largely polar, and exhibit low chemical reactivity at room temperature (Sierra-Garcia and de Oliveira 2013). The occurrence, type, and arrangement of unsaturated bonds determine the different reactivities of HCs. There are many physical, chemical, and biological methods for the degradation of HCs (Fig. 25.1). The physical methods include the use of skimmers, tilling, in situ burning, and mechanical removal, whereas chemical methods include dehalogenation, deamination, decarboxylation, hydroxylation, dispersants, and demulsifying. On the other hand, biological methods make use of microbes and include methods like phytoremediation, rhizoremediation, and degradation by enzymes; microbial degradation is also done with the help of bacteria, fungi, and protozoa.

Bioremediation is a significant approach for the removal of HCs from the contaminated sites; this approach is cost-effective and can lead to complete degradation

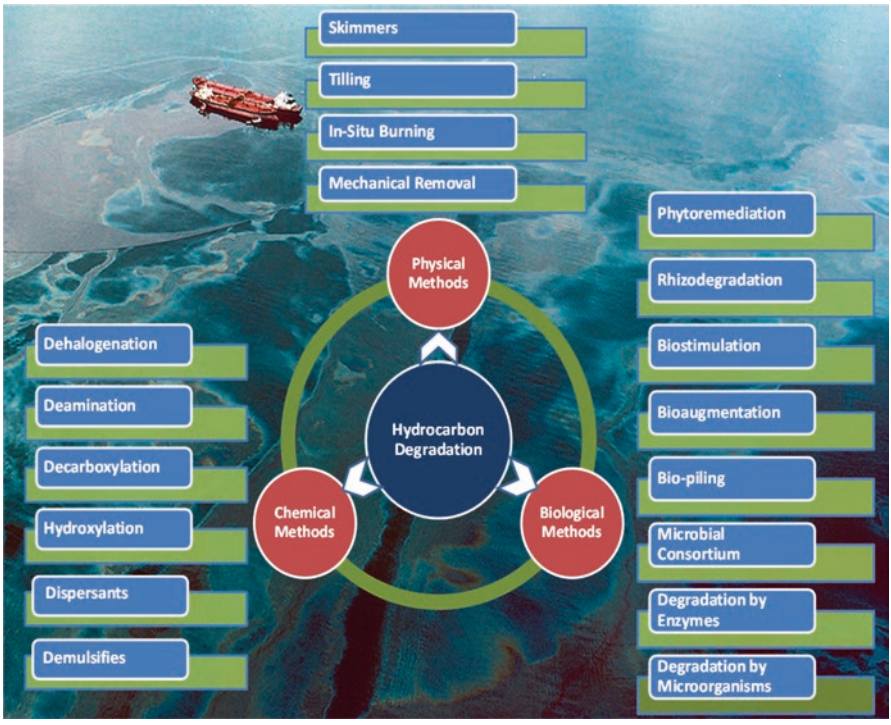

Fig. 25.1 Different methods (physical, chemical, and biological) for the degradation of HCs

and mineralization of complex HCs. From different studies, it had been proved that bacterial isolates use aliphatic and aromatic HCs as sole carbon and energy source (Söhngen 1913). Numerous aerobic and anaerobic bacteria have been studied to understand their mechanism of degrading the specific members of aliphatic and aromatic compounds. The researchers have revealed that aerobic bacteria require oxygen and anaerobic bacteria require nitrate, ferric iron, sulfate, and other electron acceptors for the degradation of most classes of HCs including aromatic compounds, alkane, alkenes, and alkynes (Widdel and Musat 2010). Microorganisms use their metabolic pathways for the biodegradation of organic pollutants into inorganic compounds, carbon dioxide, and water after their partial or complete mineralization. Organisms use HCs as their energy source. For the biodegradation or complete mineralization of complex pollutants such as petroleum or crude oil, a collaboration of more than one species is generally required. The collaboration of species increases the rate and enzymatic capacities to degrade the HC substrate. Oxygenase enzyme helps in the initial degradation of HCs (Atlas and Ronald 1991); nitrate or sulfate may serve as a terminal electron acceptor (Bartha 1986) in the successive steps.

25.2 Hydrocarbons

Hydrocarbons (HC) classified as alkanes, cycloalkanes, aromatics, polycyclic aromatics, asphaltines, and resins. N-alkanes HC is the most amenable to biodegradation. Alkanes with carbon chain C_{20} to C_{40} are less biodegradable, as they are hydrophobic in nature and have low solubility. Oxygenase enzyme acts on the terminal methyl group of alkanes and converted them into alcohol during the degradation process. Further, alcohol oxidized into the aldehyde and then into fatty acids. Fatty acids are utilized by β-oxidation of the aliphatic chain; moreover, the higher is the methyl branching, the lower is the extent of β-oxidation.

The alicyclic HCs are less degradable than alkanes, as with the increase in the number of the ring structure, biodegradability decreases. Cycloalkanes substituted with alkyl group degraded more easily by the action of oxidases into cyclic alcohol, and further, it is dehydrogenated into ketone than the non-substituted HCs. Cycloketones and cycloalkane-carboxylic acids are the primary products of the metabolism of cycloalkanes.

The aromatic HCs have benzene-based structures and are more stable because of the sharing of delocalized electrons by pi bonds. Benzene, toluene, ethylbenzene, and xylene (BTEX) compounds are relatively more mobile and water-miscible. The polynuclear aromatic HCs (PNA) specifically polycyclic aromatic HC (PAH) are organic compounds consisting of multiple aromatic rings; they are synthesized in different industries such as coke production, petroleum refining, thermosetting plastics, and wood preservation (Park et al. 1990) using temperature more than ambient conditions. PAH's compounds include 16 pollutants, and some of these are carcinogenic in nature (Dzomback and Luthy 1984; McEldowney et al. 1993). PAH's solubility and volatility decreases, whereas adsorption capacity increases with increase in molecular weight and number of rings.

The compounds containing sulfur, nitrogen, and oxygen such as asphaltines and resins are mostly high molecular weight compounds. These compounds are resistant to biodegradation because of high solubility and the presence of functional groups which get shielded from microbial attack by aromatic rings.

25.3 Requirement for Biodegradation of Hydrocarbon

Degradation of HC is very complex as aromatic components such as of petroleum dissolve in the water body and disrupt the marine flora and fauna. HCs at low concentration even at *ppb* level interfere with the chemoreceptors of marine fauna. Interference with the receptors also leads to the elimination of many marine species. Some condensed polynuclear constituents of petroleum are resistant to biodegradation and carcinogenic in nature and may enter the food chain leading to taint the marine life.

There are many ecological and economic impacts of direct or indirect exposure of HCs to biota, as described below:

- Direct exposure: There are a variety of harmful effects of oil spills to marine animals and also to human beings who work in such areas. Gaseous oil compounds of oil spill get dispersed in air and come in contact with fauna through breathing. Inhalation of contaminants causes irritation to the respiratory tract and allergies, causes asthma problem, and may lead to lung cancer. Another exposure pathway is through the skin by coming in contact with the contaminated materials. PAH exposure to pregnant women may lead to prenatal defects, the problem in the development of the fetus, and also cause a reduction in the birth weight.
- Indirect exposure: This kind of exposure happens through the food chain, by consuming the contaminated seafood such as fishes. Oil components of oil spills get biomagnified and get accumulated inside the marine organisms. Oil serves a wide diversity of purposes, which include transportation, heating, electricity, and industrial applications, and is an input into over 2000 end products. But humans get seriously affected by the concentration of oil constituents more through the intake of polluted fish meat in comparison with the environment or by ingestion of polluted water. Exposure to aromatic HCs may also cause cancer.
- Economic effect: Many industries like tourism, fishing, mining, textiles, etc. have been affected by oil spill problem. Many endangered species like sea turtles were found dead; it was reported by the National Wildlife Federation. Together these effects resulted in the widespread economic impact throughout all sectors of the economy and in all geographic areas. The major effect is the loss of endangered species due to contamination of soil, air, and oceans. Agricultural land's productivity also reduced due to HC contamination.
- Cause global warming: HCs combustion is the main source of carbon dioxide release which is a greenhouse gas. Global temperature is rising with an increase in the CO_2 level leading to global warming.

25.4 Biodegradation Mechanism

Biodegradation of an aromatic molecule involves two main steps, i.e., (1) activation of the ring and (2) ring cleavage.

25.4.1 Activation of the Ring

Ring activation in the aromatic HCs involves the inclusion of the molecular oxygen in the aromatic ring that entailed to dihydroxylation of the nucleus of an aromatic compound with the help of the enzyme oxygenase. The following are the enzymes involved in the activation of the aromatic HC ring:

1. Oxygenase enzyme helps in the dihydroxylation of aromatic nucleus ring which is activated by the inclusion of molecular oxygen.
2. Monooxygenase enzyme of eukaryotes catalyzes the addition of oxygen single atom to form an epoxide which further forms trans-dihydrodiols by hydration (Rochkind-Dubinsky et al. 1986).
3. Dioxygenases enzyme catalyzes the addition of two atoms of oxygen to form a dihydrodiol. Dihyrodiols oxidized to catechols which can be formed either by ortho-cleavage pathway or meta-cleavage pathway. The ortho-cleavage pathway involves bond cleavage between carbon atoms of the hydroxyl groups to produce muconic acid. Meta-cleavage pathway involves cleavage of carbon atom bonds with a hydroxyl group and the adjacent carbon to form 2-hydroxymuconic semialdehyde (Cerniglia 1984). The product is further degraded to form organic acids which afterward are used by microorganisms for their cell synthesis and energy generation. Dioxygenase reactions occur for toluene, para-chlorotoluene, benzene, halogenated benzenes, xylenes, biphenyls, anthracene, etc. (Gibson 1988).

25.4.2 Ring Opening

A general pathway for aromatic HC degradation has been shown in Fig. 25.2. The ring opening reaction proceeds with the oxidation of catechol either via ortho-cleavage or meta-cleavage pathways. The ortho-cleavage pathway involves the breaking of carbon atoms bonds of the two hydroxyl groups to produce muconic acid. The meta-cleavage pathway involves breaking of carbon atom bonds with a hydroxyl group and the adjacent carbon atom to produce 2-hydroxymuconic semialdehyde (Cerniglia 1984). These products are further degraded to form organic acids which get utilized by microorganisms for the ATP production.

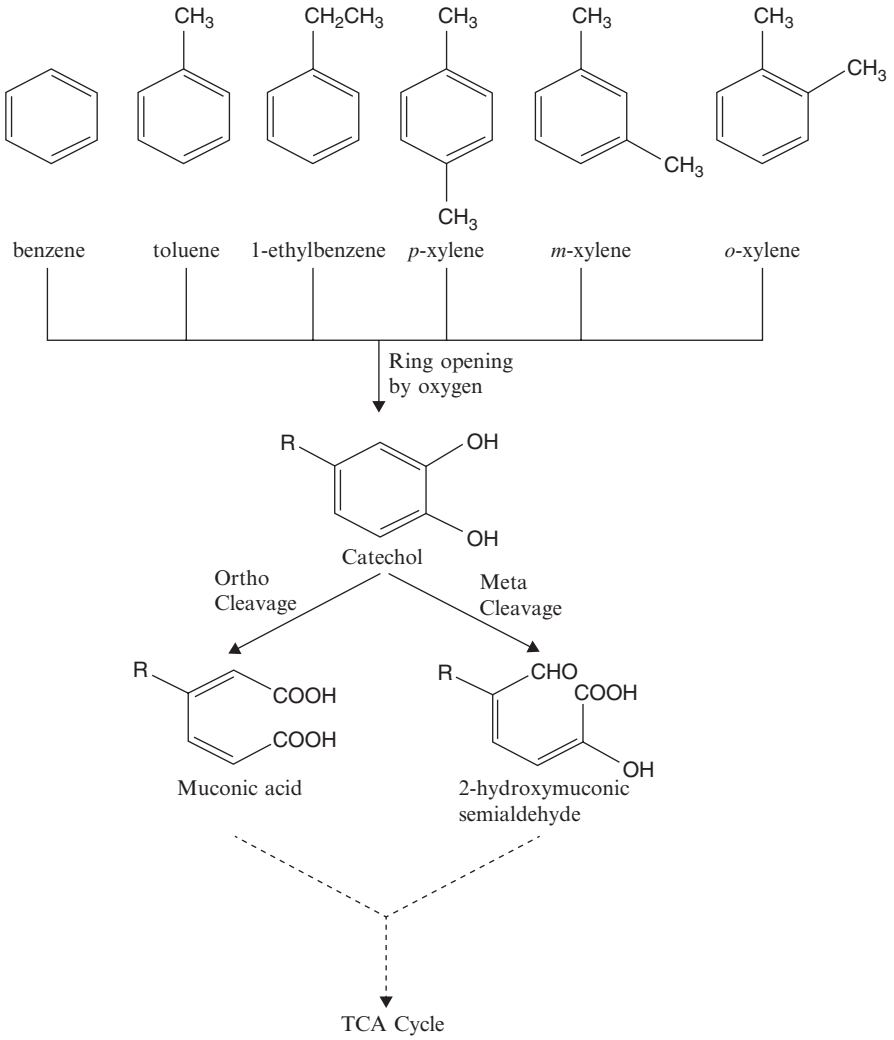

Fig. 25.2 General pathway for aromatic HC degradation (Kothari et al. 2013)

25.5 Different Methods for the Biodegradation of Hydrocarbons

This section of the chapter deals in details various methods for the biodegradation of HCs.

25.5.1 Phytoremediation

It is a bioremediation process in which different plants are used for the removal of contaminants present in soil and groundwater. PAHs and petroleum HCs are biodegraded either under aerobic or anaerobic conditions; in aerobic conditions, biodegradation takes place at a faster rate. Plants provide oxygen for the biodegradation of HC either by transporting oxygen or by creating void spaces in the subsurface for the diffusion of atmospheric oxygen (Tsao 2003). Plants have the capability of degrading the PAH by introducing oxygen in the aromatic ring of HC which increases PAH solubility and chemical reactivity (Sutherland 1992; Wilson and Jones 1993; Meulenberg et al. 1997). Aromatic compounds having two- and three-ring structures are more easily biodegradable than having more than three-ring-structure compounds. Selective plants have been used for the bioremediation of HCs; selection of plant for phytoremediation is dependent on various factors such as soil type, plant root system, and climate (Neder et al. 2004).

25.5.2 Rhizodegradation

It is the plant-assisted degradation of contaminants through microbial action in the soil. For example, phenanthrene degradation is faster in rate in the rhizosphere soil planted with slender oat (Arena barbata Pott ex Link) (Miya and Firestone 2000). Nichols et al. in 1997 reported that rhizosphere of alfalfa (*Medicago sativa* L.) and alpine bluegrass (*Poa alpina* L.) helps in the degradation of PAH. Bioremediation of phenol, toluene, and trichloroethylene (TCE) is promoted by the alfalfa growing in sandy soil (Erickson et al. 1995), and *p*-nitrophenol remediation is enhanced in the rhizosphere of rice (Reddy and Sethunathan 1994).

25.5.3 Biostimulation

This technique is also known as nutrient augmentation, which involves stimulating the bacteria that assist in the bioremediation by adding some rate-limiting nutrients and electron acceptors such as nitrogen, phosphorus, carbon, etc. Many pollutants after getting released from industries (industrial effluents) and from an underground storage tank or landfill leakage can contaminate the soil (Eweis et al. 1998). Microbial division and growth can be enhanced in the contaminated site by providing the nutrients (nitrogen and phosphorus), by providing the nutrient that is present in the limiting concentration, or by using the surfactants along with other nutrients that enhance the degradation of HCs.

25.5.4 Bioaugmentation

When the contaminated soil is deficient of endogenous oil-degrading microbes, microorganism efficient of degrading the HCs is supplemented in the soil, e.g., bacterial culture to speed up the HC's degradation, and this approach is known as bioaugmentation. This process can be done either in situ or ex situ; for ex situ treatment, soil is excavated and treated separately, whereas for in situ treatment, microbes are added at the polluted site. A combination of biological and non-biological processes is used in both in situ and ex situ treatment. In situ treatment also includes a process called bioventing; this technique involves the addition of oxygen in the polluted site which stimulates the growth of microorganisms that help in the bioremediation of contaminants (Maier 2000). Examples of ex situ treatment methods are land treatment (land farming), composting, windrows, bio-pile, slurry-phase bioreactors, etc.

Treatment of contaminated site is dependent on the solubility of the compound in water. The water-soluble contaminant is easy for microbes to degrade, and compounds with low solubility have limited degradation.

25.5.5 Bio-piling

This technique involves the grouping of composting and landfarming. In this method, engineered cells are built on the composted piles with proper aeration. Biopile comprises treatment bed, proper aeration, nutrients, irrigation system, and a leachate collection system. Temperature, pH, oxygen, nutrients, and moisture are regulated to enhance the biodegradation. Bio-piles are covered with plastic to control the volatilization, runoff, and evaporation. This method is used for the treatment of contaminated soil with spilled HC pollutants by making a conducive environment to grow native aerobic and anaerobic microorganisms (Sonawdekar 2012).

25.5.6 Use of Microbial Consortium

This involves two or more microbial groups living together symbiotically and helps in degradation of HCs. Some microbial consortium helps in the production of biosurfactants that enhance the degradation of oils. Biofilms produce by some microbial consortium can be of one type of species or of multiple species that requires energy from carbon source of organic compounds. *Marinobacter hydrocarbonoclasticus SP17* form biofilms also known as oleolytic biofilms at the interface of hydrophobic organic compounds (HCs and lipids) and water for the degradation of hydrophobic organic compounds (HOC's) (Grimaud, et al. 2012). Biofilm formation by some microbes at the interface is nutritive, and it is not observed in non-metabolizable compounds.

Some microbes show cometabolism, i.e., fortuitous metabolism at the polluted site, where microbes transform the compounds into degradable one, without deriving energy or growing on it. The coexisting microbes use this transformed compound as the source of energy and used it as a growth substrate. For example, *Mycobacterium vaccae* grows on propane while cometabolizing cyclohexane and oxidizing it into cyclohexanol, which can be utilized by some other microbes (Beam and Perry 1974).

Microorganism's uptake the HCs by three modes:

1. By utilizing the organic compound solubilized in water.
2. Microbes utilize the organic compounds which are in direct contact with the microbial cells either by cells appendages such as fimbriae (Rosenberg et al. 1982) or by modifying the cell membrane such as cell hydrophobicity. *Pseudomonas aeruginosa* bacteria reported to have high cell hydrophobicity and can degrade octadecane at a high rate (Zhang and Miller 1994).
3. Microbial contact with the submicrometer size substrate droplets in the liquid phase (Maier, 2000).

25.5.7 Recognized Bacteria to Degrade Hydrocarbons

Many microorganism and microbial consortium degrade the HCs in different environmental conditions. For example, *Archaebacteria* such as thermoacidophiles, halophiles, etc. help in the degradation processes. *Pseudomonas putida* is capable of degrading the HCs such as toluene and benzene (Gibson et al. 1970). *Pseudomonas oleovorans* help in the degradation of tetrahydrofuran (THF). THF and benzene, toluene, ethylbenzene, and xylene (BTEX) compounds are reported to be biotransformed and can be utilized by the *Pseudomonas oleovorans* DT4 species (Zhou et al. 2011). TOL plasmid of *Pseudomonas putida* has alkylbenzoate degradation genes which help in the degradation of aromatic HCs, and *Pseudomonas putida* is more suitable for HC degradation application than others (Kaldalu et al. 2000). It has been reported that *Virgibacillus salarius* bacteria use benzene, toluene, and ethylbenzene as a carbon source, catechol 2, 3-dioxygenase, and chlorocatechol 1,2-dioxygenase enzymes present in this bacterium help in the degradation of HCs (Solanki and Kothari 2012). Similarly, some more examples are cited in Table 25.1.

25.5.8 Degradation by Fungi

Several fungi are known to degrade HCs present in the contaminated sites (Haritash and Kaushik 2009). Fungi can metabolize the soluble organic compounds (Spellman 2008). Fungal enzymes involve in the breakdown of the polymeric compounds through mycelium systems (Matavulj and Molitoris 2009). There are two groups of

Table 25.1 Examples of hydrocarbon-degrading microorganisms

Microorganism	Target substrate	References
Pseudomonas putida	Toluene and benzene	Gibson et al. (1970)
Pseudomonas oleovorans	Tetrahydrofuran (THF)	Zhou et al. (2011)
Marinobacter hydrocarbonoclasticus	Several HCs	Gauthier et al. (1992)
Arhodomonasrozel	Toluene, benzene	Dalvi et al. (2012)
Thalassobacillus devorans	Various aromatic HCs and phenol	García et al. (2005)
Acinetobacter venetianus	n-alkanes	Di Cello et al. (1997)
Alcanivorax borkumensis	C_{14}-C_{15} n-alkanes	Yakimov et al. (1998)
Marinobacter vinifirmus	PAH, naphthalene, phenanthrene, pyrene	Cui et al. (2008)
Kaistiaadipata		
Pseudoalteromonas ganghwensis		
Thalassospira lucentensis		
Stappia aggregate		
Marinobacter alkaliphilus		
Oceanobacterkriegii	Petroleum	Teramoto et al. (2009)
Burkholderia cepacia F297	Naphthalene	Grifoll et al. (1995)
Comamonas testosteroni	PAH	Goyal and Zylstra (1996)
Nocardioides sp.	PAH	Iwabuchi et al. (1998)
Mycobacterium strain, PYR-I,	Pyrene and fluoranthene	Heitkamp and Cerniglia (1966)
Sphingomonas paucimobilis strain EPA 505	Fluoranthene	Mueller et al. (1990)

fungi that help in the degradation of PAHs, i.e., ligninolytic and non-ligninolytic fungi (Bamforth and Singleton 2005). Cytochrome P450 monooxygenase enzyme present in non-ligninolytic fungi such as *Cunninghamella elegans*, *Aspergillus niger*, and *Chrysosporium pannorum* helps in the breakdown of PAHs via oxidative pathway (Sutherland et al. 1995). Ligninolytic fungi have enzyme system consisting of manganese-dependent peroxidases (MnP), lignin peroxidases (LP), and laccases (Haritash and Kaushik 2009). Enzymes present in ligninolytic fungi such as *Antrodia vaillantii* and *Pleurotus ostreatus* involve in the oxidation of lignin present in organic matter (Bamforth and Singleton 2005). *Pleurotus eryngii*, i.e., white rot fungus investigated for the naphthalene degradation by deoxygenation mechanism, cleaving the C1 and C4 position of naphthalene and giving 1,4-naphthoquinone that further converts into benzoic acid and to catechol via decarboxylation and hydroxylation combination process. *Penicillium janthinellum* SFU 403 strain helps in the metabolism of pyrene via hydroxylation to 1-pyrenol hydroxylate and further to 1-pyrenol, followed by 1,6- and 1,8-pyrenequinones (Leitão 2009; Wang and Zhao 2007). Deuteromycete ligninolytic fungi are also involved in the breakdown of PAH (Clemente et al. 2001). PAH degradation by fungi is an environment-friendly approach and is more important because some products have been detected as lethal in higher forms of life (Cerniglia 1984).

25.5.9 Degradation by Algae

Many algae have confirmed their role in the degradation of PAHs such as *Scenedesmus platydiscus*, *Scenedesmus capricornutum*, *Scenedesmus quadricauda*, and *Chlorella vulgaris* (Wang and Zhao 2007). Cyanobacteria and marine algae are known to breakdown the naphthalene by different metabolites (Haritash and Kaushik 2009). Cyanobacteria fabricate four main metabolites, i.e., 1-naphthol, cis-naphthalene dihydrodiol, trans-naphthalene dihydrodiol, and 4-hydroxy-4 tetralone that play a role in the metabolism of naphthalene degradation (Cerniglia et al. 1980). *Pseudomonas migulae* and *Sphingomonas yanoikuyae* algal-bacterial microcosm have the potential of phenanthrene degradation (Haritash and Kaushik 2009). Likewise, fluoranthene, pyrene, and combination of fluoranthene and pyrene degraded by the action of some algae for, e.g., *Scenedesmus platydiscus*, *Selenastrum capricornutum*, *Scenedesmus quadricauda*, and *Chlorella vulgaris* (Ueno et al. 2008).

25.5.10 Degradation by Yeast

Yeast is a member of fungi kingdom that uptake and uses the aromatic HCs as a food source. *Trichosporon cutaneum* is a yeast present in the soil that utilizes the aromatic compounds (e.g., for phenol) (Mortberg and Neujahr 1985). Aliphatic HCs present in petroleum products and crude oil metabolized by *Candida lipolytica*, *Candida tropicalis*, *Aureobasidium* (*Trichosporon*) *pullulans*, and *Rhodotorula aurantiaca* (Miranda et al. 2007).

25.5.11 Protozoa Degradation

Compared to other microbes such as bacteria, fungi, and algae, protozoa are not a good biodegrader. Protozoa are not always significant for biodegradation as they reduce the number of bacteria available for HC removal (Stapleton and Singh 2002). *Heteromita globosa* flagellate protozoa reported to degrade the benzene and methylbenzene (Mattison et al. 2005). Protozoa infusorians reportedly fasten the biodegradation of PAHs in the environment (Chen et al. 2007). There are many theories that suggest the biodegradation acceleration mechanism of protozoa degrading the organic contaminants which include (1) mineralization of nutrients, (2) activation of bacteria, (3) reduction of the competition for resource and space and by increasing the growth of degrading bacteria by selective grazing, (4) increase in oxygen content, (5) direct degradation, and (6) cometabolism that offers carbon source to the bacteria for the degradation (Chen et al. 2007).

25.6 Role of Enzymes Involved in Hydrocarbon Biodegradation

Many enzymes are reported to degrade the HCs such as cytochrome P450 alkane hydroxylases that enhance the microbial degradation of chlorinated HCs (Van Beilen and Funhoff 2007). Under aerobic conditions, alkane oxygenase enzyme present in the bacteria, fungi, protists, plants, and animals helps in the degradation of HCs (Van Beilen and Funhoff 2005).

25.7 Factors Involved in the Biodegradation of Hydrocarbons

Numerous environment factors are involved in the biodegradation of HCs such as temperature, light, pressure, pH, salinity, oxygen, and nutrient. All these factors are described below (Maier 2000).

25.7.1 Temperature

Degradation of HCs can occur from zero to more than 30° C. Bacteria can accustom to temperature change to maintain the metabolism; on the other hand, wide temperature fluctuations can affect the rate of biodegradation.

25.7.2 Light

Photosynthetic microbes such as algae utilize the light and help in the breakdown of HCs and thus have a positive impact (Muñoz et al. 2003). By direct photochemical action, light can also degrade the petroleum compounds.

25.7.3 pH

Maximum degradation of HCs is observed at neutral pH, though microbes observed to grow on HC at pH 2–3.

25.7.4 Salinity

Salts present in soil and water have different effects on HC degradation. Salinity effect depends on the organism's type and also on the environment. Degradation process gets affected by the high concentration of salt (Kerr and Capone 1988).

25.7.5 Oxygen

HC degradation takes place in both aerobic and anaerobic conditions. In the presence of oxygen, i.e., the aerobic process is more favorable for the degradation of HC as this process involves oxygenase enzymes which enhance the rate of HC degradation.

25.7.6 Nutrient Availability

Addition of nutrients such as nitrogen and phosphorus enhances the biodegradation process. To degrade the petroleum oil spills, carbon, nitrogen, and phosphorus are added to the ratio of 100:10:1 (C/N/P) (Fig. 25.3).

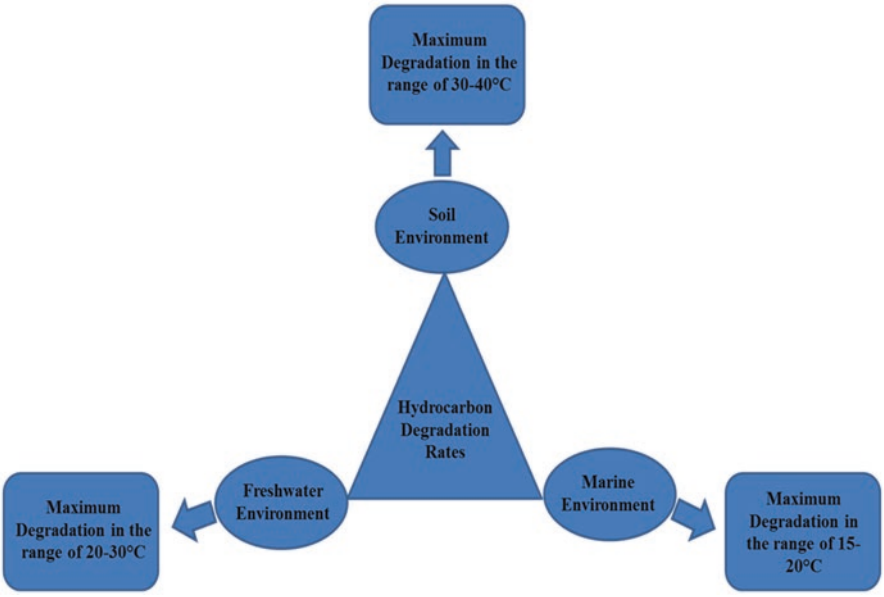

Fig. 25.3 HC degradation rates in soil, freshwater, and marine environments (Das and Chandran 2011)

25.8 Conclusion

The current technology and science offer different methods to remove or degrade the contaminants. There are many microorganisms that help in the breakdown of the contaminants such as hydrocarbons. Apart from prokaryotes, fungi, algae, protozoa, and enzymes involved also play a crucial role in the conversion of PAHs into less harmful compounds. Protozoa are not directly linked to the degradation process, but it increases the rate of remediation. Due to the accidental leakage of oil or petroleum products, there's a constant risk of contaminants to the whole biosphere, and because of this impact on flora and fauna, there's a need for an efficient strategy to deal with the HCs. It became necessary to consider the impact of HCs on the environment along with toxicological studies on risk for human health. A successful implementation of HC biodegradation studies involves grouping of different fields such as microbiology, biochemistry, genetics, environmental chemistry, and chemistry.

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Chapter 26
Hydrocarbon Degradation

Samina Siddiqui and Asghari Bano

Abstract Bioremediation involved the use of microbes to degrade petroleum hydrocarbons. Quite a number of microbial genera isolated from extreme environment mainly from petroleum-contaminated soils (mesophilic microbes), deep subsurface reservoirs (thermophilic microbes), and sediment under the permafrost (psychrophilic microbes) once inoculated may enhance the degradation/removal of petroleum in extreme environment in field. Limitation in bioremediation at field scale was that the bioavailability and biodegradation of some recalcitrant poly-aromatic hydrocarbons were not enhanced with mixed genera of microbes. Thus further improvement in bioremediation was made when biosurfactant- and bioemulsifier-producing microbial strain such as alasan, strain F2-7, strain TU was inoculated to petroleum-contaminated soils. Considerable increase in the bioavailability and biodegradability of poly-aromatic hydrocarbons was recorded. However the mode of transport and mechanism of intercellular or extracellular microbial degradation of hydrocarbons remained debatable. Similarly biosurfactants and bioemulsifier are expensive, thus limiting their use commercially. Recent advances in the use of nanoparticle to enhance the surface area and bioavailability of hydrocarbons to microbes are under experimentation. Further detail study is required to understand the response of microbes to nanoparticle and then the mode of adaptation and mechanism of degradation adopted by microbes. Appropriate maintenance of microbial population and soil conditions required for effective degradation of hydrocarbons in the field also need further investigation. To avoid this problem, plants are used with microbes and promising results have been recorded in the field. Bioremediation is successful in remediating the surface soil; however, subsurface soil or saturated zone remained unreclaimed with this technique. Thus there is need to develop such bioremediation technique that can degrade hydrocarbon from saturated subsurface zone of the soil.

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26.1 Introduction

Indecent mode of disposal of petroleum contaminants to terrestrial environment may turn numerous sites around the world unreclaimed. Productive sites are under constant threat to the living bodies. These sites are intolerable to microbiota to grow unless dealt with carefully. Gradually such sites become barren and cannot support plant growth over a long period of time. Persistent contaminants in such unreclaim sites are constant threat to aquatic lives. Further delay in the reclamation of these sites may require enormous effort, investment, and time. The current available digging, dumping, excavation, transportation, and incineration methods are very costly. It has been estimated that the cost involved to reclaim these sites is extremely high and may reach to 500 billions of dollars around the world (Tsao 2003). However, in the USA the cost may be of 6–8 billion US dollars. According to the European Environmental Protection Agency report (2000), the cost to reclaim contaminated sites is around 115 billion euros (Van Camp et al. 2004). Oil companies and the government are unable to pay this amount, thus limiting the success of these methods. Penalty is being paid by the companies to the government and the community. Thus, search for economic and rapid method with no or minimum environmental risk continued over the last several decades.

26.2 Traditional Methods to Remediate Petroleum-Contaminated Soils

Petroleum waste is composed of variable number of carbon chain length that differs structurally from each group. Petroleum waste is composed of saturate, aromatics, resins and asphaltenes (Vdovenko et al. 2015). Saturates are present at high concentration and composed of straight-chain hydrocarbons such as *n*-paraffins, whereas aromatics are benzene ring hydrocarbons present at low concentration. Resins are present in minute concentration, whereas asphaltenes in bitumens are also present in traces in petroleum waste (Alexdander 2012).

This difference in the carbon chain hydrocarbons and benzene rings had a pronounced effect on their rate of degradation. It is well documented that short carbon chain hydrocarbons structurally bound as C-H *n*-alkanes are more susceptible to degradation than long and middle carbon chain hydrocarbons (Singh et al. 2011). These hydrocarbons with carbon numbers nC_{15} to nC_{44} are considered to be non-toxic to some flora and funna in the soil. Thus can persist in the environment over centuries having no hazardous effect. Similarly one benzene ring aromatic hydrocarbons are also considered to be more degradable and toxic than two- or three-ring poly-aromatic hydrocarbons (Hu et al. 2014).

When petroleum waste spilled over the terrestrial environment, quite a numerous off-site and on-site biological techniques are applied. The off-site bioremediation techniques are mainly land farming, biopiling, and composting, whereas in situ

bioremediation techniques are bioremediation, biosparing, and bioventing (Camenzuli and Freidman 2015). Bioremediation (off site or in situ) applied under different climatic conditions (warmer and colder) differs because of the differences in the soil physicochemical characteristics and climatic conditions. Under warmer climatic condition, surface spread or land farming and biopiling for bioremediation of hydrocarbons are recommended. Marin et al. (2005) performed ex situ bioremediation experiment under semiarid climatic condition at Cartagena, Murcia (SE Spain). Three 600 m^2 plots for carrying out the bioremediation process (land farming) and another three for control soil were established; 230.7 g per kg of hydrocarbon content was remediated over a period of 11 months. Simple aeration enhanced 80% degradation in 11 months.

Hazen et al. (2003) have performed in situ bioremediation via biopiling of petroleum sludge generated from oil refinery in Poland. The petroleum sludge was placed in a biopile, and temperature was maintained at optimum necessary for microbes to degrade hydrocarbons. Enough nutrients, surfactants, and aeration were supplied to enhance degradation. They reported that around 81% (120 metric tons) of petroleum sludge was remediated over a period of 20 months. They demonstrated biopiling as a cost-worthy, rapid technique to eliminate the injurious effect of petroleum sludge impregnated soils.

Land farming is well known as an off-site biological technique applied to remediate petroleum-impregnated soils around the world. Land farming involves surface spread of petroleum-contaminated soils subsequently treated with nutrients and aerated and then was ploughed. Paudyn et al. (2008) carried out surface spread bioremediation of oil-impregnated terrestrial environment of arctic region of Canada. They reported that land farming with nutrients enhanced degradation of petroleum hydrocarbon even at low temperature (30 °C).

Land farming is a cost-effective method used to remediate petroleum-contaminated soils. However land farming is not suitable for heavily petroleum-contaminated soils. Land farming may enhance subsurface contamination, and thus complete remediation of petroleum-contaminated soils with land farming in warmer climate is debatable. For successful remediation of petroleum hydrocarbons, it is necessary to maintain the optimum soil conditions for land farming. Unluckily the soil moisture, temperature, oxygen, and nutrients are rather difficult to maintain under cold soil conditions than warm soil conditions. Thus fluctuation in soil moisture and temperature during land farming may delay the rate of degradation of petroleum hydrocarbons and thus enhance the cost involved in land farming. Saturated conditions that prevail may result in lack of oxygen, thus limiting the success of land farming in soils near permafrost table (McCarthy et al. 2004). Similarly inappropriate dose of irrigation to petroleum-contaminated soils during land farming may also limit the mineralization of petroleum waste soils. This suggests that microbes cannot survive under waterlogged conditions. For maintenance of appropriate moisture and temperature, compost was applied during land farming. Compost maintain the optimum soil conditions which is necessary for microbes to degrade hydrocarbons (Anastasi et al. 2008). The success of bioremediation to remedaite oily sludge contaminated soils under various climatic regimes have been recognized around the world (See Table 26.1).

Table 26.1 The total area reclaimed by bioremediation at oil field sites around the world

S. No	Region/oil field	Area (%) or acre	Technique	Microbe genera	Reclamation duration	Reference
1	Shengli-China	30 m^2	Bioremediation	Heterotrophic bacteria	360 days	Liu et al. (2010)
2	India (Assam, Gujrat, Tamilnudu)	30,706 tonnes	Bioremediation		33 months	Mandal et al. (2014)
3	India	48,941 tonnes	Bioremediation		12 months	Mandal et al. (2012)
4	India (Assam)	50,000 tonnes	Bioremediation	Novel yeast strain *Candida digboiensis* TERI ASN6.	175 days or 6 months	Sood et al. (2010)
5	Canada	600 mg per kg	Biopile	Aeration and moisture content	110 days	Akbari and Ghoshal (2014)
6	Antarctica	2180 mg kg^{-1}	Biopile		40 days	Martínez et al. (2017)

26.2.1 Biopiling

Biopiling is also an ex situ bioremediation technique in which optimum soil conditions are created with continuous supply of oxygen and heat through injection well, whereas moisture is maintained through irrigation. Efficient drainage system is installed to collect leachate. Further improvement was made and some accelerator such as saw dust, compost, wood chips are added to accelerate the rate of degradation of hydrocarbons. Biopiling is recommended to remediate petroleum-contaminated soils for arid to semiarid and arctic climatic condition. Under arctic condition, controlled biopiling is recommended. Contaminated soil beds are developed over the impermeable layer placed on the soil surface to avoid downward contamination (Azubuike et al. 2016; Table 26.1). However biopiling maintenance in the field throughout the remediation period is expensive. Continuous supply of heat may cause decrease in soil moisture required by microbes for remediation (Sanscartier et al. 2009).

Keeping in view the limitation faced in rehabilitation of petroleum waste contamination via land farming and biopiling, bioremediation is recommended.

26.3 Biological Method or Bioremediation

Bioremediation in which microbes are used to reclaim and reduce the threshold levels of undesirable and injurious contaminants to tolerable level without high investment, labor, and time, gaining scant attention all over the world. Success of this method is more acceptable because of lack of environmental risk. Numerous in situ and ex situ bioremediation methods were applied, and huge amount of contaminated soils has been reclaimed around the world (Table 26.1). The success of bioremediation to remediate oily sludge-contaminated soils under various climatic regimes has been recognized around the world. Nevertheless, investigations are in progress to increase the efficiency of this method in order to reclaim these sites over a short period of time.

Prior to discuss in detail the challenges faced in adaptation of biological methods to reclaim such sites completely, it is necessary to provide a general background about the composition of contaminants and their rate of degradation under various climatic regimes such as semiarid and arctic climatic conditions.

Presence of diverse nature of various groups of compounds in petroleum, it is necessary to understand their extent and degradation under certain specific climatic conditions prior to design a remediation technique to reclaim these sites completely.

26.4 Fate and Effect of Petroleum Hydrocarbon Under Extreme Environment

Petroleum hydrocarbon contamination once spilled over terrestrial environment under arid or arctic region causes severe changes in the soil microbial population which affect their activity necessary for degradation of such hydrocarbons.

The fate of petroleum hydrocarbon when spilled over the arid or arctic terrestrial environment primarily depends on the viscosity and secondarily on soil conditions and climate of the region (Bartha 1986). Highly viscous hydrocarbons tend to float over the soil surface, whereas lighter hydrocarbons penetrate downward (Zhang et al. 2012). Petroleum hydrocarbon behavior when spilled over the arid or arctic terrestrial environment primarily depends on soil conditions (Bartha 1986). Soil conditions are quite variable under arid and arctic environment.

Dryland regions with aridity index (AI) (the ratio of total annual precipitation to potential evapotranspiration, P:ET) ranged from 0.05 to 0.65 (Reynolds et al. 2007). Dryland regions occupy an area of 6.31 billion hectares (Bha) over the earth surface (Lal 2009).

Dryland soil texture is quite variable. Some dryland soils are sandy in nature with minimum water and nutrient retention capacity because of weak structure, whereas others are silty clay loam to clay loam in nature, with high water and nutrient retention capacities due to strong structure (Lal 2004).

When petroleum hydrocarbon spilled over the sandy soil of dryland, lateral distribution of petroleum hydrocarbon over the sandy soil surface of dryland is commonly observed because of the availability of greater pore size of sandy soil. However horizontal distribution of petroleum hydrocarbon is also recorded over such soils. Nonetheless petroleum hydrocarbon distribution is texture- and structure-dependent, and thus over clayey or silty clay soil horizontal distribution of such hydrocarbons occurs. Clayey soil has less pore size and may form hardpan underneath the soil surface; thus lateral distribution of petroleum is slow unless cracks or root zones are present under the subsurface. Thus horizontal distribution of petroleum is commonly observed under such soil condition. Desert where soils are sandy in nature, petroleum distribution can be lateral rather than horizontal. Arctic region snow covers the surface soil and forms the impermeable barrier, horizontal distribution of petroleum is recorded. Nonetheless the presence of cracks, fissures, or pore spaces may facilitate lateral distribution of petroleum even underneath the saturated soil. Thus downward petroleum contamination is slow under arid or arctic environment than temperate and semiarid environment (Caravaca and Roldán 2003; Filler et al. 2008).

Unlike arid region, arctic region covered with permafrost may influence the soil conditions underneath. The soil microbial processes may also become effected because of anoxic conditions, lack of nutrients availability and low temperature (Filler et al. 2008; Greer and Juck 2017). Arctic soil conditions are quite variable. Soils near permafrost table is saturated with water entire year (Drew and Tendrow 1962). Thus waterlogged conditions prevail entire year. Soil away from permafrost table covered with snow because of freeze and thaw cycle the entire year. Arctic soil is deficient in nutrient thus microbial activity is extremely slow (Walworth et al. 2007). Arctic forest soils are not deficient in organic matter. However organic matter mineralization remained a problem in such soils (Paré and Bedard-Haughn 2013). Therefore proper understanding of permafrost, physical process of freezing and thawing with in-depth study of soil conditions may necessary to evaluate when designing bioremediation technique to accelerate degradation of petroleum hydrocarbons in such soils.

Petroleum hydrocarbon distribution in arctic soil is climate dependent phenomena. Under warmer climate petroleum hydrocarbon is distributed vertical whereas during freezing and thawing the distribution is horizontal and may covered the permafrost underneath the soil surface (Yang et al. 2014). Thus become sorbed in the soil sediments and become unavailable for microbes for degradation (Aislabie et al. 2006).

Prior to discussing in detail the mode of degradation adopted by microbes under arid or arctic regions for petroleum hydrocarbons, it is necessary to understand the factors affecting the degradation of petroleum hydrocarbons under extreme environment.

26.5 Factors Effecting the Microbial Degradation of Petroleum Hydrocarbons Under Arid and Arctic Soils

26.5.1 Soil Texture

Soil texture is quite variable in arid and arctic soils. Some arid soils are sandy in nature with minimum water and nutrient retention capacity because of weak structure, whereas others are silty clay loam to clay loam in nature, with high water and nutrient retention capacities due to strong structure (Lal 2004). Arctic soils have less surface horizon of gravel, stones, or boulders, subsurface freeze and thawed permafrost horizon have lost unconsolidated material with low clay content (Margesin 2009).

Petroleum hydrocarbon distribution once spilled over the arid or arctic soils depends on soil texture. Arid sandy soils may facilitate lateral distribution of petroleum hydrocarbon. This is more likely because of the availability of greater pore size of sandy soil. Petroleum hydrocarbon horizontal distribution in clayey or silty clay soil is recorded (Abdel-Moghny et al. 2012). Clayey soil has less pore size and may form hardpan under the surface soil; the lateral distribution of petroleum is slow unless cracks or root zones are present under the subsurface. Thus horizontal distribution of petroleum is commonly observed under such environment. In the desert where soils are sandy in nature, petroleum distribution can be lateral rather than horizontal. Whereas in arctic environment snow covers the surface soil and forms the impermeable barrier underneath the surface, horizontal distribution of petroleum is recorded (Lawrence et al. 2015). Nonetheless the presence of cracks, fissures, or pore spaces may facilitate lateral distribution of petroleum even underneath the saturated soil (Erikkson et al. 2001). Thus downward petroleum contamination is slow under arid or arctic environment than temperate and semiarid environment.

26.5.2 Soil Moisture Content

Soil moisture content depends on the climatic conditions of the region. Under arid region where evaporation exceeds precipitation, soil moisture content does not follow the same pattern throughout the year. It has been recoded that soil moisture content is less than 15% in arid soils, whereas arctic soils near permafrost table remained saturated with water throughout the year (Krogh et al. 2017, Walvoord and Kurylyk 2016, Swindles et al. 2017). Arctic soils away from the permafrost have low moisture content like arid soils. Optimum soil moisture ranges from 50 to 80% necessary for microbes to degrade hydrocarbons in petroleum-contaminated soils (Bossert et al. 1984). Dibble and Bartha (1979) reported that optimum moisture content from 30 to 90% may be enough for microbes to degrade hydrocarbons.

Malakahmad and Nuramalina (2013) reported that 50–60% of moisture content was enough to degrade petroleum-contaminated soils. Braddock et al. (1997) reported that moisture content of 1–3% inhibit microbial activity in petroleum-contaminated soils.

26.5.3 Soil Temperature

Soil temperature contributes significantly to degrade petroleum waste. Optimum temperature requires for microbes to grow in soil ranges from 20 to 40 °C (Alexander 1965; Margesin and Shinner 1997; Singh and Swaranjit 2013; Alrumman et al. 2015; Müller et al. 2016). However microbes can survive under temperature below 20 °C and above 45 °C. Microbes grown under low temperature are known as psychrophilic, whereas those at high temperature are thermophilic and those at intermediate temperature range are mesophilic (Roth and Wheat 1962; Pankowski et al. 2013). Microbial degradation of petroleum hydrocarbons at all temperature ranges is recorded. Nonetheless the rate of microbial degradation of hydrocarbon is rapid at a temperature range of 20–40 °C (Anwar et al. 2017). Seckbach (2013) explained that thermophilic bacteria can increase the mineralization of petroleum waste when spilled on to desert soils. Whereas psychrophilic bacteria can degrade petroleum hydrocarbons under arctic soils (Margesin et al. 2008; Das and Chandran 2011).

26.5.4 Soil Organic Matter

Soil organic matter sequestered more than 90% of nitrogen, and around 2–4% of nitrogen is made available to plants by soil microorganisms yearly (Mooshammer et al. 2014). Thus soils with low organic matter are usually low in nitrogen (Ren et al. 2014). Nitrogen is an important nutrient for plant growth and development. The significance of soil organic matter for aggregate stability, building particle structure, holding soil moisture and nutrients, and maintaining microbial activity in soils is well understood (Baldock and Nelson 2000). Thus soils with high organic matter have good structure, and particles are strongly bonded and thus reluctant to soil loss (Schoonover and Crim 2015).

Total carbon stock in arctic cryosols varies depending on the soil type. Arctic forest ecosystem soil has high organic matter content than arctic soil saturated with water near permafrost table (Atlas, 1985). While arctic landscape soils are also high in organic matter because of plant material, this organic matter is not available (White et al. 2013; Sobak et al. 2014). Mineralization of organic matter releases soil organic carbon which is important for microbes to conduct degradation of hydrocarbons. Nearly carbon to nitrogen ratio of <30 is necessary for microbes to carry out degradation of petroleum hydrocarbons (Agamuthu et al. 2013). Agamuthu et al. (2013) performed a laboratory-scale study to study the effect of adding organic

waste (sewage sludge and cow dung) in the degradation of lubricating oil. They added 10% of sewage sludge or cow dung to the 1.5 Kg of soil spiked with used lubricating oil. They concluded that nearly 98% of oil was degraded in soil with cow dung over a period of 98 days. This is more likely because enough carbon and nitrogen were present in the soil for microbes to enhance degradation.

Liu et al. (2013) performed land farming batch study at a laboratory scale over a period of 300 days. The study was designed to understand the relationship between organic matter and degradation of diesel hydrocarbon. They reported that around 73% of diesel hydrocarbon was degraded in soil spiked with 4000–12,000 mg/kg when soil organic matter was 2.3% and inoculated with bacterial consortium with strains BA015, BA092, and BA125. They found that the increase in soil organic matter up to 11.8% reduces the concentration of diesel even when inoculated with mixed genera of microbes.

26.5.5 Soil Nutrients

Soils of arid region are low in nitrogen and carbon naturally. Thus microbial activity is slow in such soils. Petroleum hydrocarbons degradation by microbes is very slow in arid region. Virtually the same trend is observed in arctic soils because these soils are also low in nitrogen (Maslov and Makarov 2016). Snelgrove (2010) reported that the concentration of nitrogen which was five times more than phosphorus may be enough for the degradation of petroleum hydrocarbons under arctic or subarctic regions. The concentration of nitrogen which was ten times more than phosphorus was enough for petroleum hydrocarbon degradation under arid region (Smith et al. 2015).

26.5.6 Soil pH

Soil pH is quite variable under arid and arctic conditions. Arid soils are alkaline to hypersaline whereas arctic soils are acidic to alkaline in nature (Jiao et al. 2016; Nuttall 2004; Day and Ludeke 1993). Relative abundance and diversity of microbes and fungi is pH dependent (Wang et al. 2007). Under extreme acidic and hypersaline condition, inhibition of microbial growth occurs (Ghosal et al. 2016). Acidophilic microbes can survive under acidic pH, whereas alkalinophilic microbes can survive under alkaline to hypersaline pH (Ashok et al. 1995). Fungi can survive under both acidic and alkaline pH (Ghosal et al. 2016). Optimum pH necessary for microbes to degrade hydrocarbons under temperate soil is 6.0–8.0 with salt content of 20% (Goltapeh et al. 2013; Pim de Voogat 2016). Prakash et al. (2015) found that optimum pH of 7.5 was necessary for bacteria to degrade poly-aromatic hydrocarbons in arctic, arid, and temperate soil. Bell et al. (2013) and Gomez and Sartaj (2014) concluded that in polar soil, hydrocarbon mineralization by microbes was

enhanced at pH > 8.8. Further increase in pH up to 9 poses no effect on microbial mineralization of petroleum waste (Filler et al. 2008). Microbes can tolerate 20% of salt for the degradation of petroleum hydrocarbons.

26.6 Role of Microbes in Bioremediation Under Extreme Environment

Soil conditions at optimum are necessary to maintain for enhancing microbial activity, which is an important tool of bioremediation under arid or arctic environmental conditions. The role of microbes was well accepted since 1940s to overcome the catastrophic situation created because of petroleum hydrocarbon contamination. Numerous classical studies were carried out under controlled conditions when bacterial culture medium such as mineral salt media (MSM) or Bushnell and Haas medium (1944) was impregnated with few oil drops, and degradation was recorded with an increase in bacterial growth and disappearance of oil drops over a period of time. Bacteria efficiency to utilize oil in such medium with consortium was experimented in the field. Consortium prepared from mixed genera of bacteria carry genes to assimilate hydrocarbons when removed from the petroleum stress environment showed positive progress in remediation of petroleum contaminated soil in the field.

Nonetheless maintenance and survival of microbes under extreme environmental and climatic conditions to reclaim complex mixture of contaminants remained debatable. It is well documented in the previous literature that when petroleum contaminants are spilled over the terrestrial environment of semiarid region, the role of microbes is efficient than in arctic region of the world (Yang et al. 2017). This is more likely because in semiarid region, the soil conditions are conducive to microbes, whereas in arctic region, soil is deficient in nutrients and lacking enough oxygen necessary for the proliferation of microbes to increase in number essential for degradation. Similarly optimum temperature is low in arctic soil than semiarid soils. Thus, microbial activity is slow in arctic soils than semiarid soils. Suitable pH (alkaline to neutral), enough carbon source (more organic matter present), nutrient availability (nitrogen and phosphorus), and survival temperature (nearly 20–30 °C) with enough moisture are the factors that can help microbes to act efficiently to take spilled petroleum as a growth accelerator. Graj et al. (2013) and Delille et al. (2003) found that alkaline pH, with enough moisture, oxygen, and nutrients under semiarid climate, enhanced the bacterial population.

Microbial degradation of saturates, aromatics, resins, and asphaltenes varies from genera to genera. This is not necessary that single genera of microbe can degrade all groups of compounds in petroleum. This is more likely because of the difference in the microbial genetic characteristics. This problem was dealt with the development of suitable consortium of microbes (mixture of various genera of bacteria according to their suitability for degradation of wide range of groups of hydrocarbons in petroleum) in the laboratory. Success of inoculation of such consortium

to semiarid contaminated sites turned the contaminated sites in productive sites. But again the inoculation was rapid when climatic and environmental conditions was conducive to consortium.

It is acceptable that consortium can degrade hydrocarbons rapidly when inoculated to the petroleum-contaminated soil. The rapid degradation was achieved worldwide under natural conditions conducive to microbes to grow and increase in number. Consortium was added and microbial population is maintained as 10^7 during the entire period of experimentation. Aeration is also maintained for microbial population (Paudyn et al. 2008; Akbari and Ghoshal 2014).

Quite a number of consortiums were prepared from double or multiple genera of microbes isolated from diverse environment such as desert soils, arid to semiarid soil, and arctic soils. These microbes differ from each other because of the difference in the soil conditions of arid and arctic region (Mironov et al. 2015). Arid region with temperature range above 40 °C may have mesophilic bacteria, whereas most of the microbes in arctic soil are psychrophilic or cold-loving microbes (Gao et al. 2007) (Table 26.2).

Mao et al. (2012) reported the success of *Sphingobacteria* and *Proteobacteria* consortium inoculated to the soil become contaminated with poly-aromatic hydrocarbons (PAHs). They found that nearly 80–90% of PAHs were degraded over a period of 2 months. They concluded that soil condition conducive to consortium was necessary for enhanced degradation of hydrocarbons.

It is well documented that the bioavailability and biodegradation of some recalcitrant aromatic hydrocarbons remained a problem even with consortium. Further improvement in the development of consortium was made when bacteria and fungi combined genera was used as a consortium to remediate recalcitrant aromatic hydrocarbon-contaminated soil. Bacteria can degrade all type of hydrocarbons, but in some cases to enhance the biodegradability of some recalcitrant hydrocarbons such as poly-aromatic and biphenyl fungi is applied in combination with bacteria. Fungi because of the presence of extracellular lignin enzyme such as phenol oxidase (Lip) and heme peroxidase (Mnp and Laa) structurally the same as poly-aromatic hydrocarbons thus can degrade highly recalcitrant hydrocarbons faster than bacteria (Mehdi et al. 2010; Adenipekun 2008; Falade et al. 2017).

A successful combination of bacteria and fungi as a consortium was prepared by Marchand et al. (2017). They reported that bacterial strains belong to the *Sordariomycetes, Actinobacteria, Betaproteobacteria, Gammaproteobacteria*, and *Rhodococcus* sp.; fungi like *Trichoderma tomentosum* and *Fusarium* oxysporum can degrade poly-aromatic hydrocarbons and poly hydrocarbons when inoculated to petroleum-contaminated soils.

Fungi that can survive under acidic condition are mainly recommended for arctic soil where pH is 2 to 3 (Dix and Webster 1995). However the use of fungi consortium under alkaline condition or highly saline condition cannot be recommended. This limits their application under arid or semiarid condition where pH is above 9 or even 10 in some areas. Nonetheless some fungi such as *Mortierella*, *Peziza*, *Trichosporon* sp. DF-M, and *Penicillium citrinum* DF-3 survive under neutral to slightly alkaline conditions (Warcup 1951; Yamanaka 2003). Limited information is

Table 26.2 Diversity of microbial genera and strains isolated from arid and arctic regions of the world

S.No	Region	Type	Bacteria	Yeast	Fungi	technique	Carbon chain degradation	References
1	South Victoria Land, Antarctica	Polar desert soil		*Dioszegia, Leucosporidium*, and *Cryptococcus*		Plasmid DNA	Not tested	Connell et al. (2008)
2	Siberia and Alaska	Pristine Alpine soil	*Burkholderia*, *Novosphingobium*, and *Sphingomonas*				*n*-alkanes and aromatics	Yang et al. (2016)
3	Austria		*P. putida xylE*, *ndoB*, *Mycobacterium* sp. strain PYR-1 *nidA*)				Aromatics	Margesin et al. (2003a, b)
4	China	Polar Marine sediment	Arctic psychrotrophic bacterium *Pseudoalteromonas* sp. P29			Plasmid DNA	n-alkanes C12 to C20	Lin et al. (2009)
5	Chile	Temperate climate	*Pseudomonas* sp. SOD-3 and *P. putida*			Plasmid DNA	Aromatic and alkanes	Ma et al. (2006)
6	Chile	Antarctic soil	*Sphingobium xenophagum* D43FB				Phenanthrene	Gran-Scheuch et al. (2017)
7	*Canada*	*Antarctica and Arctic*	*Rhodococcus*				*All straight chain hydrocarbons*	Yang et al. (2014)
8	*Kuwait*	*Desert soil/ Arabian gulf*	*Bacillus stearothermophilus* strains				*nC10 to nC14*	Sorkhoh et al. (1993)
							nC15–nC17	
							Naphthalene, phenanthrene, anthracene	

available in the previous literature about the importance of fungi to mineralize petroleum waste under hypersaline condition. Qin et al. (2012) isolated mesophilic fungi from saline-alkaline soils sporadically become contaminated with petroleum. They found that these fungi were able to degrade crude oil from the saline soil with 5–10% of sodium salt. Unlike fungi, bacteria and archaea and a few eukaryotes can survive under 0–30% of salt content whereas salt-tolerant bacteria such as halophilic or halotolerants can survive under ≤1–13% (w/v) of salt content (Mishra and Tanna 2017; McGenity 2010).

Prior to use consortium to remediate saline petroleum-contaminated soil, the saline soil is leached with water to reduce the pH. Thereafter, the soil was inoculated with consortium and nutrients, and enhanced degradation of petroleum hydrocarbons was noted. Qin et al. (2012) reported that *Trichosporon* sp. DF-M and *Penicillium citrinum* DF-3 were able to degrade petroleum hydrocarbons when pH was reduced to 8, when excessive salt was leached down through soil washing.

Soil washing is expensive and unsuitable to reclaim petroleum waste impregnated soil in the field. Similarly the use of fungi as a consortium may degrade some specific group of hydrocarbons such as poly-aromatic hydrocarbons, whereas other group of hydrocarbons such as *n*-alkanes and poly chloro biphenyl may remain a problem in such soils. Similarly the availability of some resins and biphenyl for degradation to microbes cannot be enhanced with fungi alone or in combination with bacteria. Thus to increase the bioavailability and biodegradability of hydrocarbons, some biosurfactant and bioemulsifier bacteria were also isolated and consortium was prepared. Biosurfactant produced by bacteria is applicable in bioremediation via soil washing and flushing techniques (Bustamante et al. 2012). The success of these techniques with use of biosurfactant producing bacteria is explained by Usman et al. (2016) and Liu et al. (2018). Barin et al. (2014) reported the success of biosurfactant- and bioemulsifier-producing bacteria, namely, *Bacillus subtilis* tb1 and *Pseudomonas aeruginosa* sp., to degrade petroleum hydrocarbons. Parthipan et al. (2017) reported that *Bacillus subtilis* A1 when inoculated to crude oil is able to degrade 80% over a week of incubation.

Barkay et al. (1999) reported the efficiency of an emulsifier produced by bacteria known as alasan in degradation of poly-aromatic hydrocarbons (PAHs) at laboratory scale. *Sphingomonas paucimobilis* EPA 505 increases the solubility of two to three benzene ring aromatic hydrocarbons in culture media.

Under arctic condition when emulsifier or biosurfactants were used, they can degrade petroleum hydrocarbons under low temperature. Biosurfactant, because of their high degradability, increases surface activity, and nontoxicity enhances their application in bioremediation. Nonetheless surfactant production at laboratory scale may not be enough to be applied on large scale commercially. This may increases the cost of using biosurfactants to remediate petroleum-contaminated soils commercially.

The use of the same consortium efficiently to degrade petroleum hydrocarbons under arctic region needs further investigations. Quite a numerous studies reported that arctic soil with acidic condition may have some identical genera that occur in semiarid soil (Ferrera-Rodríguez et al. 2013). *Gammaproteobacteria* and

psychrophilic groups are found under such condition (Williams et al. 2010; Stomeo et al. 2012). Particular genera that belong to this group and commonly occur in cold and dry arctic conditions are *Pseudomonas*, *Actinobacteria*, and *Rhodococcus* (Ganzert et al. 2014). Thus when the growth temperature of microbes and soil temperature are suitable for microbial survival, enhanced degradation of hydrocarbons is recorded even under cold environment. The limiting factor in arctic soil is not the presence of efficient microbes genetically suitable to degrade complex mixture of hydrocarbons rapidly but is the lack of availability of nutrients and organic matter. Similarly the difference in the temperature necessary for maximum microbial growth and soil temperature may also be a limiting factor in the slow degradation of hydrocarbons in arctic region. When arctic microbes as a consortium were inoculated to the contaminated soil under controlled environment, may result in rapid degradation than under uncontrolled conditions.

26.7 Pathway Adopted by Microbes to Degrade Alkanes

Naturally bioremediation is carried out by microbes that carry genes having genetic encoding that respond positively when exposed to petroleum contamination under extreme environment. Recent advances in the molecular technique to understand the mode and transformation of diverse groups of hydrocarbon by specific mesophilic microbial gene encoding plasmids or enzymes or protein plasmids coupled with optimum conditions necessary for them to carry out their activity under petroleum-contaminated environment have provided us in-depth knowledge of advancement in the bioremediation. Nonetheless information about the psychrophilic microbial gene encoding enzymes involved in bioremediation is lacking. Furthermore limited information is available in the previous literature to understand the mode adopted by such microbes for intercellular or extracellular degradation of hydrocarbons. Therefore a brief description about the mineralization of diverse groups of hydrocarbon and the processes involved in the degradation under optimum condition via bacteria are presented below.

Pathway followed by microbes to degrade *n*-alkanes was presented in detail by Zobell (1950) which was further confirmed by Foster (1962), Van der Linden and Thijsee (1965), McKenna and Kallio (1965), Rosenberg and Ron (1996), and recently Zampolli et al. (2014a, b) and Kothari et al. (2016a, b). According to earlier studies, the terminal oxidation of carbon proceeds by monooxygenase protein catalyzed by NADH-rubredoxin reductase in primary alcohol and aldehyde which are subsequently converted in aldehyde and monocarboxylic acid. Further oxidation of carboxylic acid results in release of double fatty acids and acetyl coenzyme A with carbon dioxide (Atlas and Bartha 1978).

Until recently unique *alkB* gene expression in *Rhodococcus opacus* reported in a number of studies results in the formation of primary alcohol and 1-hexadecanol as terminal pathway (Zampolli et al. 2014a, b). Subterminal pathway via formation of secondary alcohol and ketone by B-4 *alkB1* and *alkB2* genes *of Rhodococcus opacus*

able to convert *n*-alkanes (*n*-pentane to *n*-hexadecane) to their respective alcohols is also reported. Holst et al. (2006) reported that encoding gene involved in the degradation of short to medium carbon chain hydrocarbons ($n\mathrm{C}_{10}$ to $n\mathrm{C}_{18}$) is alkMa and alkMb present in *Acinetobacter*.

Tribelli et al. (2017) isolated *alkB* gene carried by *Pseudomonas extremaustralis*. They concluded that *alkB* gene can degrade *n*-decane ($n\mathrm{C}_{10}$) to nonadecane ($n\mathrm{C}_{19}$) under low toxic conditions. Tribelli et al. (2018) and Tribelli et al. (2012) isolated *Pseudomonas extremaustralis* from Antarctica. This strain is resistant to cold temperature and forms biofilms to survive under freezing because of Anr (anaerobic global regulator). The growth of this strain was monitored under diesel-enriched culture media. The rapid mineralization of diesel is linked with this strain. This revealed the contention that the above mentioned bacterial strain can mineralized diesel under microaerophilic environment.

Poehlein et al. (2017) reported that *Oleovorans* strain of *Pseudomonas* rapidly degrades straight carbon chain and isoprenoids. The use of this strain in the degradation of petroleum hydrocarbon has not been reported in this study. Therefore the role of this stain in bioremediation is lacking.

The significance of *Pseudomonas* in the degradation of diverse nature of hydrocarbons was recognized nearly five decades before (Vinothini et al. 2015). Nonetheless the *Pseudomonas putida* GPo1 previously known as *Pseudomonas oleovorans* GPo1 strain GPo1 and the OCT-plasmid was first reported by Van Beilen et al. (1994, 2001). Van Beilen et al. (2002) reported the significance of alkane rubredoxins (Alk-Rds) isolated from *Pseudomonas* in the degradation of straight-chain carbon compounds.

Marginsen et al. (2003a, b) first studied the effect of inoculation of genes *alk* B, M, B1, and B2 on the degradation of *n*-alkanes under Alpine soil, which become contaminated with oil and pristine hydrocarbons. Whyte et al. (2002) found that some psychrotrophs gram-positive bacterium such as *Rhodococcus* strain is capable of degrading *n*-alkanes $n\mathrm{C}_{12}$ to $n\mathrm{C}_{32}$ under cold environment. They concluded that psychrotrophs because of the alkane degrading gene is suitable for arctic soils sporadically become contaminated with low carbon chain oil.

Das et al. (2015) while studying the performance of strain N002 Pseudomonas aeruginosa in mineralization of crude oil found that strain N002 may be used as degrader, emulsifier, and assimilator of crude oil.

26.8 Pathway of Alkane Degradation in Arctic Region

It is well accepted that arctic soil becomes impregnated with low-carbon chain refined petroleum, kerosene, and jet fuel. Diesel predominated by *n*-alkanes ranged from $n\mathrm{C}_{10}$ to $n\mathrm{C}_{26}$, whereas minute fraction of aromatic hydrocarbons was also present (Sutton et al. 2013). Arctic soils host abundance of bacteria capable of degrading *n*-alkanes and aromatic hydrocarbons (Yumoto 2013). *Pseudomonas*, *Rhodococcus*, *Sphingomonas*, and *Arthrobacter* are the dominant genera of bacteria isolated from

arctic diesel-contaminated soils (Wang et al. 2016). Yergeau et al. (2012) reported that *Pseudomonas* and *Rhodococcus* were the dominant genera of bacteria occurring in diesel-contaminated arctic soils. These genera are capable of degrading diesel hydrocarbons rapidly. Bej et al. (2000) found that the most commonly occurring *Rhodococcus* spp. strains (7/1, 5/1 and 5/14) in cold environment are capable of degrading straight chain carbon compounds usually from hexane (nC_6) to eicosane (nC_{20}) and the isoprenoid compound pristane (2,6,10,14-tetramethyl-pentadecane). *Rhodococcus* because of its biosurfactant nature can enhance the rate of degradation of *n*-alkanes under arctic soils. Apart from *n*-alkanes these bacteria were capable of degrading aromatic hydrocarbons under arctic conditions.

Further improvement in bioremediation was made when enzyme-mediated bioremediation was introduced by the researchers. Enzyme-mediated bioremediation means that consortium was prepared from a mixture of those bacteria which have alkane monooxygenase *Alk*B, encoding *Alk*B gene, an important enzyme that can enhance bacterial degradation of hydrocarbons (Jurelevicius et al. 2013). Nie et al. (2014) found that alkB genes were only found in Proteobacteria, Actinobacteria, Bacteroidetes, and Spirochaetes encoded gene carry AlkB. Liu et al. (2017) performing bioremediation experiment to degrade petroleum hydrocarbons found that alkane monooxygenase *AlkB* was increased 1000-fold and directly correlated with an increase in the disappearance of total saturated and aromatic compounds present in petroleum waste soils. They concluded that enzyme-mediated bioremediation can be a success to remediate wide range of hydrocarbons over a short period of time.

Like *AlkB*, cytochrome P450 CYP153 is usually present in those bacteria which have no *AlkB* enzyme (Liang et al. 2016).

Nonetheless degradation of long carbon chain such as above nC_{40} cannot be achieved with bacteria such as *Pseudomonas putida*, and thus further study is needed. Elumalai et al. (2017) reported that *eubacteria* or archaea can accelerate the rate of degradation of heavier paraffinic compounds mainly from nC_{32} to nC_{40}. They performed batch cultivation test of nC_{32} and nC_{40} with mixed thermophilic consortium. They found that because of the presence of catalysts mainly alcohol, such as alcohol dehydrogenase, the mineralization efficiency of nC_{32} to nC_{40} was improved. However application of such consortium in the field to remediate petroleum hydrocarbon-contaminated soil is in scarcity.

26.9 Pathway of Aromatic Hydrocarbon Degradation by Microbes

Aromatic compounds are present in minute quantity in petroleum waste. Regardless of their concentration, structurally these compounds are recalcitrant to microbial degradation even present at low concentration (Fuchs et al. 2001). Their inhibition to germination and toxicity to plants and microbes is well documented in the previous literature (Díaz et al. 2013). Aromatics with single benzene compound such as

naphthalene is rapidly degradable aromatic hydrocarbons, whereas increase in benzene ring such as 2–3 and even 4 benzene ring poly-aromatic hydrocarbons decreases their rate of degradation (Pérez-Pantoja et al. 2010). Information about the enzyme-mediated bacteria gene that can degrade 4 benzene ring such as benzo[*a*]pyrene is lacking. Limited information about the degradation of organochloro and organophosphate compounds by enzyme-mediated bioremediation is available in the literature. Therefore, there is an urgent need to design such experiment suitable for wide range of hydrocarbons degraded by enzyme-mediated bioremediation.

The mode of degradation of aromatic compounds by bacteria is quite different than *n*-alkanes (Gibson et al. 1968; Cripps and Watkinson 1978). First, diol is formed and then ring cleavage with end product of diacid such as cis,cismuconic acid (Ladino-Orjuela et al. 2016) as mentioned in Fig. 26.1.

Tomás-Gallardo et al. (2014) studied the degradative pathway of naphthalene by *Rhodococcus* strain TFB. Naphthalene was initially degraded in 1,2-dihydroxynaphthalene and finally formed salicylate. Undugoda et al. (2016) reported that phyllosphere bacterial strain, *Alcaligenes faecalis* and *Alcaligenes* sp.11SO, because of the difference in their gene encoding (*nahR* and *nahU*), can degrade a wide range of single to three benzene ring compounds.

Liu et al. (2017) noted that degradation of aromatic compounds such as naphthalene was enhanced because of the presence of enough naphthalene and tricyclic dioxygenase gene *Nah.*

The role of enzyme-mediated bioremediation to degrade long carbon chain hydrocarbons and aromatic compounds is under experimentation. This needs further investigation to develop such consortium that can degrade a wide range of saturates and benzene ring compounds. Their application in the field needs further investigation. Enzymes oxidized rapidly, and thus their sustainability during the entire duration of bioremediation remained a problem. Their rapidly oxidizing nature may increase the cost and limit their application commercially.

Regardless of the success of bioremediation, bioremediation with consortium and accelerators, enzyme-mediated bioremediation, and mixed fungi and bacterial bioremediation, there are still some gray areas which need to be address. The failure of bioremediation to remediate deep subsurface contamination opens the corridor for new experimentation.

26.10 Nanobioremediation

Recent application of elemental or zero nanoparticles in bioremediation is gaining scant attention all over the world. This is more likely because of their nano size (100 nm), greater surface area, and nontoxicity to plants.

Nanotechnology in an emerging field and is more effective due to its large surface area in the degradation of petroleum hydrocarbon. The principles of nano-based biodegradation of petroleum hydrocarbons are as below (Kumari and Singh 2016):

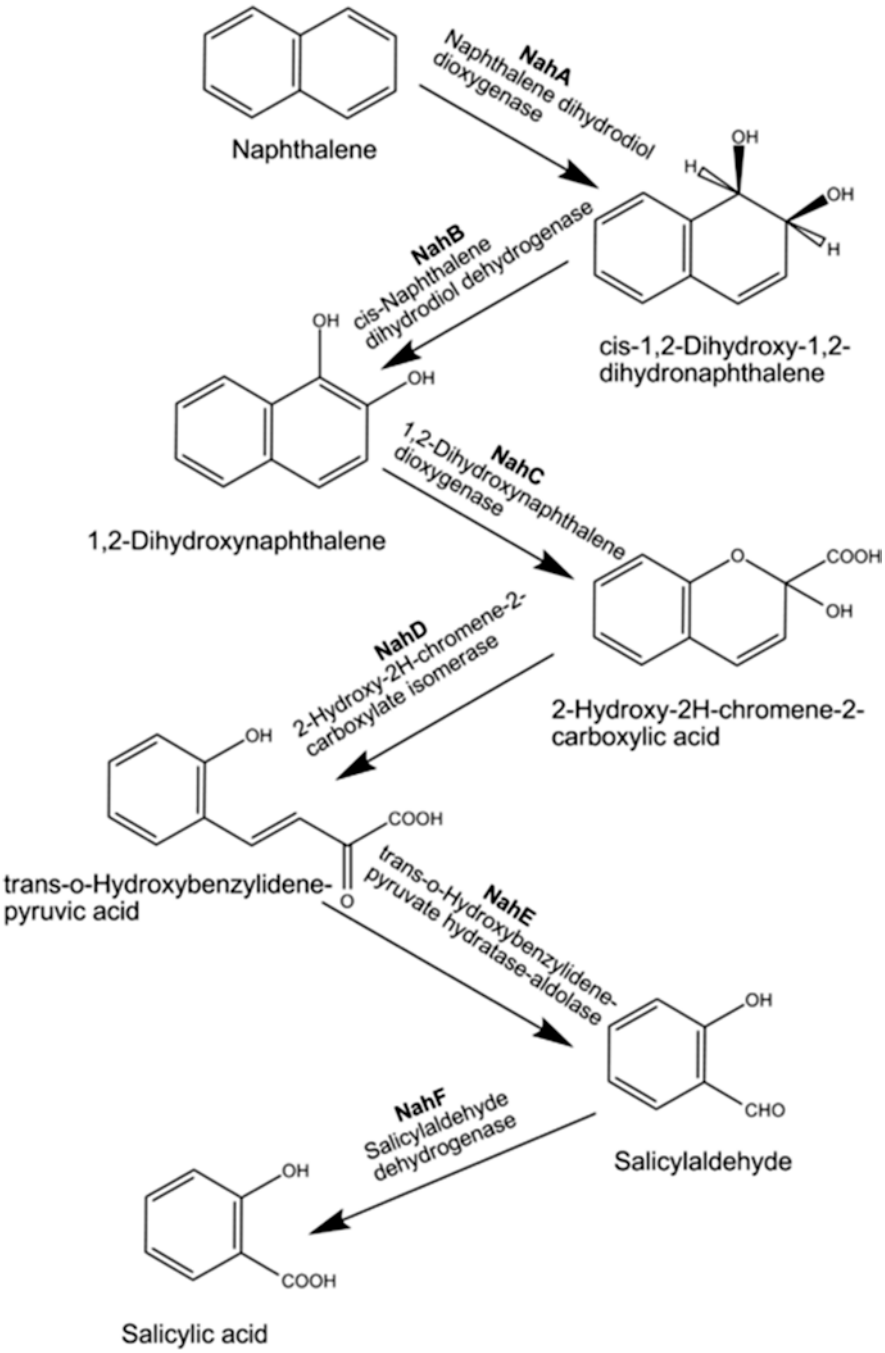

Fig. 26.1 The aerobic pathway followed by bacteria to degrade naphthalene. (Adopted from Habe and Omori 2003)

1. Adaption of organic contaminants
2. Chemical modification
3. Act as facilitator in microbial remediation of contaminates
   (a) Promoting microbial growth
   (b) Immobilization of remediating agent
   (c) Induced production of microbial enzyme to assist bioremediation
   (d) Induce production of biosurfactants by microbial
   (e) Enhanced solubility of hydrocarbon

Metal oxide, e.g., ZnO nanoparticle, can interfere with the crude oil biodegradation (Ismail et al. 2013). Cecchin et al. (2016) present a classical review on the use of nanoparticle to remediate hydrocarbon-contaminated soils. The information about nano in combination with consortium for bioremediation is lacking in this review. Limited information is available in the previous literature about the role of nanoparticles in combination with consortium in the degradation of petroleum hydrocarbon-contaminated soils. A classical review of Yadav et al. (2017) concluded that some bacteria and fungi are capable of synthesizing nanoparticle to remediate petroleum-contaminated soils. Galdamas et al. (2017) reported that application of nanoparticle of zero-valent iron (nZVI) in combination with compost to aliphatic hydrocarbons (long carbon chain hydrocarbons) in biopiles may enhance the degradation of these hydrocarbons.

There are contradictory evidences available in the previous literature regarding the difference in the rate of mineralization of petroleum waste impregnated soil by fungi or bacteria or both. The rate of mineralization depends on the type of species of fungi and bacteria and also on the extent of petroleum contamination to which such species are exposed. Al-Hawash et al. (2018) reported that fungi species such as *Penicillium* may degrade <60% of 1% of crude oil (Al-Hawash et al. 2018). Mohsenzadeh et al. (2012) found that *Penicillium* may degrade well when petroleum hydrocarbon extent is 8%.

Ghoreish et al. (2017) reported that bacteria can mineralize nearly 69% of kerosene (5% v/v). Diverse genera of bacteria have broader functional components, e.g., enzyme production and biosurfactant productions. Tang et al. (2012) found that algae can utilize mixed hydrocarbon, and this algae can also degrade *n*-alkanes, isoalkanes, and aromatic hydrocarbons. Cyanobacteria green red and brown algae as well as diatoms can oxidize naphthalene (Cerniglia et al. 1980).

26.11 Conclusion

Microbial degradation of petroleum hydrocarbon constitutes a significant role to clear up petroleum waste contamination. Recent trend is the use of genetically modified bacteria in bioremediation at large scale needs to be worked out. Further improvement in bioremediation may be made with nano particles. Nano particles with different shapes and sizes can be used. Single metal NPs, biometallic NPs.,

have large surface area and require less activation energy for the chemical reaction. Recent use of magnetic nanoparticles (Fe_3O_4) coated on the surface of *pseudomonas delafieldic* made this suitable to degrade petroleum. They can be recycled for the treatment of sure substrate.

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Chapter 27
A Review on the Bioremediation of Petroleum Hydrocarbons: Current State of the Art

Leadin S. Khudur, Esmaeil Shahsavari, Arturo Aburto-Medina, and Andrew S. Ball

Abstract Petroleum hydrocarbons (PHC) enter the environment due to exploration, transportation, usage and spills. PHC contamination is of major concern worldwide due to the damage they cause to the environment. Clean up of hydrocarbon-contaminated sites is expensive and time-consuming; however, bioremediation represents a cost-effective and environmentally safe approach to clean up PHC contamination. Many bioremediation strategies can be applied depending on the contaminated site and the surrounding environment. In addition, a variety of technologies are used to assess the efficiency of bioremediation of contaminated environments through analysis of the concentration of the pollutant. Other technologies are applied to study the microbial communities in the contaminated sites since they represent the backbone of any bioremediation process. One of the most convenient technologies in this regard is next-generation sequencing (NGS) since it is cost-effective and provides comprehensive information regarding diversity and therefore bioremediation potential of microbial communities. Bioremediation, however, is not always a straightforward approach, especially when another contaminant (e.g. heavy metals) is associated with PHC. In this chapter, the concept of bioremediation of hydrocarbon-contaminated environments is illustrated. Moreover, the most common technologies applied in bioremediation are explained. In addition, the most recent tools for assessing the microbial ecology are described. Finally, current challenges and limitations of bioremediation are presented.

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27.1 Introduction

27.2 What Happens to Petroleum Hydrocarbon After Entering the Environment?

Petroleum hydrocarbons (PHC) describe a large group of chemicals which are basically made of hydrogen and carbon. Crude oils are the main source of PHC, and the concentration of each compound varies according to the source. Four main structural groups represent the main components of PHC (Fig. 27.1). The saturate component or the aliphatic fraction which contains alkanes and cycloalkanes and extends from C1 (methane gas) up to C40; the aromatic component which is classified based on the number of the benzene rings; the asphaltene component which includes ketones, phenols and esters; and the resin component which includes sulphoxides, pyridines and amides (Colwell and Walker 1977). While most of the PHC components are combustible, they differ in their physiochemical characteristics, such as colour, odour and boiling and evaporation points.

PHC enter the environment through transportation, oil spills and leaks, industrial activities and private use. They move through the soil while some compounds might attach to soil particles, while others reach the groundwater and therefore affect the whole ecosystem including humans (ATSDR 1999). Therefore, for assessing the risks associated with PHC in any ecosystem, it is important to investigate the fate of

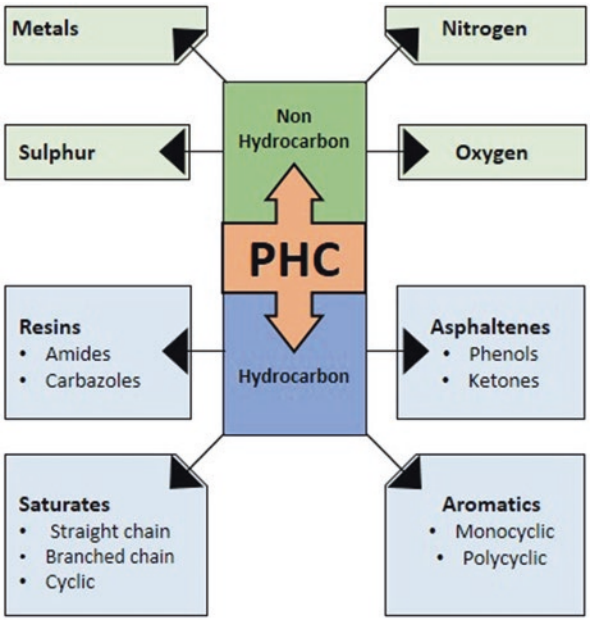

Fig. 27.1 The classification of petroleum hydrocarbons (PHC)

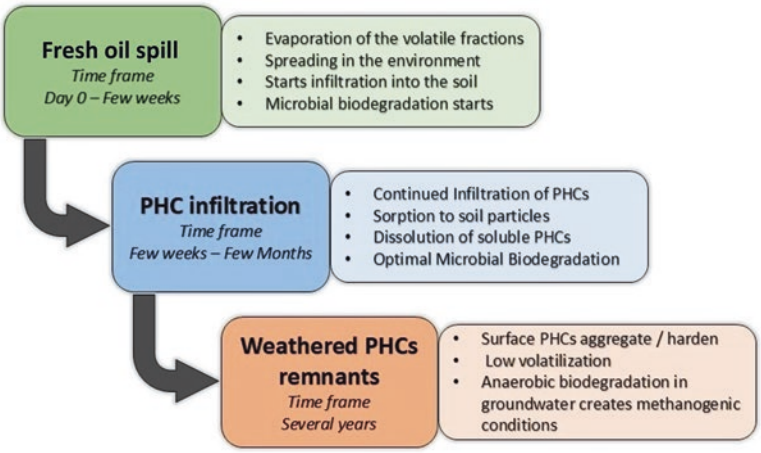

Fig. 27.2 The fate of PHC in the terrestrial environment

PHC when entering the environment. Figure 27.2 illustrates the main fate of PHC when they enter the terrestrial environment (Brown et al. 2017). Many factors affect the spreading, infiltration and biodegradation of the PHC in the soil. Some of these factors are contaminant-related, such as quantity, chemical composition and viscosity of the PHC fraction, while other factors are site-related such as type, porosity, permeability and particle size of the soil.

Biodegradation of PHC takes place through the hydrocarbon-degrading fraction of the natural microbial community, following the initial oil spill into the environment, although the soil microflora is highly affected by the presence of the contaminant (Fig. 27.2). The number of hydrocarbon-degrading microorganisms as well as the concentration and bioavailability of the PHC fraction affect the degradation rate, in addition to many other factors, such as temperature, moisture content and nutrient concentration (Baboshin and Golovleva 2012).

27.3 Bioremediation of Petroleum Hydrocarbons

Bioremediation is the application of biological agents to break down and naturalize environmental contaminants. Bacteria, fungi and plants are the most common organisms used in bioremediation of petroleum hydrocarbon-contaminated soil due to their ability to degrade petroleum hydrocarbons (Medina-Bellver et al. 2005). The application of bioremediation started last century when the hydrocarbon utilization abilities of many indigenous microorganisms were reported. However, since the beginning of the twenty-first century, research has extensively focused on bioremediation and its application.

Table 27.1 The main bioremediation strategies used to treat PHC-contaminated environment

Bioremediation strategy	Mechanism	Limitations
Natural attenuation	Using the indigenous microflora to breakdown PHC	Slow process
		Incomplete degradation
Biostimulation	Enhancing the indigenous microflora by the addition of nutrients to accelerate the degradation process	May alter the natural balance of nutrients
Bioaugmentation	Increasing the biomass of hydrocarbon-degrading microflora to increase the degradation rate	Changes the biodiversity of the natural microbial habitat
Phytoremediation	Using plants and associated microbes to break down hydrocarbons	Toxicity of pollutants towards the plants

Many bioremediation strategies can be applied to treat hydrocarbon-contaminated environmental samples (Table 27.1); these strategies include:

1. Natural attenuation can be defined as the biodegradation of the contaminant by the indigenous microflora of the soil, without any human involvement (Yu et al. 2005a). The degradation rate of the contaminant may be slow because it depends on the presence of hydrocarbon-degrading microbes, nutrient availability and the contamination level (Iwamoto and Nasu 2001). This strategy requires periodic monitoring but has the advantage of not disturbing the sensitive ecological habitats.
2. Biostimulation is a widely used strategy of bioremediation in which the degradation process of a contaminant is accelerated through the addition of nutrient to the soil microflora. Many nutrients are involved in the biostimulating process such as carbon, phosphorus, nitrogen and oxygen (Andreolli et al. 2015). Many studies have shown that biostimulation has significantly increased biodegradation of PHC (Khudur et al. 2015; Suja et al. 2014; Andreolli et al. 2015). However, the elevated concentration of the added nutrients can cause an imbalance of the natural microbial diversity in the soil resulting in a reduced biodegradation capacity (Yerushalmi et al. 2003).
3. Bioaugmentation is the increase of the hydrocarbon degraders' biomass by adding native microbial inocula and therefore increases the degradation rate of the contaminant. The effect of bioaugmentation on increasing the degradation rate of PHC has been reported in many studies (Wu et al. 2016). However, other studies have shown that bioaugmentation has no effect on hydrocarbon degradation (Yu et al. 2005b). This addition of exogenous microbial species into the contaminated soil might alter the natural composition of the microbial diversity in the treated environment (Festa et al. 2016).
4. Phytoremediation is defined to use the plant and associated microorganisms to degrade, remove or clean up the environments. In regard to PHC, plant roots and

their rhizosphere play the most important role which is defined as rhizoremediation. Reports show that many PHC compounds such as diesel and polycyclic aromatic hydrocarbons (PAH) can be subjected to phytoremediation (Shahsavari et al. 2013, 2015). However, toxicity associated with most PHC towards the plants may affect the efficacy of phytoremediation.

27.4 Microbial Degradation of PHC and Affecting Factors

The ability of microflorae to utilize PHC as their source of energy was firstly reported in 1946 (Zobell 1946). Since then, research has been conducted to investigate the biological degradation of PHC. Although most hydrocarbonoclastic biological agents are bacteria which act as primary degraders in the case of an oil spill (Brooijmans et al. 2009), hydrocarbon-degrading microflora also includes fungi and yeast, both of which are widely distributed in the environment. Many of these organisms have been isolated from different environmental samples, such as soil, marine and fresh water (Atlas 1991). Aerobic degradation of PHC is the most comprehensive process (Das and Chandran 2011). The oxidative processes of the oxygenase and peroxidase catalyse the initial step of the peripheral pathways of PHC degradation. The hydrocarbons are converted into intermediate metabolites, such as those commonly found in the tricarboxylic acid cycle (TCA), ultimately being assimilated into cell biomass. Figure 27.3 illustrates the main mechanisms involved in the aerobic degradation of PHC (Fritsche and Hofrichter 2008).

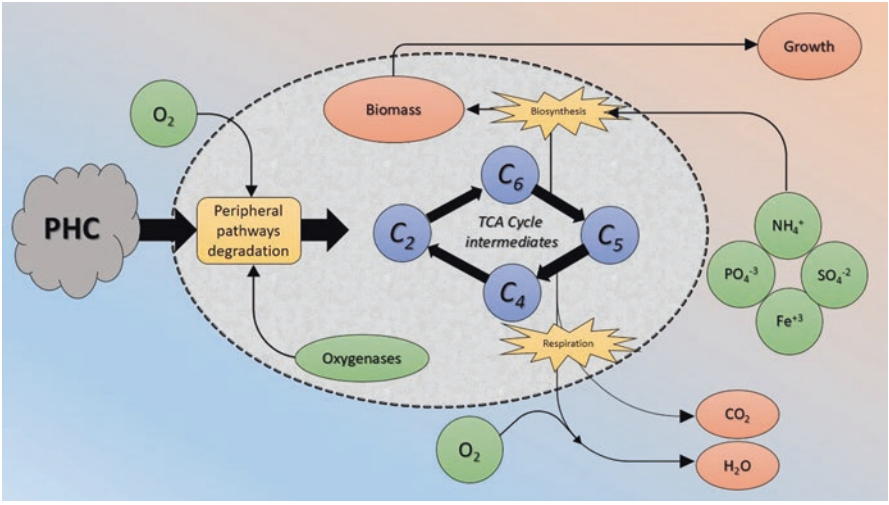

Fig. 27.3 Main pathway of microbial aerobic degradation of PHC

27.4.1 Factors Affecting the degradation Process

27.4.1.1 The Presence of Oxygen

The microbial degradation of PHC takes place optimally under aerobic conditions. In the presence of oxygen, complete degradation of the contaminant may take place (Das and Chandran 2011). During this process, the organic pollutant is attacked by activated intracellular oxygenases and peroxidases and transferred into simple organic substances, such as water, carbon dioxide as well as biomass (Das and Chandran 2011). Degradation may also take place under anaerobic conditions but at a much slower rate in comparison to aerobic conditions (Wentzel et al. 2007). In many cases when large quantities of oil are introduced into the environment, the consumption rate of the oxygen increases which creates anaerobic conditions. In such conditions, other electron receptors will be used by the microorganisms as a source of energy, such as sulphate, iron or nitrate, which will produce much less energy than using oxygen as electron receptor, thus slowing the rate of degradation of the contaminant (Thapa et al. 2012). The degradation of PHC may reach 90% under aerobic conditions, whereas the degradation may not exceed 25% under anaerobic conditions (Grishchenkov et al. 2000).

27.4.1.2 Nutrient Concentration

Nutrients are required for the biodegradation of PHC (Coulon et al. 2005). The vast majority of microorganisms require carbon (C) as the essential nutrient for their metabolism. However, many other micronutrients, such as nitrogen (N) and phosphorus (P), are also required to optimize the microorganisms' growth. The hydrocarbon-degrading microorganisms require all these elements, and the optimal nutrient molar ratio for C:N:P for most microorganisms is 100:10:1 (Straube et al. 2003). The availability of limited concentration of nutrients leads to low microbial degradation rates. Also, excessive levels of nutrients, such as nitrogen and phosphate, might reduce the PHC degradation rate (Chaillan et al. 2006). In addition, any alteration in the natural balance of the nutrients in the environment might lead to changes in the microflora natural communities and the ecological relationship between them (Hays et al. 2015).

27.4.1.3 Microbial Diversity

In a naturally balanced ecosystem, microorganisms produce a variety of substances (e.g. enzymes) which are essential for their metabolism and proliferation. The synergic relationship among different microbial communities helps them to adapt and modify the changes in their environment thus creating ideal conditions for increased growth (Sabra et al. 2010). Although some species have adopted a variety of mechanisms for breaking down contaminants entering their environment, single microorganisms are only able to metabolize a small range of hydrocarbons. Therefore, other microorganisms in the same ecosystem offer symbiotic relationships by producing

molecules aiding the pollutant degraders. For example, secreted surfactants (e.g. rhamnolipids) increase the bioavailability of hydrocarbon to hydrocarbon degraders (Gkorezis et al. 2016).

27.4.1.4 Temperature

Temperature is the most crucial environmental factor influencing the degradation rate of PHC. The role of temperature is very important since it directly affects the bioavailability of hydrocarbons during the biodegradation by changing their viscosity, volatilization and diffusion rates (Coulon et al. 2007). In addition, the diversity of microbial communities is also influenced by temperature; generally the greatest diversity is detected between 30 and 50 °C. In addition, elevated temperatures influence the activity of bacterial enzymes involved in PHC degradation (Abed et al. 2015b).

27.5 Approaches to Evaluating Bioremediation Efficiency

Many parameters should be assessed during any bioremediation process in order to evaluate the efficacy of the treatment. Different techniques can be used for this purpose which can be based on chemical, physical and/or biological indices of the treatments (Fig. 27.4).

27.5.1 Physiochemical Approaches

27.5.1.1 Gas Chromatography-Flame Ionization Detection (GC-FID)

The principles of GC technology have been extensively reported (Sherma and Zweig 1972). In this approach the PHC are extracted using solvents (e.g. hexane, dichloromethane or acetone) and absorbents (e.g. alumina or silica gel) (Wang and Fingas 1995). When the extracted samples are injected into the chromatographic column, the column temperature increases gradually, and separation of PHC components takes place based on their boiling points. The flame at the end of the column burns the separated components which can be detected, and the concentration of each component is calculated in relation to the reference PHC standards provided (Sherma and Zweig 1972; Okparanma and Mouazen 2013).

GC-FID has a detection limit of 10 mg/kg for PHC in the soil; however, this depends on the sample matrix and protocol used. Furthermore, GC-FID has the advantage of providing high sensitivity and selectivity, and it can detect and measure the *n-alkane* fraction of the PHC range C_{10}–C_{40}, as well as PAH. This technique has been extensively used in laboratory applications for the screening of environmental samples including qualitative and quantitative studies (Wang and Fingas 2003). However, this approach has many challenges, including high operational time and

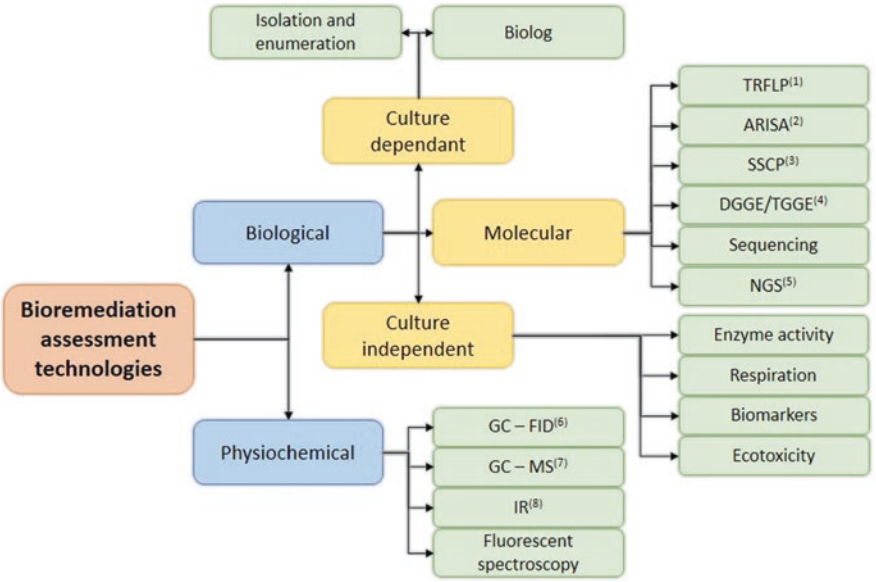

Fig. 27.4 Techniques used to evaluate the bioremediation of PHC. (1) Terminal-restriction fragment length polymorphism, (2) automated ribosomal intragenic spacer analysis, (3) single-strand conformation polymorphism, (4) denaturant gradient gel electrophoresis/temperature gradient gel electrophoresis, (5) next-generation sequencing, (6) gas chromatography-flame ionization detection, (7) gas chromatography-mass spectrometry (GC-MS), (8) infrared spectroscopy

cost (Aske et al. 2001), difficulties in the calibration of the GC instrument (Krupcík et al. 2004), and the effect of the instrument operation conditions (Saari et al. 2010).

27.5.1.2 Gas Chromatography-Mass Spectrometry (GC-MS)

GC-MS has been used for many years, and it is the most common analytical technique for measuring PHC in contaminated samples (Poster et al. 2006). GC-MS has shown a versatility in evaluating a variety of environmental samples, including PHC, and it provides specific mass spectra and retention time for each component in a sample mixture (Thornton et al. 2011); this technique is therefore known as a universal detector. Similar to GC-FID, GC-MS had a detection limit of 10 mg/kg; however, FID has shown higher sensitivity than MS. Different fractions of PHC, including Total Petroleum Hydrocarbons (TPH) and PAH, are measured using this technique (Poster et al. 2006). Many techniques are involved in the application of GC-MS, such as the conventional technique (one dimensional) and the comprehensive technique (two dimensional). In addition, gas chromatography/gas chromatography-mass spectrometry (GC/GC-MS) is usually applied using the two-dimensional technique to increase the separation of the components of any mixture (Thornton et al. 2011).

Although GC-MS is a widespread technique for assessing PHC in many environmental samples, many drawbacks have been associated with its applications. High laboratory analytical costs and labour-intensive and time-consuming extraction protocols make this approach uneconomical for commercial applications, especially when large-scale environmental contamination is studied (Peterson et al. 2002). Furthermore, only a few organic solvents can be used for GC-MS sample extraction as these solvents need to be stable thermally. They should also have minimal risk to the analyst's health (Chuang et al. 2003).

27.5.1.3 Infrared Spectroscopy (IR)

This technique uses the energy associated with molecule vibration spectra which can be absorbed in the infrared region. The overtones or combinations of the carbon-hydrogen saturated groups in the hydrocarbon molecules produce electromagnetic spectra, which can be detected by an IR-based device (Aske et al. 2001).

IR-based techniques are routinely used in detecting and measuring petroleum hydrocarbons, especially TPH. These techniques have the advantage of being simple, safe and inexpensive and have low detection limits for PHC (about 10 mg/kg) (Lambert et al. 2001); also because they have global recognition, they have been used frequently before the advent of gas chromatography-based approaches (Current and Tilotta 1997). However, the IR-based approach has some limitations, such as insensitivity to the aromatic fraction of the hydrocarbons. In addition, the hydrocarbon standards used to pre-calibrate any device do not represent the environmental contaminant and the weathered hydrocarbons (Whittaker et al. 1995).

Recently, accurate prediction of the TPH concentration using a portable handheld spectroscopy device was reported (Webster et al. 2016). This instrument, which uses "diffuse reflectance (mid)infrared Fourier transform spectroscopy (DRIFTS)" (Forrester et al. 2013), was tested on different types of contaminated soils, on a variety range of TPH concentrations (C_6–C_{40}) and in the field as well as laboratory conditions. This technology provides an accurate, time and cost-effective measurements of TPH in the contaminated soil samples and can detect TPH concentration range of 50–100,000 mg/kg in soil (Webster et al. 2016).

27.5.1.4 Fluorescence Spectroscopy

This technique is based on measuring the radiation emitted from PHC molecules during the relaxation time after they have been exposed to short wavelength radiation which causes molecule excitation. Each molecule type emits radiation at a certain wavelength, and the amount of emitted energy correlates with the concentration of PHC molecules in the samples (Aldstadt et al. 2002). Fluorescence spectroscopy employs two different types of light sources. Firstly, "ultraviolet-induced fluorescence (UVIF)" uses ultraviolet (UV) light as a source of energy (Greason 2009), whereas the other type uses the pulsed laser for energy production, "laser-induced fluorescence (LIF)" (Bujewski and Rutherford 1997). This technique has been used in field studies

to assess TPH and PAH in contaminated soil with detection limits of approximately 3 and 1 mg/kg for THP and PAH, respectively (Greason 2009).

The inaccuracy of this application in assessing complex samples, due to overlapping spectra of various sample components, is one of its disadvantages. In addition, it has high sensitivity to other non-hydrocarbon substances in the sample as well as the sample (soil) matrix (Barnes 2009).

27.5.1.5 Biological Approaches

The investigation on the microbial communities in any contaminated sample is an essential process in order to understand the potential of the microorganisms involved in the bioremediation process. To evaluate the role of hydrocarbon degraders in any contaminated environment, many factors should be studied, such as growth rate, nutrition, proliferation, respiration and the shifts in the microbial communities (Kadali et al. 2012).

27.6 Culture-Dependent Techniques

27.6.1 Microbial Isolation and Enumeration

The initial isolation and count of the total microbial communities and the hydrocarbon degraders provide crucial information about the biological profile of the contaminated soil. The standard serial dilution and direct plate counting are *standard* techniques for determining the bacterial population in any sample, which can be presented as colony-forming unit (CFU) (Balba et al. 1998). Many enrichment and selective culturing media are used in order to grow different microbial species and therefore calculate the number of culturable microorganism in samples. The most commonly used media is nutrient growth media; however, many other media are used for this purpose (Table 27.2) (Schlegel and Zaborosch 1993). For culturing hydrocarbon-degrading microorganisms, selective media are used which are enriched with a single or mixed source of hydrocarbons as the only source of energy, for example, Bushnell Haas mineral salt medium (BHMSM) (Bushnell and Haas 1941). Non-culturable microorganisms represent the biggest limitation to the isolation and enumeration technique because these organisms cannot be cultured (Balba et al. 1998).

27.6.2 Biolog™ Plates

Biolog™ plates were developed by the Biolog Corporation in the late 1980s to be used for determining the metabolic activity of single microorganism in contaminated environmental samples. The MT2 MicroPlate™, which consists of 96 wells,

Table 27.2 Media used in cultivation and isolation of microorganism from environmental samples

Medium	Type
Nutrient agar/broth (NA/NB) (LO et al. 2014)	Enrichment medium for a wide range of microorganisms
Bushnell Haas mineral salt medium (BHMSM) (Guru et al. 2013)	Selective medium for hydrocarbon degraders
Dextrose media (Gil et al. 2009)	Enrichment medium for a wide range of microorganisms
Dextrose nitrate agar (DNA) (Gil et al. 2009)	Enrichment medium for a wide range of microorganisms
Marine agar/broth (LO et al. 2014)	Selective medium for marine microorganisms
Luria-Bertani medium (LB) (Escobar-Niño et al. 2014)	Enrichment medium for a wide range of microorganisms

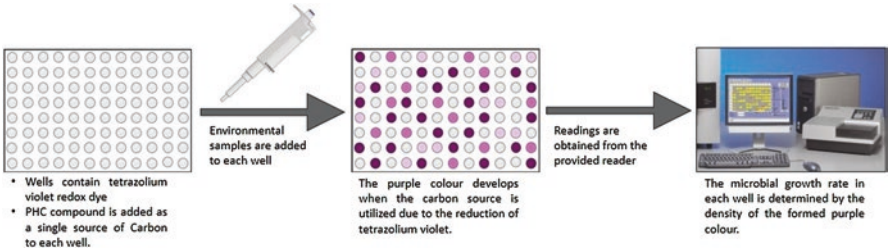

Fig. 27.5 The Biolog test

is usually used for assessing the microbial activity in hydrocarbon-contaminated soil because utilization tests for 31 carbon sources can be performed at the same time (Hill et al. 2000). After adding the microbial sample and the source of carbon into each well, the metabolic potential is assessed based on the purple colour generated due to the presence of tetrazolium violet redox dye in the wells. The aerobic growth rate of the microorganism correlates with the intensity of the produced colour (Fig. 27.5). However, insufficient cell intensity may affect the tetrazolium violet redox dye; therefore, no noticeable results are shown (Widmer et al. 2001).

27.7 Culture-Independent Techniques

27.7.1 Enzyme Activity

Evaluating the activity of key microbial enzymes is a commonly used indicator in assessing the biodegradation of PHC. Dehydrogenase enzymes, which are responsible for the oxidation of organic matter present in the majority of soil microorganisms, are widely regarded as an estimate of the oxidation potential of microbial

communities. Therefore, these enzymes can be used in an assay to assess the inhibitory impact of PHC on the microbial oxidation activity of contaminated soil (Gianfreda et al. 2005). The colorimetric-based enzyme assay uses 2,3,5-triphenyl tetrazolium chloride (TTC), which acts as an electron acceptor, and represents the most commonly used technique. Microbial dehydration activity catalyses TTC and forms triphenyl formazan which is a red colour compound. The colour intensity, which can be measured at 485 nm, correlates with the dehydrogenation activity in the soil (Fig. 27.6) (Page et al. 1982). Other enzyme activities can also be used for this purpose, such as acylphosphatase, which is responsible for the mineralization of organic phosphorus (Baran et al. 2004).

Enzyme assays have some limitations. Microbial enzymatic activities do not necessarily represent the viable microbial communities because it depends on the metabolism of the total microbial communities in the contaminated samples. In addition, the presence of some elements, such as iron, nitrite and nitrate, in the soil impacts the dehydrogenase activity due to their electron reception ability (Gianfreda et al. 2005).

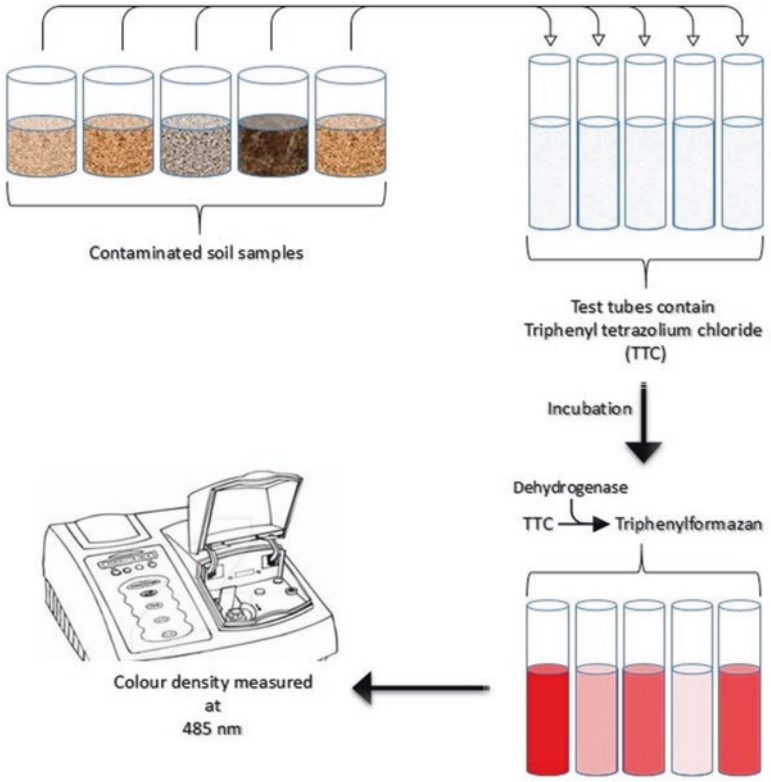

Fig. 27.6 The dehydrogenase enzyme activity test

27.7.2 Soil Respiration Tests

The complete mineralization of PHC in aerobic conditions results in the consumption of oxygen and the production of CO_2. Measuring the consumed and/or the produced gases provides reliable information regarding the potential biodegradation rate of PHC in the soil because these measurements correlate with microbial metabolic rate (Aspray et al. 2008). The most commonly used technique to measure the consumption and/or production of gases is the "simple respirometric-flask" technique; however, more advanced, automated instruments can also be used (Balba et al. 1998).

A standard respiration test commonly used is measuring CO_2 production because it represents the biodegradation of the contaminant, is easy to perform and is non-destructive.

On the other hand, for soils with pH greater than 7, biodegradation assessments measuring O_2 utilization have been shown to be more accurate than assessments measuring CO_2 production as under alkaline conditions, CO_2 is converted to carbonate (Hinchee and Ong 1992). Other types of respiration tests can also be used to evaluate the utilization of the carbon source within the contaminated soil. For example, basal respiration is a technique used to evaluate the ability of soil microorganisms to utilize organic carbon in the soil. Also, substrate-induced respiration (SIR) is another technique which can be performed to assess the ability of a certain microbial group to utilize a specific carbon source in the soil (Torstensson 1996). However, the produced CO_2 may not result from the tested contaminants only. The addition of any degradable organic matter to the soil could enhance CO_2 production due to the microbial degradation of these matters (Kim et al. 2005). In addition, the respiration rate differs according to the fraction and concentration of the PHC in the soil (Dawson et al. 2007).

27.7.3 Biomarkers

Biomarkers can be used as indicators to evaluate the biodegradation of PHC. These biomarkers can be internal petroleum indicators or microbial cellular indicators.

27.7.3.1 Internal Petroleum Biomarkers

Many PHC branched-alkane compounds can be used as internal petroleum indicators, such as hopane, pristine, octadecane and phytane. The use of such biomarkers is based on the fact that the degradation rate of the straight-chained alkanes is much faster than that of branched alkanes. Therefore, after any bioremediation process of a hydrocarbon-contaminated soil, the ratio of certain biomarkers to other components reflects the biodegradation activity in the PHC complex mixture (Wang et al. 1994).

27.7.3.2 Cellular Biomarkers

Many intracellular molecules of microbial cells are used as biomarkers. In environmental samples, many parameters can be assessed including microbial community composition, microbial biomass, the function of single microorganism in the community and the microbial response to toxicants and environmental stresses (Hill et al. 2000; Uhlík et al. 2009).

Phospholipids fatty acids (PLFAs), which can be found in the cell's cytoplasmic membrane, represent one example of intracellular biomarkers. The rapid decomposition of PLFAs after cell death and their proportional presence in the cells make them a reliable indicator of viable cells in the contaminated environment. Additionally, the composition of PLFAs can be changed by the organism as a response to any xenobiotic exposure, such as PHC and heavy metals. All these characteristics of PLFAs are employed as biomarkers to evaluate the structure and status of the microbial community in contaminated samples (Malik et al. 2008). However, this technique relies on the PLFA structure of the whole microbial community; therefore, inaccurate estimations might result using this method, especially after exposure to contaminants, which differently impacts the PLFA structure (Hill et al. 2000).

Another example of cellular biomarkers is stable isotope probing (SIP). In this technique, substrates labelled with stable isotope are incorporated into intracellular biomarkers especially DNA or rRNA, which are the most informative biomarkers (Uhlík et al. 2009). The use of whole microorganism genomes gives SIP the advantages of being a very informative technique in relation to metabolic activities of a single organism. This method mainly depends on the primary extraction, purification and labelling of the organism's nucleic acids, which increase its sensitivity (Malik et al. 2008).

27.7.4 Ecotoxicity Tests

Traditional chemical analyses are the usual way to evaluate the degradation of pollutant in contaminated sites. However, the concentration of contaminants does not necessarily reflect the associated toxicity due to a variety of reasons. Therefore, integration between concentration measurements of PHC, ecotoxicological assessment and the evaluation of the microbial community is required, though seldom carried out to evaluate the efficiency of the approach used to treat the contaminated area and the environmental outcomes in terms of its effects on ecosystems (Khudur et al. 2015). The key indicators of the risk posed by chemicals to human health and other organisms in the environment are related to the bioavailability of the contaminant, which can be defined as the difference between the amount of the contaminant to which an organism is exposed and the actual dose of the substance the organism receives (Hartemink et al. 2008).

Acute toxicity test is the most commonly used approach for evaluating the toxicity of PHC-contaminated samples; however, chronic or sublethal tests are also used but less common (Hubalek et al. 2007). Inhibition of natural bacterial bioluminescence, which is known as the Microtox test, has been developed as a cost-effective, easy prescreening test, which is based on measuring the inhibition in light emitted by the marine bacterial species, *Vibrio fischeri* (Kamlet et al. 1986).

The earthworm acute toxicity test is another universal ecotoxicological test used worldwide to examine the toxicity of a pollutant in contaminated soil (Mooney et al. 2013).The earthworms readily come into contact with the PHC in the soil during their movement. The uptake of the contaminant fractions which are environmentally bioavailable occurs either via dermal absorption, ingestion or both (Lanno et al. 2004).

A new approach, termed microbial ecotoxicology, has recently been introduced into ecotoxicity assessment of contaminants on the biota. This approach aims to accurately investigate the ecotoxicity effects of environmental contaminants on the entire community. This can be achieved by employing a variety of approaches together such as analytics, enzymatic, molecular and toxicity (Shahsavari et al. 2017).

27.8 Molecular-Based Techniques

In molecular-based techniques, the whole or part of nucleic acids (DNA and RNA) of organisms are used. These techniques provide information regarding the diversity and the changes in the microbial communities in contaminated samples, allowing assessment of the hydrocarbon degradation potential of these communities (Zhang et al. 2010). Polymerase chain reaction techniques (PCRs) are used to amplify the extracted genetic materials. The most widely applicable technique of PCR is the amplification of 16S rRNA and related genes prior to studies of the structure of microbial communities (Malik et al. 2008). During PCR, the nucleic acid template is denatured by heat, and then the desired oligonucleotide primers are annealed to a single-stranded nucleic acid which is finally extended using thermally stable polymerases. These two steps are crucial since the quality of any downstream process depends on the amount and purity of the extracted and amplified genetic material which is used as a template for microbial characterization (Malik et al. 2008).

Many molecular-based techniques have been used to assess microbial communities in contaminated environmental samples and also to evaluate the efficacy of the bioremediation process (Table 27.3).

In addition to the techniques illustrated in Table 27.3, the most current technologies used in studying microbial communities include real-time or quantitative PCR (qPCR) and next-generation sequencing (NGS). These technologies provide comprehensive qualitative and quantitative data regarding the microbial communities' structure in hydrocarbon-contaminated samples in a relatively short period of time and low cost.

27.8.1 Real-Time Polymerase Chain Reaction (qPCR)

Quantitative PCR or real-time PCR (qPCR) is a tool to provide real-time quantitative data regarding a specific gene or a certain sequence of nucleic acid. This technique relies on detecting the fluorescent-labelled PCR products and measuring them in real time during the PCR thermal cycle (Shahsavari et al. 2016). In the last decade, qPCR has been intensively used in the bioremediation of PHC to investigate genes of interest or the abundance of a functional microbial community which indicates the biodegradation potential of a microbial community (Schulz et al. 2010). For example, bacterial *alkB* gene, which encodes for an oxygenase enzyme involved in

Table 27.3 The most common molecular techniques used to evaluate the microbial communities in hydrocarbon-contaminated environmental samples

Technique	Purpose	Principles	limitations
Terminal-restriction fragment length polymorphism (TRFLP)	Provides quantitative data about the structure of microbial communities in environmental samples	Relies on the combination of the restriction digests of the isolates and fluorescently labelled primers (Dickie and FitzJohn 2007)	Expensive, labour intensive and requires long run time (Rastogi and Sani 2011)
Automated ribosomal intragenic spacer analysis (ARISA)	Estimation of the richness of microbial species in a diverse community	The differences in the length of DNA fragments which are separated by capillary electrophoresis (Kovacs et al. 2010)	Limited data obtained on phylum level because fragments between 200 and 1150 base pair can only be detected (Gobet et al. 2014)
Single-strand conformation polymorphism (SSCP)	Provides information about the complexity of the microbial communities	The separation of amplified DNA fractions with different nucleotide sequence but similar length (Schwieger and Tebbe 1998)	The accuracy of the results depends on the used instrument and the operator's experience (Malik et al. 2008)
Denaturant gradient gel electrophoresis (DGGE)/ temperature gradient gel electrophoresis (TGGE)	Assessment of the structure and the changes in the microbial communities in contaminated environmental samples	The amplified double-stranded nucleic acid fragments with various melting points are separated from each other when they passed through a thin layer of acrylamide gel (Muyzer 1999)	The inability to identify the phylogenetic of some communities due to the limited size of the amplified nucleic acid fragment limited to 500 base pair (Malik et al. 2008)
The Sanger sequencing	Profiling and phylogenies of microbial communities in the environment based on 16S rRNA	Complementary DNA strand is synthesized using DNA polymerase which is fluorescently labelled, so the four DNA bases can be differentiated (Lakshmi 2010)	Individual samples can be sequenced, so it is inadequate technique to assess a heterogonous environmental sample (Shokralla et al. 2012)

the aerobic degradation of an alkane, is a major indicator of the biodegradation potential of soil microbial communities. Therefore, the quantification of the *alkB* gene in comparison to the total microbial population in any contaminated environment is a crucial procedure before planning any bioremediation strategy.

27.8.2 Next-Generation Sequencing (NGS)

NGS is considered as a revolutionary change in sequencing technologies. Using NGS for the assessment of contaminated environmental samples (also known as metagenomics) addresses the issue of non-culturable microorganisms and also provides a vast amount of data compared to other techniques (Mukherjee and Chattopadhyay 2017). NGS provides comprehensive data regarding the structure and diversity of microbial communities, the potential roles of organisms within the community and also the interaction between the individuals in their communities (Mukherjee and Chattopadhyay 2017). The introduction of NGS to research on the bioremediation of PHC has dramatically improved our knowledge in relation to microbial communities, especially the taxonomic classification of different organisms including bacteria, fungi, algae, archaea and protozoa (Hivrale et al. 2015). Many innovations have been introduced to NGS technologies over the last decade. For example, a dramatic reduction in cost and running time has been achieved using pyrosequencing through the introduction of Illumina MiSeq (Loman et al. 2012). For all the reasons above, NGS has become the most convenient technology in the field of environmental biotechnology in general and bioremediation in particular.

The role of NGS in improving the bioremediation technology of hydrocarbon-contaminated environment has been reported in many different scenarios. The importance of specific unculturable microbial groups was identified during a bioremediation process of a marine oil spill, which involved improving the effectiveness of marine bioremediation (Techtmann and Hazen 2016). Also, the changes in the diversity of soil indigenous microbes were studied during a crude oil spill in a terrestrial environment. In this study, a number of dominant, unculturable hydrocarbon degraders were successfully identified using NGS (Abbasian et al. 2016). In a different study, NGS has been employed to evaluate the effects of environmental factors, such as temperature and salinity, on the microbial diversity in a PHC-contaminated desert soil (Abed et al. 2015a).

27.9 Mixed Contamination

One of the recent and serious challenges impacting the effectiveness of bioremediation of PHC-contaminated environments is mixed contamination. In addition to the known factors that limit the biodegradation of PHC, the presence of other contaminants alongside PHC causes severe complication during the bioremediation

process. Heavy metals are among these co-contaminants; their toxicity often causes inhibition of microbial degradation of PHCs (Thavamani et al. 2012a, b). In addition, pesticides, herbicides and chlorinated solvents are other examples of co-contaminants. The bioremediation of PHC-co-contaminated soils is a complicated process because of the differences in the remediation strategies for each group of contaminants (Sandrin and Maier 2003). Therefore, to achieve a comprehensive bioremediation strategy to treat a mixed contamination, intensive studies are required to investigate the microbial communities, their interactions and the roles of each organism in the community. Furthermore, the metabolic potential, as well as the mechanisms of microbial adaptation and resistance to the presence of more than one contaminant, should be fully investigated (Alisi et al. 2009).

In a balanced ecosystem, the indigenous PHC degraders are present in small number which increases after the PHC contaminant enters their environment. This microbe's proliferation could be inhibited by the presence of co-contaminants. Therefore, to achieve an effective bioremediation technique, many strategies can be applied in order to activate and/or accelerate the degradation potentials (Thavamani et al. 2012a, b). Biostimulation represents one such strategy which takes place by shifting the physiochemical characteristics of the contaminated environment including nutrient concentration, electron acceptors/donors, pH and temperature. This strategy was applied to bioremediate contaminated soil with PAH and cadmium (Cd) (Thavamani et al. 2012b). Bioaugmentation also provides a significant enhancement to bioremediate mixed contaminated environments by increasing the number of contaminant degraders. Soil contaminated with TPH and moderate concentrations of Pb, Zn and Cu have been remediated using bioaugmentation (Agnello et al. 2016). Over the last decade, electrokinetics (EK) or bioelectrokinetics (BioEK), which is a recent technique that combines bioremediation and electrokinetic, has been applied to remediate mixed contamination soils with PHC and heavy metals (Dong et al. 2013). Successful remediation of mixed contaminated soil with PAH (phenanthrene) and nickel (Ni) was reported using this technique (Reddy et al. 2009). Also, the concentration of TPH in soil co-contaminated with lead (Pb) has been shown to be reduced by 81.7% and 88.3%, respectively (Dong et al. 2013). EK or BioEK has the advantages of being simple, safe and cost-effective and can be applied to a variety of contaminants (Reddy 2010).

27.10 Gaps and Limitations

Bioremediation processes are preferred over others because they are more economical and they usually have better public acceptance. However, there are some limitations that should be taken into account. These limitations have been listed in several reviews (Boopathy 2000; Dua et al. 2002), and here we discuss them further.

Boopathy (2000) has grouped the factors that affect bioremediation, whether they are scientific (Table 27.4), non-scientific and even regulatory.

Table 27.4 Technical factors limiting bioremediation of PHC

Factors	Limitations
Environmental	Lack of nutrients and preferential substrates
	Inhibitory environmental conditions
Microbial	Enzyme induction
	Enrichment of capable populations
	Production of toxic metabolites
	Horizontal gene transfer
Aerobic/anaerobic processes	Oxidation/reduction potential
	Electron acceptors availability
	In situ microbial community
Growth substrate vs co-metabolism	Contaminant type, concentration
	Alternate carbon source
	Microbial interactions (competition, succession predation)
Bioavailability of contaminants	Equilibrium sorption
	Irreversible sorption
	Incorporation into humic matters
Substrate	Low concentration of contaminants
	Contaminants chemical structure
	Toxicity and solubility
Mass transfer	Oxygen diffusion and solubility
	Diffusion of nutrients
	Solubility/miscibility with water

Adapted from Boopathy (2000)

Mass transfer is very important because it controls the rate at which microbial cells can convert contaminants. Increased microbial conversion capacities do not guarantee higher biotransformation rates when the mass transfer is a limiting factor (Boopathy 2000). It also affects the bioavailability of the contaminant, and they become unavailable in the absence of mass transfer. This may lead to weathering or aging (a decrease in availability over time).

Apart from the scientific factors, there may be other non-technical obstacles that must be taken into account. These include the ability to meet time limitations and favourable regulatory perception and the ability to reach the target among others. Regulatory factors are very important because they dictate what contaminants must be cleaned, to what extent and the approaches to be used.

Other factors include liability and human resources. Bioremediation is a relatively innovative and emerging industry; therefore, tighter restrictions are imposed on it. Furthermore, there is not an abundance of specialized personnel, and successful projects require the involvement of several disciplines such as engineering, microbiology, hydrogeology, soil science and project management.

Furthermore, some authors have proposed synthetic microbiology (Ramos et al. 2011) or a system biology approach for each bioremediation project. A systems biology approach is a research approach that studies the interactions and networks at the

molecular, cellular, community and ecosystem levels in complex biological systems (Chakraborty et al. 2012). A recent paper also proposes an engineering workflow for the use of bioremediation calling it bioremediation 3.0 (Dvořák et al. 2017).

Therefore, bioremediation projects may have some obstacles that need to be addressed in order to achieve success. However, bioremediation approaches are often still more economical than others, and their efficacy has been proven in several sites around the world.

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Correction to: Microbial Degradation of Hydrocarbons in the Ecosystem

Anupreet Kaur

Correction to:
Chapter 14 in: V. Kumar et al. (eds.), *Microbial Action on Hydrocarbons*, https://doi.org/10.1007/978-981-13-1840-5_14

Figure 14.1 of this chapter was inadvertently published with errors. The correct presentation is given here.

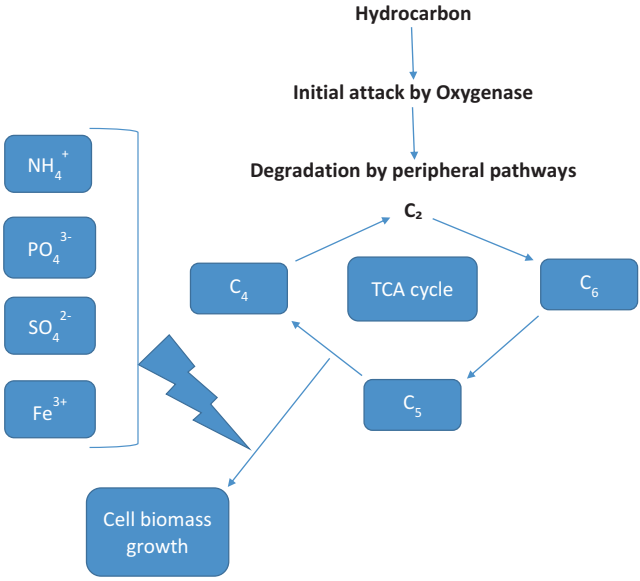

Fig. 14.1 Main principles of the aerobic degradation of hydrocarbons

The updated version of this chapter can be found at
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