2 Roles of Plants and Bacteria in Bioremediation of Petroleum in Contaminated Soils

Abbas Alemzadeh

Abstract

Large amounts of petroleum compounds are released into the environment every year as a result of industrial activities causing serious damages to the environment and human health. Various methods may be applied to remove the petroleum pollutants, but bioremediation is a cost-effective and sustainable process to remove these hazardous organic pollutants. The utilization of organisms such as plants and bacteria for biodegradation of pollutants is an inexpensive, environmentally friendly, and efficient approach to clean up polluted soils. Bacteria are ubiquitous in polluted environments and may develop different strategies to utilize pollutants. Plants may also be used to degrade the pollutants; that is called phytoremediation which is a promising method for reclaiming contaminated sites. Most plants associate with different bacteria that live around their roots, and this association can increase the biodegradation rate of organic compounds. In fact, plants and bacteria play pivotal roles in cleaning up the environment and can accelerate the remediation process of petroleum waste.

2.1 Introduction

Bioremediation, which is also referred to as biotreatment, bioreclamation, and biorestoration (Ahluwalia and Sekhon 2012), has been defined as the use of living organisms, plants, bacteria, or fungi, to detoxify, remove, or reduce the concentration of pollutants from the environment (Boopathy 2000; Vidali 2001), or may be defined as a biological response to environmental abuse (Hamer 1993). We must be

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A. Alemzadeh (✉)
Department of Crop Production and Plant Breeding, School of Agriculture, Shiraz University, Shiraz, Iran
e-mail: alemzadeh@shirazu.ac.ir

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careful in the use of our environment inasmuch as there are limited lands and resources, but studies worldwide show our carelessness in using them. Contaminated soils result from human activities when hazardous substances are produced. In the past, awareness of the potential adverse health and the environmental effects associated with these substances were less well recognized, but today, it is well known that contaminated soil is a threat to human health, leading to many efforts to remedy these sites, although the number of them is significant (Hou and Al-Tabbaa 2014; Sam et al. 2017).

The traditional techniques used for remediation of contaminated soils have usually been to excavate and remove it to landfill or cap and contain the contaminated areas of a site (Vidali 2001). Each method has some disadvantages. In the first method, it is expensive and difficult to find new sites for disposal of hazardous substances, and it may also create significant risks in the excavation, handling, and transport of these materials. The second method is an interim solution because the contamination remains on-site. A better approach is to destroy the contaminants or to transform them to innocuous substances (Vidali 2001). Hence, bioremediation, as a biological method, in which microorganisms and/or plants (phytoremediation) are used to destroy, transform, or in other ways detoxify wastes, may be considered as a substitution approach. In this method, microorganisms and plants have a critical role, and the increase in information about the parameters controlling the growth and metabolism of them in contaminated areas can predict the activity of plants or microorganisms that are involved in bioremediation and transform this technique from an empirical practice into a science (Lovley 2003).

2.2 Fate of Petroleum in Soil

The name petroleum covers both petroleum products and naturally occurring unprocessed crude oil (Jukic 2013; Matejicek 2017) and is constituted of hydrocarbons mixed with nitrogen, oxygen, and sulfur compounds and trace amounts of metals (Jukic 2013). Crude oil contains four major fractions including alkanes (paraffins), aromatics, cycloalkanes (naphthenes) and nitrogen-sulfur-oxygen (NSO) compounds (resins and asphaltenes) (El Nemr 2005; Franco et al. 2010; Jukic 2013). Alkane fraction mostly contains saturated hydrocarbons which occur as straight- or branched-chain (Jukic 2013). Aromatic fraction is composed of benzene rings, and their amounts are relatively small compared to naphthenes and paraffins in crude oil (El Nemr 2005). Cycloalkanes are saturated cyclic hydrocarbons which have one or more carbon rings, and their ratio depends on the crude oil type (Jukic 2013). NSO compounds consist of one or more aromatic rings and at least one heteroatom which can be oxygen, sulfur, or nitrogen atom (Oberoi et al. 2015).

The composition and inherent biodegradability of the hydrocarbon compounds are the most important factors in biodeterioration process. Polycyclic aromatic hydrocarbons (PAHs) and benzene, toluene, ethylbenzene, and xylene compounds (BTEX compounds) have been considered as main soil and water pollutant groups (Kuhad and Gupta 2009) inasmuch as they have low water solubility, high chemical stability, and resistance to biodeterioration. There are other factors that affect biodegradation of the compound in the soil as well as chemical characterizations. Among physical factors, temperature has an important role in biodegradation, affecting chemical characteristics of the compound and diversity of the microbial flora (Das and Chandran 2011). It has been shown that maximum degradation is obtained in the range of 30–40, 20–30, and 15–20 °C, in soil, freshwater, and marine environments, respectively (Das and Chandran 2011). In lower temperature, the volatility of low molecular weight hydrocarbons is decreased, while the viscosity of the oil increases, delaying in hydrocarbon biodeterioration (Atlas 1975). In addition, hydrocarbon biodegradation is mainly affected by the composition of the environmental pollutants (Huesemann 1995). There is a report to show that when the population of biodegrading microorganisms is more than 105 CFU/g of soil, efficient biodeterioration occurs (Forsyth et al. 1995). In addition, the rate of biodegradation is affected by the kind of microorganisms in the soil. It has been shown that the different population of microorganisms results in different rates of biodeterioration inasmuch as each microorganism has its own unique characteristics (Xu et al. 2009).

2.3 Use Bacteria in Bioremediation

Bacteria play a critical role in the sustainability of any ecosystem forasmuch as they are present everywhere and able to adapt to environmental changes. Bacteria are the most important groups in biodegradation of hydrocarbons although this process has been done by a wide range of microorganisms (Song et al. 1986). There is a wide group of bacteria that can decompose hydrocarbons derived from crude oil. They belong to different genera such as *Aeromonas*, *Alcaligenes*, *Achromobacter*, *Bacillus*, *Beijerinckia*, *Brevibacillus*, *Burkholderia*, *Corynebacterium*, *Delftia*, *Enterobacter*, *Flavobacterium*, *Gordonia*, *Lysinibacillus*, *Microbacterium*, *Mycobacterium*, *Nocardia*, *Paenibacillus*, *Paracoccus*, *Pseudomonas*, *Sphingomonas*, and *Stenotrophomonas* (Bartha and Bossert 1984; Paixão et al. 2010; Roy et al. 2014; Mojarad et al. 2016). Of these, some bacteria such as *Pseudomonas* and *Enterobacter* genera are the most predominant bacteria in environments contaminated by petroleum and have been applied in different studies as the main bacteria (Paixão et al. 2010; Mojarad et al. 2016; Ghoreishi et al. 2017). It has been found that some bacteria from the genus *Pseudomonas* are able to degrade many polycyclic aromatic hydrocarbons (Mrozik et al. 2003). The biodeterioration potential of bacteria isolated from contaminated environment is generally more than others isolated from non-contaminated environments, as these bacteria have adapted to contaminated area (Van der Meer 2006). It has been reported that the biodegradation rate of petroleum for soil bacteria is close to 70% (Mojarad et al. 2016; Ghoreishi et al. 2017), but it is more for marine bacteria, up to 100% (Mulkins-Phillips and Stewart 1974).

Bioremediation of petroleum using bacterial consortium is more effective compared to individual strain since there are various hydrocarbons in petroleum and they have various susceptibility to microbial attack, ranked as follows: linear alkanes>branched alkanes>aromatic compounds>cyclic alkanes>NSO compounds (Huesemann 1995). It should be considered that each environment harbors only a few strains and it can be an effective way to use various strains in bacterial consortium to increase the bioremediation efficiency (Lafortune et al. 2009). It has been shown that the diversity of bacterial species between different consortia can result in different rates of biodegradation. In addition to diversity, the bacterial community of different consortia is also important and can affect the biodegradation potential of a consortium (Ledezma-Villanueva et al. 2015).

2.3.1 Thermophilic Bacteria

Bacteria having an optimum growth temperature of above 40 °C are considered as thermophilic, in contrast to mesophilic bacteria that grow best in moderate temperature, between 20 and 40 °C (Margesin and Schinner 2001). These bacteria are divided into moderate thermophilic bacteria which refer to bacteria that grow optimally from about 50 to about 70 °C, extreme thermophilic bacteria which refer to bacteria that grow optimally from 70 to 80 °C, and finally hyperthermophiles which refer to bacteria having an optimum growth temperature of above 70 °C (Stetter 1998).

It has been shown that the biodeterioration of a wide range of hydrocarbons occurs in high-temperature habitats and many thermophilic bacteria have been isolated for use in petroleum-contaminated bioremediation (Margesin and Schinner 2001). Some thermophilic bacteria with potential for bioremediation are shown in Table 2.1. Of these, most bacteria belong to *Bacillus* and *Geobacillus* genera (Zhou et al. 2016; Elumalai et al. 2017; Singh et al. 2017) which are capable of degrading hydrocarbons, including all major classes, when the temperature is raised above 40 °C. This property of these bacteria has been of special interest in the bioremediation of contaminated sites mostly in naturally hot environments (Perfumo et al. 2007). Compared to mesophilic bacteria, thermophilic bacteria have great advantages in cleaning up oil-contaminated soils in terms of their properties, such as faster reaction rates and higher mass transfer rates (Feitkenhauer et al. 2003; Adhikari and Satyanarayana 2007).

It should be noted that hydrocarbons are naturally present in uncontaminated soils as a result of biotransformation of organic materials; therefore the presence of bacteria with biodegrading capacity is natural. Then biodeterioration of petroleum is a natural process. Although thermophilic bacteria naturally exist in hot environment like desert soils and hot springs (Aanniz et al. 2015), it has been reported some thermophiles exist in cool soil environments (Zeigler 2014). Both of these bacteria, isolated from hot environments or mesophilic environments, have the potential to be used in bioremediation of petroleum in contaminated soils.

Table 2.1 Some thermophilic bacteria with potential for bioremediation

Bacteria	Temperature	Carbohydrate	Reference
Bacillus licheniformis	50 °C	Alkanes	Garcia-Alcántara et al. (2016)
Bacillus licheniformis	50 °C	Alkanes	Elumalai et al. (2017)
Bacillus licheniformis	55 °C	Crude oil	Liu et al. (2016)
Bacillus stearothermophilus	60 °C	Hexadecane	Sorkhoh et al. (1993)
Bacillus thermoleovorans	70 °C	Alkanes	Kato et al. (2001)
Bacillus thermoleovorans	60 °C	Naphthalene	Annweiler et al. (2000)
Nocardia otitidiscaviarum	50 °C	Naphthalene	Zeinali et al. (2008)
Geobacillus kaustophilus	55 °C	Paraffin	Sood and Lal (2008)
Geobacillus pallidus	60 °C	Polycyclic aromatic	Zheng et al. (2011)
Geobacillus stearothermophilus	50 °C	Alkanes	Elumalai et al. (2017)
Geobacillus stearothermophilus	60 °C	Alkanes and aromatic	Zhou et al. (2016)
Geobacillus thermoleovorans	60 °C	Alkanes	Perfumo et al. (2007)
Geobacillus thermoparaffinivorans	50 °C	Alkanes	Elumalai et al. (2017)
Pseudomonas aeruginosa	45 °C	Crude oil and diesel oil	Perfumo et al. (2006)
Pseudomonas oleovorans	55 °C	Crude oil	Meintanis et al. (2006)
Pseudomonas putida	55 °C	Crude oil	Meintanis et al. (2006)
Thermus brockii	70 °C	Polycyclic aromatic	Feitkenhauer et al. (2003)

2.3.2 Bacterial Populations

In addition to abiotic factors that can affect the bioremediation rate of contaminated soils, bacterial population is also an important factor for hydrocarbons biodegradation. It has been shown that the soil bacterial population has a critical role in the biodeterioration of petroleum. Addition of organic materials instead of inorganic nutrients could not increase the degradation of hydrocarbons, indicating the lack of suitable strains in the environment (Vasudevan and Rajaram 2001). Forsyth et al. (1995) has been shown that biodegradation rate will not significantly occur when the population of biodegraders is less than 10^5 CFU (colony-forming units) g^{-1} of soil. In addition, it has also been previously reported that efficient biodegradation of crude oil was achieved when biodegraders were 1.7×10^6 CFU g^{-1} (Bello 2007). Although biodegraders are ubiquitous, the environmental conditions in petroleum-contaminated soils negatively affected the growth of these bacteria (Liu et al. 2010). It has been reported that manure increases the population of bacteria capable of degrading hydrocarbons in contaminated soils (Fallgren and Jin 2008; Liu et al. 2009). In all of these studies, the treatments led to increase in heterotrophic bacterial counts in contaminated soils. It was cleared that the bacterial counts increased in the first days after treatments and then gradually decreased, but always, the bacterial counts in treated soils were higher than control (Liu et al. 2009).

With gaining information about the bacterial communities in the contaminated soils, the bioremediation of contaminated sites can be performed in a more efficient way. It was shown that bacterial community dynamics during bioremediation of contaminated sites and the diversity of the community are decreased with increased biodegradation of petroleum (Ruberto et al. 2003; Katsivela et al. 2005; Leal et al. 2017). Different studies have showed that various biotic and abiotic factors affect the size of a bacterial community and principally depend on previous exposure to the contamination present in bacterial habitats and their adaptive capacity (Ruberto et al. 2003). It has been shown that a small fraction of indigenous flora is able to biodegrade hydrocarbons and usually needs to add exogenous bacteria (Ruberto et al. 2003).

2.3.3 Bioaugmentation

Bioaugmentation or biological augmentation is the addition of bacterial cultures to contaminated sites to speed up the rate of biodeterioration of undesired compounds (Van Limbergen et al. 1998). This method could serve as a powerful tool to improve biodegradation of recalcitrant compounds. There are several successful cases of biodeterioration of petroleum compounds that improved bioremediation efficiency in treated soils compared to control (bioremediation only by indigenous microorganisms) (Table 2.2). Bioaugmentation is often compared with biostimulation, which involves the modification of the environment to stimulate indigenous bacteria capable of bioremediation. Literature reviews indicated that both of these technologies can become environmentally friendly and economic approaches especially when applied together (Tyagi et al. 2011). These methods are the most common approaches for in situ bioremediation of contaminated environment, and in both strategies, the improvement of biodegrader population is the goal. Many factors such as applied bacteria, indigenous microbial community, competition with autochthonous microorganisms, type and concentration of pollutants, their availability to bacteria, the presence of roots that release organic compounds, and physicochemical characteristics of environment may affect the bioaugmentation process (El Fantroussi and Agathos 2005; Tyagi et al. 2011). Different strategies may be used for bioaugmentation, and the most common ones are addition of a bacterial strain, addition of a bacterial consortium, addition of genetically engineered bacteria, and biodeterioration of relevant genes into a vector to be transferred by conjugation to indigenous bacteria (El Fantroussi and Agathos 2005).

Although the introduction of exogenous bacteria into different environments is not new, bioaugmentation remains an experimental technique for in situ bioremediation of polluted soils (El Fantroussi and Agathos 2005). The success of any bioaugmentation depends on the relationship of exogenous bacteria with its new environmental conditions, and it is especially true in a dynamic and complex

Table 2.2 Successful cases of bioaugmentation of petroleum compounds

Bacteria	Strain	Percentage	Contamination	Reference
Acinetobacter sp.	B-2-2	$75\%_{35\%}$	TPH	Ruberto et al. (2003)
Acinetobacter SZ-1	KF453955	$34\%_{16\%}$	TPH	Wu et al. (2016)
Bacterial consortium	–	$61\%_{26\%}$	TPH	Xu and Lu (2010)
Bacterial consortium	–	$84\%_{47\%}$	TPH	Bento et al. (2005)
Bacterial consortium	–	$85\%_{30\%}$	Alkanes	Tahhan et al. (2011)
Bacterial consortium	–	$61\%_{6\%}$	Aromatics	Tahhan et al. (2011)
Bacterial consortium	–	$43\%_{0\%}$	Asphaltenes	Tahhan et al. (2011)
Bacterial consortium	–	$97.5\%_{62\%}$	TPH	Taccari et al. (2012)
Bacterial consortium	–	$62.1\%_{16.6\%}$	PAH	Wu et al. (2013)
Bacterial consortium	–	$75\%_{-}$	Total diesel hydrocarbons	Alisi et al. (2009)
Bacterial consortium	–	$78\%_{-}$	PAH	Jacques et al. (2008)
Indigenous bacteria	–	$\underline{95.5\%}\ 64\%_{35\%}$	C_{10}–C_{22}	Lebkowska et al. (2011)
Indigenous bacteria	–	$\underline{98.2\%}\ 52\%_{31\%}$	TPH	Lebkowska et al. (2011)
Paracoccus sp.	HPD-2	$23.2\%_{3.4\%}$	PAH	Teng et al. (2010)
Pseudomonas aeruginosa	WatG	$57\%_{35\%}$	TPH	Ueno et al. (2007)
Rhodococcus sp.	–	$97\%_{65\%}$	Diesel (C_{13}–C_{28})	Suja et al. (2014)

Subscript numbers correspond to the biodegradation percentage of control (without adding bacteria) and "–" indicates that data is not available. Underlined numbers indicate the biodegradation percentage of area where the inoculation was repeated every 3 days

PAH Polycyclic aromatic hydrocarbons, *TPH* Total petroleum hydrocarbons

environment such as soil. Therefore, it can be inferred that sterile soil is usually more hospitable to exogenous bacteria than natural soil (El Fantroussi et al. 1999). Hence, to increase the initial establishment of exogenous bacteria in natural soil, one must provide protection from harmful circumstances and predictable ecological selectively.

One way in which to overcome the problems in bioaugmentation is to use bacteria from the same niche as the contaminated soil as illustrated by the literature. The indigenous bacteria can be easily acclimatized and various studies showed this capacity is tremendous than previously thought inasmuch as environmental stresses may increase mutation rate. As these rapidly acclimating bacteria are to be used in bioaugmentation process, it should be for their applicability to remove pollutants from contaminated soils. Indigenous bacteria are often faster distributed than exogenous ones and can be better established in contaminated area. Another important concept is the distance between bacteria and target compound(s); it is thought that indigenous bacteria are closer to old contamination, while exogenous bacteria are closer to recent contamination (Vogel 1996). It has been recently shown that the addition of activated soils, soils containing indigenous biodegraders that are exposed to the pollutant, may be effective in bioaugmentation. However, the capacity of applied bacteria in bioaugmentation to survive and perform the biodegradation activity under conditions prevailing in the contaminated soil undergoing the bioremediation process is consequential.

2.3.4 Effects of Abiotic Factors

In biological process, abiotic factors usually play a pivotal role and determine the success or failure of the process. These factors are divided into two categories: those that limit the transfer of pollutants to bacteria such as soil texture and those that affect the activity of bacteria such as humidity and temperature (Zekri and Chaalal 2005; Leal et al. 2017). Here, we focus on those factors that affect the growth and activity of bacteria.

2.3.4.1 Temperature

There are some reports that showed the biodegradation increases in high temperature since increase in temperature accelerates the growth and activity of bacteria that results in increase of pollutants' deterioration (Zekri and Chaalal 2005). The results of some studies have shown that high temperature can increase removal of contaminants near to two times (Perfumo et al. 2007), although the best temperature for bacterial activity is the optimum temperature for bacterial growth (Ghoreishi et al. 2017). For example, the bacteria isolated from an area with yearly average high temperature of 32 °C could biodegrade petroleum pollutants at 28 °C very well, close to optimum temperature for their growth (Mojarad et al. 2016). Hence, when thermophilic bacterial strains are applied for biodegradation of unwanted compounds, the best result is obtained at high temperature (Elumalai et al. 2017); but the highest biodeterioration by mesophilic bacteria was obtained at 25 °C, and with increase in temperature, the biodegradation rate often decreases (Hesnawi and Mogadami 2013). In general, the optimum biodegradation has been carried out in the range of 25–40 °C for mesophilic bacteria (Kuhad and Gupta 2009) and well done at 50 °C or higher for thermophilic bacteria (Hesnawi and Mogadami 2013).

Cold temperature delays the biodegradation of petroleum-contaminated soils since microbial growth and activity are very negligible under low temperature. It means that pollutions remain for many years and natural remediation is believed not to be enough to rapidly clean up the contaminated environment. In situ bioremediation methods can be considered as a relatively low-cost way to remove pollutants in cold area with reasonable environmental safety. One of the most important factors to enhance bioremediation process in cold areas is the selection of suitable strains. It has been previously shown gram-negative bacteria, such as genus *Pseudomonas*, are the predominant bacteria in these areas, and therefore, they can be suitable candidates for petroleum bioremediation in cold environments (Labbé et al. 2007). Psychrophiles or cryophiles are the extremophilic bacteria that are capable of growth at temperature about 0 °C with an optimum growth temperature of <15 °C and are not able to grow above 20 °C and are usually found at temperature below 5 °C. Psychrotrophs or psychrotrophic bacteria are those able to grow at temperature about 0 °C with an optimum growth temperature >15 °C and are able to grow above 20 °C (Margesin and Schinner 2001).

The biodeterioration of different petroleum hydrocarbons by cold-adapted psychrophilic and psychrotrophic bacteria has been reported from various cold environments. These areas possess enough indigenous cold-adapted bacteria that can adapt rapidly to pollution inasmuch as after a contamination event, the number of biodegraders increase. As mentioned above, the temperature threshold for cold-adapted bacteria is around 0 °C, so lower temperature is not favorable for biodegradation. Bioaugmentation may be used as a useful bioremediation strategy in cold area, but the studies showed that indigenous cold-adapted bacteria can biodegrade pollutants more efficiently than exogenous strains (Margesin and Schinner 2001). One important factor that may limit pollutant biodeterioration by biodegraders in cold environments is the availability of nutrients like P and N. It was revealed that ammonium affects the biodegradation of petroleum components through progressive acidification to cause the inhibition of aromatic biodegradation. Biologically induced mineralization and biologically controlled mineralization are two models for the synthesis of minerals by bacteria that are affected by different factors. The mineralization of petroleum compounds decreases by some nutrients; for example, dodecane mineralization, any of the oily paraffin hydrocarbons having the formula $C_{12}H_{26}$, is limited by P and N at low temperature (Margesin and Schinner 2001).

Bacterial pathways responsible for deterioration of hydrocarbons, in petroleum contaminations, are spread in cold environments, and some of them can naturally coexist in the same bacterium. Some cold-adapted bacteria, such as *Rhodococcus* species, isolated from contaminated areas in cold environments are able to retain their metabolic activity even at sub-zero temperatures. The decreased availability of hydrocarbons at low temperature may be a reason for decreased biodeterioration; at 0 °C, bacteria mineralize the long-chain hydrocarbons to a greater extent than the long-chain ones because of their low bioavailability that may be responsible for their recalcitrance (Margesin and Schinner 2001).

2.3.4.2 pH

Although the optimum pH for petroleum compounds is at a range of 6–8, near-neutral pH values, biodeterioration process may occur over a wide pH range. If the pH of soil is less than 6, acidic soils, lime is generally added to increase the pH, and if the pH of soil is more than 8, alkaline soils, ammonium sulfate is generally added to decrease the pH (Kuhad and Gupta 2009). Different bacteria may have different pH optima; the soil pH plays a critical role in determining which bacterial strains become dominant during bioremediation process. Biodeterioration of aromatic petroleum hydrocarbons by bacteria seems to be sensitive to pH, which may be responsible for the persistence of aromatic hydrocarbons in environments. A pH of around 7 is often favorable for most biodegraders and can degrade the highest proportion of petroleum (Palanisamy et al. 2014).

2.3.4.3 Other Factors

In addition to temperature and pH, other factors have been shown to have a significant impact on bacterial growth and activity and consequently affect biodegradation activity. The growth of biodegraders in contaminated soils and their activity are affected by various factors; nutrient availability is one of them. Among nutrients, phosphorous and nitrogen are considered as the most important ones inasmuch as they are required for incorporation of carbon into biomass (Kuhad and Gupta 2009). The condition is unfavorable for bacterial growth when the ratio of C to N or P is increased. The optimal ratio of C/N and C/P for effective biodegradation of petroleum is usually 60/1 and 800/1, respectively (Kuhad and Gupta 2009).

Oxygen is another important factor in bioremediation process forasmuch as the main degradative pathways in most hydrocarbons in bacteria involve oxygenases for which oxygen is required (Kuhad and Gupta 2009). In addition, oxygen plays a role as an electron acceptor in the biodeterioration of petroleum compounds. Therefore, the depth of oxygen penetration in contaminated soils plays an important role in acceleration of biodegradation process, and it depends on the magnitude of the diffusion coefficient for oxygen in soils (Huesemann and Truex 1996). The oxygen availability is very important for metabolic activities of bacterial cells; hence it is necessary that oxygen supply rate should be more than oxygen consumption rate.

It has been shown that some other substances such as NaCl and chemical surfactants like Tween 80 and sodium dodecyl sulfate (SDS) affect the biodegradation rate through influence on bacterial growth (Palanisamy et al. 2014). Although it has been reported that NaCl increases biodegradation of petroleum products, but, in general, the effect of NaCl on the biodegradation process by bacterial cell somewhat depends on the strain involved (Palanisamy et al. 2014). It also showed that some surfactants, especially ionic ones, may enhance the growth of bacteria cells which leads to an increase in biodegradation rate (Palanisamy et al. 2014).

2.3.5 Genetic Engineering

Many bioremediating bacteria have been isolated and used to remove environmental pollutants; however, those that are present in high concentrations or some recalcitrant compounds do not occur in nature (xenobiotics), which are new compounds, that bacteria cannot catabolize them in an efficient way or have not usually evolved suitable catabolic pathways for them. Hence, application of genetically modified bacteria (GMB) with novel catabolic capabilities in the bioremediation of petroleum-contaminated sites can be considered as a new approach. GMB have shown high potential for petroleum biodegradation in contaminated soils although most of studies have been carried out in laboratory-based experiments and there are a few examples of GMB applications in the environment. Over the past years, application of GMB in bioremediation has been little developed, and its future is obscure for a number of underlying reasons. One of these is the high public sensitivity to genetically modified organisms (GMOs).

Recent advances in molecular biotechnology have been used to create new strains regarding bioremediation of petroleum-contaminated sites. These include development of new pathways and/or extension of existing pathways to catabolize a wider range of substrates, modification of catabolic enzyme specificity, cellular localization, substrate affinity, expression, increasing bioavailability contaminants, and creation and development of processes for monitoring and tracking of GMB (Urgun-Demirtas et al. 2006). In the case of pathways, various genes are obtained from different bacteria and combined together in one host to create an efficient pathway to catabolize xenobiotics and recalcitrant compounds. Site-directed mutagenesis has been used to increase properties of existing enzymes or alternative promoters used to enhance the expression level of enzyme genes. Oxygenases are required for the biodeterioration of many organic contaminants especially aromatic ones, and oxygen is necessary for optimal function of these enzymes. In the past decade, bacterial hemoglobin (VHB), which is synthesized in response to low-oxygen conditions, has been attracting more attention for use in bioremediation process because of their ability to provide sufficient oxygen to support enzyme activity. Expression or overexpression of VHB in bacterial cells can help them to overcome the problem in bioremediation of organic compounds under low oxygen availability (Urgun-Demirtas et al. 2006; Stark et al. 2015).

The first GMB, derived from genus *Pseudomonas*, was generated by an Indian scientist, Ananda Chakrabarty, in 1971 (Ezezika and Singer 2010). This strain harbored genes from four different *Pseudomonas* strains encoding different oil-degrading enzymes and enabled to break down crude oil very well, but unfortunately due to public concerns about genetically modified organisms (GMOs), it still sits on a shelf (Ezezika and Singer 2010). Following this effort, *Pseudomonas fluorescens* strain HK44, designed by the University of Tennessee in collaboration with Oak Ridge National Laboratory, was the first GMB released into the environment for bioremediation of a contaminated soil (Ripp et al. 2000). Strain HK44 derived from the parental strain was isolated from a polyaromatic hydrocarbon-contaminated site and harbors pUTK21 plasmid, naphthalene catabolic plasmid. In addition, this strain contains a *lux* gene fused to naphthalene catabolic promoter that shows a bioluminescent response when exposed to naphthalene (Ripp et al. 2000). Therefore, the use of *lux* gene in this GMB promises an efficient approach to monitor GMB in the environment that can facilitate the use of these bacteria in bioremediation process.

Potential environmental risks and biosafety issues for the release of GMOs should be considered as one of the most important aspects of using GMB in bioremediation process in the field. It is recommended that environmental risk assessment should be identified through the use of a model before release of GMB (Urgun-Demirtas et al. 2006). Another important reason for the limitation of application of GMB in the field may be a result of the instability of transferred genetic elements that includes two aspects: first, the functioning GMB depends on their ability to carry the genetic elements stably. Second, transfer of genetic elements to autochthonous bacteria may negatively affect other indigenous microorganisms (Ezezika and Singer 2010).

In spite of the advantages of GMB and recent advances in monitoring and tracking strategies for GMB, there are still concerns that their application in bioremediation of contaminated sites may have environmental risks such gene flow. Horizontal DNA transfer among bacteria is a widespread and natural process that plays a pivotal role in bacterial evolution. It has been suggested that this phenomenon contributes to the development of new strains with higher biodegradation potential for xenobiotics when bacterial cells are exposed to contaminants (Shintani and Nojiri 2013). It seems that horizontal transfer rate depends on both substrate concentration and growth rate of plasmid-bearing cells (Urgun-Demirtas et al. 2006). Hence, GMB introduced into contaminated sites may have unknown and undesired effects on indigenous bacterial communities because of horizontal DNA transfer, and this effect is not uniform, and therefore, it is necessary to evaluate case by case. Thus, it is required to find a way to overcome concerns about the use of GMB in bioremediation of contaminated sites. The use of antibiotic resistance genes as selectable markers is an important concern with the use of these bacteria, and replacing them with other kinds of markers, such as genes for herbicide or heavy metal resistance, can be a solution. Another solution may be use of transposons as a way for stable integration of transgenes into bacterial chromosome instead of recombinant plasmid. Suicide systems can be used as another solution for this problem. In these systems, GMB will be dead after finishing its job. Although the use of GMB in bioremediation project has been limited due to public concern and little application in the field over the past years, recent advances in molecular biology allow us to create "suicidal genetically modified bacteria" (S-GMB) to minimize the hazards and clean up the contaminated sites in a safer way (Singh et al. 2011).

2.3.6 Tracking GMB

It is very important that we are able to use some methods to track released GMB to determine the effects of them on the environment. The used methods should be simple, accurate, inexpensive, and applicable in the environment. It is also necessary and useful to monitor the recombinant plasmid and transgene by which the GMB has been genetically modified as well as GMB itself to determine the potential loss of recombinant DNA from the GMB and transferring to nontarget organisms. Growth of colonies on plates has traditionally been used as a simple method to monitor GMB, but this method has some limitations that methods based on molecular biology techniques overcome these limitations. Various molecular techniques including Southern blotting, polymerase chain reaction (PCR), reverse transcription-PCR (RT-PCR), real-time PCR, and denaturing gradient gel electrophoresis (DGGE) have been used in tracking GMB or recombinant DNA (Urgun-Demirtas et al. 2006; Han et al. 2012). Molecular methods are suitable ones because they are applied directly to contaminated sites and it is not necessary to culture bacterial cell outside the environment. Hence, in these methods bacterial communities do not change, and all microorganisms, culturable and non-culturable ones, will be used in the experiment. Anyway, using molecular methods in combination with traditional ones may give a strength method for monitoring GMB and/or recombinant DNA.

2.4 Phytoremediation: Role of Plants

The use of plants to clean up contaminated soils is called phytoremediation that has been considered as a promising method for the bioremediation of petroleum-contaminated soils. In phytoremediation technology, the plants usually absorb the pollutants from the soils, but in the case of petroleum or its products, the plants are not able to take up most of them inasmuch as they are water insoluble. Petroleum contains PAHs that may be taken up by plants through several transferring mechanisms of PAHs from soil to plant roots and leaves, including volatilization, transpiration stream, and direct absorption from soils (Watts et al. 2006). The solubility of pollutants is a critical factor to uptake, translocate, and catabolize by plants that is usually reflected by log K_{ow}, octanol-water partition coefficient of the pollutant. Plants are able to uptake pollutants with log K_{ow} values less than 3 and translocate and catabolize those pollutants with log K_{ow} values less than 1 (Alkorta and Garbisu 2001).

2.4.1 The Rhizosphere

Indeed, in phytoremediation of petroleum-contaminated sites, plants are usually used to stimulate rhizosphere bacteria, namely, plants that have an indirect effect in cleaning up petroleum-contaminated sites (Merkl et al. 2005), although they may play a pivotal role in direct removal of pollutants. The rhizosphere is the thin layer of soil that contains plant roots and is influenced by root system and associated soil bacteria. Hence, rhizosphere is a suitable environment for microorganism's life inasmuch as the moisture, oxygen, and organic matter content of rhizosphere is usually higher than other parts of the soil, and the bacterial communities in rhizosphere are usually different from non-rhizosphere area (Shrivastava et al. 2014; Prasad et al. 2015). On the other hand, some species such as bacteria belonging to the genera *Pseudomonas* and *Azospirillum* affect the plants and may increase the release of organic compounds from roots (Curl and Truelove 1986).

Bacterial numbers increase around the plant roots and decrease with increasing distance from the roots; that is called “rhizosphere effect.” Rhizosphere effect is calculated using the ratio of bacterial numbers in the rhizosphere to non-rhizosphere soil which is between 1 and more than 100 (Jones et al. 2004). Rhizosphere effect is affected by many factors that one of them is the quality of the soil, and in contaminated soils, the ratio is dramatically decreased (Jones et al. 2004). It is expected that there is a high potential for deterioration of petroleum compounds in rhizosphere for more bacterial activities, although the bacterial numbers decrease in contaminated soils.

2.4.2 Potential Use of Plants

Petroleum-contaminated soils may affect plant growth and production in different ways. Pollutants influence both abiotic and biotic factors, microorganisms, and parts of soil that leads to damage of cell membranes of plant roots and shoots. But, different plants have different potentials to use in phytoremediation of petroleum-contaminated sites, and some plants can grow well in contaminated soils, whereas the growth of some species is inhibited after germination or in other growth phases (Kaimi et al. 2007). The toxicity of petroleum compounds on the plant growth may be caused by parameters: hydrophobicity in contaminated soils and/or volatile hydrocarbons (Kaimi et al. 2007). Hydrophobicity causes the decrease of aeration rate that results in growth inhibition of plant roots, and volatile compounds cause toxic effects moving through the plant cells. In most studies, legumes and grasses have been used for phytoremediation of petroleum contaminants because grasses have well-developed root system with maximum root surface area and can deeply penetrate in the soil and legumes have the potential for nitrogen fixation (Merkl et al. 2005; Kaimi et al. 2007). However, it is expected that legumes are able to grow in contaminated soils due to their capacity for symbiotic relationship, but some of them showed poor growth in these soils (Merkl et al. 2005; Kaimi et al. 2007). In addition to these plants, some other species are also used for phytoremediation of petroleum pollutants that the result was positive. *Mirabilis jalapa* is one of these species that show a high potential for phytoremediation and can be effectively used for phytoremediation of highly contaminated soils (Peng et al. 2009). Hence, it is necessary to test the efficiency of other plants from other families for phytoremediation of petroleum-contaminated soils.

A long exposure to pollutants may prolong the toxic effects of petroleum compounds on the plant, since the pollutants can accumulate and persist for a long period of time (Merkl et al. 2005). Hence, some species may germinate and grow in contaminated soils in a short time, but they died when exposed to contaminants for a long time. The sensitivity of plant to contaminants can limit the phytoremediation of petroleum-contaminated soils due to reduced plant growth, soil fertility, and bacterial numbers. Plant root exudates including amino acids, simple carbohydrates, vitamins, alkaloids, tannins, phosphatides, and other organic materials induce some microbial metabolites and increase bacterial communities in soils which results in higher efficiency of contaminant biodeterioration (Mehmannavaz et al. 2002). Not only fertilizers can increase the growth of plants resulting in higher biomass production but also enhance the bacterial growth and their activities since sufficient fertilizers in contaminated soils reduce the competition for nutrients. However, it should be considered that the high levels of fertilizers may damage plants due to the hydrophobic nature of petroleum-contaminated soils and accumulation of the fertilizers on the soil surface (Merkl et al. 2005).

In addition to root exudates that stimulate the growth and activities of rhizosphere bacteria, some degradative enzymes including dehalogenases, laccases, nitrilases, nitroreductases, tyrosinase (monophenol monooxygenase), and peroxidase are also released from roots into the rhizosphere (Alkorta and Garbisu 2001; Gianfreda and Rao 2004). Different plants have been studied for the exudation of enzymes from their roots, and it has been revealed that several members of Solanaceae, Gramineae, and Fabaceae are able to efficiently secrete various enzymes into the rhizosphere (Gianfreda and Rao 2004). There is no doubt that these enzymes play a pivotal role in phytoremediation of organic compounds, but there is a paucity of information on the amount of secreted enzymes which lead to the deterioration of organic substrates, although the calculation of half-life of the enzymes suggests that they are able to remain active and degrade the compounds for days (Alkorta and Garbisu 2001). Frick et al. (1999) states *n*-alkanes can be assimilated in both roots and leaves of plants and the pathway of conversion for *n*-alkanes is generalized as:

$$\begin{aligned} n\text{-alkanes} &\rightarrow \text{Primary alcohols} \rightarrow \text{fatty acids} \rightarrow \text{acetyl-CoA} \\ &\rightarrow \text{various compounds} \end{aligned}$$

2.4.3 Plant-Bacteria Interactions

Although both bacteria and plants can directly and indirectly degrade hydrocarbons into products in petroleum-contaminated soils independently from each other, it is thought that the interaction between bacteria and plants is the primary mechanism in phytoremediation (Frick et al. 1999). Plant-bacteria interactions are important processes defining the efficiency of phytoremediation of petroleum-contaminated soils; however our knowledge about the mechanisms of these interactions remains very incomplete. The interactions between bacteria and plants can be divided into two types, specific and non-specific interactions depending on root exudates. Specific interactions occur when the roots exude the specific compound(s) in response to the presence of specific pollutant and the plant provides a specific carbon source for the bacteria, but in non-specific interactions, normal plant processes stimulate the bacterial cells. Both of these interactions enhance the biodegradation rate of organic compounds by bacteria associated with plant roots, and in return, the capacity of plants increases for petroleum biodegradation or reduces the phytotoxicity of the contaminated soil (Siciliano and Germida 1998). As mentioned above, the plant also releases some enzymes from roots that play a pivotal role in deterioration of organic compounds and altering the environment to promote bioremediation by bacteria (Frick et al. 1999). In fact, the external degradation of contaminants by rhizosphere bacteria is a way to reduce the harmful effects of petroleum compounds, and it is believed that plants and bacteria have coevolved to develop a mutually beneficial strategy to reduce phytotoxicity (Frick et al. 1999). Plant roots enhance the degradation of contaminants by affecting the chemical and physical properties of the soil.

The presence of roots in the contaminated soil brings plants, bacteria, pollutants, and nutrients into contact with each other.

It has been shown that the interaction between plants and biodegraders is relatively stronger during vegetative phase than reproductive phase. The roots have very important roles in interaction between plants and bacteria, and its traits vary faster than other organs in response to the presence of contaminants in the soil (Nie et al. 2011).

2.4.4 Limitations of Petroleum Phytoremediation

The depth of contamination is a limitation factor for phytoremediation and it can be effective only in the surface area (Anyasi and Atagana 2011). The roots of most plants, except trees, cannot penetrate very deeply into the soil, and root density decreases with increase in depth. In addition, there is an increase in immobile pollutants that cannot be taken up by the roots when the depth increases more than 2 m that can negatively affect the efficiency of phytoremediation.

In spite of positive aspects of phytoremediation, it is a slow process and needs a long period, usually several years, to clean up contaminated soils (Van Epps 2006). The time required will increase in the presence of hydrophobic contaminants which are tightly bound to soil particles. There are some approaches such as chemically enhanced phytoextraction, application of genetically modified plants, and developing plant-bacteria system that may be used to enhance the phytoremediation rate (Oh et al. 2013).

The high concentrations of contaminants may not allow plants to grow and extend the roots due to oxidative stress and toxic effects (Van Epps 2006). Hence, this technology can be effective when used to clean up low contaminant concentrations (Yavari et al. 2015). In highly contaminated soils, the contamination should be reduced before applying phytoremediation to remediate contaminated sites, and then it can be used as a final treatment (Yavari et al. 2015).

Environmental factors such as temperature, soil texture, oxygen availability, pH, salinity, and nutrient availability can affect the efficiency of phytoremediation (Frick et al. 1999; Brandt et al. 2006). The effectiveness of phytoremediation will be enhanced when the environmental conditions are optimum for plant growth. For example, phytoremediation is not an effective technology in low-temperature environment forasmuch as the plant growth is slow or stopped (Frick et al. 1999; Brandt et al. 2006; Yavari et al. 2015). It has been shown that the sufficient nutrient can enhance plant growth parameters in contaminated soils that results in increase of phytoremediation rate (Brandt et al. 2006).

The chemical properties of contaminants also play important roles in phytoremediation efficiency. Water solubility of contaminants is an important factor in biodegradation of them; i.e., the more water-soluble the contaminant, the more rapidly it is biodegraded (Frick et al. 1999). The compounds with smaller molecular weight are more easily dissolved in water and biodegraded by living organisms (Yavari et al. 2015). Although, water solubility of the compound is a favorable property, but it may cause leaching of compounds and resulting in contamination of underground water (Frick et al. 1999).

2.5 Conclusion

In conclusion, the removal of contaminants from the environment by bacteria (bioremediation) and plants (phytoremediation) has been used as a reasonable approach to clean up the soil in recent decades. However, this technology has developed into an acceptable alternative to physical methods, but numerous biotic and abiotic factors involved in the successful implementation of the technology. Hence, even under optimal conditions, it is unlikely that petroleum contaminants have been completely removed from contaminated sites. It is important to keep in mind that when bioremediation is applied to a contaminated site, increased biodeterioration may not immediately occur due to various factors. Some new strategies, such as the use of GMB, may increase the efficiency of bioremediation, but the stability of GMB and the horizontal transfer of the recombinant DNA should be considered. Finally, recent advances in our knowledge of associations between plants and bacteria and the mechanisms by which bioremediation occur can help us develop practical soil bioremediation strategies.

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