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Abstract	Breakdown of the crude oil contaminant by soil microbial community in the bioremediation can be enhanced in the presence of plant rhizosphere, but plant cannot grow and tolerate the high concentration of hydrocarbon. Composting of the crude oil-contaminated soil was found to be an effective technology to decrease the pollutant concentration to the acceptable level for plant growth. Phytoremediation with native plants followed with composting can be performed well, but the microbial abundance in the contaminated soil usually becomes much low as time passes. In order to enhance the degradation potential of heterotrophic microorganisms in bioremediation, different bioaugmentation techniques are used, while few researches also used plant rhizosphere along with it, but the results are ambiguous. The present chapter will explore this ambiguity by providing the complete description about the bioremediation and the facts that plant rhizosphere selects microbial community, which is more suitable for pollutant degradation than addition of microbial culture in soil. Rhizodegradation followed with composting is found to be a useful treatment method for extremely polluted soil, and bioaugmentation with immobilized and free bacterial culture was found more effective without plant.

Keywords (separated by " - ")	Rhizosphere - Bioaugmentation - Immobilization - Inoculum - Petroleum hydrocarbon

Chapter 7
Inoculum Addition in the Presence of Plant Rhizosphere for Petroleum-Polluted Soil Remediation

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7.1 Introduction

Composting of contaminated soil is one of the feasible methods to clean up petroleum hydrocarbon in the biopiles by the addition of nutrients and amendments, which enhances the hydrocarbon degradation and improve soil quality (Van Gestel et al. 2003). However, the contents of the petroleum hydrocarbons and their decomposed matter present in the soil after composting are often above environmental standards (Zhang et al. 2010). Bioremediation is the conversion of complex organic contaminant into simpler inorganic compounds including carbon dioxide, water, and cell biomass by biological agents like microorganisms (Das and Chandran 2011). Due to the presence of pollutant in the soil, microorganism including bacteria, fungi, and yeast that prefer the chemicals as a source of food and energy can be flourished. In the soil, mineralization rate contaminant depends on the microbial activity and abundance in the soils. Pollutant degradation is extremely dependent on the presence of electron provider or acceptors,

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presence of co-metabolites, absence of inorganic nutrients, plant vitamins and hormones, as well as microbial competition (ITRC 2009).

Bioremediation methods, which enhance the biodegradation rates, include natural attenuation without any interference, use of seed culture of hydrocarbon degraders, biostimulation with nutrient addition for improved microbial growth, and rhizospheric biodegradation (Odokuma and Dickson 2003; Bhatti et al. 2017). Microbes spread in the rhizosphere, and their number increases due to the availability of nutrients which are released by plants including C, N, S, and P. Few studies used bioaugmentation in the presence of plant to remediate the petroleum-polluted soil (Kirk et al. 2005; Baek et al. 2004).

Through bioaugmentation desired microbes and their enzymes increase, but this process does not work always due to the inability of culture to adjust in new environment and to compete with native microbes (Gerardi 2016; Ramos et al. 2010). Microbial entrapment in solid media is used for the protection and survival of inoculated culture, and the most effective microbial immobilization was cell entrapment into a porous matter (Partovinia and Rasekh 2018). The immobilized cells have proved stability under different environmental conditions for petroleum-contaminated soil (Gao et al. 2011). The present chapter reveals different strategies of complete biodegradation of highly petroleum-polluted soil, limitation, and outcomes of these techniques.

7.2 Different Strategies of Bioremediation

7.2.1 Composting of Highly Petroleum-Polluted Soil

Composting is the addition of biomaterial and biogenic bulking agents used for aeration and to adjust water, nutrient, and pH of the contaminated soil (Kästner and Miltner 2016). Composting of highly petroleum-contaminated soil is a very encouraging technique, and it can be performed at the first step before phytoremediation to lower down the concentration to acceptable level for plant growth. Maize straw, pine wood chips, and soybean cake were used as bulking agent in composting to lower down the total petroleum hydrocarbon (TPH) concentration from 17,900 to 3700 mg kg^{-1} (Wang et al. 2011). This composted soil was further used to lower down the concentration up to 673 mg kg^{-1} using *Sesbania cannabina*, a coastal halophyte (Table 7.1 shows all results of the project) (Farhana et al. 2012). Jørgensen et al. (2000) used bark chips as the bulking agent with inoculum to compost the lubricating oil-contaminated soil; 70% removal rate was observed with organic matter addition and no effect of inoculum addition. Another study used cow bed and potato peelings for high asphaltenic fuel oil composting, and the result showed that with

Table 7.1 Different bioremediation approaches for the Shengli Oil Field petroleum-polluted soil and their maximum removal percentages

S. no.	Technique	Maximum bioremediation duration (days)	Removal percentage of petroleum hydrocarbon (%)
1	Bioaugmentation with free culture inoculation	70	39
2	Bioaugmentation with immobilized culture inoculation	70	47
3	Addition of compost in contaminated soil	90	45
4	Phytoremediation with seepweed	90	42
5	Phytoremediation with *S. cannabina*	90	73
6	*S. cannabina* in the presence of free culture inoculation	120	73
7	*S. cannabina* in the presence of immobilized culture inoculation	120	68

[1, 2]Wang et al. (2012), [3, 4](Wang et al. (2011), [5,6,7]Farhana et al. (2012)

autochthonous bacteria, fragmentation into easily degradable smaller structure took place (Martin-Gil et al. 2008).

7.2.2 *Enhanced Degradation of Pollutants with Bioaugmentation*

Sometimes biodegradation fails due to insufficient indigenous oil degrader population or their incapability of degrading the broad range of toxic fractions present in the environment (Hussein 2006). In this situation, bioaugmentation was found to be a promising low-cost technique in which effective bacterial culture or microbial community capable of degrading hydrocarbons is added to the contaminated soil (Zawierucha and Malina 2011). Bioaugmentation can be achieved in three ways, first, autochthonous bioaugmentation in which seed cultures are obtained from the original contaminated site, re-cultured in the lab, and reused in the same soil which needs treatment (Ueno et al. 2007). The second method is that seed culture is taken from one site and used in different contaminated site, and the third method is the use of genetically modified organism with degradative potential (Vogel and Walter 2001). Among these autochthonous bioaugmentation is of great interest because it is found to be best adopted with the contaminated environment.

Many researchers used bioaugmentation alone for crude oil degradation (Bento et al. 2005; Yu et al. 2005), but few researches are found in the field of rhizosphere bioaugmentation (Peng et al. 2009; Ma et al. 2010; Cai et al. 2010; Tang et al. 2010; Thangarajan et al. 2011) (Table 7.2). The use of higher plants to enhance

removal of contaminants from soil although low cost than other remediation techniques often does not result in complete removal of contaminants (Banks et al. 2003); therefore some researchers tried to use inoculum addition in the presence of plant. Bioaugmentation required successful and safe introduction of microbial cells into uncontained environments (Trevors et al. 1993).

Ramos et al. (2010) explained that inoculated cultures sometimes fail to resist and survive in contaminated soil or in their native living place soil. Seed culture should be highly stable and resistant to different environmental conditions (Burken 2004). These obstacles can be overcome using some suitable inoculation techniques.

7.2.2.1 Liquid Microbial Culture Inoculation

In previous studies for bioaugmentation in soil, broth culture inoculation such as free culture spraying procedures or culture in semisolid media, i.e., immobilization techniques, was used (Trevors et al. 1994). The use of inoculated culture is not always acceptable, since inoculation experiments have shown ambiguous results in comparison to degradation by native microbial population (Leahy and Colwell 1990). Kastner et al. (1998) used several strains of PAH-degrading bacteria, but after introduction into artificially PAH-contaminated soil, no degradation was observed; further experiment suggested that inoculation with mineral salt medium inhibited bacterial survival and growth as well as their degradation activity; while using water without increasing salinity, no inhibition of autochthonous bacterial strain took place (Hosokawa et al. 2009).

7.2.2.2 Inoculation with Immobilized Microbial Culture

Captivity of enzyme or whole bacterial cell inside or outside the carrier matrix is known as immobilization (Partovinia and Rasekh 2018). For petroleum hydrocarbon biodegradation, microbial attachment on solid surface, entrapment in a gel or membrane, or encapsulation into carrier was used (Hussein 2006). Microbial culture for bioaugmentation in an immobilized form may offer more complete and more rapid degradation, which is easy to handle, can be reutilized, and has high tolerance to pH and temperature changes (Partovinia and Rasekh 2018). Immobilized matrices provide sustained microbial population by continuous release of bacteria in the soil, water, and sediments. Although in many studies culture used in immobilized beads form, still most of the studies have not addressed the growth dynamics of different bacterial strains inside the beads (Bazot and Lebeau 2009).

There are two types of hydrogel material for cell immobilization, natural and synthetic. Natural material includes agar, agarose, polyacrylamides, carrageenan, and alginate (Leenen et al. 1996) (Table 7.3).

Table 7.2 Researches on bioaugmentation in bioremediation of petroleum-contaminated soil

S. no.	Strategies	Pollutants	Outcome	References
1	Natural attenuation	TPH	Effective than biostimulation	(Mishra et al. (2001)
2	Phytoremediation (grasses)	Diesel and heavy oil	Noneffective in old contaminated soil	Banks et al. (2003)
3	Bioaugmentation	TPH	Effective than NA*/B[a]	Bento et al. (2005)
4	Bioaugmentation	PAH	Noneffective than NA*	Yu et al. (2005)
5	Bioaugmentation	PAH	Noneffective	Herwijnen et al. (2006)
6	Phytoremediation	TPH	Effective than NA*	Peng et al. (2009)
7	Phytoremediation	PAH	Effective in freshly contaminated soil than old	Ma et al. (2010)
8	Phytoremediation (*Impatiens balsamina L*)	TPH	Effective than natural degradation	Cai et al. (2010)
9	Phytoremediation and bioaugmentation	Petroleum	Combine treatment effective than single	Tang et al. (2010)
10	Bioaugmentation	TPH	Noneffective than NA*/B[a]	Thangarajan et al. (2011)
11	Bioaugmentation	Aromatic and asphaltic	Effective than indigenous	Gonzalez et al. (2005)
12	Bioaugmentation	Crude oil	Mix culture effective than single spp.	Rahman et al. (2002)

∗*NA* natural attenuation, *B*[a] bioaugmentation

Alginate is used widespread due to its nontoxic and nutritional behavior for bacterial cell immobilization. The incorporation of some adsorbents into alginate beads is used to transport pollutants inside and outside of the beads (Zhang et al. 2008). Sodium alginate matrix has hindrance to mass and air transfer inside the dense gel layer (Mikkelsen and Elgsaeter 1995). Diatomite was incorporated to overcome this problem (Farhana et al. 2012). Diatomite (SiO_2 nH_2O) is off-white to white color, soft, light in weight sedimentary rock composed mainly of silica microfossils of aquatic algae. It is highly porous with good sorption ability, is nonreactive with other chemicals, and has low density and large surface area; due to these unique properties, this material is used in cell immobilization for hydrocarbon degradation (Nenadovic et al. 2009). Wang et al. (2012) in their study found that after 20 days of oil degrader inoculation in the soil, the maximum degradation rate in the sodium alginate diatomite (SAD)-immobilized systems reached up to 29.8%, significantly higher ($P < 0.05$) than free cells (21.1%). Moreover, both microbial number and total microbial activity reached to highly significant level ($P < 0.05$) in the immobi-

lized culture than free culture inoculation systems at a same initial inoculation amount. In the presence of plant rhizosphere, this immobilized inoculum was ineffective (Table 7.1) (Farhana et al. 2012) because sodium alginate diatomite carrier has short life and degraded within 20 days and plant growth takes more time (Mikkelsen and Elgsaeter 1995).

7.2.3 *Rhizoremediation of Pollutants*

Pollutant breakdown in the rhizosphere is improved due to the increase in the microbial activity than non-rhizosphere; this process is generally known as phytostimulation or plant assisted microbial degradation (Donnelly et al. 1994). Rhizodegradation involves the immobilization and removal of the pollutants which is strongly dependent on the rhizospheric processes including enhancement of microbial degradation. The number of PAH degraders and total microbial count were found higher in the rhizospheric region of ryegrass and clover than in the un-vegetated soil (Ma et al. 2010).

Symbiotic association is observed around the plant rhizosphere and their associated microbes due to the release of root exudates which provide necessary nutrients for the survival of microbes and in turn microbes responsible for improved soil conditions for plant growth. Especially, plants help in softening of soil due to aeration and water transport into the rhizosphere (Fig. 7.1). Plants also release allelopathic agents which suppress the growth of other plants in the same soil and protect them from competition, soil pathogens, toxins, and chemicals released from the unwanted plants.

In specific plant-microbe interaction, plant detects toxicants and secretes specific compounds in response to that stimuli and subsequently promotes the growth and activity of specific bacterial community (Fang et al. 2001). Due to this reason sometimes inhibition of inoculated bacterial culture was observed which interferes with the natural plant-microbe interaction and leads to the failure in enhanced degradation by bioaugmentation (Farhana et al. 2012) (Fig. 7.1). Nonspecific interaction occurs when the contaminants themselves may have similarities to the phytochemical which are released by the plant naturally (Phillips et al. 2012). These phytochemicals are used by the rhizospheric microbes as a primary carbon source, and co-metabolic process starts which slows down the pollutant degradation. Sometimes pollutants' degradative enzymes produced by microbes can also be produced by the plant itself. These secondary plant metabolites (SPMEs) are very diverse in nature, having structural similarities with organic pollutant which play an important role in the production of many degradative enzymes (Jha et al. 2015). Examples of analogy included PAH, their structures are similar to the root exudates morin (Donnelly et al. 1994), and pyrene resembles SPME confusarine released by plants (Singer et al. 2003). The degradation capacity increased in the presence of exudates acetate and alanine while decreased when malonate was

AU7

Table 7.3 Summary of the researches on different immobilization strategies for bioaugmentation t3.1

Material for immobilization	Target pollutant	Microorganisms type	Effect	Reference	t3.2 t3.3
Biofix and Drizit	Petrol	*Pseudomonas fluorescens*	Best results than	Wilson and Bradley (1996)	t3.4 t3.5
Sodium alginate	Phenol, ortho- and Para-cresol	Methanogenic consortium	Twofold higher than free culture	Guiot et al. (2000)	t3.6 t3.7
Polyvinyl alcohol and Drizit	Diesel	Mix hydrocarbon degraders	Best results than commercial liquid containing surfactants	Cunningham et al. (2004)	t3.8 t3.9 t3.10 t3.11
Vermiculite	PAH	*Bacillus* and *Mucor*	Best results than free culture	Dan et al. (2006)	t3.12 t3.13
Ca alginate	Cd	*Streptomyces* and *Bacillus*	Less effective than culture type	Jeˇzeˇquel and Lebeau (2008)	t3.14 t3.15
Alginate-lignin	Phenanthrene	*Phanerochaete chrysosporium*	Best results than free culture	Zhang et al. (2008)	t3.16 t3.17
Phosphorylated polyvinyl alcohol	Atrazine	*Agrobacterium radiobacter* and mix culture	Best results than free culture	Siripattanakul et al. (2008)	t3.18 t3.19 t3.20
Alginate and biofilm on tezontle	Organophosphate pesticides (OP)	Bacterial consortium OP adopted	Best than suspension culture	Yañez-Ocampo et al. (2009)	t3.21 t3.22 t3.23
Ca alginate	Diuron herbicide	*Delftia acidovorans* and *Arthrobacter*	One immobilized strain has better result than free culture	Bazot and Lebeau (2009)	t3.24 t3.25 t3.26 t3.27
Sodium alginate and diatomite	TPH	Hydrocarbon degrader mix consortium	Less effective than free inoculum and control plant	Farhana et al. (2012)	t3.28 t3.29 t3.30 t3.31

produced by plant roots. It inferred that the degradation potential is associated with functional changes in microbial community. 179 180

Rhizospheric biodegradation is the smart technique which utilizes sunlight through plant to degrade pollutants with indigenous microbes. Due to limited methodological capabilities, there are many challenges which are difficult to explore; these includes which mechanisms influence microbial community composition in the rhizosphere, its type, degradative genes involved, and the effect of bioaugmentation in the rhizosphere. In order to assess the influence of plants on pollutant degradation, appropriate experimental design is an essential part of research study so that the conclusive results of pot experiments can be applied in the field plots or in scientific field studies (Mackova et al. 2006). 181 182 183 184 185 186 187 188 189

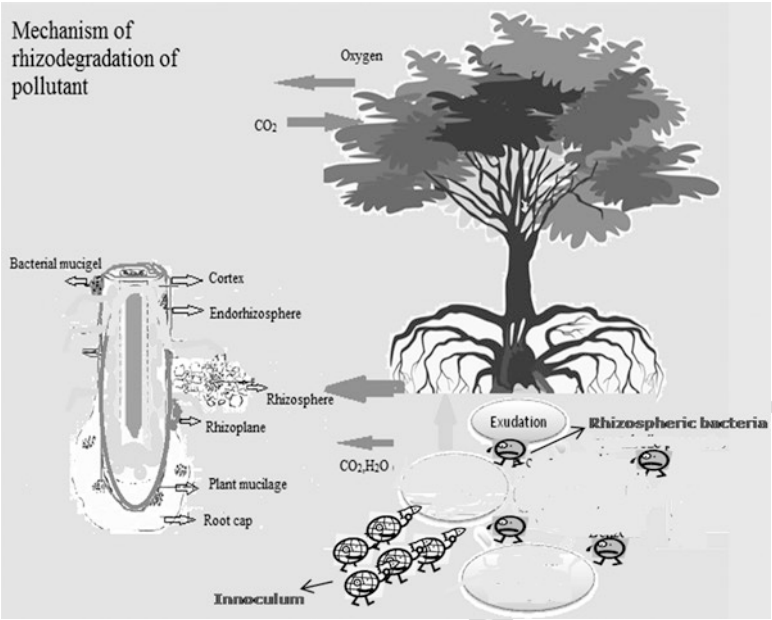

Fig. 7.1 Bacterial community attaches with rhizosphere due to mucilage, and negative interaction of rhizospheric and inoculated bacteria takes place due to competition for nutrients and niche

7.2.4 Plant Species Selection Criteria for Rhizodegradation

Plant root growth parameters are particularly important in the petroleum degradation. The large 0 roots penetrate deeply in the impermeable soil layers, and its root-associated microbial flora is exposed to more contaminants; similarly higher number of root tips provides more binding site for contaminants (Fig. 7.2). The monocot plant species have great potential of contaminant degradation due to its dense root systems which provide a high surface area to soil-microorganism interaction for biodegradation (Hussein 2006). In order to improve the efficiency of rhizospheric pollutant degradation, bacterial inoculation on plant's seed could be an important strategy (Kuiper et al. 2004).

The successful study of rhizodegradation of petroleum hydrocarbon was achieved with *Sesbania cannabina* plant; this plant tolerates anoxia in the root zone which is a prevailing condition of petroleum-polluted soil.

Observation of natural revegetation at the site can provide additional information on potential plant species. The use of native versus non-native plants for the rhizoremediation of contaminated sites is an important point of concern. In most situations, plants that are native to the region of contamination have shown to be most appropriate for rhizoremediation (Merkl et al. 2004). Species chosen for rhizoremediation should have good adaptability to new environment and climatic conditions of the region. This means that average temperature, annual rainfall, and length of growing season are important considerations in rhizoremediation

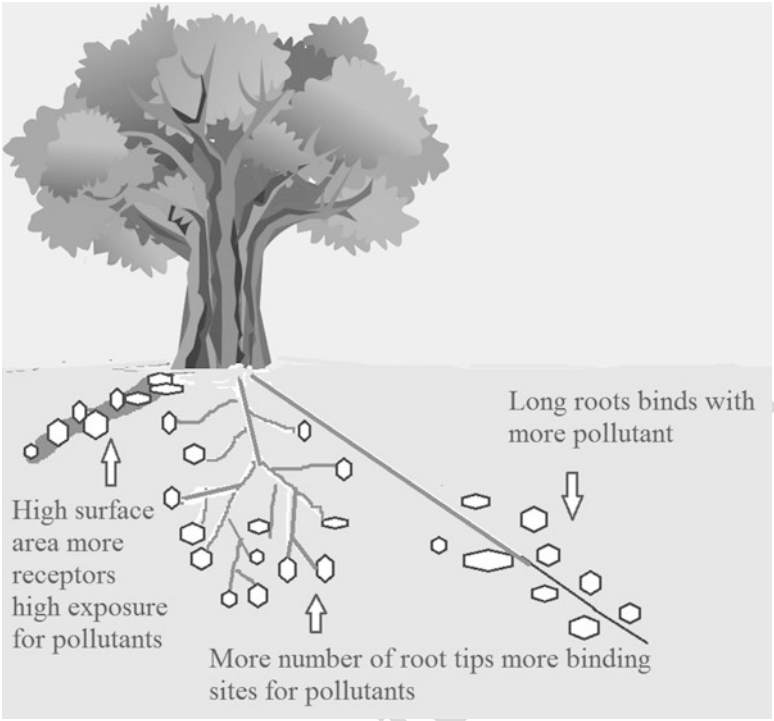

Fig. 7.2 Effect of root growth in petroleum rhizodegradation, morphology of roots plays an important role in binding of pollutants with roots which ultimately increases the pollutant degradation potential either by co-metabolic process or by pollutant uptake

planning (Frick et al. 1999) and each country have to recognize indigenous plants that can be utilized for phytoremediation (Robson et al. 2003). The introduction of non-native plants into any agricultural ecosystem is not always possible, and practical considerations such as cost and availability of seed are also very important.

7.3 Conclusion

In the bioremediation breakdown of the crude oil contaminant due to microbial activity enhanced in the presence of plant rhizosphere, the selection of native dominant plant species is required which can tolerate the exposed crude oil concentration. Bioaugmentation by using autochthonous microbes is of great interest because it is found to be best adopted with the contaminated environment. Inoculum addition or biostimulation techniques appeared to be effective in enhancing biodegradation of oil hydrocarbons in soil when used alone. However, in the presence of plant, bioaugmentation with free and sodium alginate-immobi-

lized cultures was ineffective as sodium alginate degrades very quickly and plant growth required long time. One other reason was presence of root exudates which select bacterial species and their subsequent activity. Natural plant-microbe interaction seems to be responsible for higher hydrocarbon degradation directly and indirectly by promoting plant growth. The reason for ineffectiveness of bioaugmentation was that the inoculated culture was isolated from the freshly contaminated site while the soil sample was composted and weathered having more recalcitrant fraction, so the stage of degradation was different which required different microbial community with relevant substrate-utilizing pathway which can degrade petroleum hydrocarbon in a better way.

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Author Queries

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AU1	Please check and confirm if the author names and affiliations are presented correctly.	
AU2	Please check if edit to sentence starting "Sometimes biodegradation…" is okay.	
AU3	Please check if edit to sentence starting "The second method…" is okay.	
AU4	Please check if edit to sentence starting "Among these autochthonous…" is okay.	
AU5	Please check whether the presentation of legend is fine for Table 7.2.	
AU6	Please check if edit to sentence starting "It is highly…" is okay.	
AU7	Please check if edit to sentence starting "Examples of analogy…" is okay.	
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