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Review

Bioremediation of polyaromatic hydrocarbons (PAHs) using rhizosphere technology

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Abstract

The remediation of polluted sites has become a priority for society because of increase in quality of life standards and the awareness of environmental issues. Over the past few decades there has been avid interest in developing *in situ* strategies for remediation of environmental contaminants, because of the high economic cost of physicochemical strategies, the biological tools for remediation of these persistent pollutants is the better option. Major foci have been considered on persistent organic chemicals *i.e.* polyaromatic hydrocarbons (PAHs) due to their ubiquitous occurrence, recalcitrance, bioaccumulation potential and carcinogenic activity. Rhizoremediation, a specific type of phytoremediation that involves both plants and their associated rhizospheric microbes is the creative biotechnological approach that has been explored in this review. Moreover, in this review we showed the significance of rhizoremediation of PAHs from other bioremediation strategies *i.e.* natural attenuation, bioaugmentation and phytoremediation and also analyze certain environmental factor that may influence the rhizoremediation technique. Numerous bacterial species were reported to degrade variety of PAHs and most of them are isolated from contaminated soil, however few reports are available from non contaminated soil. *Pseudomonas aeruginosa*, *Pseudomons fluoresens*, *Mycobacterium* spp., *Haemophilus* spp., *Rhodococcus* spp., *Paenibacillus* spp. are some of the commonly studied PAH-degrading bacteria. Finally, exploring the molecular communication between plants and microbes, and exploiting this communication to achieve better results in the elimination of contaminants, is a fascinating area of research for future perspective.

Key words: PAH, bioremediation, rhizoremediation, enzyme, plant microbe pair.

Introduction

Increase in industrialization over the last century has led to elevated releases of anthropogenic chemicals into the environment. Prevalent contaminants include petroleum hydrocarbons (PHCs), polycyclic aromatic hydrocarbons (PAHs), halogenated hydrocarbons, pesticides, solvents, metals, and salt. There resulting stresses on human and ecosystem health are well documented (CCME, 2001). Polyaromatic hydrocarbons (PAHs) have been identified as hazardous chemicals by different State and Central Pollution Control Boards, because of their toxic, carcinogenic and mutagenic effects on living body. At present, hydrocarbon fuels (mainly diesel) contain an excessive quantity of PAH, causing abundant distribution of the same in the ecosphere (Ruma *et al.*, 2007). They occur colorless, white/pale yellow solids with low solubility in water, high melting and

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boiling points and low vapour pressure. The physiochemical properties of some PAH given in (Table 1). These compounds enter the environment in many ways like incomplete combustion of organic materials arising from natural combustion such as forest fires and volcanic eruptions, but mainly it spread through anthropogenic activities like industrial production, transportation, refuse burning, gasification and plastic waste incineration. Release of residual PAH in air causes serious hazards to human for example, phenanthrene had been reported to affect human skin as it act as a photo sensitizer and mild allergen (Fawell and Hunt, 1988). In soil PAHs also sorbs to organic-rich sediments and in water ecosystem it accumulate in fish and other aquatic organisms, and may be transferred to humans through seafood consumption (Meador *et al.*, 1995).

PAHs has been divided into two category *i.e.* low-molecular weight (LMW) PAHs (two or three rings) which are relatively volatile, soluble and more degradable than are the higher molecular weight compounds and other were high molecular weight (HMW) PAHs (four or more rings) which sorbs strongly to soils and sediments and are more resistant to microbial degradation because of it's high molecular weight and hydrophobicity, while these PAHs were also toxic to bacteria cells (Sikkema *et al.*, 1995). The ability of microbes to degrade oil components was already recognized at the start of the twentieth century and mainly bacteria were isolated from oil contaminated sites which able to degrade PAH [Cerniglia and Heitkamp, 1987; Juhasz and Naidu, 1996; Wilson and Jones, 1993) while bacteria from non contaminated soil was also found which have the ability to degrade PAH (Bisht *et al.*, 2010). The concentration of PAHs in the environment varies widely, depending on the level of industrial development, proximity of the contaminated sites to the production source and the mode of PAH transport. Kanaly and Harayama (Kanaly and Harayama, 2000) reported that in soil and sediment PAHs concentrations varies from 1µg/kg to over 300 g/kg.

In rhizorermediation technique the rhizosphere microbial communities were used for biodegradation of pollutants. Earlier, rhizospheric and endophytic bacteria were also reported for rhizoremediation of PAH using *Populus* sp. as an inoculation system in soil (Bisht *et al.*, 2010; 2014). However, as comparison to bioaugmentation, microbe-assisted phytoremediation *i.e.* rhizoremediation, appears to be particularly effective for removal and/or degradation of organic contaminants from impacted soils, particularly when used in conjunction with appropriate agronomic techniques (Zhuang *et al.*, 2007) because the chemical condition of the rhizosphere differs from bulk soil as a consequence of various processes induced by plants roots as well as by rhizobacteria (Marschner, 2001). The synergistic effect of plant roots and rhizospheric microbial communities like secretion of organic acids followed by reduction in soil pH, production of siderophores, phytochelains, amino acids and 1-Aminocyclopropane-1-carboxylate (ACC) deaminase by plant growth promoting rhizobacteria (PGPR) were also effective for ecorestoration of polluted site as clearly depicted in (Figure 1). Rhizoremediation defined as the degradation of recalcitrant pollutants by bacteria in the rhizosphere is an attractive process since plant roots provide a large surface area for a significant population of bacteria and transport the root-colonizing, remediating microorganism to pollutants 10 to 15 m deep in the soil (Kingsley *et al.*, 1994). Apart from these beneficial effects of rhizormediation over bioremediation, rhizobacteria have considerable biotechnological potential to improve the applicability and efficiency of remediation of pollutants (Weyens *et al.*, 2009a).

The roots supply nutrients like amino acids, carbohydrates, and organic acids (Anderson *et al.*, 1993; Kravchenko *et al.*, 1997) so no exogenous carbon source must be added, and they may also supply bacteria with cofactors required for the activation of bacterial enzymes involved in the pollutant degradation pathway. With the wide range of catabolic reactions mediated by microbes and its enzymes,

Table 1 - Physico- chemical properties of PAH.

S. No.	Name	M.F.	CAS registry No[a]	B.Pt. (°C)[a]	M.Pt. (°C)[a]	V.P. (Pa at 25 °C)	Aqueous solubility (mg/L)[b]	IARC[c] group
1	Benzo[k] flouroanthene	$C_{20}H_{12}$	207-08-09	480	215.7	5.2×10^{-8}	-	28
2	Anthracene	$C_{14}H_{10}$	120 -12- 7	342	216.4	1×10^{-3}	0.015	3
3	Benzo[b] flouroanthene	$C_{20}H_{12}$	205 - 99 - 2	481	168.3	6.7×10^{-5}	-	28
4	Flouroanthene	$C_{16}H_{10}$	206 - 44 - 0	375	108.8	1.2×10^{-3}	0.25	3
5	Napthalene	$C_{10}H_{8}$	91 - 20 - 3	218	80.2	11	30	n.e
6	Phenanthrene	$C_{14}H_{10}$	85 - 01 - 8	340	100.5	2×10^{-2}	1- 2	3
7	Benzo[ghi] perylene	$C_{22}H_{12}$	191 - 24 - 2	500	277	6×10^{-8}	-	3
8	Benzo[e] pyrene	$C_{20}H_{12}$	192 - 97 - 2	493	178.7	4×10^{-7}	-	3
9	Pyrene	$C_{16}H_{10}$	129- 00 - 0	150.4	393	6×10^{-4}	0.12 - 0.18	3

a: (WGPAH, 2001); b: (Mackay *et al.*, 1991); c: (IARC, 1983).

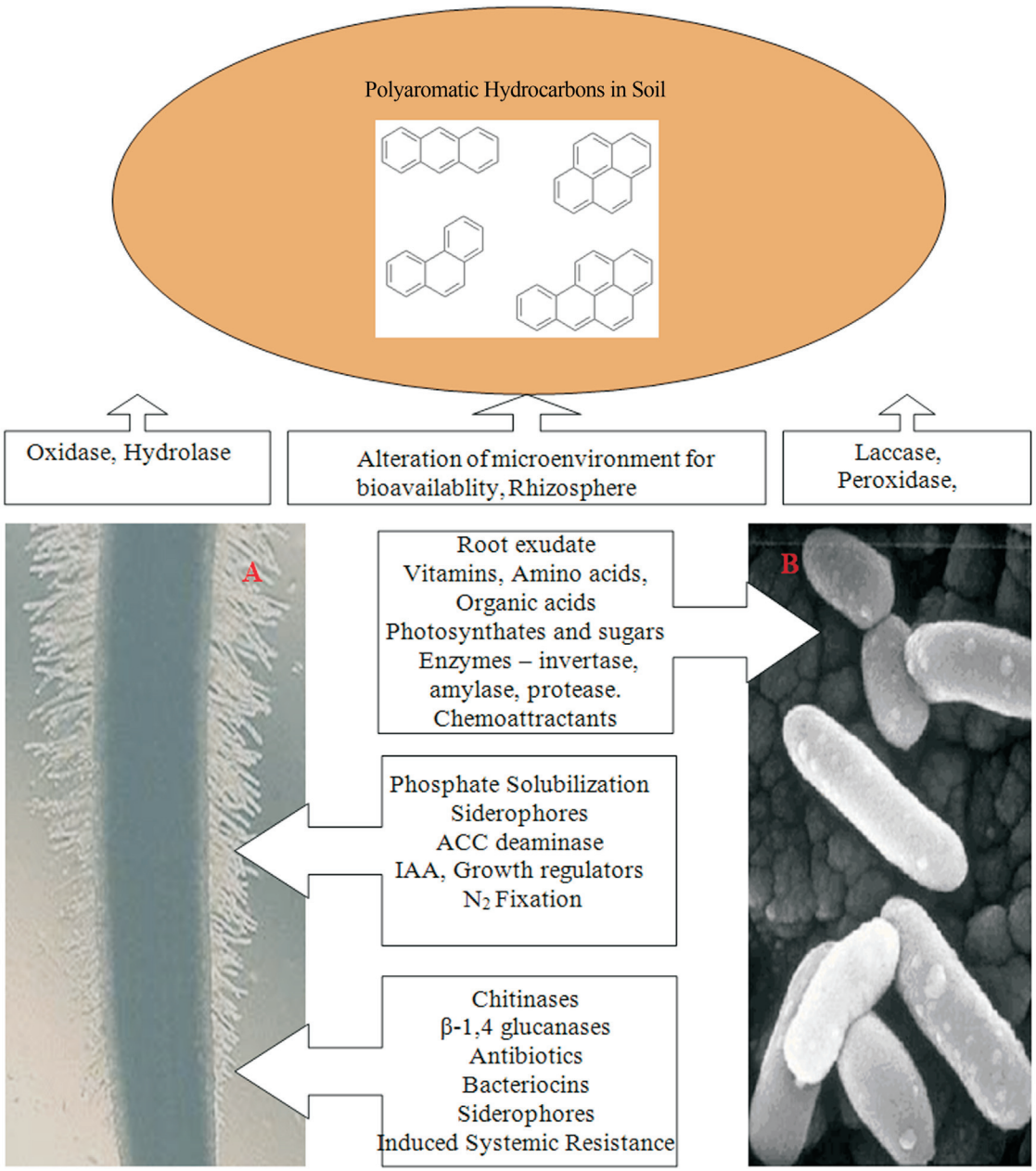

Figure 1 - Synergistic effect of plant root and rhizobacteria in biodegradation of polyaromatic hydrocarbon contaminated soil. (A) Factor affecting rhizoremediation of PAH by plant roots: support microbial growth at the root surface as well as in the rhizosphere with the help of root exudates which provide nutrients vitamins, sugars, organic acid, enzymeslike protease, amylase etc which act as a chemoaatractant for bacteria. (B) PGPR, plant growth promoting rhizobacteria; provide growth hormone (IAA), minerals, nutrient cycling, ACC deaminase and various enzymes for bioprotecting from fungal pathogen to roots.

bioremediation techniques till date are the most economical and ecofriendly strategies for removal of organic and inorganic contamination. Although rhizoremediation occurs naturally, it can also be optimized, by deliberate manipulation of the rhizosphere. It can be accomplished by using suitable plant-microbe pairs. There may be either combinations of plants and plant growth promoting rhizobacteria (PGPR), or combinations of plants and contaminant-degrading microbes. Kuiper *et al.* (2004) described the pair of a grass species with a naphthalene degrading microbe which protected the grass seed from the toxic effects of naphthalene, and the growing roots exploited with the naphthalene degrading bacteria into soil. In rhizodegradation of PAHs, other main processes were also involved as the rhizoremediation of PAH in which plant root system aerates the soil, distributes the rhizobacteria through soil and penetrates impermeable soil layers, solubilizing the pollutants in soil-water and making it bio available to the

plant and microbes. Researchers have exploited this symbiotic relationship for rhizoremediation of hazardous and xenobiotic compounds like (PCBs) (Narasimhan *et al.*, 2003), (PAHs) (Shah *et al.*, 1994; Bisht *et al.*, 2014), (TCE) (Walton and Anderson, 1990). As reviewed by Mackova *et al.* (2006), suggested that rhizoremediation can be successfully used for restoration of contaminated sites by choosing right type of plant cultivar with right rhizobacteria or by inoculating efficient rhizobacterial strains on plant seeds/roots. Mechanical injection of contaminated sites with pollutant-degrading bacteria has been used to clean polluted sites in an inexpensive and less labor-intensive way than the removal and/or combustion of polluted soils (Timmis and Pieper, 1999). However, bioaugmentation technique is limited for a number of reasons. Firstly, high beneficial bacterial population numbers cannot be maintained, presumably because of low nutrient availability and low energy yield from the degradation of the polluting compounds (Ramadan *et al.*, 1990), while the addition of nutrients though increase bacterial activity, yet this method is expensive and too labor-intensive for larger contaminated sites. Secondly, injected microbes cannot penetrate less porous layers and do not reach the deeper layers of the soil, and third, the plasmids that usually encode the ability to degrade pollutants can be lost in soil. The use of plants as bio-injectors of pollutant-degrading bacteria has a number of advantages such as a highly branched, deep root system that can be used as a vector for the more-or-less homogeneous bio-injection of root-colonizing bacteria and for the penetration of layers normally not permeable to bacteria. Additionally, because roots can exude up to 35% of their photosynthate as exudate carbon (Gregory and Atwell, 1991) and release oxygen or provide better redox conditions (Bodelier *et al.*, 2000) which can become limiting for bacterial growth in soil, the rhizosphere is a good environment for bacteria to survive and proliferate (Brazil *et al.*, 1995; Liste and Alexander, 2000; Nichols *et al.*, 1997; Yee *et al.*, 1998). In this review, we tried to focus on the importance of rhizoremediation technique over other remedial strategies and use of plant -microbe pair with biodegradation of PAH pollutants from soil.

Bioremediation by Microbes in Polluted Sites

Growing awareness of the harmful effects of PAH pollutants to the environment and human health has led to a marked increase in research into various strategies that might be used to clean up contaminated sites. Many conventional decontamination methods are expensive partially because of the cost of excavating and transporting large quantities of contaminated materials for treatment, such as soil washing, chemical inactivation, and incineration (Chaudhry *et al.*, 2005). The increasing costs and limited efficiency of these traditional physicochemical treatments, the biological treatment of soil have come into existence of alternative technologies for *in situ* applications (Singh and Jain, 2003). The term bioremediation refers to the use of living organisms to degrade environmental pollutants (Dua *et al.*, 2002; Barea *et al.*, 2005).

Natural Attenuation/Intrinsic Bioremediation: A Natural Process for Biodegradation of Pollutants

The simplest form of bioremediation is natural attenuation (NA) or bioattenuation, during which the indigenous microbial populations degrade recalcitrants or xenobiotics compounds based on their natural metabolic processes (Kuiper *et al.*, 2004; Widada *et al.*, 2001). According to the Environmental Protection Agency in the United States (USEPA, 1999) NA or intrinsic bioremediation processes include a variety of physical, chemical, and biological processes that act to reduce the mass, toxicity, mobility, volume, or concentration of contaminants. These processes include aerobic and anaerobic biodegradation, dilution, sorption, volatilization decay, and chemical or biological stabilization, transformation of contaminants (Gentry *et al.*, 2004). When time is not a limiting factor, NA can be well being applied on sites with low environmental pollutants, where no other remedial techniques are applicable (Kuiper *et al.*, 2004). As a limiting factor, no other microbes with suitable catabolic genes might not be available on the contaminant sites.

Bioaugmentation by Modified Microbes

Bioaugmentation is a method to improve degradation and enhance the transformation rate of xenobiotics by introducing either wild type or genetically modified microbes into soil (Kuiper *et al.*, 2004). A bioinoculant is a beneficial, natural microbe introduced into soil for *e.g.* by bacterization of plants seeds. The laboratory scale results of seeding microbes for degradation of soil pollutants have been vague (Kuiper *et al.*, 2004). Problems associated with bioaugmentation (Gentry *et al.*, 2004) observe that the number of exogenous microorganisms decreases shortly with the increase in time of inoculation after addition on to the site. There are several reasons for the death of introduced organisms, including both abiotic and biotic stresses; it may include fluctuations or extremes in temperature, water content, pH, and nutrient availability, along with potentially toxic pollutant levels in contaminated soil. In addition, the added microorganisms almost always face competition from non indigenous bacteria (NIB), limited nutrients, along with antagonistic interactions including antibiotic production by competing organisms, and predation by protozoa, bacteriophages and fungi. Rahman *et al.* (2002) suggested that co-inoculation of a consortium of bacteria, each with different catabolic degradation route, involved in biodegradation of a certain pollutant is often found to be more efficient than the introduction of one individual strains with the complete catabolic pathway. More-

over maximum degradation pathways were reported from *Mycobacterium* sp. because of its exceptional ability to degrade a great variety of low and high molecular weight PAH and it follows mainly oxidative and hydroxylation mechanism (Kim *et al.*, 2004). Intermediates metabolites were separated and identified via HPLC, UV and analyzed using mass and NMR spectral data.

Phytoremediation: As a Remedial Strategy for PAH Pollutants

Phytoremediation is mean to be the use of plants and their associated microbes for environmental cleanup (Salt *et al.*, 1998; Singh and Jain, 2003). Phytoremediation has potential versatility to treat a diverse range of hazardous polllutants, may be used in much large scale clean-up, as it is environmental friendly ecotechnology and is easy to implement to soil pollutants. This non invasive method is also sustainable and can be visually attractive technology for eco restoration of polluted sites. Earlier, to this phytoremediation field trials, extensive research was performed in vitro condition and many of the work explored the effects of plants on removal of contaminants from spiked soil and soil excavated from contaminated sites (Huang *et al.*, 2004) and most of these experiments provided valuable insights into the specific mechanisms of phytoremediation of organic contaminants (Reed and Glick, 2005). Organic pollutants that have been successfully phytoremediated include organic solvents such as TCE (trichloroethylene), herbicides such as atrazine, explosives such as TNT (trinitrotoluene), PHC, BTEX (mono aromatic hydrocarbons) and PAHs, the fuel additive MTBE (methyl tertiary butyl ether), and PCBs (polychlorinated biphenyls) (Pilon-Smits, 2005) Along with these Pilon-Smits (2005), also remediate some inorganic pollutants such as nitrate, phosphate, plant trace elements like Cr and Zn, heavy metals, plant macronutrients and radioactive isotopes. With these experiments allowed researchers to explore methods for overcoming contaminant stress, without the confounding effects of environmental condition such as weather and nutrient limitation. Earlier, Boyajian and Carreira (1997) reported that plants can have more than 100 million miles of roots per acre, which suggests great potential for phytoremediation in natural environments but Harvey *et a* . (2002) conclude that the in phytoremedition problem lies in removal of high concentrations of contaminants which tend to inhibit plant growth, including root growth, or due to oxidative stress. The resulting stress will limit the rate of phytoremediation in *situ* condition (Huang *et al.*, 2005). The main application for phytoremediation has so far been successful to remove toxic heavy metals from soil (Chaudhry *et al.*, 2005; Salt *et al.*, 1998). The demerits related to this strategy is that it needs longer time for remediation due to slow growth of plants, limited by climate change and soil characteristics, the pollutants may enter onto ground again by litter effects and root exudates may increase the solubility of pollutants to increase their distribution rates in soil environment (Pilon-Smits, 2005). However, there is a growing extensive research in broadening applications of this eco technology to remove/degrade organic pollutants in the environment.

Rhizoremediation: The Use of Beneficial Plant-Microbe Interaction (PMI) for Ecorestoration of Polluted Sites

In many of the studies an important contribution to the degradation of pollutants is ascribed to microbes, without the microbial contribution, phytoremediation alone may not be a viable technology for many PAH (Chaudhry *et al.*, 2005). Rhizoremediatioin term consists of both phytostimulation and rhizodegradation describing the beneficial interaction of both the plant and the rhizobacteria. The first study towards degradation of compounds in the rhizosphere was mainly focused for herbisie and pesticides (Jacobsen, 1997). Today, there are many reports regarding the rhizoremediation of PAH were studied and many of them listed in (Table 2) but the composition of microbial population were not analyzed in details. Plant-microbial interactions in the rhizosphere offer very useful means for remediating environments contaminated with recalcitrant PAH compounds (Chaudhry *et al.*, 2005). Rhizoremediation occurs naturally for one reason *i.e.* of flavonoids and other compounds released by roots which can stimulate growth and activity of PAH degrading bacteria (Thoma *et al.*, 2003; Leigh *et al.*, 2006). Furthermore, root growth and death promotes soil aeration, which can enhance oxidative degradation of recalcitrant PAHs compounds (Leigh *et al.*, 2002). Notably, Siciliano *et al.* (2003) reported that some plant species appear to increase the numbers of degradative microbes in a large volume of soil that extends beyond the rhizosphere. The success of plant species as the successful rhizoremediation might depend on its highly branched root system to harbor large numbers of bacteria, primary and secondary metabolism and establishment, survival, and ecological interactions with other organisms (Kuiper *et al.*, 2004; Salt *et al.*, 1998). Plant roots can act as a substitute for the tilling of soil to incorporate additives (nutrients) and to improve aeration (Kuiper *et al.*, 2004; Aprill and Sims, 1990). Plant species belonging to the genera *Populus* sp. (poplar) and *Salix* sp. (willow) have been used successfully for rhizoremediation of PHC contaminated soils probably due to introduction of oxygen into deeper soil layers through specialized root vessels, aerenchyma (Zalesny *et al.*, 2005). The mucigel secreted by root cells, lost root cap cells, the starvation of root cells, or the decay of complete roots provides nutrients in the rhizosphere (Kuiper *et al.*, 2004; Lugtenberg and de Weger, 1992). In addition, plants release a variety of photosynthesis derived organic compounds (Salt *et al.*, 1998; Pilon-Smits, 2005). These root exudates contain water soluble, insoluble, and volatile

Table 2 - Rhizoemediation of pollutants using plant - microbe pair.

Plants	Pollutants	Microbes	References
Prairie grasses	PAHs	Not identified	(Aprill and Sims, 1990)
Prairie grasses	PAHs	Not identified	(Qiu *et al*., 1994).
Alfalfa	Pyrene, Anhracene, Phenanthrene	Not identified	(Schwab *et al.*, 1995).
Sugar beet	PCBs	*Psudomonas flourscens*	(Brazil *et al.*, 1995).
Senecus glaucus	Oil	*Arthrobactor*	(Radvan *et al*., 1995).
Barley	2, 4- D	*Bulkhurderia cepacia*	(Jacobsen, 1997).
Wheat	2 4-D	*Pseudomonas putida*	(Kingsley *et al*., 1994).
Grasses	Napthalene	*Pseudomonas putida*	(Kuiper *et al*., 2001).
Oat, lupin, rape, dl, pepper, radish, pine	Pyrene	Not identified	[Liste and Alexander, 2000).
Populus deltoids niger	1 4-dioxane	Actinimycetes	(Schnoor *et al*., 1998).
Populus deltoides	PAH	*Kurthia* sp.	(Bisht *et al*., 2010; 2014).
		Micrococcus sp.	
		Bacillus sp.	
		Dienococcus sp.	
		endophytic *Bacillus* sp.	

*PAHs- Polyaromatic hydrocarbons, PCBs- Polychlrinated biphenyls and 2, 4-D- 2,4-Dichlorophenoxy acetic acid.

compounds including sugars, alcohols, amino acids, proteins, organic acids, nucleotides, flavonones, phenolic compounds and certain enzymes (Yee *et al.*, 1998; Salt *et al.*, 1998; Kuiper *et al.*, 2004; Anderson *et al.*, 1993). The rate of exudation changes with the age of a plant, the availability of mineral nutrients and the presence of contaminants (Yee *et al.*, 1998). The nature and the quantity of root exudates, and the timing of exudation are crucial for a rhizoremediation process. Plants might respond to chemical stress in the soil by changing the composition of root exudates controlling, in turn, the metabolic activities of rhizosphere microorganisms (Yee *et al.*, 1998). Some organic compounds in root exudates may serve as carbon and nitrogen sources for the growth and long-term survival of microorganisms that are capable of degrading organic pollutants (Anderson *et al.*, 1993; Kuiper *et al.*, 2004; Salt *et al.*, 1998). For instance, plant phenolics such as catechin and coumarin may serve as co-metabolites for PCB-degrading bacteria (Kuiper *et al.*, 2004; Salt *et al.*, 1998). Rhizomicrobial population is a major soil ecological environment for plant-microbe interactions involving colonization of different microorganisms in and around the roots of growing plants (Pandey and Maheshwari, 2007). Microbes living in the rhizosphere, rhizomicrbia, in turn, can promote plant health by stimulating root growth (regulators), enhancing water and mineral uptake, and inhibiting growth of pathogenic or other, non-pathogenic soil microbes (Kuiper *et al.*, 2004; Pilon-Smits, 2005). Rhizomicrobial population may also accelerate remediation processes by volatilizing organics such as PAHs or by increasing the humification of organic pollutants (Salt *et al.*, 1998). In particular, the release of oxidoreductase enzymes (*e.g.* peroxidase) by microbes, as well as by plant roots, can catalyze the polymerization of contaminants onto the soil humic fraction and root surfaces. In contrast to the limited studies of rhizoremediation (Kuiper *et al.*, 2004), Barea *et al.* (2005) reviewed that beneficial plant growth promoting, root-colonizing rhizobacterial strains (PGPR) have been extensively described for processes such as biocontrol of plant pathogens, and nutrient cycling by non symbiotic N_2- fixing bacteria and phosphate solubilizing bacteria (PSB), *i.e.* act as a biofertilizer. The success of beneficial processes is based on the rhizosphere competence of the microbes, which is reflected by the ability of the microbes to survive in the rhizosphere, compete for the exudate nutrients, sustain in sufficient numbers, and efficiently colonize the growing root system (Kuiper *et al.*, 2004; Lugtenberg and de Weger, 1992). Recently, it was shown that chemotaxis by *P. fl uorescens* WCS365 toward some organic acids and amino acids (but not to sugars) present in tomato root exudates plays an important role during root colonization (Kuiper *et al.*, 2004; de Weert *et al.*, 2002). Another example of the importance of the efficient use of exudates was shown for *P. putida* PCL1444. The selection of this strain for survival and proliferation on grass roots coincided with very efficient use of the main organic acids and sugars from the grass root exudate and with a high expression level of its catabolic genes for naphthalene degradation during the use of these substrates (Kuiper *et al.*, 2002; Kuiper *et al.*, 2004). Usually, several bacterial populations degrade pollutants more efficiently than a single species/strain due to the presence of partners, which use the various intermediates of the degradation pathway more efficiently (Joint metabolism) (Pelz *et al.*, 1999; Kuiper *et al.*, 2004). During rhizoremediation, the degradation of a pollutant, in many cases, is the result of the action of a consortium of bacteria (Kuiper

et al., 2004). The colonization of different niches of plant roots by different strains has also been recognized (Kuiper *et al.*, 2004; Kuiper *et al.*, 2001; Dekkers *et al.*, 2000). However, very few studies report the directed introduction of a microbial strain or consortium for xenobiotic degradation activities (bioaugmented rhizoremediation), which is able to efficiently colonize the root (Kuiper *et al.*, 2004; Kuiper *et al.*, 2001; Ronchel and Ramos, 2001; Sriprang *et al.*, 2002).

Environmental Factor Affecting PMI in PAHs Biodegradation

In many ways environmental factor may alter the mechanisms of rhizoremediation of PAH in soil for *e.g.* soil type *i.e.* texture, particle size, nutrients and organic matter content which can limit the bioavailability of pollutants (Walton *et al.*, 1994). Water content in soil and wetland affect plant/microbial growth, availability of oxygen required for aerobic respiration and temperature affects the rate at which various processes take place because temperature change in the environment due to sunlight can transform parent compounds into other compounds, which may have different toxicities than original compounds. Nutrient availability to the plant can influence the rate and extent of degradation in contaminated sites. Finally, these environment factor causes degradation of certain fraction of the contaminant mixture with end result being that only the more resistant compounds remain in soil.

Microbial Bioavailability of Pollutants

With respect to the environmental factor the bioavailability of organic compounds is also utmost important factor that determines the overall success of bioremediation process (Chaudhry *et al.*, 2005; Salt *et al.*, 1998). It depends on the physio - chemical properties of the pollutant, soil properties, environmental conditions and biological activity (Salt *et al.*, 1998; Pilon-Smits, 2005). Rentz *et al.* (2003) suggested that overcoming oxygen limitation to plants should be considered in phytoremediation projects when soil contamination exerts a high biochemical oxygen demand, such as in former oil refinery sites. Plants with aerenchyma such as reed (*Phragmites australis*) can release oxygen into the rhizosphere and are used for rhizoremediation (Muratova *et al.*, 2003). Chemotaxis has been shown to promote bioavailability in bacteria isolated from a polluted. rhizosphere that degrade PAHs (Paul *et al.*, 2005; Ortega-Calvo *et al.*, 2003). Chemotaxis is the movement of microorganisms under the influence of a chemical gradient that helps them to find optimum conditions for growth and survival (Paul *et al.*, 2005). Similarly, Bisht *et al.* (2010) isolated chemotactically active rhizobacteria from the rhizosphere of *P. deltoides* which has immense importance in biodegradation of anthracene and naphthalene. In the past few years, several microbes have been reported to be chemotactic towards different environmental pollutants, for instance toluene acting as chemoattractant to *Pseudomonas putida* F1 (Paul *et al.*, 2005; Parales *et al.*, 2000). Chemotactic bacteria might be more competent for bioremediation than their non chemotactic counterparts (Paul *et al.*, 2005).

PAHs Biodegradation: Role of Microbial Diversity

Bacteria are the class of microorganisms actively involved in the degradation of organic pollutants from contaminated sites. A number of bacterial species are known to degrade PAHs. Most of them, representing biodegradation efficiency, are isolated from contaminated soil or sediments. Long-term petrochemical waste discharge harbours bacteria capable of degrading PAH to a considerable extent. Among the PAH in petrochemical waste, Benzo (a) pyrene (BaP) is considered as the most carcinogenic and toxic. Earlier, studies have shown that bacteria can degrade BaP when grown on an alternative carbon source in liquid culture experiments. Ye *et al.* (1996) observed a 5% decrease in BaP concentration after 168 h during incubations with *Sphingomonas paucimobilis* strain EPA 505 and with the immobilization of *S. paucimobilis* strain EPA 505 grown on nutrient agar supplemented with glucose, resulted in significant evolution of $^{14}CO2$ (28%) indicating hydroxylation and ring cleavage of the 7,8,9,10-benzo ring. Aitken *et al.* (1998) isolated 11 strains with the ability to degrade BaP from a variety of contaminated sites (oil, motor oil, wood treatment, and refinery) and they were mainly from five species of *Pseudomonas*, *Agrobacterium*, *Bacillus*, *Burkholderia* and *Sphingomonas* species The other bacteria like *Rhodococcus* sp., *Mycobacterium*, and mixed culture of *Pseudomonas* and *Flavobacterium* species were also reported to degrade BaP (Walter *et al.*, 1991; Trzesicka-Mlynarz and Ward, 1995).

Heitkamp *et al.* (1988) described a bacterial isolate which was able to mineralize pyrene. Similarly, Rehmann *et al.* (1998) isolated a *Mycobacterium* spp., strain KR2 from a PAH contaminated soil of a gaswork plant, which was able to utilize pyrene as sole source of carbon and energy. The isolate metabolized up to 60% of the pyrene added (0.5 mg mL^{-1}) within 8 days at 20 °C. Earlier, also Dean-Ross *et al.* (2002) isolated two bacterial strains (*Mycobacterium flavescens* and *Rhodococcus* spp.) from sediments of River Grand Calumet from two different locations. Both the bacteria were found to be capable of PAH degradation with the initial reaction rates of 0.044 mg L^{-1} for the *K*s for pyrene mineralization by *M. flavescens* and 0.470 mg L^{-1} for the *K*s for anthracene mineralization by *Rhodococcus species*. Romero *et al.* (1998) isolated *Pseudomonas aeruginosa* from a stream polluted by petroleum refinery. It was found to be actively growing over high dosages of phenanthrene with comlete removal of the pollutant

in a period of 30 days. Yuan *et al.* (2002) isolated six gram negative strains of bacteria from a petrochemical waste disposing site having the capacity of degrading acenaphthene, fluorene, phenanthrene, anthracene, and pyrene by 70-100% in a period of 40 days of initial treatment. Two of the six strains isolated were *Pseudomons fluoresens* and *Haemophilus* spp. while others were rod-shaped bacteria.

The first presentation of the metabiolic pathway and the enzymatic reactions resulting in mineralization of naphthalene was by Davis and Evans (1964). Since then, the degradation of PAH by microbes has been studied intensively and this ability was identified to be present in a wide variety of bacteria, fungi and algae (Cerniglia, 1997; Dagher *et al.*, 1997; Shuttleworth and Cerniglia, 1995). The first PAH degrading bacteria were reported from oil contaminated soil by (Cerniglia and Heitkamp , 1987, Juhasz and Naidu, 1996; Wilson and Jones, 1993). Naphthalene biodegradation is the best studied of the PAHs because it is the simplest and most soluble PAH, and naphthalene-degrading microorganisms are relatively easy to isolate. Bacterial strains that are able to degrade different PAH have been repeatedly isolated, mainly from soil. These are usually gram-negative bacteria; most of them belong to the genus *Pseudomonas*. The biodegradative pathways have also been reported in bacteria from the genera *Mycobacterium*, *Corynebacterium*, *Aeromonas*, *Rhodococcus*, and *Bacillus* (Cerniglia, 1984; Annweiler *et al.*, 2000). The tricyclic aromatic hydrocarbons are widely distributed throughout the environment. So, they have been used as model substrates in studies on the environmental degradation of PAHs, since both structures are found in carcinogenic PAHs such as benzo[a]pyrene, benz[a]anthracene and 3-ethylcholanthrene. Pure cultures and mixed cultures of bacteria isolated from fresh-water and marine environments have the ability to metabolize anthracene and phenanthrene as the sole source of carbon. Anthracene can be completely mineralized by *Pseudomonas*, *Sphingomonas*, *Nocardia*, *Beijerinckia*, *Rhodococcus* and *Mycobacterium* with the initial oxygenated intermediate being a dihydriol (Moody *et al.*, 2001). Growth of *Sphingomonas yanoikuyae* JAR02 on plant root exudates and root extracts was observed along with removal of benzo[a]pyrene from solution through constitutively expressed degradative enzymes, corroborating field observations of increased PAH degradation in the rhizosphere (Aprill and Sims, 1990; Banks *et al.*, 1999; Binet *et al.*, 2000; Paquin *et al.*, 2002). Hedlund and Staley (2006) found that *Pseudoalteromonas* strains with identical 16S rRNA gene sequences varied in the suite of PAHs that they could degrade and had likely acquired a gene coding for a naphthalene-degrading enzyme by horizontal transfer. Secondly, isolates can be identified that degrade PAHs but do not contain genes with high homology to known genes implicated in PAH degradation (Widada *et al.*, 2002). Danne *et al.* (2001) reported the isolation of spore-forming, PAH-degrading bacteria and subsequent genetic studies of their degradation pathways which may lead to the discovery of novel genes involved. In addition, they evaluate the biodegradation potential of select isolates and determine if reinoculation of the rhizosphere would enhance degradation of contaminated marine sediment. Filonov *et al.* (2006) assessed the efficiency of the naphthalene degradation process performed by different microbial strains of the genera *Pseudomonas* and *Burkholderia* in soil model systems. Moreover, the effect of salicylate (2-hydroxybenzoic acid) on the naphthalene biodegradation process was also developed in such systems. Finally, a mathematical description of the process was also proposed to check the efficiency of degradation in field site.

Numerous studies have demonstrated the importance of the rhizosphere effect on degradation of organic contaminants. Most of these studies have examined terrestrial plants and agricultural chemicals (Arthur *et al.*, 2000); few have looked at the influence of plant-associated microorganisms on the fate PAHs (Schwab and Banks, 1994). Many of the bacterial species and genera that we isolated have been reported to degrade PAHs previously. Few reports have been made regarding *Paenibacillus* and its ability to degrade PAHs. Pichinoty *et al.* (1986) described *Bacillus gordonae* sp. nov., later to be emended as *P. validus* (Heyndrickx *et al.*, 1995) which was able to utilize *p*-hydroxybenzoate, phthalate, isophthalate, protocatechuate, trimellitate, quinate, phenol, *p*-cresol, and naphthalene. Although, Meyer *et al.* (1999) reported the isolation of a PAH degrading tentative *Paenibacillus* sp. from tar oil-contaminated soil. Bacterial strains belonging to a few genera such as *Sphingomonas*, *Pseudomonas*, and *Mycobacterium* have been observed to dominate PAH degradation in soil (Kim *et al.*, 2004). Recently, Bisht *et al.* (2010, 2014) reported four bacteria and endophytic strain from non contaminated rhizospere of *P. deltoidesa* which able to degrade 80 - 90% degradation of anthracene and naphthalene as well as in PAH conducive soil. The maximum degradation pathways were reported from *Mycobacterium vanbaalenii* because of its exceptional ability to degrade a great variety of low and high molecular weight PAHs oxidatively in soil. Versatility of this species makes it a probable inoculants in the remediation of PAH contaminated sites (Kim *et al.*, 2004).

Enzymatic Biodegradation

To search for catabolic genes involved in degradation of PAH, we have to correlate them with enzymes involved in degradation pathways. Mainly, enzymes involved in the degradation of PAHs are mainly oxygenase, dehydrogenase, phosphatases, d± and lignolytic enzymes (Table 3). These enzymes require optimum temperature ad it was reported that mostly degradative enzymes work at mesophilic temperatures and it activity decreases with very high and low temperatures. While, some of the enzymes are reported to be active even at extremes of temperatures expect,

Table 3 - Microbial degradation enzymes involved in biodegradation of PAH.

Enzymes	Catalytic action	Source	References
Dehaloginase	Hydrolyse halogen compound from PAH with halogen derivatives (Cai and Xun, 2002).	*Xanthobacter autotrophicus*	(Mena-Benitez *et al.*, 2008)
Sphingobium chlorophenolicum			
Laccase	Degradation of various PCB's	*Mycobacterium* sp.	[Novotny *et al.*, 1997).
Dioxygenase	Degradation of various PAH's	*Pseudomonas* sp.	(Pieper et al., 2004).
Mycobacterium sp			
Peroxidase	Rreductive dehalogenation of aliphatic hydrocarbons (Khindaria *et al.*, 1995).	*Phanerochaete chrysosporidium*	(Van Aken *et al.*, 2000).
Phanerochaete lae			
Nitrilase	Cleaves cyanide groups from aromatic and aliphatic nitriles	*Aspergillus niger*	(Kaplan *et al.*, 2006).
Nitroreductase	Reduces nitro groups on nitroaromatic compounds (e.g., 2,4,6-trinitrotoluene); removes N from ring structures (Caballero *et al.*, 2005)	*Comamonas* sp.	(Liu *et al.*, 2007)
Pseudomonas *putida*			
Dioxygenase	Degeradation of various PAH's	*Psudomonas* sp.	(Tsuda and Iino, 1990).
		Bacillus sp. ,	
Cytochrome P450 monooxygenase	Hydroxylation of aromatic and aliphatic hydrocarbons	Most aerobic bacteria, all fungi	(McLean *et al.*, 2005).

laccase activity was detected at 5 C. The optimum temperature is 45 °C for laccase, but its activity drops to 30% at 5 °C however, 31% activity is found at 75 °C. On the other hand, the activity of Mn-dependent peroxidase is high even at 75 °C (Farnet *et al.*, 2000). Enzymes also show substrate specificity but lignolytic enzymes are non-specific acting on phenolic and non-phenolic organic compounds via the generation of cation radicals after one electron oxidation (Wilson and Jones, 1993). A phthalic derivative is produced as one of the ring fission products of PAHs by bacteria (Kotterman *et al.*, 1998). The derivatization of phthalate results into release of carbon-di-oxide or highly complex metabolites and the lignolytic enzymes and ozonation/photocatalytic oxidation also act by free radical attack on the organo pollutants (Machado *et al.*, 2000). Thus, the intermediates of these three methods are ring opening phthalic derivatives and aliphatics such as pentadecane, hexadecane, and nonadecane. A higher dose of PAHs in the substrate may also affect the activity/rate of microbial degradation (Verrhiest *et al.*, 2002) while studying the interaction between a PAH mixture and microbial communities in natural freshwater sediment established that PAH dose has no effect on the microbial community in sediments up to a range of 30 mg PAH/kg.

The PAHs had an effect at higher concentration owing to partial inhibition of the leucine-aminopeptidase activity. The β-glucosidase activity was stimulated by the organic pollutants at the same concentration. Schutzendubel *et al.* (1999) found that during only 3 days of incubation, *Bjerkandera adusta* removed 56% and 38% of fluorene and anthracene, while *Pleurotus ostreatus* degraded 43% and 60% of these compounds; other PAH were degraded to a lower extent. Except for anthracene in cultures of *P. ostreatus*, all PAH were removed uniformly during the cultivation time but Fluorene and anthracene were degraded faster than other PAH. The fungi produced valuable activity of manganese dependent peroxidase (MnP) but laccase was secreted only by *P. ostreatus* and was strongly induced by the addition of milled wood.The production of the oxidative enzymes did not correlate directly to the metabolism of PAH. Both fungi showed a very low activity of LiP during the degradation of PAH in the BSM medium (basal salt medium). Laccase activity was detected only in cultures of *P. ostreatus* in the BSM showed activity of MnP only at the end of cultivation. The addition of wood inhibited production of the enzyme in younger cultures and increased the activity after 27 days. The first maximum of activity was reached after 22 days in BSM cultures and BSMW (basic medium with milledwood) cultures, respectively. In cultures of both fungi, only a very low and no significant cresolase activity of the tyrosinase could be detected. In the case of *P. ostreatus*, the highest level of anthracene elimination was observed in 12-dayold cultures (62%). A second maximum in removal of anthracene was detected after 39 days (18%). Fluorene was degraded to a

high degree in a 7-day-old culture (42%) and was practically uniformly removed over the whole cultivation time. Other PAHs were degraded at an almost constant rate during the 48 days of cultivation (1 ± 12%). Cultures supplemented with milled wood showed much lower degradation values: only anthracene (max 23% after 17 days), fluorene (19 ± 30%) and partially phenanthrene (0 ± 8%) were degraded. Barnsley (1983) reported the naphthalene dioxygenase, the first key enzyme of naphthalene degradation from *Psudomonas* sp. and showed that salicylate is the inducer of this enzyme. Smith *et al.* (2006) described the metabolism of naphthalene by a strain of *Mycobacterium* sp. involves both monooxygenation and dioxygenation with the formation of both *cis*- and *trans*-1,2-dihydrodiols (the ratio of *cis*- to *trans*- diol 25:1). The reaction is catalized by cytochrome P-450 monooxygenase that forms naphthalene 1, 2-oxide which is further converted to the *trans*-diol by an epoxide hydrolase enzyme. However, numerous studies were going on the biodegradation of PAH but still little is known about complete metabolic enzymatic pathways of the biodegradation processes.

Improvement in Rhizosphere Bioremediation

Although the biodegradative abilities of the bacteria, and the expression and maintenance of bacterial genes in the rhizosphere are extremely important for the effective removal of contaminants in phytorhizoremediation, several other aspects can improve the effectiveness of the process. As mentioned previously, the root exudate composition changes with the developmental stage of the plant and depends on plant species; these variations obviously exert different effects on the rhizospheric community (Smalla *et al.*, 2001; Berg *et al.*, 2002; Garbeva *et al.*, 2004). *Salix* sp. plants are used in many phytorhizoremediation experiments because they produce salicylic acid and related compounds that induce the degradation of PAHs and PCBs (de Cárcer *et al.*, 2007). Flavonoids are produced by plants as a defence mechanism against pathogens. However, plants with a higher content of flavonoids will be efficiently colonized by tolerant bacteria (Palumbo *et al.*, 1998). The root exudate composition will also favour proliferation of bacteria that will degrade them efficiently for *e.g. Pseudomonas putida* PML2 can grow using plant flavonoids (it is also a PCB degrader) and it has been demonstrated that it colonizes the rhizosphere of wild-type *Arabidopsis thaliana* (or a mutant that overproduces flavonoids) better than the rhizosphere of a mutant that does not produce flavonoids (Narasimhan *et al.*, 2003). Rhizospheric bacteria can be better equipped to colonize the rhizosphere and are the best option for degradation. Shim *et al.* (2000) introduced the toluene *o*-monooxygenase genes (TOM) from *B. cepacia* G4 into several bacteria isolated from the poplar rhizosphere and they showed that when they introduced recombinant strains to coat poplar tree roots in non-sterile soil, recombinants that were derived from the plant rhizosphere were able to thrive, while non-rhizospheric recombinant strains were not maintained in the rhizosphere. These strains were also able to express the TOM and degrade trichloroethylene (TCE). Selection of rhizobacteria, which are able to produce biosurfactants in the rhizosphere of the plants, is an interesting alternative to improve the removal efficiency (Plociniczak *et al.*, 2011).

Future Outlook for PMI in Rhizoremediation

Further studies on improving the expression of catabolic genes in the rhizosphere and in the selection of the best plant-microbe combinations will have to be translated into field strategies that can demonstrate the benefits of this approach. The utilization of endophytes in the biodegradation of pollutants is an emerging field that has not been widely explored. Advances in this field will have to be followed by better knowledge about the biotransformation and bioaccumulation of contaminants together with disposal of contaminated biomass and transport of the toxic chemical by plants. However, this can pose a problem if the compound is then translocated to the shoot where it can become available to animals and humans. The fate of contaminants should be extensively studied during rhizoremediation processes to avoid undesired effects if field tests are performed. Exploring the molecular communication between plants and microbes, and exploiting this communication to achieve better results in the elimination of contaminants, is a fascinating area of research. These studies may reveal the mechanisms of plant- microbe interactions and we predict that this approach will now be adopted to study the induction of catabolic pathways in polluted soils undergoing rhizoremediation. The new genomics techniques will also allow the monitoring or selection of catabolic genes to improve remediation strategies (Kiely *et al.*, 2006) The improvement of metagenomic analysis will probably reveal new degradative capacities (genes) that will be worth introducing into strains with other interesting traits (*i.e.* good root colonization abilities). The signals that plant and microbes exchange when they recognize each other will have to be interpreted and the molecular basis of the specific interactions between certain plant genotypes with specific bacteria will need to be dissected. Non-cultivation based molecular techniques for detecting microbial genes in environmental samples are considered a powerful tool to study the presence of a PAH-transforming bacterial community. These technologies allowed the discovery of genes encoding the metabolism and catabolism of various constituents of PAHs that would otherwise be impossible by traditional culture techniques. Furthermore, as mentioned earlier (Sayler and Rip, 2000), the marriage of modern recombinant DNA technology and the chemical industries may produce new strains capable of not only broad hydrocarbon metabolism, but also adaptability to contaminated environments (van Hamme *et al.*, 2003). The studies pertaining to molec-

ular signaling meachanism for biodegradation are still missing in literature. Information that can be derived from these studies may provide further insights on how to design a successful rhizoremediation strategy. Finally, more studies about the impact of using recombinant microorganisms over indigenous microbial communities are needed to meet with safety requirements, especially with the increasing need for recombinant microbes to deal with highly toxic chemicals, such as PAHs and PCBs.

Conclusion

Bioremediation is recognized as a suitable tool to restore PAH contaminated sites. The synergistic use of plant and associated microbial communities for bioremediation resulting in rhizoremediation, could solve some of the problems encountered during the application of both individual techniques *i.e.* bioaugmentation and phytoremediation. Although the studies described above clearly indicate the significance of rhizoremediation of PAHs from other remediation strategies and also analyze certain environmental factor that may influence this rhizormediation technique. However, reports about the actual selection of a suitable plant- microbe pair for rhizoremediation system, consisting of a plant inoculated with a bacterium or a consortium with different degradation capacity are in scarce. Therefore, further studies of the selection of suitable rhizosphere bacteria or communities, able to sustain and proliferate on the root system of a plant which is suitable for rhizoremediation or phytoremediation, can yield useful novel (engineered) systems. These systems then can be an interesting tool to further improve and develop bioremediation into a widely accepted technique.

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