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, Pseudomonas fluorescens, 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). Polynuclear polycyclic aromatic 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
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., 1988). In soil PAHs also sorbs to organic-rich 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 have considerable biotechnological potential to improve the applicability and efficiency of remediation 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 rhizorremediaion 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 catalytic reactions mediated by microbes and its enzymes,

### Table 1 - Physico-chemical properties of PAH.

| S. No. | Name             | M.F.       | CAS registry No* | B.Pt. (°C)* | M.Pt. (°C)* | V.P. (Pa at 25 °C) | Aqueous solubility (mg/L) | IARC* group |
|--------|------------------|------------|------------------|-------------|-------------|--------------------|--------------------------|--------------|
| 1      | Benzo[k] fluoranthene | C_{20}H_{12} | 207-08-09       | 480         | 215.7       | 5.2 X 10^4        | -                        | 28           |
| 2      | Anthracene       | C_{14}H_{10} | 120-12-7        | 342         | 216.4       | 1 X 10^3          | 0.015                    | 3            |
| 3      | Benzo[b] fluoranthene | C_{20}H_{12} | 205 - 99 - 2    | 481         | 168.3       | 6.7 X 10^4        | -                        | 28           |
| 4      | Fluoroanthene    | C_{16}H_{10} | 206 - 44 - 0    | 375         | 108.8       | 1.2 X 10^3        | 0.25                     | 3            |
| 5      | Naphthalene      | 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 X 10^2          | 1- 2                     | 3            |
| 7      | Benzo[ghi] perylene | C_{12}H_{12} | 191 - 24 - 2    | 500         | 277         | 6 X 10^8          | -                        | 3            |
| 8      | Benzo[e] pyrene  | C_{16}H_{12} | 192 - 97 - 2    | 493         | 178.7       | 4 X 10^7          | -                        | 3            |
| 9      | Pyrene           | C_{18}H_{10} | 129 - 00 - 0    | 150.4       | 393         | 6 X 10^4          | 0.12 - 0.18              | 3            |

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

Figure 1 - Synergistic effect of plant root and rhizobacteria in biodegradation of polynuclear aromatic 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, enzymes like protease, amylase etc which act as a chemiotactant 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.
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., 1997; Yee et al., 2005), (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 meant 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 pollutants, 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 al. (2002) conclude that the in phytoremediation 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 ascribed to microbes, without the microbial contribution, phytoremediation alone may not be a viable technology for many PAH (Chaudhry et al., 2005). Rhizoremediation term consists of both phystimulation 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 - Rhizoremediation of pollutants using plant - microbe pair.

| Plants            | Pollutants | Microbes                  | References                  |
|-------------------|------------|---------------------------|-----------------------------|
| Prairie grasses   | PAHs       | Not identified            | (Aprill and Sims, 1990)     |
| Alfalfa           | PAHs       | Not identified            | (Qiu et al., 1994).         |
| Sugar beet        | PCBs       | Psudomonas flouroscens    | (Brazil et al., 1995).      |
| Senecus glaucus   | Oil        | Arthrobacter              | (Radvan et al., 1995).      |
| Barley            | 2, 4-D     | Bulkhderia cepacia        | (Jacobson, 1997).           |
| Wheat             | 2, 4-D     | Pseudomonas putida        | (Kingsley et al., 1994).    |
| Grasses           | Naphthalene| 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). |

*PAHs- Polyaromatic hydrocarbons, PCBs- Polychlorinated 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, rhizomicrobia, 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 N2-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).
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; Srirang 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, Bish 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 chemotactrant 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 immobilization of S. paucimobilis strain EPA 505 grown on nutrient agar supplemented with glucose, resulted in significant evolution of ¹⁴CO₂ (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; Trzesiecka-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 Ks for pyrene mineralization by M. flavescens and 0.470 mg L⁻¹ for the Ks 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 complete 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 degradingacenaphthene, fluorene, phenanthrene, anthracene, and pyrene by 70-100% in a period of 40 days of initial treatment. Two of the six strains isolated were Pseudomonas fluorescens and Haemophilus spp. while others were rod-shaped bacteria.

The first presentation of the metabolic 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 rhizosphere of P. deltoides 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,
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. Schutzen-dubel 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

| Enzymes | Catalytic action | Source | References |
|---------|------------------|--------|------------|
| Dehalogenase | Hydrolyse halogen compound from PAH with halogen derivatives | Xanthobacter autotrophicum | Mena-Benitez et al., 2008 |
| Splignobacter aliphaticus | Degradation of various PCB’s | Xanthobacter autotrophicum | Mena-Benitez et al., 2008 |
| Laccase | Oxidation of various PAH’s | Phanerochaete chrysosporium | Van Aken et al., 2000 |
| Dioxigenase | Degradation of various PAH’s | Pseudomonas sp. | Pieper et al., 2004 |
| Mycobacterium sp | Degradation of various PAH’s | Xanthobacter autotrophicum | Novotny et al., 1997 |
| Peroxidase | Degradaion of various PAH’s | Phanerochaete lae (Khindaria et al., 1995) | |
| Phenoloxidase | Degradation of aromatic and aliphatic hydrocarbons | Phanerochaete lae | |
| Nitrilase | Cleaves cyanide groups from aromatic and aliphatic nitriles | Aspergillus niger | Kaplan et al., 2006 |
| Nitroreductase | Reduces nitro groups on nitroaromatic compounds | Comamonas sp. (Liu et al., 2007) | |
| Dioxygenase | Degradation of aromatic and aliphatic hydrocarbons | Bacillus sp. | |
| Cytochrome P450 monoxygenase | Hydroxylation of aromatic and aliphatic hydrocarbons | Mycobacterium sp | |

Table 3 - Microbial degradation enzymes involved in biodegradation of PAH.
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 Pseudomonas 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 monoxygenation 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 catalyzed 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 phytoorhizoremediation, 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-monoxygenase genes (TOM) from B. cepacia G4 into several isolates 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 (Plochiniczak 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 molecu-
ular signaling mechanism 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 phyto remediation. 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 rhizoremediation 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 phyto remediation, 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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