Rev Environ Sci Biotechnol (2015) 14:241–269
DOI 10.1007/s11157-014-9353-3

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REVIEW PAPER

Molecular approaches for biodegradation of polycyclic aromatic hydrocarbon compounds: a review

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Published online: 12 October 2014
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Abstract The waste generated from industrial processes and operations including domestic wastes when treated partially and disposed in soil–water environment enter to lakes, streams, rivers, oceans and other water bodies. The pollutants get dissolved or lie suspended in water or get deposited on soil sediment beds. This results on aquatic and terrestrial pollution which ultimately impact ecosystems causing toxicity to biota and human beings. Industries such as petrochemical, pharmaceutical, insecticides and fertilizers generates the hazardous waste comprising of inorganic and organic compounds. Organic compounds mainly composed polycyclic aromatic hydrocarbons (PAHs), are one of the toxic environmental pollutant. This paper highlights the physicochemical properties, bioremediation treatment and its mechanism for the waste containing PAH. The process of biological remediation depends upon the metabolic action of microbe toward the contaminant which can be achieved by optimum water and nutrient supply and some other limiting factors. The enzymatic degradation gives the molecular approaches for bioremediation. The study also highlighted the molecular approaches which are helpful in revealing functional, structural and communal information about microbial diversity for exploring the routes of degradation pathway of bioremediation process and future scope to bioremediation of PAHs.

Keywords Polycyclic aromatic hydrocarbons (PAHs) · Biodegradation · Co-metabolism · Molecular techniques

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1 Introduction

Industrial development and excessive use of synthetic chemicals has led to increase in the concentration of persistent organic compounds in the environment, which causes adverse effect in the environment. Aromatic hydrocarbons are the most common pollutant found in the soil and ground water. Polycyclic aromatic hydrocarbons (PAHs) are one class of toxic environmental pollutants that have accumulated in the environment due to several natural and anthropogenic activities. The largest release PAHs is due to incomplete combustion of organic compounds during the course of industrial processes and other human activities. PAHs are a type of organic compounds that consist of two or more fused benzene rings and/or pentacyclic molecules that are arranged in various structural configurations. They are mostly used as intermediaries in pharmaceuticals, agricultural products, photographic products, thermosetting plastics, lubricating materials, and other chemical industries.

The main concern with PAHs compounds is with respect to potential health risk. Some members of these compounds are regulated as carcinogens and mutagens reported by Mastral and Callen (2000). Removal of PAHs from the environment by microbial biodegradation is one of the promising tools and has been studied extensively (Yuan et al. 2000; Zhang et al. 2004a, b). Biodegradation is a natural process that helps to remove PAHs compound from the environment by microorganisms. It is one of the cost effective methods amongst remedial approaches. Bioremediation has been shown to be effective in remediating soils contaminated with low molecular weight PAHs (Mueller et al. 1991; Banerjee et al. 1995; Kastner and Mahro 1996). However, PAHs having high molecular weights are generally recalcitrant to microbial attack (Park et al. 1990; Cerniglia 1992; Erickson et al. 1993). Microbes thriving in contaminated environment are able to use the contaminant as source of energy because of their genetic adaptability which leads to bioremediation. The lack of microbial activity towards high molecular weight PAHs may be attributed to site specific environmental factors, such as bioavailability of the contaminant, nutrients, redox potential, etc., the limiting factor may be the scarcity of micro-organisms capable of degrading the more highly condensed compounds. Process of bioremediation undergoes to some complex stages and accessibility towards the mechanism and metabolic pathway of microbes is quite difficult but with rapid advancement in biotechnological approaches and molecular techniques it become possible i.e. some of the techniques are polymerase chain reaction (PCR), fingerprinting technique based on PCR Presently, 16S rRNA gene liabrary, denaturing gradient gel electrophoresis (DGGE), single strand conformation polymorphism (SSCP), terminal restriction fragment length polymorphism (T-RFLP) rRNA intergenic spacer analysis, DNA hybridization such as fluorescence in situ hybridization (FISH) and DNA microarray, gene reporters and biosensors were also frequently used. However, as for PAHs biodegradation investigation, the techniques extensively employed were PCR, fingerprinting technique (mainly DGGE), DNA hybridization technique and gene reporters. New sequencing technologies i.e. metagenomics, metaproteomics and metatranscriptomics studies were also highlighted. Metaproteomics approaches utilizing two-dimensional electrophoresis (2-DE), mass spectroscopy (MS) have aided in global analysis of catabolic enzymes involved in microbial biodegradation pathways (Kim et al. 2006a, b; Wilmes et al. 2008). One of the upcoming "omics" technologies known as metabolomics refers to the colossal analyses of primary and secondary proteinaceous metabolites produced by microbial cells under defined physiological conditions (Mapelli et al. 2009). The nucleic acid extraction from soil is the first crucial step in the application of most of the molecular techniques, which have mainly been dominated by diverse variations of PCR. These methods can provide new insights into bacterial and fungal community compositions, their associations and their responses to each other with respect to environmental conditions. Technological advancement, sensitivity and specificity PCR-based finger printing techniques have proved enormously useful in assessing the changes in microbial community structure. Fluorescent in situ hybridization (FISH) can be used to evaluate the distribution and function of bacterial population in situ. DNA microarray techniques have also been developed and used for the evaluation of ecological role and phylogenetic affiliations of bacterial population in the soil (Dubey et al. 2006). 16S ribosomal RNA (rRNA) sequencing is a common amplicon sequencing method is helpful in the phylogenetic and taxonomic comparison and identification of bacteria present within a contaminated environment as discussed by Legge (2012). Scope of this review paper is limited to polycyclic aromatic hydrocarbon and role of microorganisms involving characterization of specific enzymatic activity and gene expression during PAHs degradation through molecular approaches.

1.1 Sources and physico-chemical properties

Sources of polycyclic aromatic hydrocarbons (PAHs) can be natural: forest and grass fires, oil seeps, volcanoes, chlorophyllous plants, fungi, and bacteria and anthropogenic: petroleum, electric power generation, refuse incineration, home heating, production of coke, carbon black, coal tar, asphalt and internal combustion engines. In areas remote from urban or industrial activities, the levels of PAHs found in unprocessed foods reflect the background contamination, which originates from long distance airborne transportation of contaminated particles and natural emissions from volcanoes and forest fires. In the

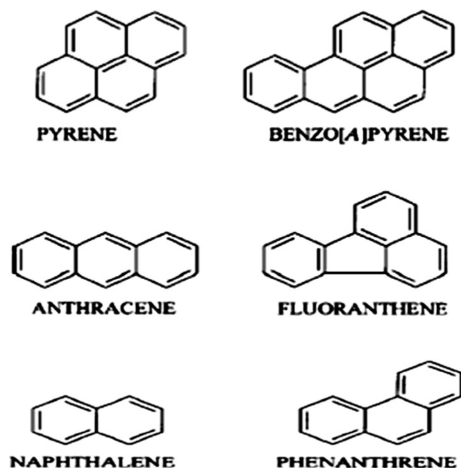


Fig. 1 Structural representation PAH's

neighbourhood of industrial areas or along highways, the contamination of vegetation can be ten-fold higher than in rural areas (Larsson and Sahlberg 1982). Processing of food (drying and smoking) and cooking of foods at high temperatures (grilling, roasting, frying) are major sources generating PAHs (Guillen et al. 1997). The general characteristics of PAHs are high melting and boiling points, low vapor pressure, and very low aqueous solubility (toxipedia). Generally solubility decreases and hydrophobicity increases with an increase in number of fused rings (Wilson and Jones 1993). PAHs are highly lipophilic and therefore very soluble in organic solvents, they also have high octanol water coefficient (K_{ow}), i.e. Naphthalene, Anthracene, Pyrene, Chrysene, Benzo (α) pyrene. Structural representation of PAHs is shown in Fig. 1. PAHs also manifest various functions such as light sensitivity, heat resistance, conductivity, emittability, corrosion resistance, and physiological action. Most PAHs are also fluorescent, emitting characteristic wavelengths of light when they are excited. PAHs have been identified as being of greatest concern with regard to potential exposure and adverse health effects on humans (Table 1).

1.2 Persistence of PAHs compounds in the environment and its toxicity

There are a variety of mechanisms by which PAHs are degraded in the environment, including chemo-oxidation photooxidation and microbial degradation which is considered as the primary route of degradation of PAHs in soils mentioned by Juhasz and Naidu (2000). Emission into the air of complex mixture of different PAHs including particulate matter as in smoke, PAHs in vapors phase can be absorbed onto airborne particles or to diffusion. Due to low vapor pressure naphthalene and fluorine can exist as vapor phase in the atmosphere, near surface water. Because of their high K_{ow} values they tend to become adsorbed to the organic material of sediments and are taken up by aqueous organisms. The potential for photochemical oxidation of structural isomers benzo[α]pyrene in the atmosphere was studied and a significant difference in photochemical oxidation rate was observed. Benzo[α]pyrene was not photochemically oxidise whereas benzo[α]pyrene was photochemically oxidised (Takata and Sakata 2002). PAHs can be bio-concentrated or bio-accumulated by certain aqueous invertebrate, but such species are less in number which are able to effectively biotransform polycyclic aromatic hydrocarbons. Phytoplankton play an important role in the fate and transport of persistent organic pollutants like PAHs and their consumption as an important initial phase in bioaccumulation was studied by Fan and Reinfelder (2003). Many PAHs are carcinogenic and therefore, of significant concern as environmental contaminants. Numerous studies have indicated that one-, two- and three-ring compounds are acutely toxic as documented by Sims and Overcash (1983), while higher molecular weight PAHs are considered to be genotoxic (Phillips 1983; Lijinsky 1991; Mersch-Sundermann et al. 1992; Nylund et al. 1992). BaP is one of the most potent carcinogenic PAHs, and as such, is the most studied compound of the PAH class (Collins et al. 1991). Metabolism and activation of BaP in mammalian systems was studied by Cooper et al. (1983). Reports states that these intermediates undergo through one of at least four different mechanisms of oxidation and/or hydrolysis before the intermediates combine with and/or attack DNA to form covalent adducts with DNA. DNA adducts can lead to mutations of the DNA, resulting in tumours (Harvey 1996). In mammals when ingested, PAHs are rapidly absorbed into the gastrointestinal tract due to their high lipid solubility (Cerniglia 1984). Vanrooij et al. (1993) were estimated 75 % of the total absorbed amount of PAHs (specifically pyrene) entered the body through the skin, highlighting this as a major exposure route of PAHs. The rapid absorption of

Table 1 PAHs with potential exposure and adverse effects on environment and on human health

Compound	Number of rings (5 or 6)	Melting points (°C)	Boiling points (°C)	Vapour pressure pa at (25 °C)	Density	N-octanol water partition coefficient (log K_{ow})	Solubility in water at 25 °C (μg/l)	Henry law constant at 25 °C (Kpa)	Ionization potential (eV)
Acenaphthylene	2(1)	92–93	–	8.9×10^{-1}	0.899	4.07	–	1.14×10^{-3}	8.22
Acenaphthene	2(1)	95	279	2.9×10^{-1}	1.024	3.92	3.93×10^{-3}	1.48×10^{-4}	7.68
Fluorene	2(1)	115–116	295	8.0×10^{-2}	1.203	4.18	1.98×10^{-3}	1.01×10^{-2}	7.88
Anthracene	3	216.4	342	8.0×10^{-4}	1.283	4.5	73	7.3×10^{-2}	7.439
Phenanthrene	3	100.5	340	1.6×10^{-2}	0.98	4.6	1.29×10^{-3}	3.98×10^{-3}	8.19
Fluoranthene	3(1)	108.8	375	1.2×10^{-3}	1.252	5.22	260	6.5×10^{-4}	7.9
Pyrene	4	150.4	393	6×10^{-4}	1.271	5.18	135	1.1×10^{-3}	7.5
Benzo(A)Anthracene	4	160.7	400	2.8×10^{-5}	1.226	5.61	14	–	7.54
Chrysene	4	253.8	448	8.4×10^{-5}	1.274	5.91	2.0	–	7.8
Benzo(B)fluoranthene	4(1)	168.3	481	6.7×10^{-5}	–	6.12	1.2 (20 °C)	5.1×10^{-5}	Na
Benzo(K)fluoranthene	4(1)	215.7	480	1.3×10^{-8}	–	6.84	0.76	4.4×10^{-5}	Na
Benzo(A)pyrene	5	178.1	496	7.3×10^{-7}	1.351	6.5	3.8	3.54×10^{-5}	7.23
Benzo(G,H,I)perylene	6	278.3	545	1.4×10^{-8}	1.329	7.1	0.26	2.7×10^{-5}	Na
Indeno(1,2,3-Cd]pyrene	5(1)	163.6	536	1.3×10^{-8}	–	6.58	62	2.9×10^{-5} (20 °C)	Na
Dibenzo(A,H)anthracene	5	266.6	524	1.3×10^{-8}	1.282	6.5	0.5 (27 °C)	7×10^{-6}	7.57

Source Agency for Toxic Substances and Disease Registry

Table 2 List of bacterial strains and sensory behaviors against polycyclic aromatic hydrocarbons

Bacterial strain	Chemo effector	Chemoreceptor	References
P. putida G7	Naphthalene salicylate Biphenyl	Nah y, encoded on the Nah 7 catabolic plasmid	Grimm and Harwood (1997)
P. putida 10D	Naphthalene phenanthrene	Unknown	Ortegocalvo et al. (2003)
P. stutzeri 9A	Naphthalene	Unknown	Ortegocalvo et al. (2003)
Pseudomonas alcaligenes 8A	Naphthalene	Unknown	Ortegocalvo et al. (2003)
Azospirillum bransilense sp 7	Benzoate catechol	Unknown	Lopez de Victoria and Lovell (1993)
P. putida FU1	Furan, Furfural, 5-hydroxyl methyl furfural, furfural alcohol, 2-furoic acid	Transposon in psfb and lysr (regulatory gene)	Nichols et al. (2012)

PAHs by humans results in a high potential for biomagnification in the food chain. In general, higher the number of benzene rings, the greater the toxicity of the PAHs (Cerniglia 1992).

1.3 Chemotaxis toward aromatic compounds

Biodegradation research has been focused on catabolic pathway and identification, characterization of catabolic enzyme gene that encode them, identification of pathway intermediates and determination of appropriate condition for pathway induction. The applicability in biodegradation process, there is need to be consider other aspect such as substrate specificity, which determines that whether the organic compounds are accessible to bacteria or fungi for biodegradation process (bioavailability); it can be termed as chemotaxis (bacterium or fungus sensory) which can be conceptualize as how the microorganism find the chemical compound in the environment and how this chemical compound transport into a cell for degradation. It allows the detection of compound and uptake system for these chemical compounds. This is a prerequisite factor for biodegradation. Several factors which affect the bioavailability include hydrophobicity, aqueous solubility, polarity of the compound and characteristic of the medium (soil or water) where the contaminant is present. Recent studies have clearly demonstrated that many bacteria have sensory system that allows them to detect and respond behaviorally to chemical pollutant (Table 2). Fungi also play important role in assessing bioavailability of organic compounds. The strategy of filamentous fungi is to enlarge their external surface and to develop mycelia of high fractal dimension that optimally exploit the three-dimensional space containing the substrate was studied by Nakagaki et al. (2004). Furono et al. (2009) observed the movement of polycyclic aromatic hydrocarbon-degrading *Pseudomonas putida* PpG7 (NAH7) along mycelium of *Pythium ultimum*. Some dispersal was also observed in the absence of a chemoattractant. The non-chemotactic derivative strain *P. putida* G7.C1 (pHG100) was used. The bacterial movement became fourfold more effective when wild type chemotactic was used and salicylate was present as a chemoattractant. No dispersal of bacteria was found in the absence of the fungus. The role of mycelia for the translocation of chemicals was observed. Study suggested that fungi improve the accessibility of contaminants in water.

2 Biological treatment of PAH compounds

Biological treatment mainly relies on microbial remediation strategies which are developed to promote the microbial metabolism of contaminants, by providing appropriate water, air and nutrient supply. This is accomplished by the biostimulation (the addition of a bulking agent such as wood chips and/or nutrients such as N/P/K) and bioaugmentation (often an inoculum of microorganisms with known pollutant transformation abilities) of the contaminated environment. The pathways of microbial degradation tend to show broad substrate specificity and occur both aerobically and anaerobically (Harayama 1997).

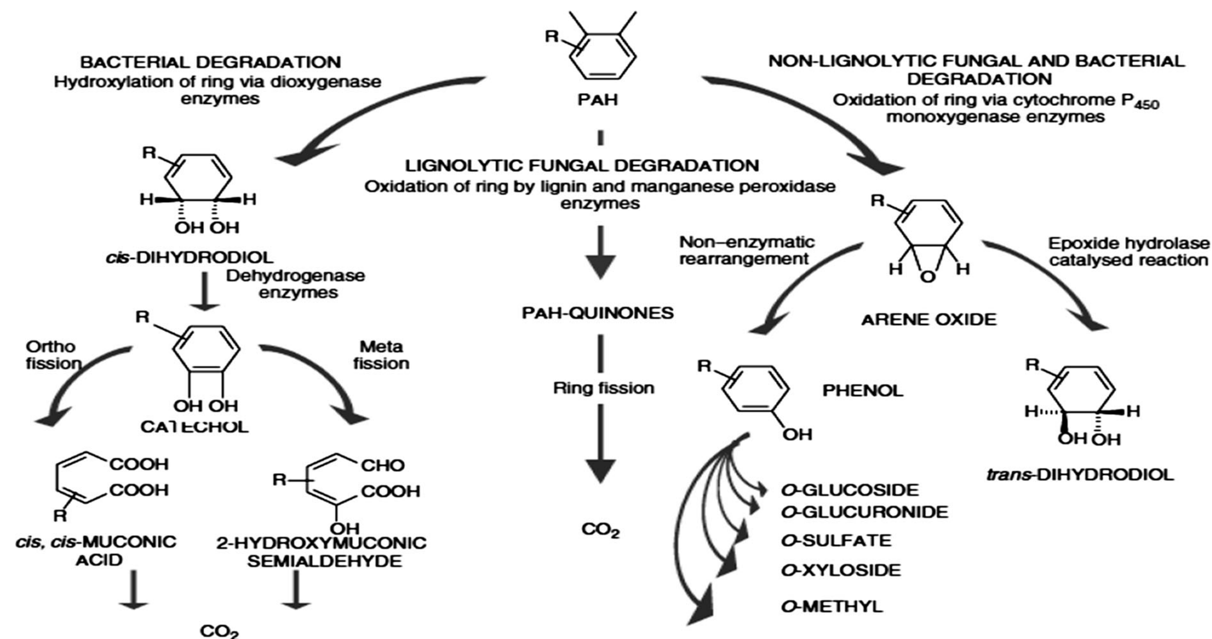


Fig. 2 Three main pathways for PAH degradation by bacteria and fungi

Bioremediation of PAHs contaminated soils, sediments, and water can be accomplished in a variety of ways, e.g. in situ treatment or ex situ methods such as bio-piling and composting. Several bacterial strains, have been isolated were identified on the basis of phospholipid analysis, FAME analysis, G1C content and 16S rRNA gene sequence analysis as species of *Pseudomonas, Microbacterium, Paracoccus, Arthrobacter* and *Mycobacterium* from Greek soils contaminated with PAH-containing waste from a wood processing industry, a steel industry and an oil refinery industry (Zhang et al. 2004a, b; Kallimanis et al. 2007). Generally unfavorable site conditions may cause variability in the applicability of bioremediation; therefore a thorough understanding of site conditions will allow optimization of bioremediation and subsequently more effective results. The basis of microbial metabolism of PAHs includes the oxidation of the aromatic ring, followed by the systematic breakdown of the compound to PAHs metabolite and/or carbon dioxide. PAHs degrading microorganisms are ubiquitously distributed in the natural environment, such as in soils (bacteria and non-ligninolytic fungi) and woody materials (ligninolytic fungi) as mentioned by Lee and Lee (2001). A study concluded by Sinha et al. (2009; Khan et al. 2001) during bacterial degradation of pyrene, in *M. vanbaalenii* PYR-1 cultures, First pathway indicates that pyrene hydroxylation takes place at the 1,2 positions, leading to the formation of 4-hydroxy-perinaphthenone which is the ultimate product so far found. Several key metabolites obtained were pyrene-4,5-dione, *cis*-4,5-pyrene-dihydrodiol, phenanthrene-4,5-dicarboxylic acid, and 4-phenanthroic acid are obtained during biotransformation of pyrene by gram-negative bacteria and accumulates Pyrene-4,5-dione as a final product and is further utilized as growth substrate (Liang et al. 2006). Fungal variation occurs in wide range of habitats in freshwater and the sea, soil, litter, in dung and in living organisms. It play very important role in mineralization and transformation of complex organic compounds into simpler compounds. According to (Pozdnyakova 2012) ligninolytic fungi, such as *Phanerochaete chrysosporium, Bjerkandera adusta*, and *Pleurotus ostreatus*, have the capacity of PAH degradation and the enzymes involved in the degradation of PAHs are ligninolytic and include lignin peroxidase, versatile peroxidase, Mn-peroxidase, and laccase. There are three main pathways followed by bacteria and fungi (Bamforth et al. 2005) shown in Fig. 2.

Algal species including *Chlamydomonas* sp., *Dunaliella* sp., and *Scenedesmus obliquus*, *Selenastrum capricornutum* have been reported as being able to

biotransform and bioaccumulate naphthalene, phenanthrene and pyrene and biotransform benzo[a]pyrene (Semple et al. 1999). Microbial consortia isolated from aged oil-contaminated soil were used to degrade 16 polycyclic aromatic hydrocarbons in soil and slurry phases. The three microbial consortia (bacteria, fungi and bacteria–fungi complex) could degrade polycyclic aromatic hydrocarbons (PAHs), and the highest PAH removals were found in soil and slurry inoculated with fungi. PAHs biodegradation in slurry was lower than in soil for bacteria and bacteria–fungi complex inoculation treatments. Degradation of three- to five-ring PAHs treated by consortia was observed in soil and slurry, and the highest degradation of individual PAHs (anthracene, fluoranthene, and benz(a)anthracene) appeared in soil and slurry. Inoculation of microbial consortia (bacteria, fungi and bacteria–fungi complex) isolated from in situ contaminated soil to degrade PAHs could be considered as a successful method (Li et al. 2008a, b).

2.1 Role of enzyme for biological remediation

Enzymology may be regarded as being at the interface between, on the one hand, biological discovery and protein engineering (Harford-cross et al. 2000) and environmental biotechnology. Enzymatic treatment of PAHs-contamination is an alternative to conventional bioremediation (Gianfreda and Bollag 2002; Gianfreda and Rao 2004; Ruggaber and Talley 2006). Microorganisms can be genetically 'engineered' to express specific xenobiotic metabolizing enzymes that would degrade even the most recalcitrant pollutants. The advantages of enzymatic remediation over microbial treatment include high reaction activity towards recalcitrant pollutants, lower sensitivity to the concentrations of contaminants, coverage of a wide range of physicochemical gradients in the environmental matrix and easy control of field application. With a necessity of improvement in biological remediation techniques, enzyme technology has been receiving increased attention. Fungal enzymes, which include lignin and manganese peroxidase and laccase, are responsible for the oxidative biodegradation of PAHs into CO_2 and H_2O. In the past several years, several oxido-reductases such as laccases and cytochrome P450 monooxygenases (CYPs) have been exploited for the enzymatic degradation of PAHs. Laccases belong to a group of multicopper enzymes that can catalyze the oxidation of a wide variety of phenolic compounds including PAHs (Majcherczyk et al. 1998; Alcade et al. 2002). Like other ligninolytic enzymes, laccase is difficult to express in non-fungal systems and knowledge of structure–function relations underlying the key functional properties of laccase is limited. Hence, directed evolution holds exciting potential for improving the performance of the enzyme. In a study undertaken by Bulter et al. (2003) the laccase gene from *Myceliophthora thermophia* (*MtL*), which was previously expressed only in *Asperigillus oryzae*, was transformed into *Saccharomyces cerevisiae* and subjected to directed evolution. PAHs can be oxidized by CYP enzymes to form catechols, which are then degraded by other enzymes, including catechol dioxygenases to harmless products and incorporated into the tricarboxylic acid cycle of microorganisms. Wild-type CYP101 (P450cam) from *Pseudomonas putida* has been shown to have an inherently low activity ($<0.01\ min^{-1}$) towards the PAH substrates phenanthrene, fluoranthene, pyrene and benzo[a]pyrene (Harford-cross et al. 2000). Therefore, CYP enzymes have been subjected to a number of rational design studies to enhance their catalytic performance. Based on the crystal structures of CYP101, selective mutations were performed on the active site residues F87 and Y96 of the enzyme. For all PAH substrates studied, the absolute oxidation rates (approximately $1\ min^{-1}$) of the mutants, Y96A, Y96F, F87A/Y96A and F87L/Y96F, were increased by two to three orders of magnitude relative to the wild-type enzyme. Carmichael and Wong (2001), introduced two mutations into CYP102, R47L and Y51F, and found that the oxidation activity of the enzyme for phenanthrene and fluoranthene was increased by 40- and ten-fold, respectively. The double mutant was then used as a basis for further engineering of the active site. When the A264G mutation was introduced to the base mutant, NADPH turnover, PAH oxidation and coupling efficiency of the enzyme was greatly improved. The most active mutants showed more than a 200-fold increase in PAH oxidation activity compared the wild type enzyme. Another mutation, F87A, resulted in a larger space in the substrate binding pocket of the enzyme, leading to better accommodation of larger fluoranthene and pyrene molecules in the vicinity of the haem site, and hence a more efficient PAH oxidation.

2.2 Aerobic degradation of PAHS

The basic mechanism behind the aerobic microorganisms to overcome the problem of degradation is linked with oxygenases that primarily reduce elemental oxygen to activate it, and allow it to insert into substrate. Aerobic condition favors the electron acceptor has lower standard reduction potential values and convert into less standard Gibbs free energy change when coupled with any given substrate.

The initial catabolic step in aerobic oxidation of a PAH molecule occurs via formation of dihydrodiol by a multicomponent dioxygenase enzyme system which is further metabolized by either an ortho-or a meta pathway, leading to intermediates such as protocatechuates and catechol. These intermediates compounds are processed through either ortho-meta cleavage leading to central intermediate compounds such as protocatechuates, catechols, gentisates, homoprotoocatechuates, homogentistases, hydroquinones and hydroxyquinols which are further transformed to tricarboxylic acid cycle intermediates (Harayama and Timmis 1992) finally channeled into intermediates of kreb's cycle. The principal mechanism for the aerobic bacterial metabolism of PAHs is the initial oxidation of the benzene ring by the action of dioxygenase enzymes to form *cis*-dihydrodiols. These dihydrodiols are dehydrogenated to form dihydroxylated intermediates, which can further metabolised via catechols to carbon dioxide and water. The ability of the microorganisms to degrade PAHs is reported the involvement of *Beijerinkia* sp. in oxidation of benzo(*a*)pyrene and benzo(*a*)Anthracene to dihydrodiols (Gibson and Subranian 1984; Jain et al. 2005).

2.2.1 Advantages and disadvantages

The aerobic pathway releases a substantial amount of energy. A portion is used by the microorganisms for synthesis and growth of new microorganisms. Aerobic treatment yields better effluent quality. Aerobic cometabolism has unique benefits derived from the advantages of aerobic respiration. These advantages and benefits can reduce costs and risks associated with soil and ground water remediation. There is a drawback of aerobic degradation process that it requires high power Supply and energy consumption.

2.3 Anaerobic degradation of PAHS

Anaerobic degradation occurs in the absence of oxygen, under anaerobic conditions, aromatic compounds are able to serve as electron donating substrates of primary metabolism, several compound acts as terminal electron acceptor (TEA) and oxidation of aromatic compounds support the growth of microorganisms. A basic pathway for biotransformation of PAHs under anaerobic condition is shown in Fig. 3. (Widdle and Rabbus 2001). Phthalate compound degradation is mainly carried out by anaerobic methanogens (*Methanospirillum hungatei*, *Methanosaeta concilii, Syntrophobacter fumaroxidens*), producing acetate and methane as end products by decarboxylation initially, then reduction followed by ring cleave and ultimately pave to the β-oxidation pathway (Qiu et al. 2004; Zhang and Bennet 2005). The biotransformation of pyrene and benzo[a]pyrene (BaP) is well studied in different bacterial species such as *Mycobacterium vanbaalenii* PYR-1, *M. flavescens* PYR-GCK, *Mycobacterium* RJGII-135, *Mycobacterium* KR2 and *Mycobacterium*AP1. Different pathways have been anticipated. *Mycobacterium* KMS has been used to study the metabolites produced during pyrene transformation. First pathway indicates that pyrene hydroxylation takes place at the 1,2 positions, leading to the formation of 4-hydroxy-perinaphthenone which is the ultimate product so far found only in *M. vanbaalenii* PYR-1 cultures (Khan et al. 2001; Sinha et al. 2009).

2.3.1 Advantages and disadvantages

Anaerobic bioreactors have several potential advantages over aerobic processes. Due to omission of aeration and the conversion of organic matter to methane, which is an energy source by itself and can be used for temperature control so, less energy is required in this process. High energy recovery, increase in hydrolysis rate at increasing biodegradability suggests that the rate of hydrolysis of particulate organic matter is determined by the adsorption of hydrolytic enzymes to the biodegradable surface sites and the gas generated after through the various stages of breakdown of complex organic compounds can be used as biofuel (Veeken and Hamelers 1999). Apart from the benefits of anaerobic process there is one

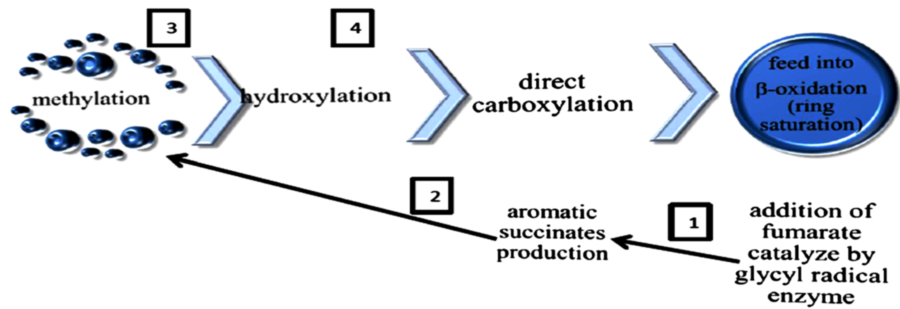


Fig. 3 Simplified representation of biotransformation of PAH's via anaerobic mechanism

shortcoming that it need for a longer start-up Period time to develop necessary biomass and the fact that they are much more sensitive to the temperature value.

2.4 Co-metabolism

Co-metabolism is especially important for the degradation of mixtures of polycyclic aromatic hydrocarbons (Chauhan et al. 2008). Co-metabolism entails the parallel oxidation of a non-growth substrate during growth of bacteria on a compatible carbon energy source. It also describes oxidation of non-utilizable substrates by resting cell suspensions grown at the disbursement of substances that are capable of supporting microbial growth. Co-metabolism refers to oxidation of substrate without utilization of energy derived from the oxidation to support microbial growth. Cometabolism is often observed for PAHs compounds which do not enable growth as a single carbon- and energy source (Janke and Fritsche 1985). This is due to long degradation pathways and unnatural structures. Cometabolism of benzo-α-pyrene is significantly increased with enrichment of the soil with phenanthrene as growth substrate for the non growth substrate of pyrene, 3,4 bezopyrene, 1,2 benzoanthracene, 1,3,5,6 dibenzanthracene (McKenna and Heath 1976).

On the other hand, microorganisms with a restricted metabolism are dependent on cometabolism of essential natural compounds. Another distinguishing feature is its high cellular demand, is additionally required for glycine, cysteine, tryptophan, and phospholipid synthesis. The short-chain fatty acid is more suitable than the carbohydrates as the carbon sources for cometabolism (Xie et al. 2009). Dioxygenase and monooxygenase enzymes are responsible for mineralization of various PAHs, including pyrene and B*a*P. Although the degradation of pyrene increases in organic nutrients to the microcosm inhibited pyrene degradation (Heitkamp and Cerniglia 1989). Study showed that in gram positive bacteria especially mycobacteria, genetical and biochemical data of high molecular weight, polycyclic aromatic hydrocarbon degradation is relatively lower since it possesses extremely resistant cell wall, significantly low growth rate and triggers the activity of cell clumping. In gram positive bacteria, *nid*, *pdo* genes encode high molecular weight polycyclic aromatic hydrocarbons (HMWPAH) dioxygenases, whereas in gram negative bacteria *nah*, *pah* and *phn* genes encode low molecular weight polycyclic aromatic hydrocarbons (LMWPAH) dioxygenases. The initial reactions in both the aerobic metabolism and co-metabolism of PAHs are catalyzed by a variety of oxygenase-type enzymes which generate mono- and di-hydroxylated products. It is important to look at the possibilities and extent of both metabolism and cometabolism of those high molecular weight PAHs. Co-metabolic pathway of Napthalene (Denome et al. 1993; Goyal and Zylstra 1997; Kiyohara et al. 1994), Phenanthrene (Moody et al. 2001; Seo et al. 2006, 2007; Prabhu and Phale 2003; Mallick et al. 2007; Krivobok et al. 2003; Pinyakong et al. 2000; Kanaly and Harayama 2000), Fluoranthene (Cerniglia 1992; Kelley et al. 1993; Weissenfels et al. 1991; Kanaly and Harayama 2000) and Benzo-α-pyrene (Schneider et al. 1996; Cerniglia 1992; Heitkamp and Cerniglia 1988; Seo et al. 2009) is shown in Figs. 4, 5, 6, and 7. Gene clusters that code for the catabolism of aromatic compounds are frequently found in mobile genetic elements, as transposons and plasmids, which assist their horizontal gene transfer, and consequently enhance adaptation of specific bacterial genera to novel pollutants. This technique generally employed for the biochemical study of microbial aromatic metabolism (Horvath 1972). Strains that exhibit the phenomenon of co-metabolism in a well-organized manner include *Nocardia*, *Pseudomonas*, *Xanthomonas*, *Bacillus*, *Brevibacterium*, *Flavobacterium*, *Aspergillus*,

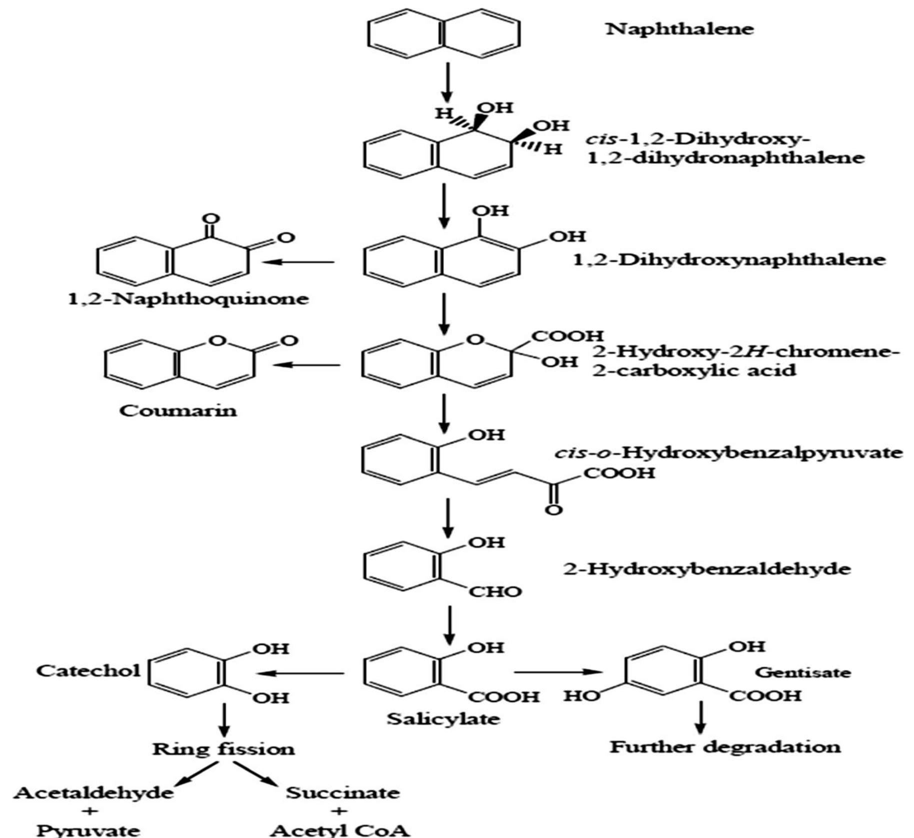


Fig. 4 Cometabolic pathway of napthalene

Azotobacter, *Trichoderma*, *Vibrio*, *Achromobacter*, *Arthrobacter*, *Hydrogenomonas*, *Microbacterium*, *Micrococcus* and *Streptomyces* (Beam and Perry 1973). The manipulation of the catabolic genes from degradative enzymes could solve the problem and boost up the process. The degradation mainly depends upon the adapting response of the microbial communities which include both selective enrichment (resulting in amplification of genes) and genetic changes (mainly includes gene transfer or mutation). Recent approaches have been made to characterize and compare the gene involved in degradation of identical or similar aromatic compounds in nearly diverse or more isolated bacterial genera from diverse topologies (Liang et al. 2006; Kim et al. 2006a, b). The influence of growth medium on cometabolic degradation of PAHs is more effective in nutrient rich medium for enhancement of cometabolic degradation of mixed PAHs concomitant with a rapid removal of metabolites, which could be useful for the bioremediation of mixed PAHs contaminated sites (Zhong et al. 2007).

3 Bacterial degradation of PAHS

Intense research pertaining to bacterial degradation of PAHs which composed of three rings has been well documented. The most commonly reported bacterial species include *Acinetobacter calcoaceticus, Alcaligens denitrificans, Mycobacterium* sp., *Pseudomonas putida, Pseudomonas fluorescens, Pseudomonas vesicularis, Pseudomonas cepacia, Rhodococcus* sp., *Corynebacterium renale, Moraxella* sp., *Bacillus cereus, Beijerinckia* sp., *Micrococcus* sp., *Pseudomonas paucimobilis* and *Sphingomonas* sp. (Kanaly et al. 2000; Arun et al. 2008). Although most of these bacterial species are reported to degrade low molecular weight PAHs, there are limited reports on degradation of high molecular weight PAHs with more than four benzene rings. Bacterial degradation predominantly occurs via aerobic oxygenase mediated pathways; although an alternative pathway of degradation involving utilization of nitrate as the alternative electron acceptor has been reported. Degradation of

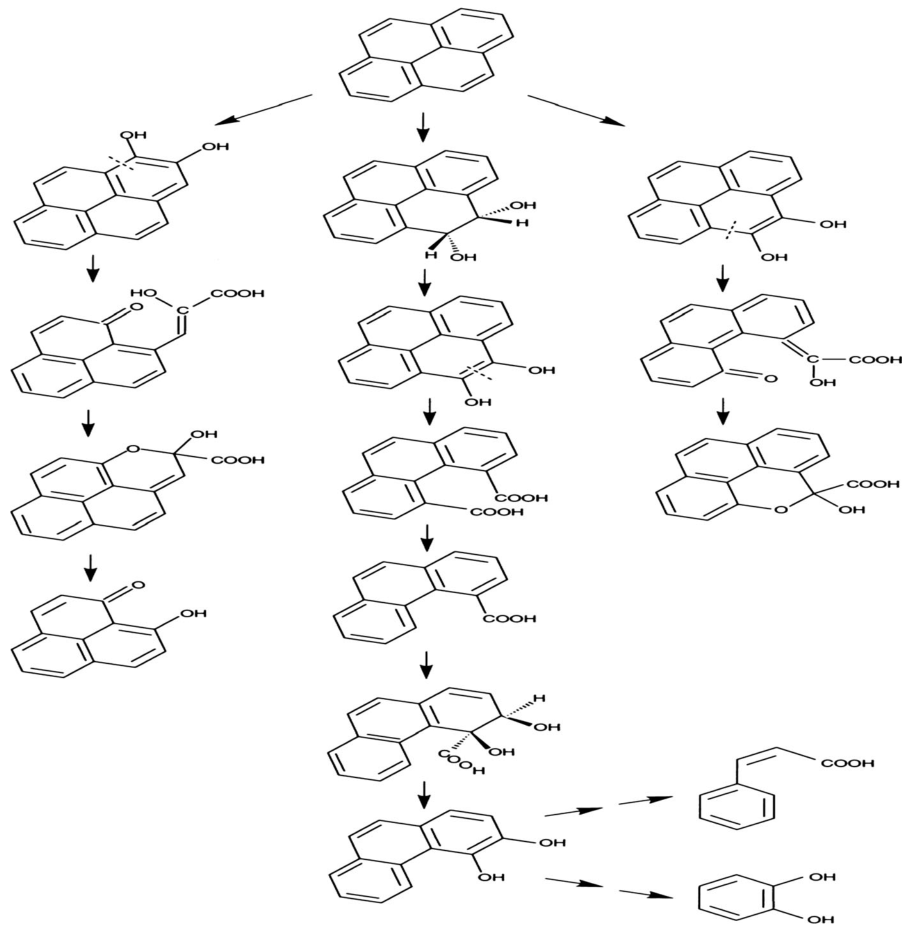


Fig. 5 Cometabolism of phenanthrene

PAHs compounds and formation of intermediates by bacterial action (Cerniglia 1992; Juhasz and Naidu 2000; UMBBD 2004) are listed in Table 3. As for mangrove environment, Zhou et al. (2009) utilized 16S rRNA PCR and DGGE to assess the effects of PAHs (before and after PAHs exposure) on the bacterial community. Sequencing of DGGE bands showed that marine bacteria from the genera of *Vibrio, Roseobacter,* and *Ferrimonas* were most abundant after PAHs exposure, which suggests that both marine and terrestrial bacteria coexisted in the mangrove sediment (Zhou et al. 2009; Shi et al. 2010). Molina et al. (2009) obtained PAHs degrading microbial consortium C2PL05 from a petrochemical complex in Puertollano (Ciudad Real, Spain) which possessed highly efficient degrading capacity and DGGE analysis revealed uncultured *Stenotrophomonas* ribotypes as a possible PAHs degrader in the microbial consortium (Molina et al. 2009; Shi et al. 2010). Although several PAHs degrading bacterial species have been isolated, it is not expected that a single isolate would exhibit the ability to degrade completely all PAHs. PCR–DGGE of 16S rRNA gene sequences was used to monitor the bacterial population changes during PAHs degradation of the consortium when pyrene, chrysene, and benzo[a]pyrene were provided together or separately in the TLP cultures (Lafortune et al. 2009; Shi et al. 2010).

3.1 Application of immobilized bacterial cell

Immobilized cells have been used and studied for the bioremediation of numerous toxic chemicals. Immobilization not only simplifies separation and recovery of immobilized cells but also makes the application reusable which reduces the overall cost. Wilson and Bradley (1996) used free suspension and immobilized *Pseudomonas* sp. to degrade petrol in an aqueous

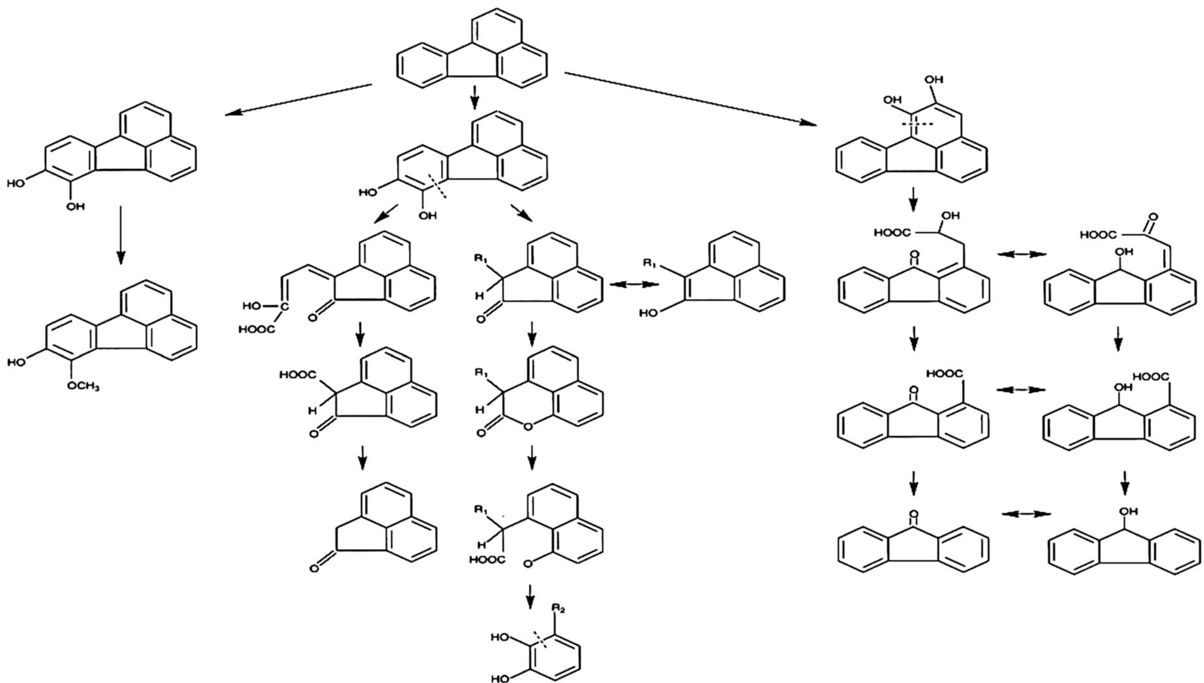


Fig. 6 Metabolic pathway of Fluoranthene

system. Immobilization increase contact between cell and hydrocarbon droplets and enhanced level of rhamnolipids production was studied by Wilson and Bradley (1996; Das and Chandran 2011). Díaz et al. (2002) reported that immobilization of bacterial cells enhanced the biodegradation rate of crude oil compared to free living cells in a wide range of culture salinity. Immobilization can be done in batch mode as well as continuous mode. Packed bed reactors are commonly used in continuous mode to degrade hydrocarbons. Polyvinyl alcohol (PVA) cryogelation was used as an entrapment matrix by Cunningham et al. (2004). Laboratory biopiles was constructed to compare immobilised bioaugmentation with liquid culture bioaugmentation and biostimulation. Immobilised systems were found to be the most successful in terms of percentage removal of diesel after 32 days and immobilized bacteria in alginate beads were able to degrade hydrocarbons, there was no decline in the biodegradation activity was observed in microbial consortium on the repeated use study conducted by Rahman et al. (2006). It can be concluded that immobilization of cells are a promising application in the bioremediation of hydrocarbon contaminated site.

4 Fungal degradation of PAHS

Fungal remediation or mycoremediation is a promising technique for cleanup of contaminated soil. Two main types of fungal metabolism of PAHs degradation; these are mediated by the ligninolytic (also known as the white-rot fungi) and non-ligninolytic fungi. The majority of fungi are non-ligninolytic, as they do not grow on wood, and therefore have no need for the lignin peroxidase enzymes that produced by the ligninolytic fungi. However, many ligninolytic fungi such as *Phanerochaete chrysosporium* and *Pleurotus ostreatus* can produce both non-ligninolytic and ligninolytic type enzymes (Hammel et al. 1992; Bezael et al. 1997). The main mechanism involved in the fungal degradation of PAHs is enzymatic transformation by intracellular cytochrome P450 enzymes and extracellular ligninolytic enzymes (Cerniglia 1997). Molecular fingerprinting profiles and selective enumeration showed biostimulation with ground corn cob increased both number and abundance of indigenous aromatic hydrocarbons degraders and changed microbial community composition of soil, which is beneficial to natural attenuation of PAHs. At the same time, bioaugmentation of PAHs with *Monilinia* strain W5-2 imposed

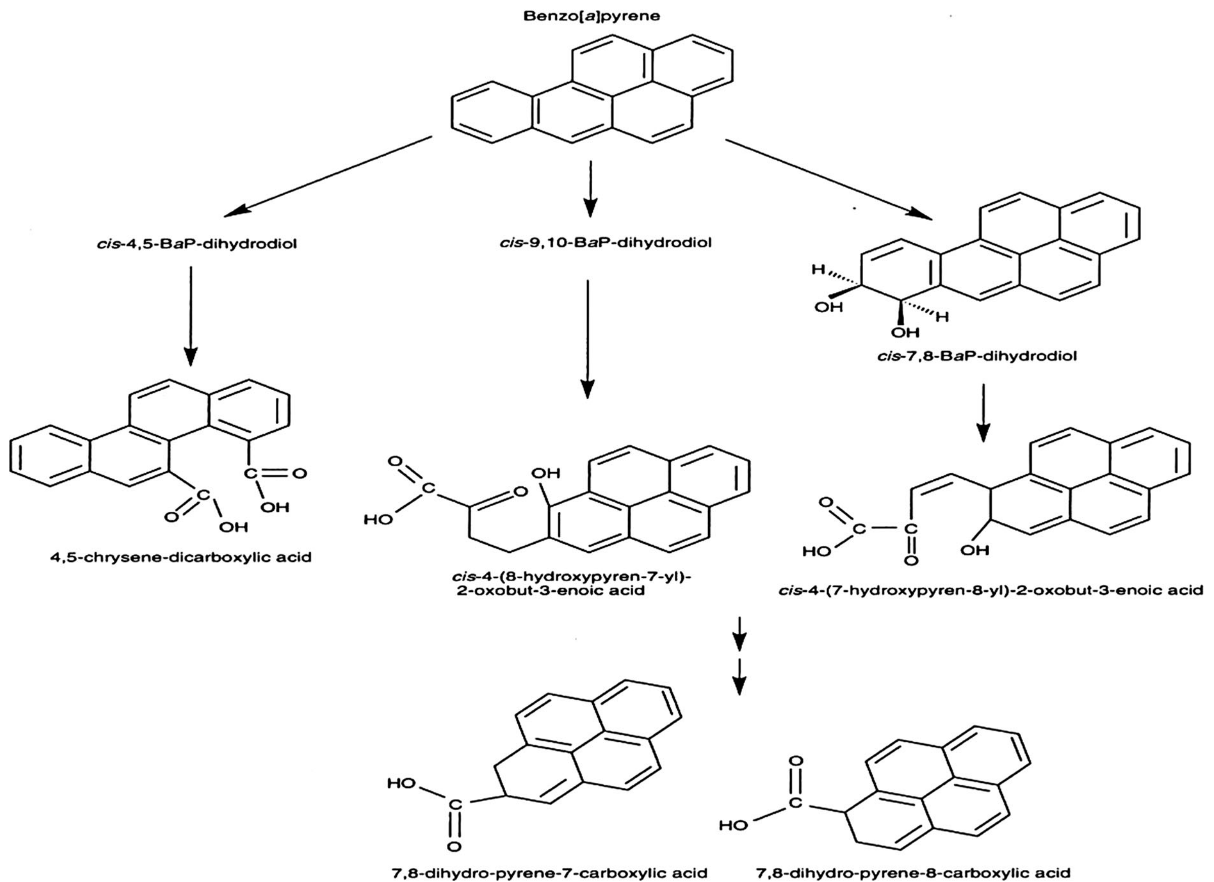


Fig. 7 Pathways proposed for the metabolism of Benzo-α-pyrene (B*a*P) by *Mycobacterium* sp. strain RJGII-135

negligible effect on indeginous microbial community. Wu et al. (2008a, b) suggested that fungal remediation is promising in eliminating PAHs especially the part of recalcitrant and highly toxic benzo[α]pyrene, in contaminated soil. It is also the first description of soil bioremediation with *Monilinia* sp.

4.1 Lygnolytic fungal degradation

There are two types of ligninolytic enzymes; these being peroxidases and laccases. These enzymes are secreted extracellularly, and oxidise organic matter via a non-specific radical based reaction. There are two main types of peroxidase enzyme depending on their reducing substrate type, lignin peroxidase (LP) and manganese peroxidase (MnP), both of which are capable of oxidising PAHs. Laccases, which are phenol oxidase enzymes, are also capable of oxidizing PAHs (Mester and Tien 2000).

Two possible roles of ligninolytic system have been discussed by Collins and Dobson (1996; Steffen et al. 2003): (a) LiP, MnP, and LAC were found to have a pivotal role in the degradation of PAHs, catalyzing the first attack of molecule. (b) Cytochrome P-450 monooxygenase could be responsible for this initial step (Bezalel et al. 1996) White-rot fungi, such as *Phanerochaete chrysosporium* and *Trametes versicolor*, were able to mineralize PAHs, and indicated the complete breakdown of PAHs observed by Renner (1999; Pozdnyakova 2012). Enzymes involved in the degradation of PAHs are oxygenase, dehydrogenase and lignolytic enzymes. Fungal lignolytic enzymes are lignin peroxidase, laccase, and manganese peroxidase. They are extracellular and catalyze radical formation by oxidation to destabilize bonds in a molecule. White-rot fungi are a group of fungi that produce ligninolytic enzymes involved in the oxidation of lignin present in wood and other organic matter as mentioned by Haritash and Kaushik (2009). Lignolytic fungal strains responsible for the PAHs degradation and their metabolites listed in Table 4.

Table 3 Polycyclic aromatic hydrocarbons oxidised by different species/strains of bacteria

PAH compounds	Bacterial strain	Functional gene	Intermediate compounds	References
Phenanthrene	Aeromonas sp., A. faecalis, A. denitrificans, Arthrobacter polychromogenes, Beijernickia sp., Micrococcus sp., Mycobacterium sp., P. putida sp. paucimobilis, Rhodococcus sp, Vibrio sp., Nocardia sp., Flavobacterium sp., Streptomyces sp., S. griseus, Acinetobacter sp., P. aeruginosa, P. stutzeri, Bacillus sp., Sphingomonas sp., Pseudomonas sp., Rhodotorula glutinis, Nocardioides sp., Flavobacterium gondwanense, Sphingomonas sp Strain ZL5, Microbacterium sp.	phnAa/phnAc/phnAd/ phnB/ phnC/phnD/phnE/ phnF/ phnG phnH/phnI (Alcaligenes faecalis AFK2), p h d (p h d Nocardioides KP7)	2-carboxy-cis, cis-muconate by protocatechuate, 1-hydroxy 2-napthoic acid, pthalic acid	Allen et al. (1999), Aitken et al. (1998), Moody et al. (2001), Yamazoe et al. (2004), Miyata et al. (2004), Liu et al. (2004), Zhang et al. 2004a, b, Liu et al. (2004), Habe and Omori (2003), Chauhan et al. (2008)
Fluorene	Terrabacter sp., Staphylococcus auriculans, Arthrobacter, Mycobacterium gilvum, Sphingomonas aromaticivorans, Sphingomonas sp. LB216, Leclercia adecarboxylat	–	1-fluorenol, 2-fluorenone and 1-indanone	Wattiau et al. (2001), Sharma et al. (2004)
Pyrene	A. denitrificans, Mycobacterium sp., Rhodococcus sp., Sp. paucimobilis, Stenotrophomonas maltophilia, Acinetobacter calcoaceticus, Gordona sp., Sphingomonas sp., P. putida, Bu cepacia, P. saccharophilia, Mycobacterium gilvum, Leclercia adecarboxylata, Mycobacterium pyrenivorans	NidAB dioxygenase (nidA and nidB), pbhD (Sphingomonas paucimobilis Var.EPA505)	cis-4,5-dihydro-4,5-dihydroxypyrene (PYRdHD), pyrene-4,5-dione (PYRQ)	Chen and Aitken (1999), Boldrin et al. (1993), Sharma et al. (2004), Derz et al. (2005), Kanaly and Harayama (2000)
Benzo[a]pyrene	Paucimobilis, Beijerinckia sp., Mycobacterium sp., Rhodococcus sp. UW1, Pseudomonas sp., Agrobacterium sp., Bacillus sp., Burkholderia cepacia	CytochromeP-450 and epoxide hydrolase	cis-4,5-dihydroxybenzo[a]pyrene (benzo[a]pyrene 4,5-dihydrodiol), cis-11,12-dihydroxybenzo[a]pyrene (benzo[a]pyrene cis-11,12-dihydrodiol), trans-11,12-dihydro-11,12-dihydroxybenzo[a]pyrene	Gibson et al. (1975), Schneider et al. (1996), Walter et al. (1991), Aitken et al. (1998), Boonchan et al. (2000)
Naphthalene	Pseudomonas putida, sulfate reducing bacteria (anaerobic)	pahA/pahAb/pahB/pahC/pahD/pahE/pahF (Pseudomonas putida), nahAc, alkB and xylE	2-napthoic acid, naphthyl-2-methylsuccinic acid	Habe and Omori (2003), Chauhan et al. (2008), Musat et al. (2009), Abu Laban et al. (2010), Bergmann et al. (2011)

Table 4 List of PAHs compound degraded by lignolytic fungal strains

PAH compounds	Lignolytic fungal strain	Enzyme	Metabolite	References
Fluorene	*Pleurotus ostreatus, Phanerochaete* sp., *Cunninghamella elegans, Laetiporus sulhureus, Penicillium sp., Trametes versicolor*	ND	9-fluorenol and 9-fluorenone	Sack and Fritsche (1997), Bezalel et al. (1996), Sack and Gunther (1993)
Phenanthrene	*P. chrysosporium* (*WRF*) *P. sordida* (*WRF*) *P. ostreatus* (*WRF*) *T. versicolor* (*WRF*) *Agrocybe* sp. (*WRF*) *Ganoderma lucidum* (*WRF*)	Monooxygenase; epoxide hydrolase, Cytochrome P-450; MnP MnP Cytochrome P-450; epoxide hydrolase, LAC	PHE-*trans*-9,10-dihydrodiol; PHE-*trans*-3,4-dihydrodiol; 9-phenantrol, 3-phenanthrol; 4-phenanthrol; 9-phenanthryl-D-glucopyranoside PHE-9,10-quinone; 2,2diphenic acid, PHE-*trans*-9,10-dihydrodiol	Collins and Dobson (1996), Casillas et al. (1996), Sutherland et al. (1993), Mori et al. (2003), Da-Silva et al. (2003)
Fluoranthene	*Agrocybe* sp. *B. adusta* *P. ostreatus*	ND	9-fluorenol;9-fluorenone	Pothuluri et al. (1990), Schutzendubel et al. (1999), Salicis et al. (1999), Sack and Gunther (1993)
Pyrene	*I. lacteus* (WRF); *P. ostreatus* (WRF) *Ganoderma lucidum* (WRF)	Mn-inhibited Peroxidase, MnP LAC LAC, VP	Quinonic metabolites PYR-4,5-dihydrodiol PYR-4,5-dihydrodiol; phthalic acid	Launen et al. (1995), Bezalel et al. (1996), Schutzendubel et al. (1999), Wunder et al. (1997), Sack and Fritsche (1997), Boonchan (1998), Da-Silva et al. (2003)
Benzo(a)pyrene	*P. chrysosporium Bjerkandera* sp. (WRF), *P. ostreatus* (WRF), *Stropharia coronilla* (LDF), *Stropharia rugosoannulata* (LDF)	LiP; MnP	Quinone metabolite; CO_2	Kapoor and Lin (1984), Launen et al. (1995), Sack and Fritsche (1997), Steffen et al. (2003), Verdin et al. (2003), Da-Silva et al. (2003)
Anthracene	*Agrocybe* sp. (WRF) *B. adusta* (WRF) *P. ostreatus* (WRF) *I. lacteus* (WRF) *Trametes versicolor* *Coriolopsis polyzona* (WRF) *P. chrysosporium* (WRF) *Stropharia coronilla* (LDF)	MnP; LAC, LiP; MnP MnP LAC	9,10-anthraquinone 9,10-anthraquinone; phthalic acid; CO_2	Vyas et al. (1994)

Table 5 List of PAHs compounds degraded by non lignolytic fungal strains

PAH compounds	Non lignolytic fungal strain	Enzyme	Metabolites	References
Fluorene	*Cunnighmella elegans*	Dioxygenase, cytochrome P450 monoxygenase	4-hydroxy-9-fluorenone, 9-fluorenone	Pothuluri et al. (1990)
Phenanthrene	*C. elegans* *Curvularia turberculata, A. niger, Curvularia lunata*	Cytochrome P450 monoxygenase	4,5-dihydropyrene; CO_2 from PHE, Phthalic acid	Cerniglia and Heitkamp (1989), Sutherland et al. (1991), Mori et al. (2003)
Fluoranthene	*C. elegans* *C. blackesleema* *C. echinuta* *Aspergillus terreus*	Dioxygenase, cytochrome P450 monoxygenase	9-fluorenol; 9-fluorenone, 3-fluoranthene-β-glucopyranoside, trans2-3,dihydrodiol	Schutzendubel et al. (1999), Salicis et al. (1999)
Pyrene	*C. elegans*	Dioxygenase, cytochrome P450 monoxygenase	Phthalic acid	Cerniglia et al. (1986), Hammel et al. (1986), Da-Silva et al. (2003)
Benzo[a]pyrene	*C. elegans* *Aspergillus ochareus*	Dioxygenase, cytochrome P450 monoxygenase	4,5-dihydropyrene; CO_2 from PHE, phthalic Acid	Collins and Dobson (1996), Rama et al. (1998)

4.2 Non lignolytic fungal degradation

The first step in the metabolism of PAHs by nonligninolytic fungi is to oxidise the aromatic ring in a cytochrome P450 monoxygenase enzyme catalyzed reaction to produce an arene oxide (Bezael et al. 1997). In comparison to the oxidation of the aromatic ring by dioxygenase enzymes to form *cis* dihydrodiols, the monoxygenase enzyme incorporates only one oxygen atom into the ring to form an arene oxide. This is subsequently hydrated via an epoxide-hydrolase catalysed reaction to form a *trans* dihydrodiol (Baldrian et al. 2000). In addition, phenol derivatives may be produced from arene oxides by the non-enzymatic rearrangement of the compound, which can act as substrates for subsequent sulfation/methylation, or conjugation with glucose, xylose, or glucuronic acid. Most non-ligninolytic fungi are not capable to complete mineralisation of PAHs. These PAHs conjugate are generally less toxic and more soluble than their respective parent compounds. Some of non lignolytic fungal strains are listed in Table 5.

4.3 Application of immobobilized fungal strains

Immobilized form of microorganisms will promote degradation as immobilization is known to offer protection from extremes of pH and toxic compounds in the contaminated soil (Su et al. 2006; Wang et al. 2012). Among all the immobilization methods, physical adsorption on farm byproducts was found to be the optimum method owing to its properties of being highly granular, absorbent, biodegradable and inexpensive (Xu and Lu 2010; Wang et al. 2012). In conclusion, the use of immobilized microorganisms with farm byproducts and nutrients as carrier materials and bulking agents for the remediation of PAH-contaminated site in situ is a promising technology. Wang et al. (2012) studied the degradation of pyrene by the immobilized microorganisms *Mucor* sp. F2, fungal consortium MF and co-cultures of MB + MF was increased by 161.7 % ($P < 0.05$), 60.1 % ($P < 0.05$) and 59.6 % ($P < 0.05$) after 30 days, respectively. When compared with free F2, MF and MB + MF. Results indicated that immobilization improved stability of laccase to temperature, pH, inhibitors and storage time compared with the free enzyme. Tao et al. (2009) were done research work on strain *Sphingomonas* sp. GY2B is a high efficient phenanthrene-degrading strain isolated from crude oil contaminated soils that displays broad-spectrum degradation ability towards PAHs and related aromatic compounds. The immobilization of strain GY2B possesses a good potential for application in the

Table 6 Comparative study of immobilized fungal strains

Fungal strain	Responsible enzyme	Comments reported	References
Sphingomonas sp.	Immobilization of strain GY2B in calcium alginate gel beads	Is a high efficient phenanthrene-degrading strain isolated from crude oil contaminated soils	Tao et al. (2009)
Trametes versicolor	Laccase was immobilized on kaolinite	Its potential to oxidize anthracene and benzo[a]pyrene in a sole-substrate system in the presence of 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS)	Dodor et al. (2004)
Mucor sp. *F2*	Co-cultures of MB + MF (Mycobacterium and Mucor sp. F2)	Immobilized microbial technique on pyrene remediation in saline alkaline soil, as the interspace of the carrier material structure was relatively large, providing enough space for cell growth	
Pycnoporus sanguinues	Laccase ecomat was found to be the best support material compare with Coconut husk and grey scouring sponge	Free mycelia culture rapidly degrades pyrene. > Anthracene > phenanthrene	Low et al. (2009)

treatment of industrial wastewater containing phenanthrene and other related aromatic compounds. A Comparative study of different immobilize fungal strains is shown in Table 6.

5 Factors affecting PAHs degradation

A number of limiting factors have been recognized to affect the biodegradation of PAHs. It is apparent that environmental factors that vary from site to site (such as soil pH, nutrient availability and the bioavailability of the contaminant) can influence the process of bioremediation by inhibiting growth of the pollutant-degrading microorganisms. The main environmental factors that could affect the feasibility of bioremediation are summarized in the following sections.

5.1 Temperature

Temperature has a considerable effect on the ability of the in situ microorganisms to degrade PAHs and, in general, most contaminated sites will not be at the optimum temperature for bioremediation during every season of the year. According to (Margesin and Schinner 2001) the solubility of PAHs increases with an increase in temperature, which increases the bioavailability of the PAH molecules. In addition, oxygen solubility decreases with increasing temperature, which will reduce the metabolic activity of aerobic microorganisms. Biodegradation of PAHs can occur over a wide temperature range however, most studies tend to focus on mesophilic temperatures rather than the efficiency of transformations at very low or high temperatures. Lau et al. (2003) were reported that the laccase and manganese peroxidase enzymes of ligninolytic fungi have a temperature optimum of ~50 and >75 °C respectively in spent-mushroom compost during the degradation of PAHs, with over 90 % degradation of the contaminating PAHs occurring at these temperatures.

5.2 pH

Many sites contaminated with PAHs are not at the optimal pH for bioremediation. For example, Phenanthrene degradation in liquid culture has been investigated by Wong et al. (2002) at a range of pH values (pH 5.5–7.5) with *Burkholderia cocovenenas*, an organism isolated from a petroleum-contaminated soil, bacterial growth was not significantly affected by the pH. Phenanthrene removal was only 40 % at pH 5.5 after 16 days, whereas at circum-neutral pH values, phenanthrene removal was 80 %. *Sphingomonas paucimobilis* (strain BA 2) was however more sensitive to the pH of growth media, with the degradation of the PAHs phenanthrene and anthracene significantly inhibited at pH 5.2 relative to pH 7 reported by Kastner et al. (1998).

5.3 Oxygen

For the reduction of complex hydrocarbons into substrates, require an electron acceptor, molecular

oxygen being most common. In the absence of molecular oxygen, nitrate, iron, bicarbonate, nitrous oxide and sulfate, have been shown to act as an alternate electron acceptor during hydrocarbon degradation. Bioremediation of organic contaminants (PAHs) can proceed under both aerobic and anaerobic conditions; most work has tended to concentrate upon the dynamics of aerobic metabolism of PAHs. This is in part due to the ease of study and culture of aerobic microorganisms relative to anaerobic microorganisms. To maintain adequate oxygen levels for aerobic metabolism for in situ treatments include hydrogen peroxide for sub-surface contamination. The aerobic biodegradation of hydrocarbons has been reported to be highly relative to anaerobic biodegradation (Rockne and Strand 1998).

5.4 Nutrient availability

Basically, microorganisms require mineral nutrients such as nitrogen, phosphate and potassium (N, P and K) for cellular metabolism and successful growth. In contaminated sites, where organic carbon levels are often high due to the nature of the pollutant, available nutrients can become rapidly depleted during microbial metabolism. Therefore it is common practice to supplement contaminated land with nutrients, generally nitrogen and phosphates to stimulate the in situ microbial community to enhance bioremediation. The amounts of N and P required for optimal microbial growth and hence bioremediation have been previously estimated from the ratio of C:N:P in microbial biomass (100:15:364 and 120:10:165). However, a recent study has shown that optimal microbial growth and creosote biodegradation occurred in soil with a much higher C:N ratio (25:1) than those predicted from the ratio in microbial biomass, with lower C:N ratios (5:1) causing no enhancement in microbial growth (Alexander 1977; Atagana et al. 2003).

5.5 Bioavailability

The effect of physico-chemical and microbiological factors on the rate and extent of biodegradation and is believed to be one of the most important factors in bioremediation. PAH compounds have a low bioavailability, and are classed as hydrophobic organic contaminants. The larger the molecular weight of the PAH, the lower its solubility, which in turn reduces the accessibility of the PAH for metabolism by the microbial cell. In addition, PAHs can undergo rapid sorption to mineral surfaces (i.e. clays) and organic matter (i.e. humic and fulvic acids) in the soil matrix. The longer that the PAH is in contact with soil, the more irreversible the sorption, and the lower is the chemical and biological extractability of the contaminant. This phenomenon is known as 'ageing' of the contaminant. Therefore the bioavailability of a pollutant is linked to its persistence in a given environment (Semple et al. 2003; Peltola 2010).

5.6 Surfactant: bioavailability enhancer

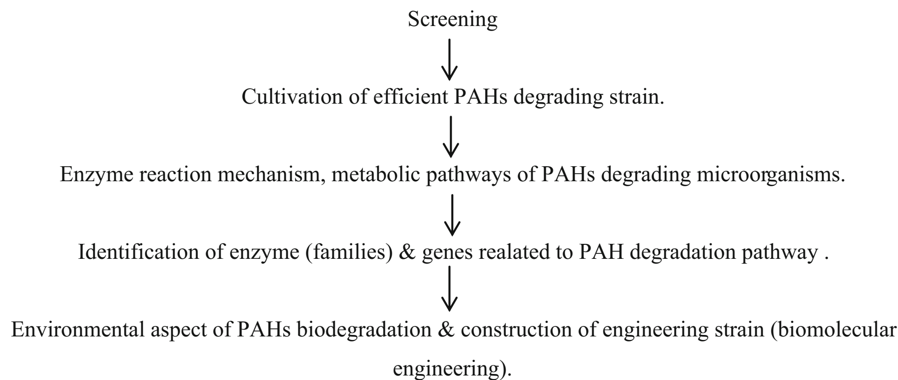
Reduction of PAHs from the surface of minerals and organic matter can be achieved by the use of surface-active agents (also known as surfactants or detergents). The common chemical surfactant such as triton x-100, tween 80 and sodium dodecyl sulfate are petroleum derived products. The salient mechanism which are involved in the surfactant—amended remediation are: lowering of interfacial tension, surfactant solubilization of hydrophobic organic compounds and, the phase transfer of organic compounds from soil sorbed to pseudo aqueous phase (Laha et al. 2009).

5.7 Salinity

Few reports have been published documenting the effect of salt on hydrocarbon biodegradation in soil. High salt concentrations can inhibit the activity of microbes that are not adapted to salt. Possible reasons for this effect include direct inhibition of metabolic activity because of unfavorable high osmotic potential of the microbe's environment (Amatya et al. 2002), and altered solubility or sorption of toxic or essential ions. The inhibitory salt effects observed included longer lag times and decreased rates and extents of mineralization. Børresen and Rike (2007) found that low levels of NaCl (0.3 % w/w NaCl) slightly stimulated rates of hexadecane mineralization in an Arctic soil. This stimulation may be explained if the salt provides a more ironically balanced medium for the microbes, or a medium which disperses clays and thus provides a larger surface area for attachment of cells, or for access to trace nutrients.

6 Molecular techniques for characterization of PAH's degrading microorganisms

A variety of molecular methods based on direct isolation and analysis of nucleic acids, proteins, and lipids from environmental sample and revealed structural and functional information about microbial community. PAHs microbial degradation were carried out by the following pathway (Source-Zhang et al. 2002).



6.1 Polymerase chain reaction (PCR) amplification technique

With the rapid development of molecular-biology, modern taxonomy prefer sequencing technologies of molecular markers such as 16S rRNA (prokaryots) or 18S rRNA (eukaryots). These technologies allow the identification of colonies isolated from microbial consortia and the establishment of phylogenetic relationships between them (Molina et al. 2009). In addition to taxonomy, PCR combined with other approaches could also be used to estimate in situ bacterial community how pollution affects structure of bacterial community and composition of sediments (Ding et al. 2013). Function and application of different PCR techniques is presented in Table 7. The recent development of real-time PCR devices has made quantitative PCR much easier. Besides single-cell level detection, the quantitative PCR approach utilizing bulk DNA from natural bacterial communities may be an effective approach to monitor target bacteria. Distinguishing closely related strains is difficult because of high conservation of 16s rRNA in prokaryotes, 16s rRNA PCR amplification fragment could not be relied on completely sometimes (Tomotada and NaSu 2001).

6.2 Fingerprinting techniques based on 16s rRNA

Microbial fingerprinting methods are a category of techniques that differentiate microorganisms or groups of microorganisms based on unique characteristics of a universal component or section of a biomolecule (e.g. phospholipids, DNA, or RNA; ITRC EMD team 2011). Fingerprinting techniques based on 16S rRNA such as DGGE, SSCP, T-RFLP and RISA could analysis the diversity and dynamics of the whole community at molecular level. Fingerprinting methods are used to provide an overall view of the microbial community, indications of microbial diversity, and insight into the types of metabolic processes occurring in the microorganisms present in the sample. Microbial fingerprinting methods can identify when adverse conditions (e.g. low pH), either natural or following a remedy (e.g. chemical oxidation). Microbial fingerprinting methods can be used to determine whether the overall microbial community has recovered or responded to remedial actions. Several microbial fingerprinting techniques can be used to identify the predominant microorganisms present in the sample and to describe the microbial community. Information about complex dynamic in microbial communities can undergo by PAHs changes and seasonal fluctuation fingerprinting technique based on 16s rRNA-DGGE, SSCP, T-RFLP and RISA can

Table 7 Function and application of various PCR techniques

Type of PCR	Function and application	References
Reverse transcription polymerase chain reaction (RT-PCR)	This technique is commonly used for qualitative analysis of RNA expression levels through creation of complementary DNA (cDNA) transcripts from RNA	Freeman et al. (1999)
	RT-PCR is used to clone expressed genes by reverse transcribing the RNA of interest into its DNA complement through the use of reverse transcriptase, useful in gene transfer and genomic study	
Quantitative PCR or real-time PCR (q-PCR)	Measure the abundance and expression of taxonomic and functional gene markers (Bustin et al. 2005; Smith and Osborn 2009), accumulation of amplicons in real time during each cycle of the PCR. Quantitative measure the amplification of DNA using fluorescent probes	Joyce (2002), Kang et al. (2010), Varkonyi-Gasic and Hellens (2010)
	For qualitative study of gene expression, RT-PCR can be utilized for quantification of RNA, termed as RT-PCR (qRT-PCR)	

analyze the diversity and dynamics of the whole community at molecular level. Function and application are discussed in Table 8.

6.3 Fluorescence in situ hybridization

Fluorescence in situ hybridization (FISH) enables in situ phylogenetic identification and enumeration of individual microbial cells by whole cell hybridization with oligonucleotide probes (Amann et al. 1995). The FISH probes are generally 18–30 nucleotides long and contain a fluorescent dye that allows detection of probe bound to cellular rRNA by epifluorescence microscopy. FISH can be combined with flow cytometry for a high resolution automated analysis of mixed microbial populations. The FISH method was used to follow the dynamics of bacterial populations in agricultural soils treated with s-triazine herbicides (Caracciolo et al. 2010).

FISH can detect microorganisms across all phylogenetic levels, whereas immunofluorescence techniques are limited to the species and sub-species levels. FISH is more sensitive than immunofluorescence because non-specific binding to soil particles does not typically occur (Amann et al. 1995). FISH probes can be generated without prior isolation of the microorganism, whereas pure cultures are needed in immunofluorescence studies for generating specific antibodies (Hahn et al. 1992; Hill et al. 2000). FISH is a powerful tool that can be used not only for studying individuals within a population, but also has potential uses for studying population dynamics, tracking microorganisms released into the environment (e.g. for biological control or bioremediation), epidemiology, and microbial ecology of economically important plant pathogens in agricultural soils (Hahn et al. 1992; Kirchhof et al. 1997; Wullings et al. 1998; Hill et al. 2000).

6.4 DGGE

Is commonly used in PAHs biodegradation this technique is based on the electrophoretic separation of PCR generated double stranded DNA acrylamide gel. The use of denaturing gradient and temperature gradient gel electrophoresis (DGGE/TGGE) for separating individual amplicons has been described (Muyzer et al. 1993; Ferris and Ward 1997; Heuer et al. 1997; Muyzer and Smalla 1998). This technique allows to separate mixtures of PCR products that are of the same length but differ in sequence. The separation power of this technique rests with the melting behavior of the double stranded DNA molecule.16s rRNA and DGGE profiling use to assess the effects of PAHs (before and after PAHs exposure) on the bacterial community.

Microbial diversity is underestimated because it is difficult to extract and amplify all microorganism-genome and DNA. Sometime microbial diversity is overestimated when a single strain might presence multiple bands in DGGE gel. 16s rRNA presents multiple copies of chromosome.

Table 8 Function and application of fingerprinting techniques

Various techniques	Function and application	References
Denaturing- or temperature-gradient gel electrophoresis (DGGE)	Commonly used method in PAH degradation, A sequence-dependent partial separation of the double strands occurs results in a DNA band pattern representative of the sampled microbial community	Zhang et al. (2002)
Single-strand conformation polymorphism (SSCP)	The environmental PCR products are denatured followed by electrophoretic separation of single-stranded DNA fragments on a non-denaturing polyacrylamide gel separation is based on subtle differences in sequences (often a single base pair), which results in a different folded secondary structure leading to a measurable difference in mobility in the gel	Schwieger and Tebbe (1998)
Terminal Restriction Fragment Length Polymorphism (T-RFLP)	Simplify the banding pattern and allowing analysis of complex microbial communities	Thies (2007)
Ribosomal intergenic spacer analysis (RISA)	RISA provides a community-specific profile, with each band corresponding to at least one organism in the original community	Fisher and Triplett (1999)

6.5 DNA hybridization technique (DNA microarray, DNA probe technique)

DNA microarrays have been used primarily to provide a high-throughput and comprehensive view of microbial communities in environmental samples. The PCR products amplified from total environmental DNA is directly hybridized to known molecular probes, which are attached on the microarrays (Gentry et al. 2006). After the fluorescently labeled PCR amplicons are hybridized to the probes, positive signals are scored by the use of confocal laser scanning microscopy. DNA microarrays has the advantage that the relative amount of transcripts from the whole genome may be easily determined compared to proteomics. Membrane-bound proteins are problematic and roughly 2/3 of *E. coli* proteins have not been identified by non-gel proteomic techniques (Han and Lee 2006; Wood 2008). However, transcriptome profiling often assumes that changes in transcription may be used to predict changes in protein formation that may not always be correct but is often true for procaryotes since regulation occurs primarily at the level of transcription. DNA microarrays used in microbial ecology could be classified into different types of microarrays for environmental studies (DeSantis et al. 2007) Detailed applications is presented in Table 9.

6.6 DNA shuffling

Directed evolution or DNA shuffling (Crameri and Stemmer 1995; Crameri et al. 1996; Stemmer 1994a, b; Wood 2008) is a powerful mutagenesis technique that mimics the natural molecular evolution of genes. It can introduce multiple mutations into a gene in order to create new enzymatic activity. It is still difficult to rationally predict the amino acid changes that occur during DNA shuffling and that are necessary to create the new activity. DNA shuffling has been used successfully to create a biocatalyst with higher degradation rates for polyaromatic hydrocarbons (naphthalene, phenanthrene, fluorene, and anthracene).

6.7 Applications of new sequencing technologies: meta-approaches

Particularly meta-transcriptomics, and meta-proteomics are new approaches which in the coming years will be more and more routinely used in environmental studies. These refer to the collection and analysis of transcription (mRNA) and protein profile information from microbial communities. Metagenomic libraries are a powerful molecular technique which has been flourished for the identification of the desired catabolic genes. Basically, metagenomic is a culture dependent genomic analysis; it is either function driven approach or sequence driven approach, of total microbial communities, which provides access to retrieve unknown sequences (Schloss and Handelsman 2003). Metagenomics provides a view not only of the community structure (species phylogeny, richness, and distribution) but also of the functional (metabolic) potential of a community because virtually about all genes are captured and sequenced. In principle, metagenomic techniques are based on the concept

Table 9 Type of DNA microarray technique

16S rRNA gene microarrays (PhyloChip)	Applied for rapid profiling of environmental microbial communities during bioterrorism surveillance, bioremediation, climate change, and source tracking of pathogen contamination	*Limitation* microarray hybridization issues related to specificity, sensitivity and quantitation
Functional gene arrays (FGA)	Detect specific metabolic groups of bacteria, community structure, in situ community metabolic potential, biological functions	
Phylogenetic oligonucleotide arrays (POA)	POA used primarily for phylogenetic analysis of microbial communities, phylogenetic relationships between different organisms, microbial community structure in natural environments, developing microarray based detection approaches	*Limitation* requires availability of individual pure isolates, although CGA-based hybridization itself does not require culturing (Voordouw 1998; Zhou 2003)
Community genome array	Detection and identification of microorganisms in environmental samples, mixed microbial community	

that the entire genetic composition of environmental microbial communities could be sequenced and analyzed in the same way as sequencing a whole genome of a pure bacterial culture.

Metaproteomics commonly known as environmental proteomics, deals with the large-scale study of proteins expressed by environmental microbial communities at a given point in time (Wilmes and Bond 2006; Keller and Hettich 2009). Compared to other cell molecules such as lipids and nucleic acids, protein biomarkers are more reliable and provide a clearer picture of metabolic functions than functional genes or even the corresponding mRNA transcripts of microbial communities (Wilmes and Bond 2006). Proteins involved in the degradation of phenanthrene regulated proteins of strain proteome analysis of Sphe3 cells grown on phenanthrene or glucose by two-dimensional gel electrophoresis (2DE) in combination with mass spectrometry (MALDITOF MS and MS/MS). Several PAH-degrading proteins were identified including 1-hydroxy-2-naphthoate dioxygenase and protocatechuate dioxygenase (Koukkou and Drainas 2008). Proteomic profiling of microbial communities provides critical information on protein abundances and protein–protein interactions, which could not be achieved by DNA/RNA molecular techniques such as metatranscriptomics and metagenomics (Keller and Hettich 2009). Once the proteins are identified, they could be linked to corresponding metagenomic sequences to link metabolic functions to individual microbial species. The limitation of metaproteomic and metagenomic has been overcome by combining these two approaches together under the name of "proteogenomics". In community proteogenomics, total DNA and proteins are extracted from the same sample, which allows linking of biological functions to phylogenetic identity with greater confidence. The metagenomic part of the proteogenomic approach plays a very significant role and increases the identification of protein sequences by metagenomic analysis of the same sample from which the proteins were extracted (Rastogi and Sani 2011). The proteogenomics approach was applied to decipher phyllosphere bacterial communities in a study by Delmotte et al. (2009). Metatranscriptomics (or environmental transcriptomics) allows monitoring of microbial gene expression profiles in natural environments by studying global transcription of genes by random sequencing of mRNA transcripts pooled from microbial communities at a particular time and place (Moran 2009). Metatranscriptomics is particularly suitable for measuring changes in gene expression and their regulation with respect to changing environmental conditions. A method for selectively enriching mRNA by subtractive hybridization of rRNA has been developed and evaluated for the gene transcript analysis of marine and freshwater bacterioplankton communities, which revealed the presence of many transcripts that were linked to biogeochemical processes such as sulfur oxidation (*soxA*), assimilation of C1 compounds (*fdh1B*), and acquisition of nitrogen via polyamine degradation (*aphA*) (Poretsky et al. 2005).

7 Conclusion

Removal of persistent organic pollutants from the environment is a real world problem. Various research

studies have been proven that bioremediation is a promising tool for degradation of PAHs and many more compounds. But this field needs better understanding of the mechanism of biodegradation pathways has a high ecological significance that depends on the indigenous microorganisms to transform or mineralize the organic contaminants. There is a urgent need to address this issue for future research on expanding our knowledge on the practical application of cometabolic processes, bioaugmentation and bacterial–fungal co-cultures. As more specific gene probes are developed, improved DNA extraction techniques could provide a much more in depth image of microbial metabolic function, diversity and interdependence on each other. A combination of several techniques should be applied to investigate the diversity, metabolic function, and ecology of microorganisms. Therefore based on the present review, it may be concluded that along with cleaning strategy of bioremediation, enzyme play vital role in enhancing the microbial degradation and at genomic level molecular tools provide specific gene location in quantitative manner which deals with microbial diversity and function analysis.

7.1 Future challenges

Studies about PAHs biodegradation by microbes is a most focused branch in environmental research field and research emphasis has been changed from finding PAHs degrading microorganisms into metabolic pathways of microbes, genetic regulation and construction of high efficiency engineering microorganisms. The efficiency of PAHs degradation can be significantly improved by addressing key issues as tolerance to various compounds of this family, constitutive expression of the catabolic genes and substrate-specificity; kinetics and the stability of the encoded enzyme. Apart from quantitative assessment qualitative analysis of microbial communities is the greatest challenge due to significant biases associated with nucleic acid isolation. It requires more advanced DNA/RNA extraction techniques for environmental samples such as soil and water. All of the molecular approaches available for analysis of microbial community structure and functions have advantages and limitations associated with them. Biomolecular engineering can be successfully used to improve the capabilities of the enzymes or microorganisms in bioremediation systems. However, there are several limitations i.e. creation of enzymes with novel functions represents an overwhelming challenge in biomolecular engineering. Research is usually focused on altering enzymes that can perform a reaction similar to the desired one. Thus, it might be difficult to apply biomolecular engineering to the bioremediation of xenobiotic compounds, which are not known to be biodegradable. An effort can be made with rational design in the future when our knowledge of the protein structure–function, folding, mechanism and dynamics is significantly improved. However, the utility of constructed organisms in dealing with problems related to environmental pollution in yet to be tested.

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