Chapter 6
Biodegradation of Polycyclic Aromatic Hydrocarbons (PAHs): A Sustainable Approach

Shaili Srivastava and Madan Kumar

Abstract Polycyclic aromatic hydrocarbons (PAHs) are aromatic hydrocarbons having two or more fused benzene rings. PAH are found in environment from natural as well as anthropogenic sources. They are widely distributed environmental contaminants that have detrimental biological effects, including toxicity, mutagenicity, and carcinogenicity. PAHs are thermodynamically more stable and resistant to microbial degradation due to their hydrophobic nature and their stabilization due to presence of multiple benzene rings and low aqueous solubility. Despite these properties, a variety of bacterial, fungal and algal species are reported for biodegradation. Most of studies involved in PAH microbial degradation is based on enzymes involved in PAH metabolism and their mineralization. Several bacteria have been found to degrade PAH such as *Sphingomonas* sp., *Psedomonas* sp., *Alcaligens eutrophus, Burkhelderia* sp. *Mycobacterium, Rhodococcus, Nocardioides, Mycobacterium, Rhodococcus, Nocardioides* and *Novosphingobium,* etc. There are several biochemical pathways and gene reported which are responsible for bacterial degradation of PAHs. Many fungi metabolize polycyclic aromatic hydrocarbons with enzymes that include lignin peroxidase, manganese peroxidase, laccase, cytochrome P450, and epoxide hydrolase. The products include *trans*-dihydrodiols, phenols, quinones, dihydrodiol epoxides, and tetraols, which may be conjugated to form glucuronides, glucosides, xylosides, and sulfates. The fungal and bacterial metabolites generally are less toxic than the parent hydrocarbons. Cultures of fungi that degrade polycyclic aromatic hydrocarbons may be useful for bioremediation of contaminated soils, sediments, and waters. Microalgae and eukaryotic algae sp. have been also reported for their bioaccumulation, biotransformation and degradation capability of PAH. While mechanism of biodegradation pathways from algae are not very specific and vary from species to species. In case of algal biodegradation of PAH it works more precisely in combination with bacterial co-culture.

S. Srivastava (✉)
Amity School of Earth and Environmental Sciences, Amity University, Gurugram, Haryana, India

M. Kumar
School of Environmental Sciences, Jawaharlal Nehru University, New Delhi, India

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6.1 Introduction

Aromatic hydrocarbons are most abundant and diverse organic pollutants present in the environment. Aromatic compounds are defined as the organic molecules with at least one benzene ring. The major sources of aromatic compounds are living organisms (aromatic amino acids), plants (secondary metabolites), lignin and fossil reserves (Fuchs et al. 2011). The aromatic compounds exist as complex mixtures in the environment. Aromatic compounds can be grouped into three major classes: polycyclic aromatic hydrocarbons (PAHs), heterocyclic compounds and substituted aromatics (Seo et al. 2009). These compounds are of great environmental concern and their removal from the polluted system is essential due to their recalcitrance, bioaccumulation potential and toxicity to ecosystems including humans. Their removal from the contaminated system has led to development of various physical, chemical, thermal and biological processes. Among these, biological processes are the most common, cost effective and sustainable approach for degradation (Haritash and Kaushik 2009; Vila et al. 2015; Kuppusamy et al. 2017). In this chapter, we have focused on sources, properties, and biodegradtion of PAHs.

6.2 Sources of PAHs

PAHs are ubiquitous recalcitrant environmental pollutants with known mutagenic, teratogenic and carcinogenic properties. PAHs comprise of fused benzene rings having only carbon and hydrogen in the structure. On the basis of benzene rings present in the structure: PAHs can be classified as low molecular weight (LMW) and high molecular weight (HMW). The LMW consists of 2–3 rings (naphthalene, phenanthrene anthracene fluorine) and HMW contains 4–7 rings such as pyrene, chrysene, coronenes (Kuppusamy et al. 2017). These benzene rings are arranged in linear, angular and cluster form in the structure. PAHs are mainly produced as complex mixture and there are more than 100 types of PAHs present in the environment. The United States Environmental Protection Agency (US EPA) report 2008 labeled 28 compounds as hazardous (Gan et al. 2009). The 16 PAH are categorized as pollutants of high concern by US EPA. The World Health Organization also documented 8 PAHs (benzo[a]anthracene, chrysene, benzo[b]fluoranthene, benzo[k] fluoranthene, benzo[a]pyrene, indeno[1,2,3-cd], pyrene, Dibenz(a,h)Anthracene (DBahA) and benzo[ghi]perylene) has potential expected to be carcinogenic pollutants (Bansal and Kim 2015).

PAHs are group of organic compounds that are produced naturally by volcanic eruptions, forest fires, oil seeps, exudates from trees and through anthropogenic sources such as incomplete burning of crude oil, coal, gas, wood, garbage, cooking at high temperatures, exhaust from vehicles, discharge from industry and treatment plants (DHHS 1995; Gehle 2009). On the basis of their source of origin, PAHs can be classified as petrogenic, pyrogenic and biogenic PAHs. The pyrogenic PAHs are produced from the incomplete combustion of organic compounds at high temperature or prolonged heating at low temperature either at low or absence of oxygen. Incomplete combustion taking place either by natural or anthropogenic means are the largest contributor of PAHs to the environment. The example of pyrogenic processes is thermal cracking of petroleum, distillation of coal for coke and coal tar production, forest fire, vehicular emission, etc. Petrogenic PAHs are generated from crude oil, crude oil products such as petroleum product and oil spills. The biogenic sources include plants and microbes and their degradation products, industries, incinerators, wastewater treatment plants, emission from burning of motor fuels, grilled and smoked food, cigarette, tobacco, wood burning (Haritash and Kaushik 2009; Abdel-Shafy and Mansour 2016).

6.3 Properties, Fate and Exposure of PAHs

Physical and chemical property of PAH depends on the number of rings or molecular weight. These compounds are solids having relatively high molecular weight, low volatility at room temperature, less or insoluble in water, soluble in organic solvents and prone to photo-oxidative degradation (DHHS 1995). Their aqueous solubility, volatility and reactivity decreases with increase in molecular weight (Seo et al. 2009). In pure form these chemicals are colorless, white or pale yellow-green with faint or pleasant odour. These are used in synthesis of medicine, dyes, pesticides and plastics (DHHS 1995). The physical and chemical properties of 16 most priority PAHs are presented in Table 6.1 as listed by US EPA. These are present ubiquitously in interstellar space and the environment i.e., air, water, and soil or sediment. The soil is the reservoir of PAHs with almost 90% of total PAH burden in the environment (Kuppusamy et al. 2017). The global annual emission of 16 PAHs was 504 Gg in 2007 and the major contributors were biomass (residential/ commercial) burning, open field biomass burning, deforestation, wildfire and petroleum combustion by motor vehicles. The HMW carcinogenic PAH emission was 6.19% of total PAH emission. The carcinogenic PAH emission was higher in developing countries (6.22%) compared to developed countries (5.735) due to difference in advancement in technology and energy structure (Shen et al. 2013). The exposure of PAHs takes place through air, water, soil and food sources and the route of exposure is inhalation, ingestion and dermal contact in both occupational and non-occupational conditions (Abdel-Shafy and Mansour 2016).

Table 6.1 Physical and chemical property of 16 high priority pollutants documented by US EPA

PAHs Name	Formula	Cas registry	B. P. (°C)	M.P. (°C)	Solubility (mg/L)	V.P. (mmHg at 25 °C)	Toxic equivalent factor (TEF)	Estimated half-lives (days)
Naphthalene	C10H8	91-20-3	218	80.2	31	8.52×10^{-2}	n.d.	5.66
Acenaphthene	C12H10	83-32-9	279	93.4	3.93	2.5×10^{-3}	0.001	18.77
Acenaphthylene	C12H8	208-96-8	280	91.8	1.93	6.68×10^{-3}	0.001	30.7
Anthracene	C14H10	120-12-7	342	216.4	0.076	6.53×10^{-6}	0.01	123
Phenanthrene	C14H10	85-01-8	340	100.5	1.2	1.2×10^{-4}	0.001	14.97
Fluorene	C13H10	86-73-7	295	216–7	1.68–1.98	6×10^{-4}	0.001	15.14
Fluoranthene	C16H10	206-44-0	375	108.8	0.20–0.26	9.22×10^{-6}	0.001	191.4
Benzo[a]anthracene	C18H12	56-55-3	438	158	0.01	4.11×10^{-3}	0.1	343.8
Chrysene	C18H12	218-01-9	448	254	1.5×10^{-3}	6.23×10^{-9}	0.01	343.8
Pyrene	C16H10	129-00-0	150.4	393	0.132	4.5×10^{-6}	0.001	283.41
Benzo[a]pyrene	C20H12	50-32-8	495	179	3.8×10^{-3}	5.49×10^{-9}	1	421.6
Benzo[b]fluoranthene	C20H12	205-99-2	481	168.3	0.0012	5×10^{-7}	n.d.	284.7
Benzo[k]fluoranthene	C20H12	207-08-9	480	215.7	7.6×10^{-4}	9.7×10^{-10}	0.1	284.7
Dibenzo[a,h] anthracene	C22H14	53-70-3	524	262	5×10^{-4}	9.55×10^{-10}	n.d.	511.4
Benzo[g,h,i]perylene	C22H12	191-24-2	500	277	2.6×10^{-5}	1×10^{-10}	n.d.	517.1
Indenol[1,2,3-cd] pyrene	C22H12	193-39-5	536	161–3	0.062	1.25×10^{-3}	n.d.	349.2

6.4 Biodegradation of PAHs by Bacteria

The remediation of PAHs in the environment takes place by various established physical, chemical, thermal and biological processes and their integration such as chemical oxidation, incineration, solvent extraction, thermal conduction, phytoremediation, bioaugmentation, biostimulation, bioreactors and composting (Haritash and Kaushik 2009; Kuppusamy et al. 2017). Compared to various physic-chemical processes, biological processes is cost effective, sustainable and green approach to environment. Physico-chemical processes only cause transformation from one compound to another less toxic form but in case of biological processes, complete mineralization of contaminants in the form of CO_2 and water can be observed. In this chapter, we are discussing biological processes for pollutants remediation. The contaminants are utilized by microorganisms as source of energy and carbon in the environment. The bioremediation of PAHs pollutants in the environment can be done by *ex situ* and *in situ* treatment methods. The bioremediation of pollutants by microorganisms is very promising technology and this research field is very active as new contaminants and microbes are being identified and reported on regular basis. The versatility and adaptability of microbes to utilize diverse range of contaminants present in diverse environment for energy and carbon source is the key to bioremediation. The biological degradation of contaminants can be aerobic and anaerobic process.

6.4.1 Aerobic Degradation of PAH by Bacteria

The microbial degradation of recalcitrant environment pollutants such as PAHs takes place favorably under aerobic conditions and are extensively documented. In aerobic degradation process, oxygen molecule is the final electron acceptor and also acts as co-substrate for hydroxylation and oxygenolytic cleavage of aromatic rings (Ghosal et al. 2016). There are various microorganisms identified as PAH degraders and these microbes belongs to *Pseudomonas, Alcaligenes*, *Sphingomonas*, *Mycobacterium*, *Streptomyces*, *Burkholderia*, *Polaromonas*, *Ralstonia*, *Rhodococcus*, *Stenotrophomonas Flavobacterium, Bacillus, Rhodotorula, Acenitobacte*, *Klebsiella* and *Arthrobacter* (Haritash and Kaushik 2009; Seo et al. 2009). The degradation of four or more ring HMW PAHs containing four or more ring was commonly observed in genus *Sphingomonads, Actinobacteria* and *Mycobacterium* as sole energy and carbon source (Vila et al. 2015).

6.4.1.1 Pathways and Enzymes Involved in Aerobic Degradation of PAHs

Microorganisms can degrade PAHs through both aerobic and anaerobic pathways but the aerobic pathway is studied extensively. In the aerobic degradation pathway, the PAHs is activated by incorporating molecular oxygen directly with the help of

mono or di oxygenases. The multicomponent dioxygenase usually consists of reductase, ferrodoxin and terminal iron sulfur containing terminal oxygenase (Haritash and Kaushik 2009; Ghosal et al. 2016). These ring hydroxylating oxygenases (RHD) belongs to rieske-type non-heme iron oxygenase family. PAH specific RHD can be distinguished phylogenetically in Gram positive and Gram negative bacterial strains. The dioxygenase hydroxylates the aromatic ring of PAH and results in formation of *cis*-dihydrodiol and gets rearomatized to diol intermediate by dehydrogenases. These diol are cleaved by intadiol or extradiol ring cleaving dioxygenase by ortho or meta cleavage pathway and forms common intermediate catechol followed by their conversion in TCA intermediates (Seo et al. 2009; Fuchs et al. 2011). Bacterial monooxygenase or cytochrome P450 activates catabolic process by introduction of one oxygen molecule in the aromatic ring and convert into arene epoxide intermediate and its subsequent conversion into*trans*-dihydrodiols (Moody et al. 2004).

Naphthalene is the simplest PAH and there are several microorganism discovered and reported with detailed mechanism of degradation, metabolic intermediates formed, enzymes involved and regulation of genes. The proposed pathway for degradation of naphthalene has been represented in Fig. 6.1.

The gene for naphthalene degradation can be divided into *nah*-like in *Pseudomonas* and non *nah*-like gene in other genus. In *Pesudomonas putida* strain G7, the catabolic gene is present in three operons encoding enzymes for upper pathway (naphthalene to salicylate), lower pathway (salicylate to TCA by meta-cleavage) and third *nah*R (a LysR trans-acting positive transcriptional regulator). In this operon, a terminal dioxygenase is reported with highly conserved structure having alpha and beta chains. The *nah*Ac gene (naphthalene 1,2 dioxygenase) was used as biomarker for PAH degradation as well as to establish correlation between PAH degradation and its levels present in the environment (Cébron et al. 2008; Lu et al. 2011). *Ralstonia* sp. U2 contains *nag* gene cluster similar in order to that of *nah* from *Pseudomonas with additional nagG* and *nagH* gene for salicylate to gentisate conversion (Zhou et al. 2001). The PAH catabolic gene reported from other bacteria are *phn* gene from *Burkholderia* sp. strain RP007, *phd* genes of *Nocardioides* sp. KP7, *nar* genes from *Rhodococcus* sp. NCIMB12038, *phd* genes from *Comamonas teststeroni* strain GZ39. These genes were detected while degradation of PAH (naphthalene, phenanthrene and anthracene). The involvement of *nid* genes in PAH such as fluoranthene, phenanthrene, pyrene and benzo(a) pyrene degradation has been reported from *Mycobacterium* sp. strain PYR-1 (Moody et al. 2001).

Phenanthrene is a LMW, three ring containing PAH pollutant ubiquitously present in an environment. It is a model compound for the degradation study of carcinogenic compounds (PAH) as its structure contains reactive 'K-' region and '-bay' regions commonly observed in other carcinogenic compounds (Seo et al. 2009; Ghosal et al. 2016*)*. The catabolic pathway and enzymes for phenanthrene degradation has been extensively studied in *Mycobacterium* sp. strain PYR-1 (Fig. 6.2). The degradation of phenanthrene has been widely studied and the catabolizing microbes isolated are *Burkholderia, Arthrobacter, Mycobacterium*, *Pseudomonas*, *Sphingomonas, Acidovorax* and *Pseudomonas* (Tian et al. 2002). The utilization of phenanthrene has

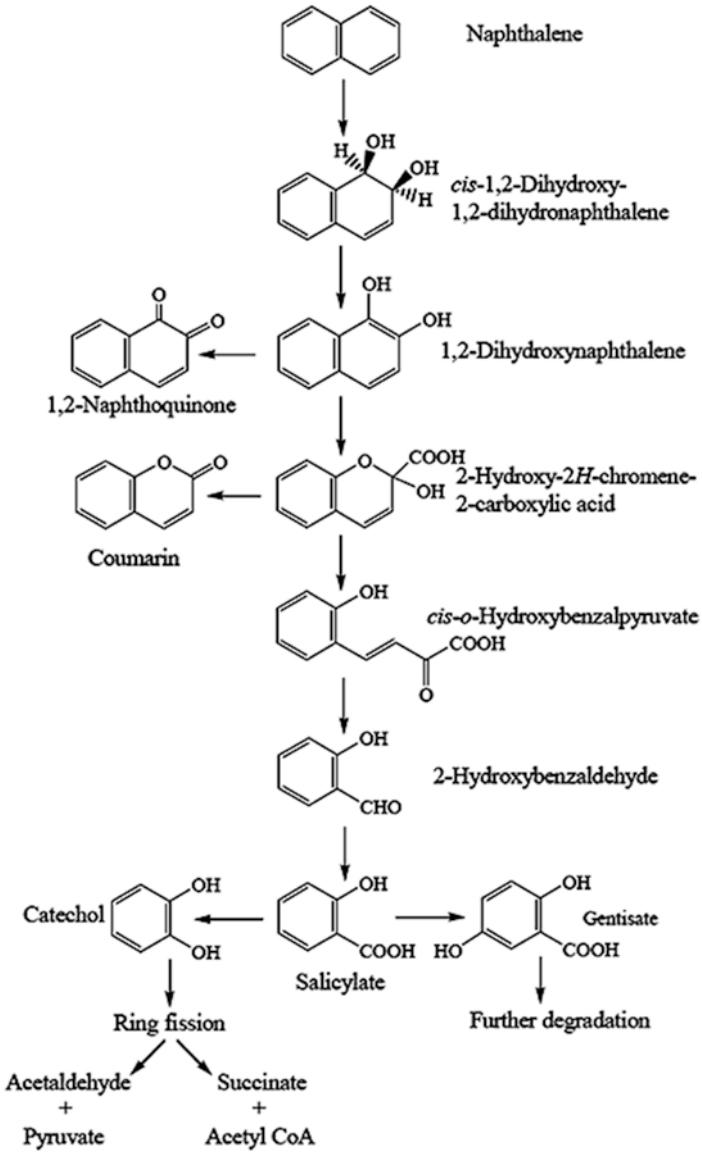

Fig. 6.1 Proposed pathway for aerobic degradation of Naphthalene. (Adapted from Seo et al. 2009)

been studied in detail from *Mycobacterium* sp. strain PYR-1. The catabolic activation of ring occurs at K- region by mono and dioxygenases, followed by ortho and meta ring cleavage (Moody et al. 2001; Haritash and Kaushik 2009; Seo et al. 2009).

Pyrene is the model compound used for the degradation study of HMW PAH. This compound contains four benzene rings in the structure and is highly

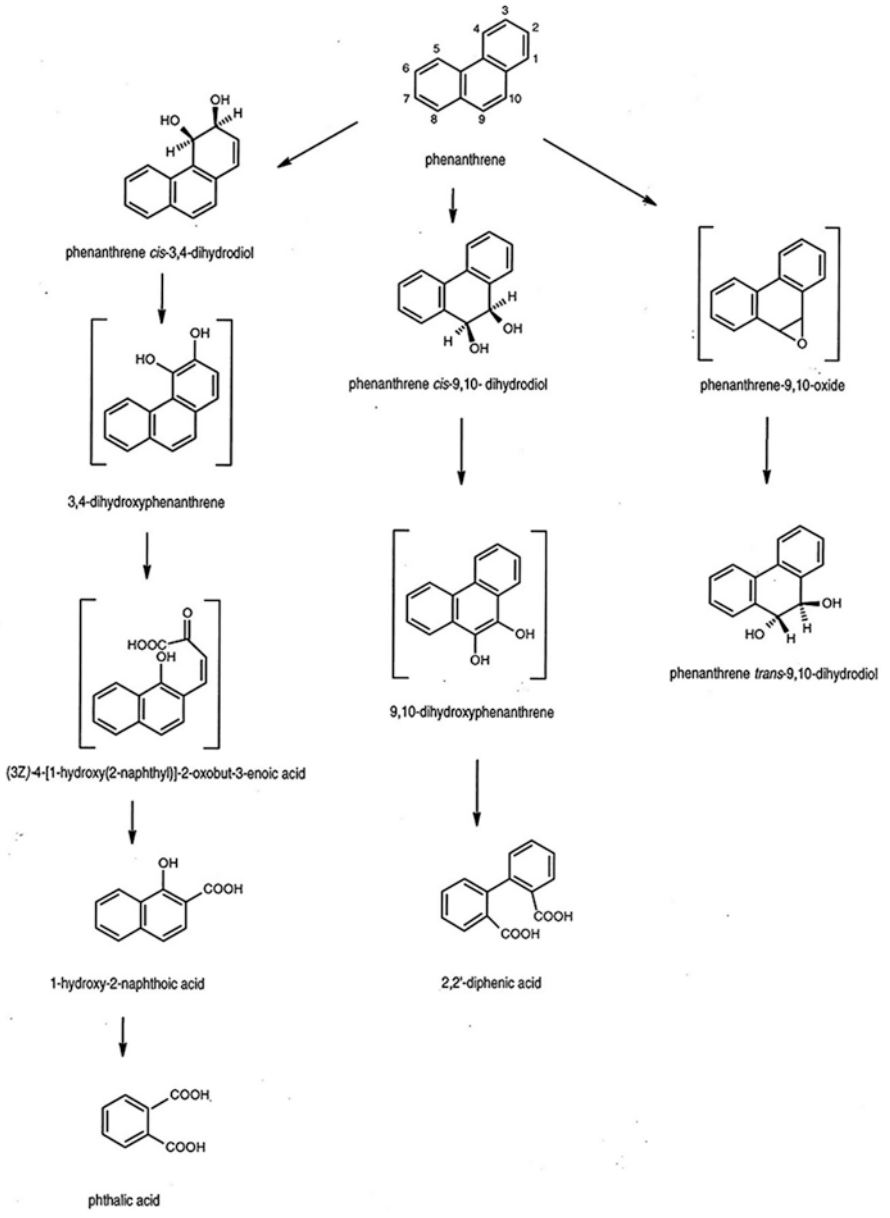

Fig. 6.2 Proposed pathway for aerobic degradation of phenanthrene by *Mycobacterium* sp. strain PYR-1. (Adapted from Moody et al. 2001)

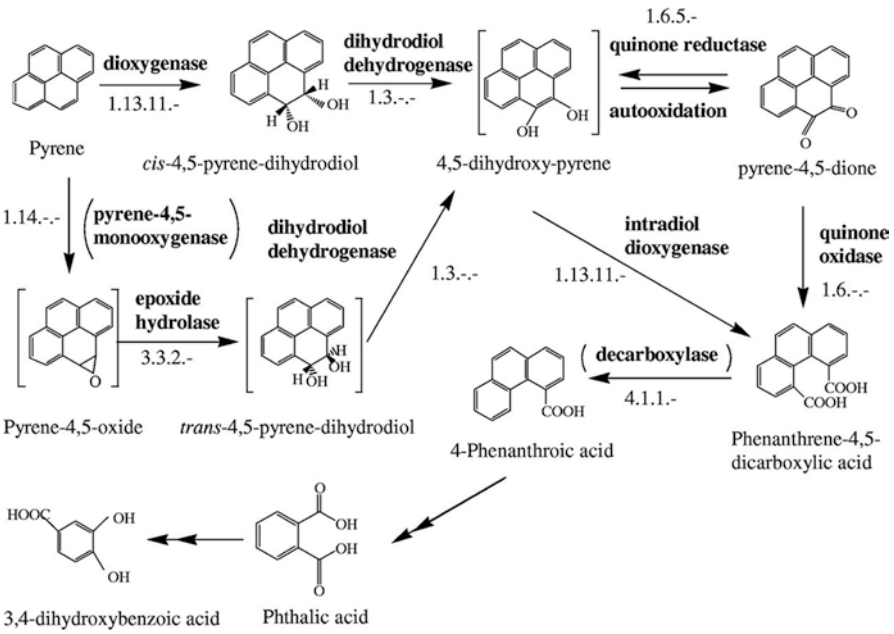

Fig. 6.3 Proposed pathway for aerobic degradation of pyrene by *Mycobacterium* sp. strain KMS. (Adapted from Liang et al. 2006)

hydrophobic in nature. The catabolism of pyrene as sole carbon and energy source has been studied in *Mycobacterium, Pseudomonas*, *Burkholderia, Bacillus, Cycloclastics* and *Sphingomonas* (Seo et al. 2009). The enzymes and pathways involved in pyrene degradation from *Mycobacterium* sp. strain KMS has been comprehensively studied and the degradation pathway was proposed (Fig. 6.3). The enzymes such as aromatic-ring-hydroxylating dioxygenase, dihydrodiol dehydrogenase, oxidoreductase, and epoxide hydrolase was found to be induced in presence of pyrene and the catabolic intermediates have also been detected (Liang et al. 2006; Seo et al. 2009).

Benzo[*a*]pyrene (BAP) is a highly recalcitrant five membered aromatic ring PAH and one of the most potent carcinogen. The genera reported for degraded of BAP are *Mycobacterium* sp., *Stenotrophomonas* and *Sphingomonas.* The metabolism of BAP has been extensively studied in *Mycobacterium vanbaalenii* PYR-1 and the enzymes and pathways involved in degradation has also been elucidated (Fig. 6.4). The catabolic activation of BAP ring (C-4,5, C-9,10, and C-11,12) takes place by stereo- and regioselective dioxygenases and monooxygenases and the degradation intermediates and pathways also proposed (Moody et al. 2004; Seo et al. 2009).

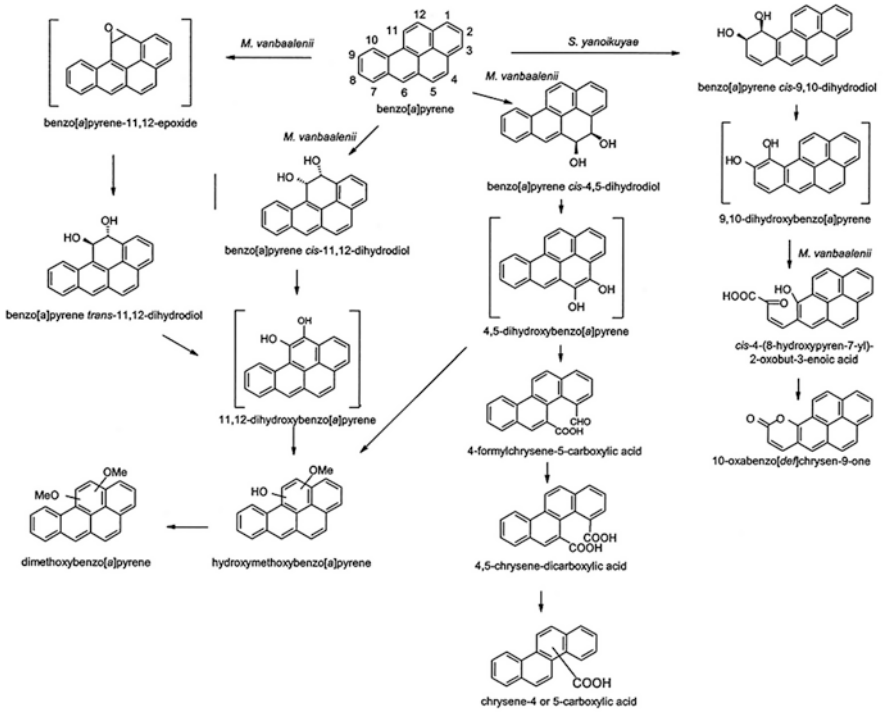

Fig. 6.4 Proposed pathway for the aerobic degradation of benzo[a]pyrene by *Mycobacterium vanbaalenii* PYR-1. (Adapted from Moody et al. 2004)

6.4.2 Anaerobic Degradation of PAHs by Bacteria

Aerobic degradation of PAH is most extensively studied from various environments and the pathway of degradation has also been elucidated. In aerobic process, the degradation takes place in presence of oxygen where oxygen helps in ring opening and acts as terminal electron acceptor (Meckenstock and Mouttaki 2011). The degradation of contaminants in places where there is less or lack of oxygen such as ocean sediment, aquifers, lakes and lower soil layer takes place by anaerobic mechanism. In anaerobic degradation process, nitrogen, sulphur, metal ions (ferric and manganese), CO_2, chlorate, perchlorate, trimethylamine oxide and fumarate can act as terminal electron acceptor (TEA) in place of oxygen (Meckenstock and Mouttaki 2011; Nzila 2018). The TEAs capture electron released during the degradation of organic compounds and helps in synthesis of ATP from ADP (Fig. 6.5). The ananerobic degradation of polyaromatic compounds is an important bioremediation process in the environment. In 1988, first report of anaerobic degradation can be of naphthalene was reported by Mihelcic and Luthy (1988) and later on described with sulphate, ferric ion and manganese as electron acceptor (Mihelcic and Luthy 1988; Meckenstock and Mouttaki 2011).

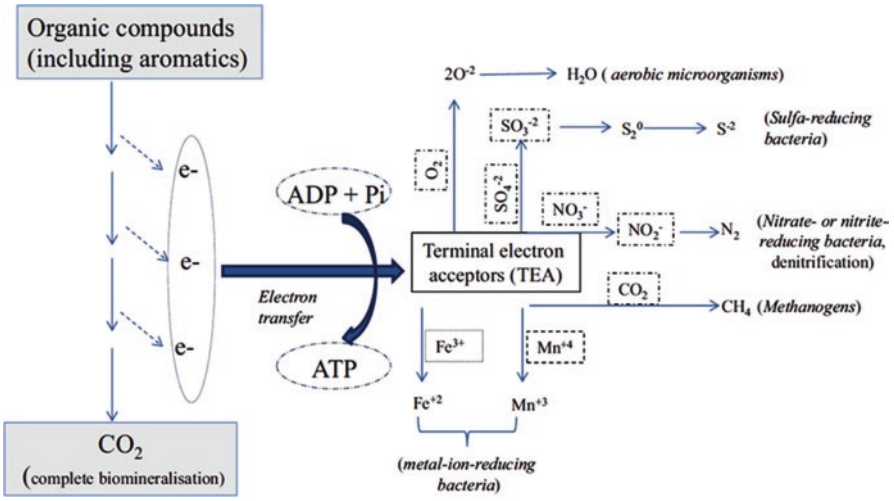

Fig. 6.5 Representation of anaerobic biodegradation of organic pollutants including PAHs. (Adapted from Nzila 2018)

But since then the degradation of aromatic pollutants under anaerobic conditions is limited to LMW PAH and very few studies have reported HMW PAH degradation. Limited biotransformation of naphthalene, phenanthrene, anthracene, pyrene, acenaphthylene and acenaphthene by methanogenic consortia was observed (Christensen et al. 2004; Maillacheruvu and Pathan 2009). Biodegradation of ^{14}C-labelled pyrene was studied under nitrate reducing condition and the emission of $^{14}C\ CO_2$ was observed (Nieman et al. 2001). Gas chromatography (GC) or high-pressure liquid chromatography (HPLC) was used to study the degradation of anthracene using sediment from aquifer under nitrogen reducing conditions (Wang et al. 2012). The PAH degradation by single strain *Microbacterium* sp., *Cellulosimicrobium cellulans* CWS2, *Pseudomonas* sp. and *Pseudomonas* sp. JP1 under nitrate and sulphate reducing conditions were observed (Nzila 2018). The mixture of PAHs and their degradation by consortia from sludge sample of a municipal sewage treatment plant, petrochemical sludge, sediments from a rice field, marine sediments, mangrove sediment, municipal solid waste compost with different TEAs and their combinations were studied (Nzila 2018).

6.4.2.1 Pathways and Enzymes for Anaerobic PAHs Degradation

The degradation of PAHs by anaerobic process is challenging due to stability of C-C and C-H bonds in benzene ring. The metabolic activation of PAH takes place by carboxylation, methylation and fumarate addition in absence of oxygen by anaerobic microorganisms (Zhang and Young 1997; Meckenstock and Mouttaki 2011). ^{13}C-labelled buffer study under sulphate reducing conditions identified major

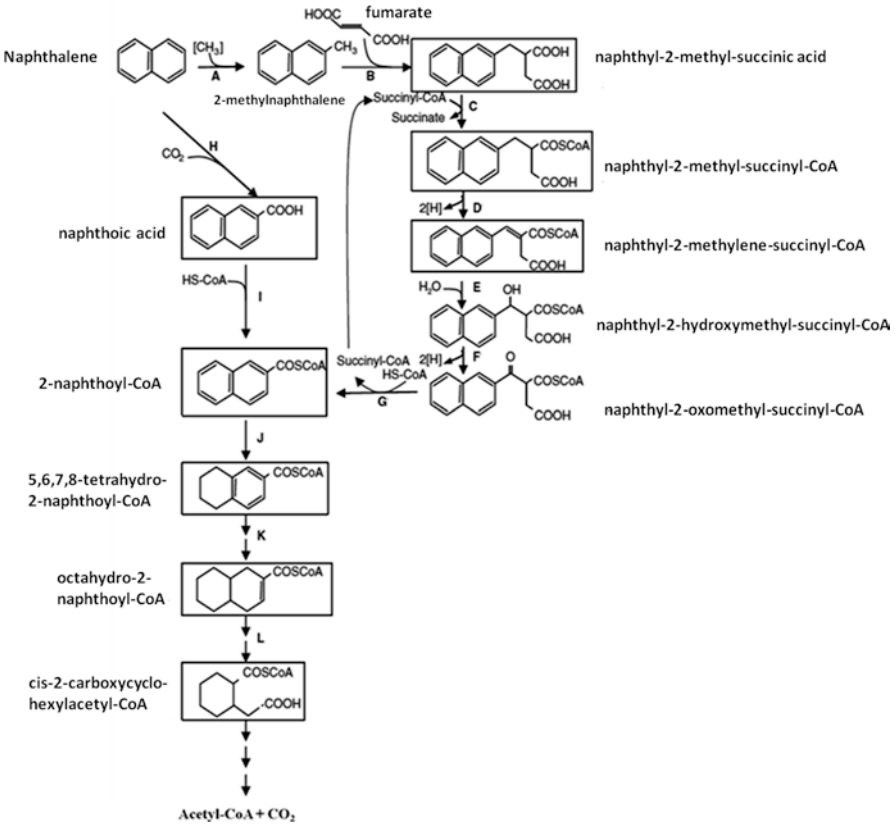

Fig. 6.6 Pathways and enzymes involved in anaerobic degradation of proposed anaerobic naphthalene and 2-methylnaphthalene degradation pathways
Enzymes represented as G, naphthalene carboxylase; H, naphthoyl-CoA ligase; (*A*) naphthalene methyl-transferase; (*B*) napthyl-2-methylsuccinyl synthase; (*C*) napthyl-2-methyl-succinyl-CoA transferase; (*D*) naphthyl-2-methyl-succinyl-CoA dehydrogenase; (*E*) naphthyl-2-methylene succinyl-CoA hydratase; (*F*) naphthyl-2-hydroxymethyl-succinyl-CoA dehydrogenase; (*G*) napthyl-2-oxomethyl-succinyl-CoA thiolase; (*H*) naphthoate carboxylase; (*I*) naphthoyl-CoA ligase; (*J* and *K*) 2-napthtoyl-CoA reductase; (*L*) enoyl-CoA hydratase. Cis-2-carboxycyclo-hexylacetic acid is then further degraded to form acetyl-CoA and CO2. (Adapted from Meckenstock and Mouttaki 2011)

metabolite as 2-napthonic acid and phenanthrene-2-carboxylic acid from naphthalene and phenanthrene respectively with labelled carboxylic acid in the structure (Zhang and Young, 1997).Methylation of napthalene and other hetercyclic compounds by methyltransferases followed by fumarate addition to 2-naphthoyl-CoA production was also proposed pathway for degradation of PAH (Annweiler et al. 2001; Safinowski and Meckenstock 2006). The carboxylases perform initial carboxylation of naphthalene to 2-naphthoic acid followed by 2-naphthoyl CoA, cyclohexane ring formation, ring cleavage and its subsequent multistep conversion to CO_2 as shown in Fig. 6.6 (Meckenstock and Mouttaki 2011).

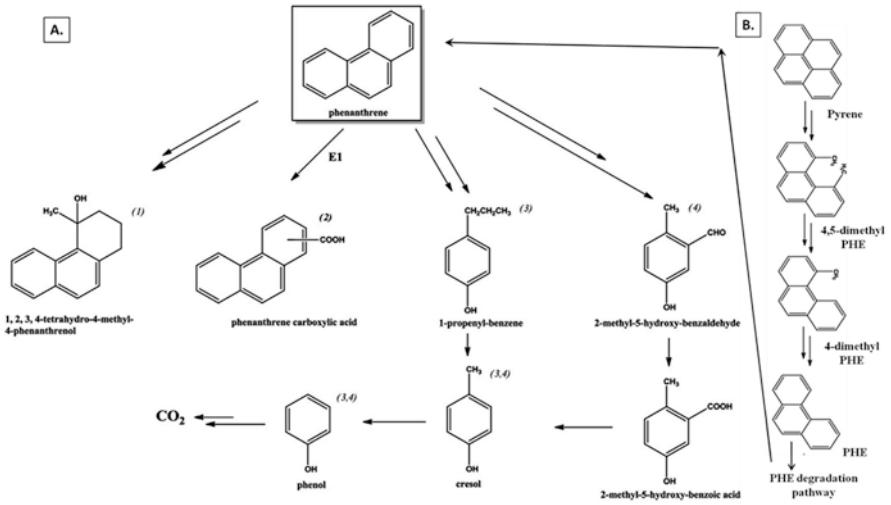

Fig. 6.7 Pathways for degradation of phenanthrene (**a**) and pyrene (**b**) and their metabolites detected during degradation.PHE (Phenanthrene). (Modified from Nzila 2018)

The mechanism of phenanthrene degradation takes place first by hydroxylation, methylation and carboxylation of first ring while other rings remains intact and then same process goes for other rings till all are cleaved. The metabolites detected from phenanthrene and pyrene degradation are 1,2,3,4-tetrahydro-4-methyl-4-phenanthrenol, p-cresol, phenol, 2-methyl-5-hydroxy-benzaldehyde 1-propenyl-benzene, anthraquinone and 1-anthraquinone-carboxylic acid (Tsai et al. 2009; Liang et al. 2014). Phenanthrene and pyrene degradation products are shown in Fig. 6.7.

Anthracene degradation detected anthraquinone and 1-anthraquinone-carboxylic acid as catabolic intermediates during degradation. The degradation of benzopyrene resulted in generation of ethyl chrysene, 1,12-dimethylbenz[a]anthracene, 7,8,9,10-tetrahydrobenzo[a]pyrene pyrene, phenanthrene, four naphthalene derivatives [1-(2-hydroxypropyl)-naphthalene, 1,7-dimethyl-naphthalene, 1-methyl-naphthalene, 2-hydroxy-3-(3-methyl-2-butenyl)-1,4-naphthalenedione] and two benzoic acid derivatives i.e., diethyl phthalate, 2-acetyl-3-methoxybenzoicacid by *Pseudomonas* sp. JP1 and *Microbacterium* sp. CSW (Liang et al. 2014; Qin et al. 2017). The anaerobic degradation of benzopyrene by *Microbacterium* sp. CSW is represented in Fig. 6.8.

The anaerobic process is advantageous compared to aerobic process i.e., removal or degradation of pollutants, nitrate and sulphate removal from site and opportunity for production of biomethane as alternate energy source while degradation. But the major disadvantage of anaerobic process is extremely slow growth rate and very less efficiency. The limitations of anaerobic degradation can be overcome by bio-augmentation, surfactant or biosurfactant addition, pH, introduction of consortia of microorganism, co-metabolism and improving biomethane production.

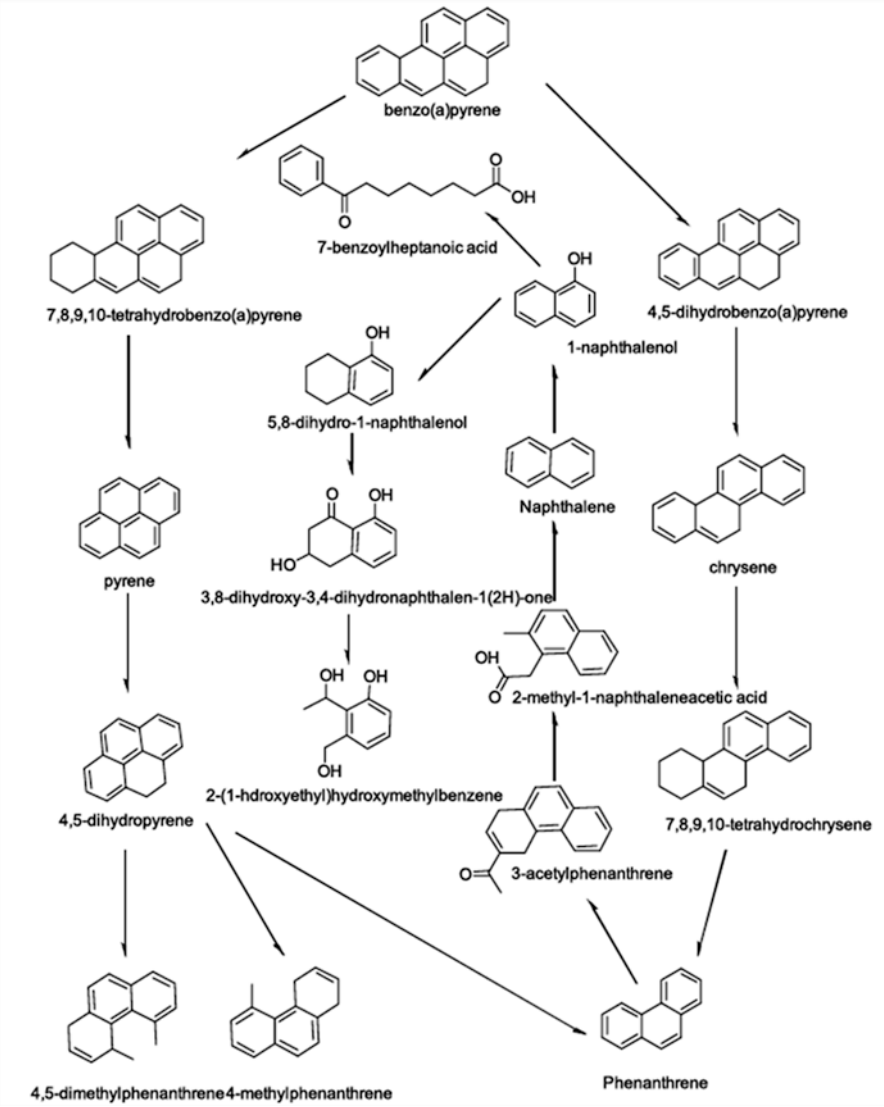

Fig. 6.8 Proposed pathway of anaerobic benzopyrene degradation by M.CSW3 CSW3 under nitrate-reducing conditions. (Qin et al. 2017)

6.5 PAHs Degradation by Complex Microbial Communities

The contaminants emitted and present in the environment are in mixture form and there are several microbes present in the environment to degrade them. Pure culture based methods ignore the interaction going on between the diverse microbial communities in the environment so culture independent approach is applied to study the

microbial community. These approaches identify the microbial communities and their abundance on the polluted site. The microbes in communities are interconnected through metabolic pathways. With the advancement in 'omics' approach such as genomics, proteomics and metabolomics and their application in environment sample will help to understand the functionally relevant communities, their interaction and construction of their metabolic network in the environment.

The techniques commonly used for identification of microbial communities are denaturing gradient gel electrophoresis (DGGE), pyrosequencing, Stable Isotope Probing (SIP), Microarrays, functional gene (RHD) as molecular marker and their combination RHD-SIP, BACTRAPS (in situ protein-SIP and 16S rRNA gene pyrosequencing), metagenomics, mettranscriptomics, metaproteomics in combination with metabolomics (Vila et al. 2015).

Massive marine oil spills are usually followed by intensification in hydrocarbon degradation research. Analyses of sand from a beach affected by the Prestige oil spill had shown high relative abundances of Sphingomonadaceae and Mycobacterium that could be associated to PAH degradation (Vila et al. 2015). The link of community dynamics to depletion of specific fuel components and to single PAH exposure revealed a role in PAH utilization for the gammaproteobacteria *Methylophaga* and *Marinobacter,* and members of Actinobacteria (Vila et al. 2015). The detection of a NidA dioxygenase gene in subcultures with pyrene identified an uncultured Gordonia as a key HMW PAH degrader. A similar community composition was found in a beach affected by the surface oil slick from Deepwater Horizon oil spill. The reduced complexity of microbial consortia enriched from natural communities facilitates the correlation between specific populations and functions. Jones et al. (2014) used substrate enrichment, co-incubation experiments and pyrosequencing to identify the genera *Cupriavidus* (Betaproteobacteria) and Luteimonas (Gammaproteobacteria) as the most likely related to benzo(a)pyrene cometabolism in a PAH-polluted soils. However, the most relevant studies on the identification of soil and groundwater PAH-degrading phylotypes are based on DNA-SIP.

6.6 Biodegradation by Fungi

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous contaminants in the environment with potential mutagenicity and carcinogenicity, which are generated from natural combustion processes as well as from human activities (Cerniglia 1992). Some PAHs are acutely toxic, mutagenic and carcinogenic (Boonchan et al. 2000). The deleterious properties of PAHs have made their remediation a critical need. Bioremediation is one of the promising technologies to reclaim PAH-contaminated sites due to its relatively low cost and limited impact on the environment (Liebeg and Cutright 1999). Diverse fungi capable of utilizing PAHs have been investigated as well. Some filamentous fungi, basidiomycetes, white-rot fungi and deuteromycetes have been shown to remove PAHs more competently than bacteria. PAHs susceptible to fungal biodegradation include naphthalene, phenanthrene, anthracene,

pyrene, benzo[a]pyrene, fluorene, dibenzothiophene, catechol, benzo[α]anthracene, chrysene, benzo[β]fluoranthene and benzo[k]-fluoranthene (Zheng and Obbard 2002). At least two mechanisms are involved in PAH biodegradation: one utilizes the cytochrome P-450 system (Yadav et al. 2006) and the other uses the soluble extracellular enzymes of lignin catabolism, including lignin peroxidase, manganese peroxidase (Steffen et al. 2003) and laccase (Gianfreda et al. 1999).

6.6.1 Fungal Species

In contrast to bacteria, fungi do not utilize PAHs as their sole sources of carbon and energy but transform PAHs co-metabolically to detoxified chemical products. Recent studies have reported several fungal species with the capacity to degrade a series of PAHs, such as naphthalene, phenanthrene, fluoranthene, chrysene, pyrene and benzo[a]pyrene (Kiehlmann et al. 1996; Saraswathy and Hallberg 2002; Mollea et al. 2005; Mineki et al. 2015). Compared to bacteria, there are advantages in using fungi for bioremediation as they possess extracellular enzymes and their mycelia provide deeper penetration and larger surface area for absorption in soil. PAHs biodegradation has been reported by using different species of White rot fungi (WRF) such as *Phanerochaete chrysosporium, Pleurotus ostreatus, Bjerkandera adusta, Irpex lacteus, Trametes versicolor*, etc. (Wang et al. 2008; Mir-Tutusaus et al. 2014). A diverse group of ligninolytic and non-ligninolytic fungi (Marco-urrea et al. 2015) are able to oxidize PAHs (Table 6.2).

Many of bacterial strains are also able to degrade five-benzene-ring PAHs partially, forming oxidized products. In contrast to bacteria, fungi generally do not utilize PAHs as their sole carbon and energy source but transform these compounds cometabolically to detoxified metabolites (Sutherland 1992). The most extensive studies have focused on white rot fungi such as *Phanerochaete chrysosporium, Pleurotus ostreatus and Trametes versicolor*. These fungi are able to degrade some five-benzene-ring PAHs and detoxify PAH-polluted soils and sediments due to the production of extracellular lignin-degrading enzymes. Nonlignolytic fungi, such as *Cunninghamella elegans, Penicillium janthinellum,* and *Syncephalastrum* sp., can transform a variety of PAHs, including pyrene, chrysene, and benzo[a]pyrene, to polar metabolites (Pothuluri et al. 1994). Biodegradation of PAHs by the white rot fungus *Phanerochaete chrysosponum* has clearly been verified in several studies that report mineralization of ^{4}C-labeled PAH (Sanglard et al. 1986).

Extensive biodegradation of PAH, e.g., fluorene and benzo[a]pyrene (B[a]P) was reported from *P. chrysosponum, Trametes versicolor TV1* and *Chrysosponum lignorum* CL1 (Morgan et al. 1991). Field et al. (1992) has been studied on different isolates of fungal strains such as *Bjerkandera adusta* CBS 595.78, *Polyporus, pinsitus* CBS 678.70, *Trametes* sp. strain Naald 11; *Trametes* sp. strain Eik 39; *Trametes*

Table 6.2 Polycyclic aromatic hydrocarbon utilized by different fungal species

S. no.	Compound	Fungal sp./Strain
1	Acenaphtene	*Cunninghamella elegans*
2	Anthracene	*Bjerkandera* sp., *Cunninghamella elegans, Naematoloma frowardii, Phanerochaete chrysosporium, Phanerochaete laevis, Pleurotus ostreatus, Pleurotus sajor-caju*
3	Phenanthrene	*Aspergillus niger, Cunninghamella elegans, Naematoloma frowardii, Phanerochaete chrysosporium, Phanerochaete laevis, Pleurotus ostreatus, Syncephalastrum racemosum, Trametes versicolor*
4	Fluorene	*Cunninghamella elegans, Laetiporus sulphureus, Phanerochaete chrysosporium, Pleurotus ostreatus, Trametes versicolor*
5	Fluoranthene	*Cunninghamella elegans, Naematoloma frowardii, Laetiporus sulphureus, Penicillium* sp., *Pleurotus ostreatus*
6	Pyrene	*Aspergillus niger, Agrocybe aegerita, Candida parapsilopsis, Crinipellis maxima, Crinipellis perniciosa, Crinipellis stipitaria, Crinipellis zonata, Cunninghamella elegans, Fusarium oxysporum, Kuehneromyces mutablis, Marasmiellus ramealis, Marasmius rotula, Mucor* sp., *Naematoloma frowardii, Penicillium janczewskii, Penicillium janthinellum, Phanerochaete chrysosporium, Pleurotus ostreatus, Syncephalastrum racemosum, Trichoderma harzianum*
7	Benzo[a] anthracene	*Candida krusei, Cunninghamella elegans, Phanerochaete chrysosporium Phanerochaete laevis, Pleurotus ostreatus, Rhodotorula minuta, Syncephalastrum racemosum, Trametes versicolor*
8	Benzo[a] pyrene	*Aspergillus ochraceus, Bjerkandera adusta, Bjerkandera* sp., *Candida maltosa, Candida tropicalis, Chrysosporium pannorum, Cunninghamella elegans, Mortierella verrucosa, Naematoloma frowardii, Neurospora crassa, Penicillium janczewskii, Penicillium janthinellum,*
9	Chrysene	*Cunninghamella elegans, Penicillum janthinellum, Syncephalastrum racemosum*
10	Benzo[a] pyrene	*Cunninghamella elegans*

Modified from Kadri et al. (2017)

sp. strain Berk 41; an unidentified strain of the order *Aganicales*, strain Beuk 47; *Bjerkandera* sp. strain Bos 55; *Daedaleopsis confragosa* GM 2; and *Stereum* sp. strain Schim 22. These strains were originally isolated from rotting pine needles, rotting oak wood, rotting birch wood, rotting beech wood, forest litter, forest soil, and forest litter, respectively. He found that PAH biodegradation is a ubiquitous phenomenon among white rot fungi. All strains tested degraded anthracene, and some of the strains degraded Benzo alpha pyrene, significantly beyond the level in the poisoned mycelium controls. Although PAH biodegradation was found to be a universal characteristic of the white rot fungi tested, two distinct patterns of PAH biodegradation could be distinguished.

6.6.2 *Fungal Enzymes and Degradation Pathways*

Fungi is found to possess inherent potential and efficiency to degrade PAHs. Ligninolytic fungi was of much interest to the scientists as they produce extracellular enzymes with reduced substrate specificity. This feature of ligninolytic fungi provided a necessary fillip for the organisms to degrade various organopollutants (Hammel 1995). Extracellular peroxidases produced by these fungi causes the initial oxidation of PAHs (Zhang et al. 2015). While fungal lignin peroxidases oxidize quite a number of PAHs directly, fungal manganese peroxidases co-oxidize them indirectly through enzyme-mediated lignin peroxidation. Further, it was reported that white rot fungi oxidize anthracene to anthraquinone (Vyas et al. 1994). Basically, the ligninolytic system contains three principal enzyme groups namely lignin peroxidase (LiP), Mn-dependent peroxidase (MnP), phenol oxidase (laccase, tyrosinase), and H_2O_2 producing enzymes (Novotný et al. 2004). Ligninolysis is an oxidative process and it is found to operate under nutrient limiting conditions for instance nitrogen limiting conditions (Novotný et al. 2004). In effect, the fungal mediated degradation of PAHs is slow and it was found that fungi have potential to degrade diverse group of xenobiotics. Ability of many soil fungi to live, grow, propagate (Lee et al. 2015a, b) and capability to degrade PAHs through fungal enzymes like cytochrome P450 monooxygenase, epoxide hydrolases, lipases, proteases and dioxygenases have been studied in depth (Balaji et al. 2014).

The two main enzyme groups involved in the initial attack on PAHs by fungi are the cytochrome P-450 monoxygenases and the lignin peroxidases (Fig. 6.9). Both

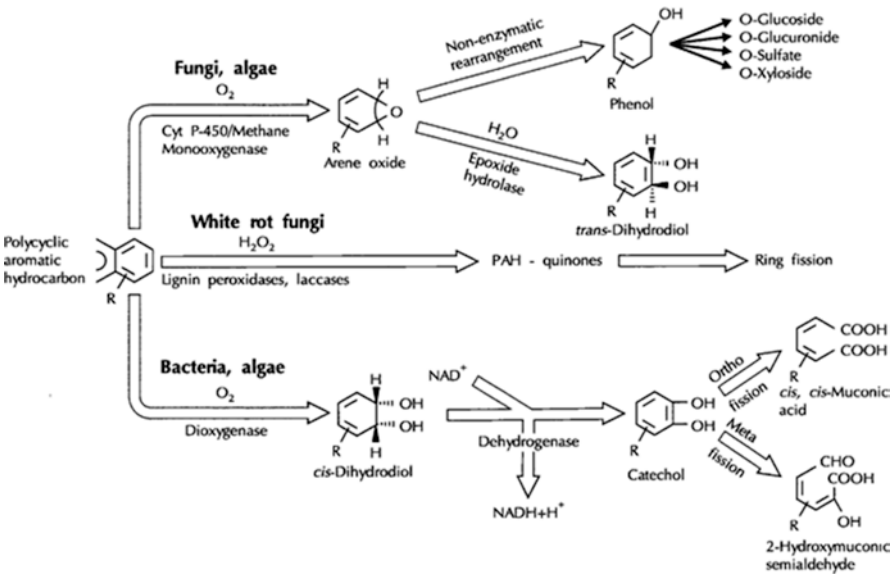

Fig. 6.9 Initial steps in the microbial pathways for oxidation of polycyclic aromatic hydrocarbons. (Adopted from Cerniglia 1993)

enzymes are relatively non-specific for the PAHs that they metabolize. Cytochromes P-450 incorporate one atom of molecular oxygen into the PAH molecule to form an arene oxide, which then undergoes either spontaneous isomerization to form a phenol, with subsequent conjugation with sulfate, glucuronic acid, glucose or xylose, or enzymatic hydration to form a trans-dihydrodiol. A non-ligninolytic fungus, *Cunninghamella elegans*, has been shown to metabolize PAHs that range in size from naphthalene to benzo[a]pyrene (Sutherland 1992).

Lignin peroxidases and laccases are extracellular enzymes produced by the white rot fungi (Field et al. 1992). The lignin peroxidases have been shown to oxidize PAHs that have ionization potentials of less than about 7.6 eV. The extracellular lignin peroxidases initiate a free radical attack on PAHs, by a single electron transfer, to form quinones. *Phanerochaete chrysosporium* was reported by Hammel et al. (1991) to oxidize anthracene via 9,10-anthraquinone to phthalate. In a similar study, they showed that *P. chrysosporium* oxidizes phenanthrene at its C9 and C10 positions to give 2,2′-diphenic acid as a ring cleavage product (Hammel et al. 1991). These results suggest that both lignin peroxidase and other enzymes may be involved in the degradation of PAHs. Sutherland (1992) reported that *P. chrysosporium* under non-lignolytic conditions metabolizes phenanthrene to phenols and trans-dihydrodiols, which suggests a cytochrome P-450 mediated reaction. Thus, *P. chrysosporium* contains several enzymes that may be involved in the degradation of PAHs.

Ligninolytic enzymes enable a one electron radical oxidation of contaminant resulting in the formation of aryl cation radicals and subsequently quinones (Vyas et al. 1994). It was reported that pure culture of *P. chrysosporium* degrade anthracene to anthraquinone (Hammel et al. 1991) which was broken down into phthalic acid and carbon dioxide. Further, it was found that purified forms of lignin peroxidase and manganese peroxidase were found to oxidize anthracene, pyrene, fluorene and benzo[a]pyrene to quinones (Hammel et al. 1991, 1992; Hammel, 1995; Bogan and Lamar 1996) (Fig. 6.10).

Hence, several systems are involved in the degradation of PAHs with fungal enzymes including intracellular cytochrome P450 and extracellular lignin peroxidase, manganese peroxidase and laccase. The pathways of degradation of PAHs vary for each enzymes and dependent on nutritional and environmental conditions of fungal strains (Fig. 6.11).

PAHs biodegradation occurs both under aerobic and anaerobic conditions. Clemente et al. (2001) reported the highest degradation of naphthalene (69%) and phenanthrene (12%) by a fungal strain that had manganese peroxidase enzyme system. Soil fungi like *Aspergillus* sp., *Trichocladium canadense,* and *Fusarium oxysporum* coulddegrade polycyclic aromatic hydrocarbons of low-molecular-weight PAHs (2–3 rings) and produce ligninolytic enzymes and activities (LiP, MnP, laccase). Under anaerobic conditions, ligninolytic enzyme activity was not normally observed (Silva et al. 2009).

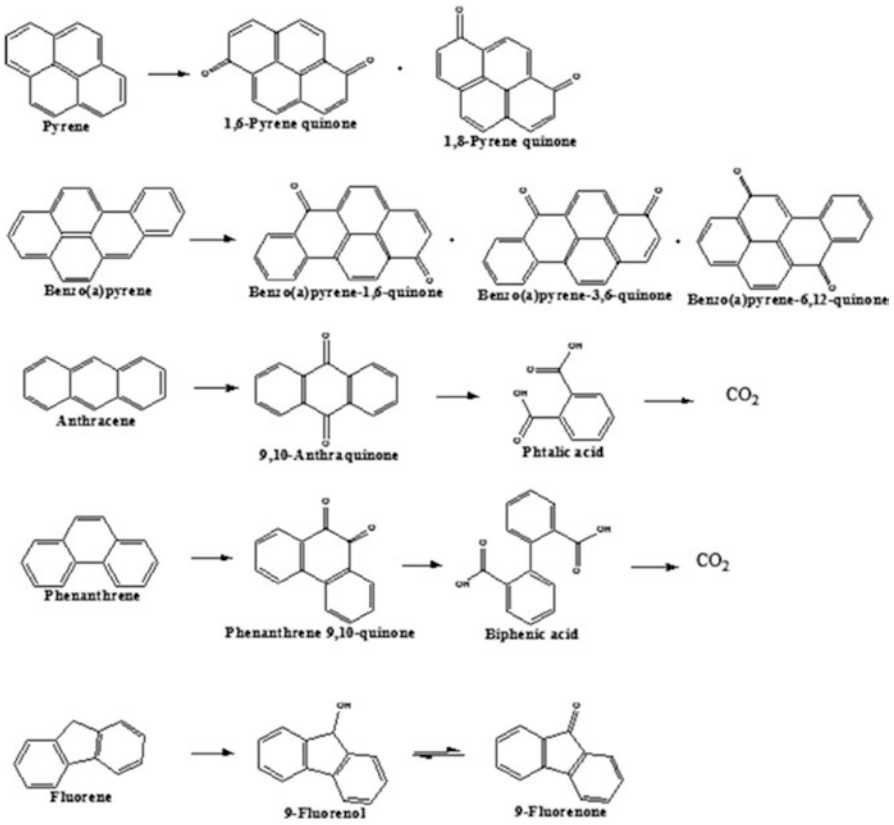

Fig. 6.10 Oxidation of PAHs by lignolytic fungi. (Kadri et al. 2017)

6.7 Algal Degradation of PAHs

Currently, the use of microalgae in bioremediation of colored wastewater has attracted great interest due to their central role in carbon dioxide fixation. In addition, the algal biomass generated has great potential as feedstock for biofuel production. These bioremediation capabilities of microalgae are useful for environmental sustainability. Compared to bacteria and fungi, relatively little attention has been paid to the biodegradation of PAHs by microalgae (cyanobacteria, diatoms etc.). Microalgae are one of the major primary producers in aquatic ecosystems, and play vital roles in the fate of PAHs in those environments. Several strains of microalgae are known to metabolize naphthalene, phenanthrene, anthracene, BaP and other PAHs (El-sheekh et al. 2012) (Table 6.3).

The biotransformation pathway of naphthalene by microalgae *Oscillatoria* sp., strain JCM are shown in the Fig. 6.12 (Cerniglia et al. 1980). Under photoautotrophic growth conditions, strain JCM has been reported to oxidize naphthaleneto1-

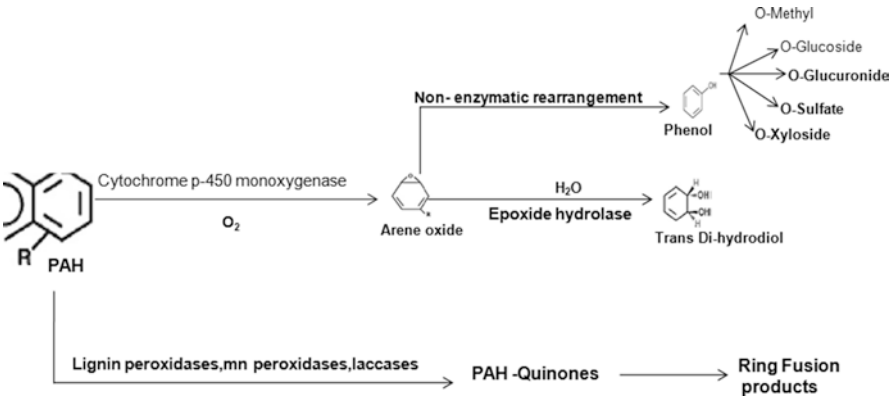

Fig. 6.11 Different pathways for the fungal metabolism of polycyclic aromatic hydrocarbons. (Modified from Kadri et al. 2017)

Table 6.3 The bioaccumulation and biotransformation of selected pesticides by eukaryotic algae

Algae	Bioaccumulation	Biotransformation
Chlamydomonas	Mirex	Lindane, naphthalene, phenol
Chlorella sp	Toxaphene, methoxychlor	Lindane, chlordimeform
Chlorococcum sp.	Mirex	
Cylindrotheca	DDT	
Dunaliella	Mirex	DDT, naphthalene
Euglena gracilis	DDT, parathion	Phenol
Scenedesmus obliquus	DDT, parathion	Naphthalene sulfonic acids
Selenastrum capricornutum	Benzene, toluene, chlorobenzene, 1,2-dichlorobenzene, nitrobenzene, naphthalene, 2,6-dinitrotoluene, phenanthrene, di-n-butylphthalate, pyrene	Benzo[a]pyrene

Adapted from Kobayashi and Rittman (1982)

naphthol, whereas marine cyanobacterium *Agmenellum quadruplicatum* strain PR-6 can convert phenanthrene to phenanthrene trans-9,10-dihydrodiol and 1-methoxyphenanthrene (Narro et al. 1992a, b).

The microalgae *Scenedesmus obliquus* GH2 is used to construct an artificial microalgal-bacterial consortium for crude-oil degradation (Tang et al. 2010). Addition of the bacterial consortium in different amendments significantly enhanced degradation efficiency of both aliphatic and aromatic hydrocarbons of crude oil. Another consortium of pre-isolated oil-degrading bacteria in association with three species of plants effectively remediated contaminated silt-loam soil more than silt, loam and sandy loam with an average 80% reduction of total petroleum hydrocarbon (Ghosh and Syed 2001).

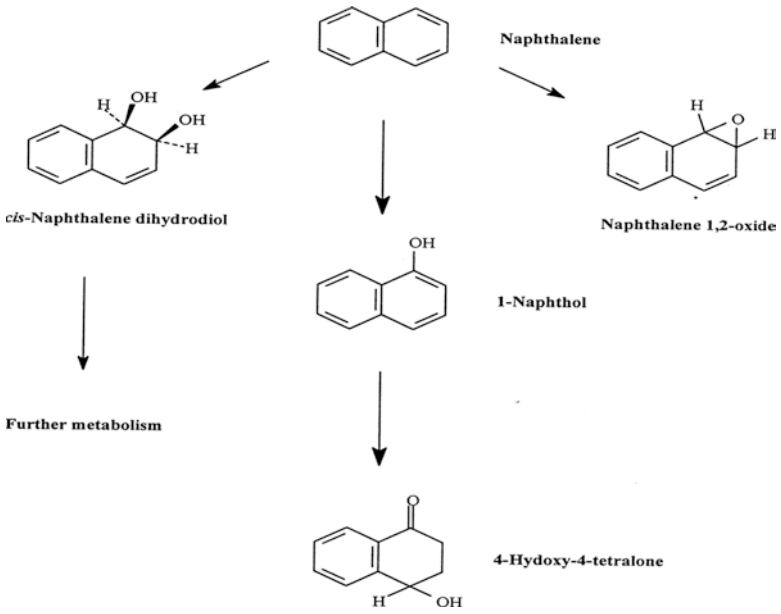

Fig. 6.12 Proposed naphthalene biotransformation pathway by the cyanobacteria, *Oscillatoria* sp. strain JCM. (Adapted from Cerniglia et al. 1980)

The phytoremediation of PAHs by algae have limited success due to the high toxicity (Dhankher et al. 2012). The accumulation and biodegradation of two typical polycyclic aromatic hydrocarbons (PAHs), phenanthrene (PHE) and fluoranthene *(FLA),* by the diatoms was studied by Hong et al. (2008), using two algal species *Skeletonema costatum* and *Nitzschia* sp. It was found that the accumulation and degradation abilities of *Nitzschia* sp. were higher than those of *S. costatum*. Degradation of FLA by the two algal species was slower, indicating that FLA was a more recalcitrant PAH compound. The microalgal species also showed comparable higher efficiency in the removal of the PHE and FLA mixture compared with PHE or FLA alone, suggesting that the presence of one PAH stimulated the degradation of the other.

Muñoz et al. (2003) suggested that it is possible to use microalgae to produce the O_2 required by acclimatized bacteria to biodegrade hazardous pollutants such as polycyclic aromatic hydrocarbons, phenolics, and organic solvents. When PAHs are taken up by microorganisms, they activated in aerobic metabolism by insertion of two oxygen atoms by bacteria and green algae to produce either cis-dihydrodiols or phenols.

Jinqi and Houtian (1992) investigated the degradation of azo dyes by *Chlorella vulgaris* and *C. pyrenoidosa* and found that certain dyes, such as Eriochrome blueSE and blackT, could be decolorized and actually used as carbon and nitrogen sources, but this was dependent on the chemical structure of the dyes (Fig. 6.13). The degradation was found to be an inducible catabolic process. They also found that the

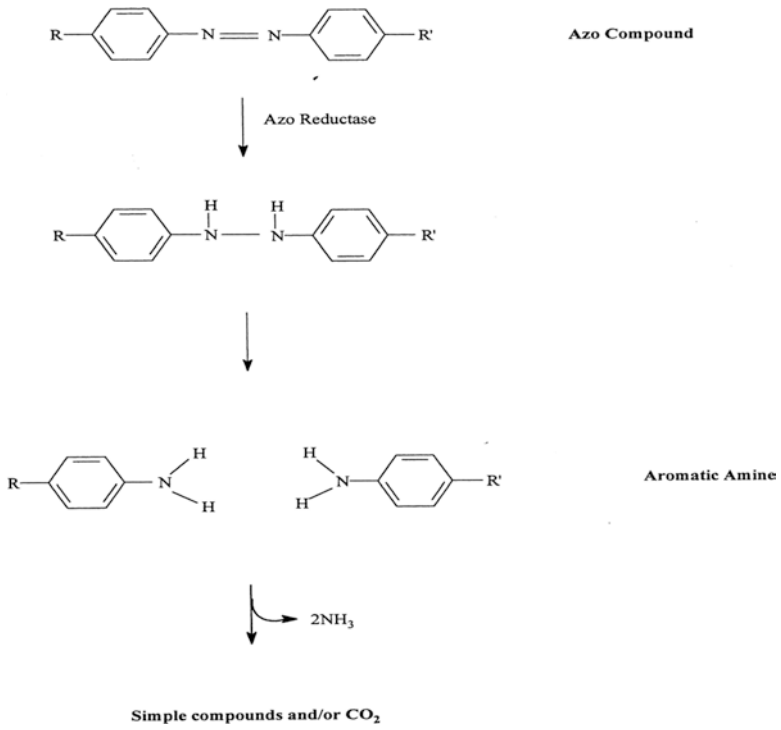

Fig. 6.13 Proposed degradation of azo dyes by eukaryotic algae. (Adapted from Jinqi and Houtian 1992)

algae degraded aniline, a potential degradation product of the azo dye breakdown. In another study, *Ochromonas danica*, a nutritionally versatile chrysophyte, grew heterotrophically on phenol or p-cresol as the sole source of carbon up to concentrations of 4 mM.

There are few examples of algae degrading aromatic compounds. (1980) and Jinqi and Houtian, (1992) have shown the removal of pollutants, accumulation of catabolic intermediates and the involved processes (Figs. 6.12 and 6.13, respectively). More detailed studies were carried out by some researchers (Lindquist and Warshawsky 1985a, b; Warshawsky et al.1995; Schoeny et al. 1988) who examined the effects of the chlorophyte alga, *Selenastrum capricornutum*, on benzo[a]-pyrene. They found that the alga used a dioxygenase system to oxidize the compound to cis-dihydrodiols which were then converted to sulfate ester and α and β-glucoside conjugates. The presence of this ring hydroxylating dioxygenase system is of particular importance as this mechanism is typically found only in bacteria and not in eukaryotes, where trans-dihydrodiols typically originate from epoxidation by the action of cytochrome P-450 monooxygenases and epoxide hydrolases on the PAH molecule.

6.8 Conclusion

During the past decade, a variety of microorganisms have been isolated and characterized for their ability to degrade different PAHs. Furthermore, many metabolic enzymes for the degradation of different PAHs have been isolated from microorganisms and several novel pathways have been elucidated based on the identification of initial ring oxidation and ring cleavage products. The genes responsible for PAHs catabolic pathways are always localized as gene clusters, and some gene clusters have been cloned and sequenced. The advancement in genetic, genomic, proteomic and metabolomic approaches, which are employed to study catabolism of organic pollutants have contributed remarkably in our understanding on the physiology, ecology, biochemistry of PAHs degrading microorganisms. However, detailed research is a prerequisite to determine exactly what is going on in PAH-contaminated environment. In addition, there are still various aspects of bioremediation of PAHs that remain unknown or otherwise have insufficient information, which requires further study. Enzymatic bioremediation is the tool to convert PAHs to less harmful/non-harmful forms with less chemicals, energy, and time. It is a solution to degrade/remove contaminants in an eco-friendly way. Microbial degradation represents the major mechanism responsible for the ecological recovery of PAH-contaminated sites. Some microorganisms are known to excrete biosurfactants which enhance the bioavailability of organic pollutants. Many microorganisms exhibit chemotaxis toward pollutants. These strategies lead to enhanced degradation of organic pollutants. The addition of small amount of biosurfactant, which increases the bioavailability of PAHs, or some merely toxic chemicals, like salicylicacid, which induce PAHs catabolic operons may enhance biodegradation of PAHs in the environment. It has been seen that organic amendments influence the indigenous microbial community as well as efficiency of bioremediation of PAHs in contaminated soil. Research has brought to the light the ability of diverse group of fungi in the bioremediation of PAHs contaminated sites through enzyme systems like MnP, LiP, laccase and other fungal enzymes, such as cytochrome P450 monooxygenase, epoxide hydrolases, lipases, protease and dioxygenase. Eukaryotic algae are capable of biotransforming and biodegrading aromatic pollutants commonly found in natural and wastewaters. However there are still some persistent organic pollutants that are difficult to degrade by the microalgae. The genetic engineering can solve this problem and offers a promising tool to improve the absorption and bioremediation of many organic pollutants and increase microalgal tolerance to these pollutants. From this chapter, we can conclude that the organisms like bacteria, fungi, and microalgae has immense potential in the biodegradation of many organic pollutants.

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