Chapter 17

Mycobacterium as Polycyclic Aromatic Hydrocarbons (PAHs) Degrader

Dushyant R. Dudhagara and Bharti P. Dave

Additional information is available at the end of the chapter

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Abstract

The genus *Mycobacterium* has the ability to degrade various environmental pollutants including polycyclic aromatic hydrocarbons (PAHs). *Mycobacterium* has an ability to withstand adverse environmental conditions and it has been considered for future bioremediation applications for the removal of PAH contaminants from crude oil–polluted sites. The degradation of PAHs using a cost-effective laboratory microcosm system was discussed. The various conditions such as environmental habitat, degradation behavior, enzymatic mechanisms, and ecological survival are thoroughly discussed in this chapter. Based on the above study, *Mycobacterium* has proved to be a better candidate in bioremediation of PAH-contaminated sites.

Keywords: *mycobacterium*, PAHs, microcosm, bioremediation

1. Introduction

As a result of anthropogenic activities, toxic chemicals have become ubiquitous contaminants of soils and groundwater worldwide. Thus, they are omnipresent in the environment due to rapid industrialization, urbanization, and modernization. This type of pollution is now being taken seriously by various industries, governments, environmental agencies, and non-governmental organizations. They are now always looking for an eco-friendly and cost-effective approach toward the removal of emerging environmental contaminants. Consequently, biodegradation is recognized as an efficient, economic, and versatile alternative to physico-chemical treatment of oil contaminants. Hence, microbial biodegradation plays a crucial role in the removal of polycyclic aromatic hydrocarbons (PAHs) specifically actinobacteria, which are a group of diverse bacteria, having the ability to degrade a wide range of organic compounds particularly hydrophobic compounds as PAH polychlorinated biphenyls (PCB), BTEX, pesticides, and so on [1].

Members of the genus *Mycobacterium* are of great interest due to their multiple PAH degradation capability, specifically high molecular weight (HMW), especially polycyclic aromatic hydrocarbons (PAHs) containing four or more fused benzene rings [2].

These compounds are persistent in environment due to high hydrophobicity and high stereochemical stability. They are known to possess mutagenic, genotoxic, and carcinogenic properties, causing deleterious effects on plants, aquatic organisms, animals, and humans. In contrast to low molecular weight (LMW) PAHs that can be degraded by various microorganisms (bacteria, actinobacteria, etc.), enrichment culture methods with HMW PAHs as sole sources of carbon and energy often lead to the isolation of *Mycobacterium* spp.

The goal of this chapter is to provide an outline of the current knowledge about biodegradation of PAHs using *Mycobacterium*. Moreover, various conditions as physiology of mycobacteria, environmental habitat, degradation behavior, enzymatic mechanisms, and ecological survival strategies toward organic compounds such as PAHs have also been discussed.

2. Calligraphy

2.1. Polycyclic aromatic hydrocarbons

Polycyclic aromatic hydrocarbons (PAHs) are a class of organic compounds that are widely distributed in the environment. PAHs is a predominant term describing hundreds of individual chemical compounds containing two or more fused aromatic rings and are known to persist or accumulate in the environment. PAHs in the soil have recently become a matter of great concern due to their potential toxicity, mutagenicity, and carcinogenicity. Therefore, 16 PAH compounds have been identified by the United States Environmental Protection Agency (USEPA) as priority pollutants [3, 4]. They are ubiquitous compounds that are formed either naturally during thermal geological reactions, fossilization, and biological reactions or anthropogenically during mineral production, combustion of fossil fuels, refuse burning, forest and agricultural fires, and so on. On the basis of physical and chemical properties of PAHs, they are classified into two groups: low molecular weight (LMW PAHs, including 2–3 rings) and high molecular weight (HMW PAHs, including four or more rings). **Table 1** shows the physico-chemical properties of 16 PAHs as a number of benzene rings, vapor pressure, aqueous solubility, and octanol-water partitioning coefficient (K_{ow}) values. Therefore, LMW PAHs are greatly more soluble and volatile as compared to HMW PAHs due to their higher hydrophobicity than the LMW PAHs [5]. The K_{ow} values also reflect the hydrophobicity of the PAHs. These properties regulate the environmental behavior of PAHs. Therefore, HMW PAHs are persistent in the environment specifically in soil due to their high hydrophobicity.

Generally, the rate of degradation of PAHs is inversely proportional to the number of rings in PAH molecule [6]. LMW PAHs, such as naphthalene, fluorene, phenanthrene, and anthracene, are more easily degraded and usually utilized as the model PAHs for further understanding the degradative mechanisms on the HMW PAHs. HMW PAHs are more persistent in the environment as they exhibit higher hydrophobicity and toxicity [7] than LMW PAHs.

Sr. no.	PAH	No. of rings	M_r	Melting point (°C)	Boiling point (°C)	Water solubility (mg L^{-1})	Vapor pressure (Pa)	K_{ow} value
1	Naphthalene	2	128.17	80.6	218	31	10.4	3.37
2	Acenaphthene	3	154.21	95	279	3.47	3.0×10^{-1}	3.92
3	Acenaphthylene	3	152.20	93.5–94.5	265	3.93	8.93×10^{-1}	4.07
4	Fluorene	3	166.22	116	295	0.190	8.0×10^{-2}	4.18
5	Anthracene	3	178.23	217.5	340	0.0434	1.0×10^{-3}	4.54
6	Phenanthrene	3	178.23	99.5	340	1.18	2.0×10^{-2}	4.57
7	Fluoranthene	4	202.26	110.8	375	0.265	1.23×10^{-3}	5.22
8	Pyrene	4	202.26	156	404	0.013	6.0×10^{-4}	5.18
9	Benz[a]anthracene	4	228.29	159.8	437.6	0.014	2.8×10^{-5}	5.91
10	Chrysene	4	228.29	255.8	448	0.0018	5.70×10^{-7}	5.86
11	Benzo[k]fluoranthene	5	252.31	215.7	480	0.00055	7.0×10^{-7}	6.04
12	Dibenz[a,h]anthracene	5	278.35	266	524	0.0005	1.33×10^{-8}	7.16
13	Benzo[a]pyrene	5	252.31	176.5	495	0.0038	1.40×10^{-8}	6.25
14	Indeno[1,2,3-cd]pyrene	6	276.34	162.5	536	0.0620	1.0×10^{-10}	6.58
15	Benzo[b]fluoranthene	6	252.31	167	357	0.0012	6.67×10^{-5}	6.57
16	Benzo[g,h,i]perylene	6	276.34	278.3	500	0.00026	1.39×10^{-8}	7.10

Table 1. Physico-chemical properties of 16 PAHs as classified by USEPA.

With increase in the number of benzene rings, PAH solubility decreases while hydrophobicity increases. The K_{ow} values of the 16 PAH priority pollutants are in the range from 3.37 to 6.5, which is generally considered moderate-to-higher lipophilic (**Table 1**). Thus, PAHs tend to adsorb onto organic fractions in soil sediment and biota and are also accumulated in the food chain [8].

2.2. Characteristics of *Mycobacterium*

The genus *Mycobacterium* comprises aerobic, rod-shaped, acid-fast, mycolic acid (lipid moieties)-containing bacteria; they are common saprophytes, distributed in different environmental pools. The distinguishing characteristic of all *Mycobacterium* species is that the cell wall is thicker than in many other bacteria, being hydrophobic, waxy, and rich in mycolic acid content. As per the Floyd et al. [9] data collection, the abundance of *Mycobacterium* genera accounted for 2.6% of total soil microbial diversity present in the environment. On the basis of growth cycle, they are divided into two categories such as slow and fast growers exhibiting growth within seven and after seven days, respectively. These phenomena are further supported by the difference in 16S rRNA sequences. Fast growing strains have two copies of the 16S rRNA gene, whereas slow growing strains normally have a single copy of the gene [10].

Moreover, properties of one or two 16S rRNA genes are assumed to be comparatively slow growth and lower metabolic activities, which require more time for adaptation into the environment [11].

Mycobacteria are high G + C-containing genera; they possess many properties that make them good candidates for application in bioremediation of soils contaminated with organic pollutants. *Mycobacterium* sp. is frequently found in environmental habitats including PAHs-contaminated soil. Nocardio-forming Actinobacteria has a unique enzymatic mechanism that degrades a wide range of complex organic compounds and their spores are resistant to desiccation. In addition, these groups of microorganisms have the ability to degrade a wide range of hydrophobic compounds and produce biosurfactants. Biosurfactant is useful for the adhesion of microbial cells to the hydrophobic compound. Therefore, many mycobacterial stains have the capability to degrade organic compounds as pesticides, PAHs, polychlorinated biphenyls (PCB), and so on. The nocardio-forming actinomycetes such as *Mycobacterium* sp., *Rhodococcus* sp., *Gordonia* sp., and so on have been reported to possess hydrocarbon degradation capability in PAHs-contaminated soil. Many *Mycobacterium* species have been reported

Strain	Compound degraded	Source	Reference
Mycobacterium spp. NJS-1 and NJS-P	pyrene	PAH-contaminated farmland soil, China	Zeng et al., [19]
Mycobacterium sp.	Phenanthrene, pyrene, fluoranthene	PAHs-contaminated soil	Johnsen et al., [38]
Mycobacterium sp. AP1	Phenanthrene, pyrene, fluoranthene	Crude oil-contaminated sand, Spain	Vila et al., [21]
Mycobacterium sp.	benzo[a]pyrene, pyrene, fluoranthene, and phenanthrene		Hennessee et al., [39]
Mycobacterium sp. CP1/CP2/CFt2/CFt6	Naphthalene, phenanthrene, anthracene, acenaphthene, fluorene, pyrene, fluoranthene,	Creosote-contaminated soil, Spain	López et al. [32]
Mycobacterium sp. S65	Phenanthrene, pyrene, fluoranthene	Soil contaminated with jet fuel, Quebec	Sho et al. [40]
Mycobacterium sp. SNP11	pyrene, fluoranthene, phenanthrene, and fluorene		Pagnout et al., [20]
Mycobacterium sp. 1B	Phenanthrene, pyrene, fluoranthene	Manufactured gas plant-contaminated soil, Australia	Dandie et al. [41]
Mycobacterium sp. MHP-1	Pyrene	Contaminated soil sample, Japan	Habe et al. [42]
Mycobacterium austroafricanum GTI-23	Pyrene, Fluoranthene, Phenanthrene	Manufactured gas plant site, Iowa	Bogan et al., [15]
Mycobacterium litorale	Fluoranthene, Phenanthrene	Oil-contaminated soil, India	[3, 4]

Table 2. Global scenario of PAH degradation in different environments by *Mycobacterium* strain.

as high molecular weight (HMW) PAH degraders, specifically pyrene, fluoranthene, benzo[b] pyrene, and so on. Thus, they are promising candidates for environmental bioremediation because of their ubiquitous presence in soils and their ability to catabolize aromatic compounds. *Mycobacterium* sp. has an ability to operate the unique catabolic pathway of HMW PAHs as compared to gram-negative bacteria. Cerniglia [12] has reported the *Mycobacterium* sp. PYR-l in enhanced degradation of four aromatic rings of PAHs when inoculated into microcosms-containing sediment. The scientific community worked on biodegradation of PAHs in different habitats as marine sediment, agriculture soil, and soil with alkaline or acidic conditions [1, 4, 13, 14] as listed in **Table 2**.

3. PAH biodegradation using *Mycobacterium*

3.1. *Mycobacterium* degradation ability

Our laboratory has worked on degradation of HMW PAHs as pyrene and fluoranthene using *M. litorale* on solid agar and liquid medium. Multiple PAHs-degrading bacterial strains were isolated from the PAHs-contaminated site near Bhavnagar. Preliminary culture was enriched in Bushnell Haas (BH) broth and further isolated on PAH-coated BH agar plate. Isolate showed a zone of clearance on PAH (fluoranthene)-coated BH plate and growth of bacteria in liquid culture (BH broth) supplemented with PAHs as the carbon source (**Figure 1**), which indicated that *Mycobacterium litorale* had the ability to utilize fluoranthene, a four-ring HMW PAH, as the sole source of carbon and energy [3]. Similar results have also been reported by Bogan et al. [15] who reported that *M. austroafricanum* utilized phenanthrene, pyrene, and fluoranthene as the sole source of carbon and energy.

Many *Mycobacterium* strains have been isolated from different environmental habitats (**Table 2**). Recently, culture-independent molecular techniques and PCR-based amplification of 16S rRNA gene were used to compare the diversity and abundance of indigenous *Mycobacterium* populations in different historically contaminated soils [16]. A wide variety of *Mycobacterium*

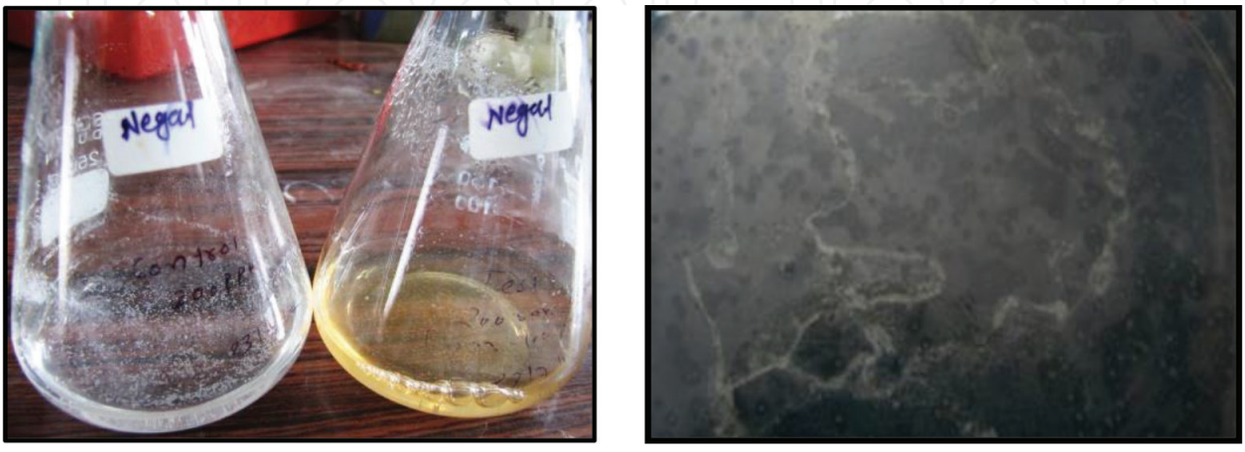

Figure 1. Fluoranthene degradation by *Mycobacterium litorale.*

genera are extensively used for removal of PAHs from contaminated sites by bioremediation techniques. It has been well established that *Mycobacteria* have exceptionally lipophilic surfaces which makes them a suitable candidate for the uptake of complex bound pollutants (i.e., PAHs) from the heavy contaminated soil particles. Thus, they have good catabolic properties toward the PAH molecule up to five benzene rings [17, 18]. Therefore, it indicates the PAH-degrading *Mycobacterium* strains are diversely distributed in the environmental soil.

The ability of the soil microbial community to degrade hydrocarbons depends on the number of microbes and its catabolic activity. *Mycobacteria* are metabolically versatile and are able to metabolize LMW and HMW PAHs. They have been reported to degrade HMW PAHs as pyrene, fluoranthene, and benzo[a]pyrene. Zeng et al. [19] demonstrated that *Mycobacterium* sp. NJ1 has an ability to degrade anthracene, pyrene, fluoranthene, and benzo[a]pyrene to various extents. Pagnout et al. [20] described *Mycobacterium* sp. SNP11 as possessing unique characteristics such as a cell wall rich with mycolic acids and the capacity to adhere strongly to hydrophobic compounds such as the HMW PAHs. This adhesion strongly facilitates the mass transfer of PAHs into the cells. Furthermore, Vila et al. [21] also reported that *Mycobacterium* sp. AP1 has the ability to degrade pyrene and produce intermediate metabolites.

3.2. Microcosm study

Soil microcosm is an approach to study microbial interactions with organic pollutants, in controlled and reproducible environmental conditions. Laboratory microcosms permit measuring of biodegradation and mineralization (CO_2 production) rates and can be used to study the effect of bioaugmentation and biostimulation on bioremediation process [22, 23]. Dave et al. [23], in our laboratory have constructed an efficient microcosm system for the enhancement of soil bioremediation process, which resulted in the improvement of HMW PAH degradation in simulated soil conditions (**Figure 2**). Addition of glucose, Triton X-100, and beta-cyclodextrin in presence of chrysene resulted in enhanced biodegradation of LMW and HMW PAHs up to six rings. In our previous study (unpublished work), we conducted a microcosm experiment in the laboratory using *M. litorale* as a bioaugmenting agent and addition of various biostimulating agents such as Triton X-100, agricultural compost, Bushnell Haas medium, and mixture of all agents, which exhibited significant biodegradation of PAHs (phenanthrene, anthracene, pyrene, fluoranthene, and chrysene) from PAH spiked soil. Actinomycetes are well known to grow under conditions ranging from obstructive to unfavorable environmental conditions for a long time. *Mycobacterium* AP1 is able to utilize pyrene, fluoranthene, and phenanthrene as a carbon source. *Mycobacterium* sp. AP1 plays a significant role in degradation of PAHs such as phenanthrene in soil microcosm conditions [22]. All over, bioaugmentation treatments showed better results than monitored natural attenuation treatments in remediating PAH-contaminated soils.

3.3. Bacterial enzymatic routes

In the aerobic degradation, cytochrome P-450 monooxygenases are complex multicomponent systems present generally in fungi and are like the bacterial aromatic ring dioxygenases. These enzymes are generally membrane bound and have broad substrate specificities. PAH

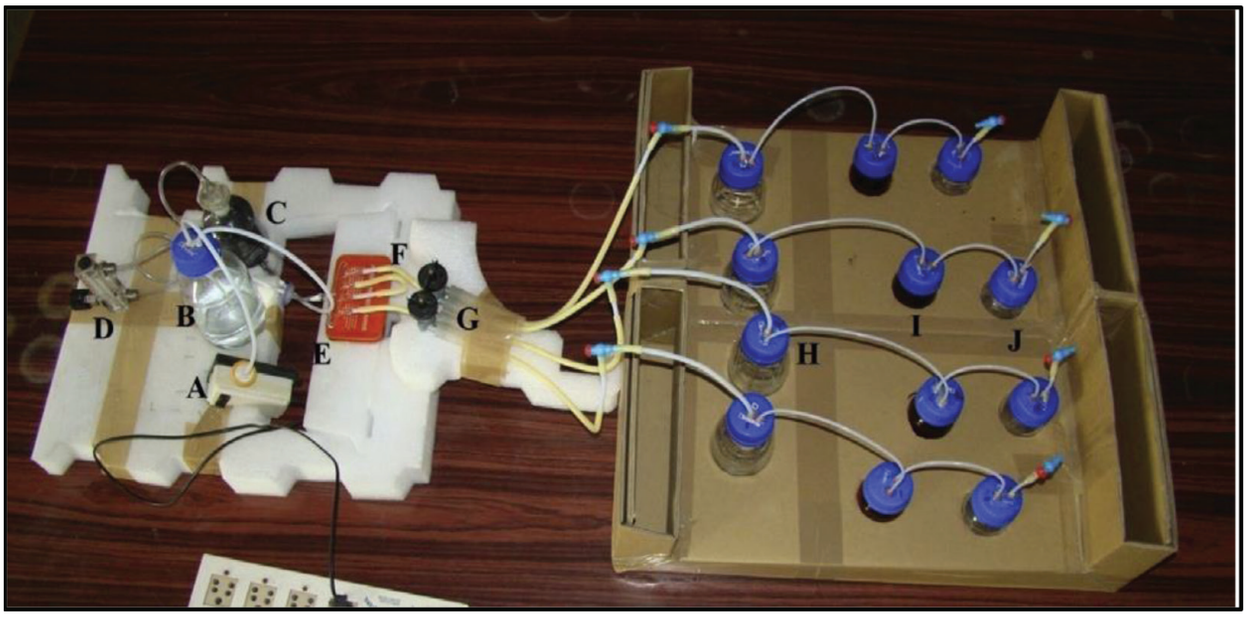

Figure 2. Microcosm system constructed in the laboratory [23]. Air pump (A), 2 M NaOH (B), activated charcoal (C), rotameter (D), 0.2 μ cellulose acetate filter (E), glass manifold (F), air regulator (G), sterile MilliQ water bottle to maintain humidity (H), microcosm flask (I) and CO2 trap (J).

is converted into arene oxide by addition by one atom of molecular oxygen by the monooxygenase (**Figure 3**), while the other atom is reduced to water.

The bacterial aerobic degradation of PAHs is generally initiated by the action of multicomponent dioxygenases that can catalyze the incorporation of both atoms of oxygen and two electrons from NADH to form *cis*-dihydrodiol. These multicomponent dioxygenases usually consist of reductase, a ferredoxin, and a third component consisting of two proteins, large and small iron-sulfur proteins [24]. Subsequent dehydrogenation by dehydrogenase forms dihydroxylated intermediates, which can further be degraded through *ortho-* or *meta-* (intradiol or extradiol) ring cleavage pathway which then eventually enters the TCA cycle (**Figure 3**). Dioxygenases have a number of applications such as in various clean-up technologies for wastewater treatments, biodegradation/bioremediation of PAHs, and other organic compounds in various contaminated niches.

Majority of dioxygenase enzymes were studied with Gram-negative bacteria but certain reports are also on gram-positive bacteria, specifically actinobacteria [25]. Silva et al. [26] reported that *M. fortuitum* has an ability to degrade anthracene maximally and increase their metabolic activity by changing various physical conditions, that is, pH and temperature. The other route of PAH degradation is accomplished by the action of monooxygenases. Initial oxidation by monooxygenases in bacteria forms trans-dihydrodiols; this activity is slower than dioxygenases. The cytochrome P-450 monooxygenase is a complex multi-enzyme protein of fungal origin that shares similarities to its bacterial counterparts.

Figure 3 represents the major routes for the degradation of PAHs by various enzyme systems. Among these, degradation of PAHs by dioxygenase-dehydrogenase enzyme system

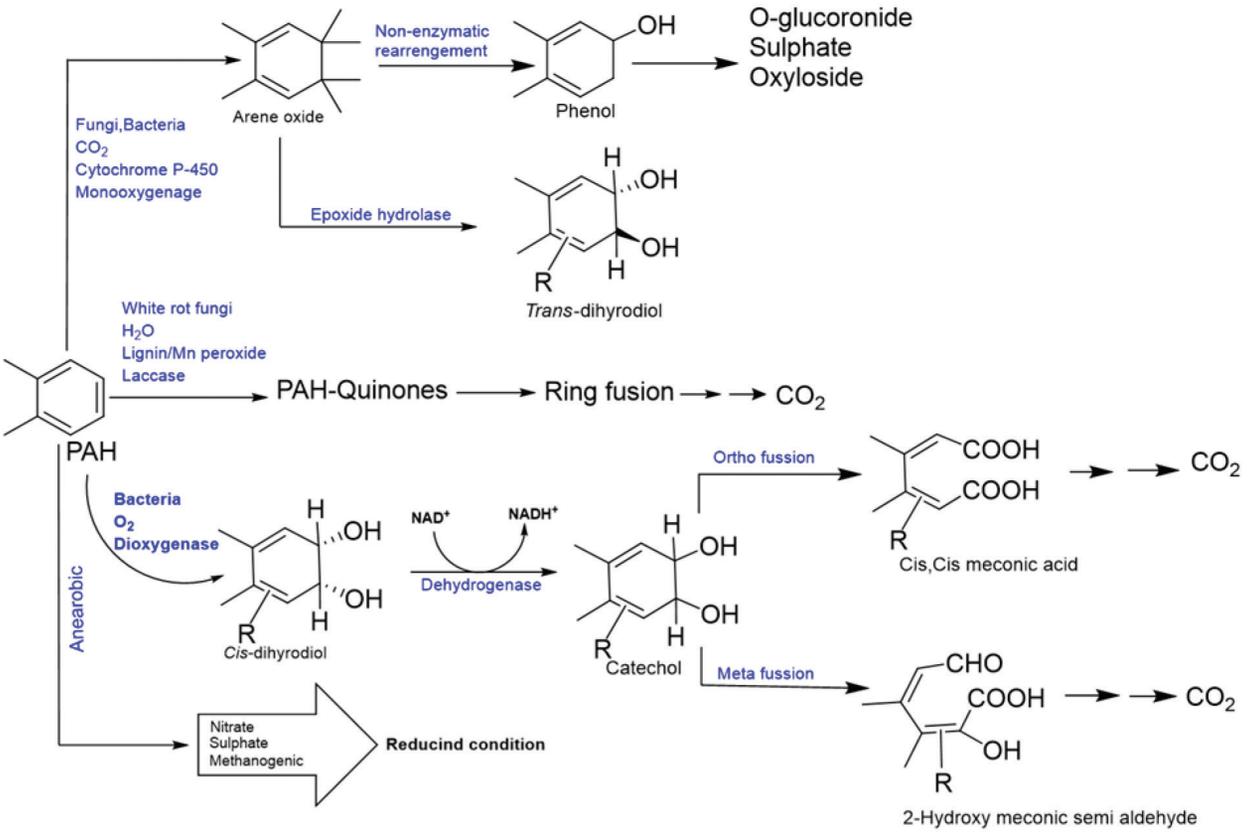

Figure 3. Microbial metabolisms of PAHs by various routes [17].

is commonly used by bacteria. Bacterial genera, capable of degrading PAHs commonly, include species of *Rhodococcus*, *Nocardia,* and *Mycobacterium*. This is a relatively small range of genera considering the prevalence of PAHs in the environment. Gram-positive actinobacteria as *Mycobacterium* spp. have been reported for the degradation of PAHs containing four or more fused aromatic rings at various extents. This is probably due to the hydrophobic cell surface which allows their adhesion to hydrophobic PAHs, thus facilitating mass transfer of the substrates inside the cells [27].

3.4. Biotransformation by *Mycobacterium* species

Many *Mycobacterial* species as *M. vanbaalenii* PYR-1 have been elucidated for the degradation of naphthalene, phenanthrene, anthracene, fluoranthene, pyrene, benz[a]anthracene, and benzo[a]pyrene, which produces key intermediate metabolites during degradation [28]. These results are significant because they have expanded our understanding of the enzymatic capabilities of bacteria to biodegrade HMW PAHs.

Mycobacterium strains have ability to degrade PAHs via either monooxygenase or dioxygenase enzymatic mechanisms, which form *trans*- and *cis*-dihydrodiol as an intermediate metabolite. Dean-Ross [29] recognized biodegradation of fluoranthene via fortuitous metabolism by an *M. flavescens* strain through *meta*-cleavage.

M. holderi was isolated from PAH-contaminated soil and was reported to grow on fluoranthene and co-oxidize pyrene in the presence of fluoranthene. It produced 29 metabolites during fluoranthene biodegradation [30]. Therefore, generated intermediate metabolite by *Mycobacterium* sp. showed a significant reduction of genotoxic potential after biodegradation of pyrene, fluoranthene, and phenanthrene [20, 31, 32].

M. vanbaalenii PYR-1 has been studied in detail with respect to enzymatic functions of various genes involved in PAH degradation [33–36]. Gene-encoding PAHs ring-hydroxylating oxygenases as *nidA, nidB*, and *nidD* are involved in PAH biodegradation [33]. These genes are expressed in *Mycobacterium* cells, which actively participated in phenanthrene, pyrene, and fluoranthene degradation. Guo et al. [37] also described that the dioxygenase *nidA* genes are involved in biodegradation of PAHs such as phenanthrene, pyrene, and fluoranthene.

4. Conclusion

Organic pollutants such as PAHs, PCB, and pesticides are resistant to degradation and are predominantly present in the environment; thus, they cause severe toxicological effects on humans as well as marine biota. Therefore, there has been growing interest in mycobacterial strains as potential bioremediation agents and as important components of indigenous PAH and other xenobiotic compound degradation. Various researchers reported the use of *Mycobacterium* for PAH degradation in different environmental conditions. Mycobacterium possesses peculiar characteristics for degradation of HMW PAHs due to their potential enzymatic mechanisms, which encoded the PAH ring-hydroxylating oxygenases genes, participating in PAH biodegradation. A member of the genus *Mycobacterium* is responsible for HMW PAH removal and their catabolic enzyme like monooxygenase/dioxygenase, which is converted into less harmful and simpler end products. Thus, *mycobacteria*, isolated from different habitats in the environment, can be exploited for their potential to remediate contaminated sediment/soil. Based on the study, interpretations will aid notable information to the scientific community for future research on bioremediation of recalcitrant high molecular weight (HMW) PAHs. Based on the previous study *Mycobacterium* has tremendous capability to remediate the contaminated sites and transform them to less toxic end products. Biodegradation is considered as the best approach to restore PAH-contaminated soils. Therefore, bioremediation is a feasible option for cleaning up PAHs because it is simple, applicable over large areas, cost-effective, and eco-friendly green approach.

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Author details

Dushyant R. Dudhagara[1,2] and Bharti P. Dave[2]*

*Address all correspondence to: bpd8256@gmail.com

1 Analytical and Environmental Science Division and Centralized Instrument Facility, CSIR-Central Salt and Marine Chemicals Research Institute, Bhavnagar, India

2 Department of Life Sciences, Maharaja Krishnakumarsinhji Bhavnagar University, Bhavnagar, India

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