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POLYCYCLIC AROMATIC HYDROCARBONS: CHARACTERISTICS AND ITS DEGRADATION BY BIOCATALYSIS REMEDIATION

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Abstract

An excessive released of polycyclic aromatic hydrocarbons (PAHs) to surroundings is one of the major factors that cause environmental pollution to increase globally. This issue had gained scientist's attention to study PAHs biodegradation pathways and their toxicity towards humans and the environment. They found that the major mechanism responsible for the ecological recovery of PAH-contaminated sites happened to be from the microbial degradation process. However, there are a few limitations faced by the PAHs degrading bacteria where the bacteria die due to extremely polluted areas. This leads the researchers to utilize genetic engineering to produce enzymes that can withstand and survive in extreme environments. Recent information and technology such as path sources, properties and biochemical pathways by means to produce the simplest and less harmful components in polluted ecosystems are discussed in this review. In-depth studies in regards to bacteria biocatalysis involving bacterial-produced-enzymes to degrade PAHs help develop new methods to enhance the bioremediation effectiveness in the future.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous and widespread pollutants in various ecosystems. Released of PAHs into the environment for example from incident of petroleum spillage usually will be settled into wastewater and atmospheric deposition. PAHs alone and their intermediate products have the potential to generate toxic or mutagenic effects to marine [1-3] and human life [4]. PAHs from environments bound to particulates in soil then sedimented, making them more complicated and harder for biological uptake because of their hydrophobicity. To overcome PAHs contamination, PAHs degrading enzymes

were discovered from fungi, and bacteria, for instance, laccase producer from *Trichoderma* sp., *Bacillus* sp., *Streptomyces* sp. and *Pseudomonas* sp. [5,6]. The first bacterial laccase was expressed from *P. putida* KT2440, followed by *P. putida* CA-3 and *P. putida* F6 [134,135].

The mechanisms involved in PAHs degradation involved series of oxidation and reduction reactions from oxygenase, dehydrogenase, isomerase, hydrolase, decarboxylase, transferase and many other enzymes that may be added, depends on the type of the PAHs and its reaction condition. The reaction will produce new products and then will be introduced into the citric acid cycle consortium with the production of electrons in the electron transport chain. The final products from this serial of degradation process will result in the hydrocarbons to become CO_2 and water. Improvements for better bioremediation enzymes to overcome PAHs contamination in the environment may be achieved by utilizing genetic engineering technology, which may help to reduce the remediation process's cost and fasten the recovery period of the contaminated site. In this review, we gathered information regarding PAHs characteristics, biodegradation by bacteria and the latest studies regarding methods development in producing excellent PAHs degrading enzymes (Figure 1).

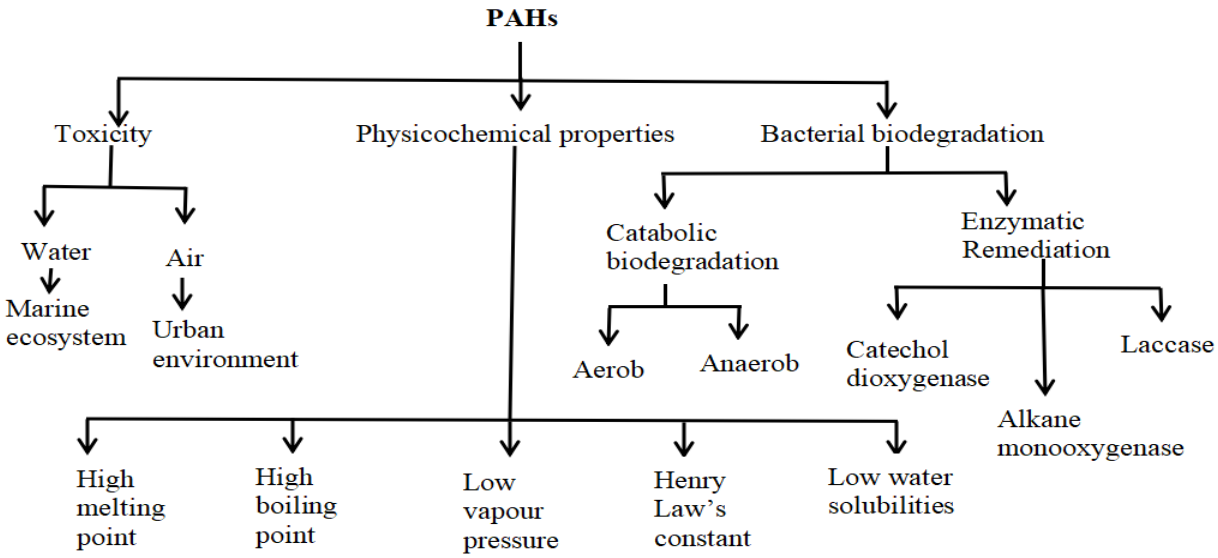

Figure 1. PAHs characteristics as factors that contribute to environmental toxicity

POLYCYCLIC AROMATIC HYDROCARBONS (PAHs)

PAHs are hydrophobic, aromatic hydrocarbons with more than one benzene ring, exist in linear, angular or cluster arrangements [134]. They are composed of carbon and hydrogen atoms only with molecular weights ranging from 128 to 278 Da, thus, characteristically they are non-polar organic compounds. The bioaccumulation tendency, hydrophobicity, resistance to biodegradation, and overall environmental perseverance of the compounds theoretically increased with an increase in molecular weight [7,135]. PAHs were classified into low molecular weight PAHs (LPAHs) and high molecular weight PAHs (HPAHs) groups. LPAHs, for instance, naphthalene, anthracene, phenanthrene, acenaphthene, fluorene, and acenaphthylene usually comprised of a basic structure of two to three benzenoid rings, while HPAHs consisted of molecular structures of four or more benzenoid rings, for instance, pyrene, benzo[a]pyrene, fluoranthene, and benzofluoranthenes (Figure 2). PAHs mostly exist as colorless, white, or pale yellow-green solids, characteristically low vapour pressure and are globally distributed in atmospheric, terrestrial and aquatic systems [8-10].

PAHs can be found in nature, for example from volcanoes and anthropogenic sources (petrogenic and pyrolytic). Pyrolytic PAHs are PAHs that originated from industrial or other human activities that involved partial combustion of organic matter (such as fossil fuels and biomass). While the petrogenic PAHs are constituted of petroleum products, such as oil spills [11-13].

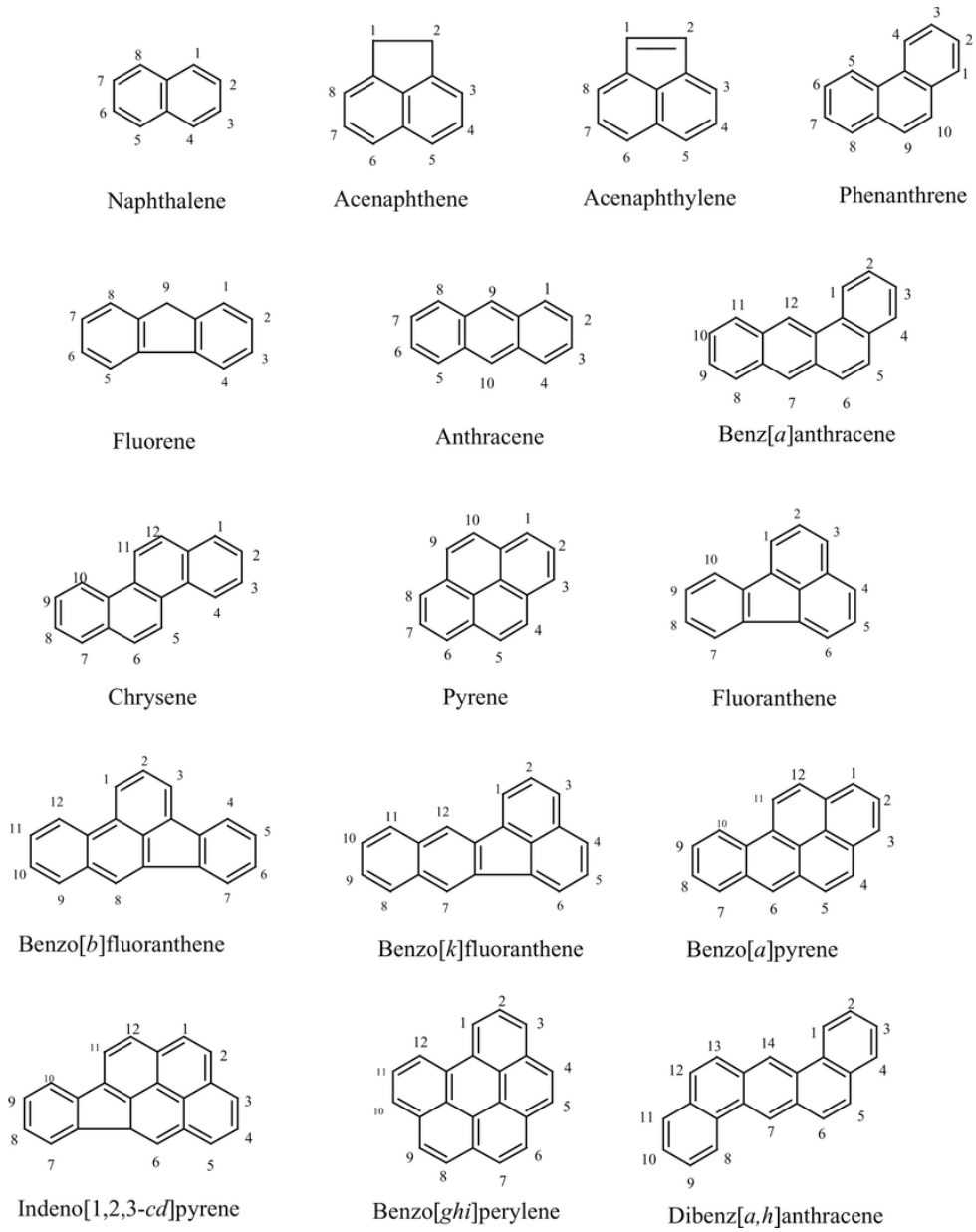

Figure 2. Rings arrangements and structures of PAHs [14]

Physicochemical properties of PAHs

PAHs are low in water solubilities, high boiling and melting points, low vapor pressures, and Henry's Law constants [15]. PAHs solubility will decreases as the molecular weight increases, while their boiling and melting point increases following the increase of molecular weight [16] (Table 1). Most of four-ring and five-ring aromatic hydrocarbons such as chrysene and benzo[a]pyrene are water-insoluble [17,18]. As ring structure increases, the degree of substitution increases, vapour pressure decreases, molecular weight increases but reduces its solubility [19]. Molecules with a linear arrangement are mostly less soluble than the angular or perifused molecules. The aromatic ring with Alkyl group substitution will results in an overall decrease in the solubility of PAHs. Because of their hydrophobicity properties, PAHs tend to attached to the organic matter in soil. Whilst due to their low solubility in water, PAHs in aquatic environments will associated with the particulate matter or organic substances such as biopolymers, and black carbon in sedimentation [9,19]. This PAHs-organic matter association caused PAHs to be more stable than its pure compounds and more resistant to oxidation and nitration reactions, the reactions that they supposedly quite sensitive due to photochemical processes [17].

Table 1: Physicochemical characteristics of PAHs according to molecular weights and their toxicity effects to the environment.

PAHs	Molecular weight (g/mol)	Melting point (°C)	Boiling point (°C)	Toxicity effect
Naphthalene	128	80.2	218	haemolytic anaemia and methaemoglobinaemia [119], peripheral neuropathy and renal failure [120]
Acenaphthylene	152	92.5	280	NR
Acenaphthene	152	93.4	279	Lung tumors[118]
Fluorene	166	115	295	NR
Phenanthrene	178	99.2	340	embryonic heart failure [116]
Anthracene	178	215	340	NR
Fluoranthene	202	108	384	NR
Pyrene	202	151	404	NR
Benzo[a]anthracene	228	167	435	Lung tumors[118]
Chrysene	228	258	448	Lung tumors[118]
Benzo[b] fluoranthene	252	168	481	Lung tumors [118]
Benzo[k] fluoranthene	252	217	480	Lung tumors[118]
Benzo[a]pyrene	252	177	495	Carcinogenic effect to lung, cervix, bladder, breast, and prostate [115], induced a loss of bone mass and bone strength [117], eye irritation and skin sensitization [118]
Dibenzo[a,h] anthracene	278	270	524	Lung tumors[118]
Indeno[1,2,3-cd] pyrene	276	164	536	Lung tumors[118]

Note: NR (Not Reported)

Environmental processes such as photo-oxidation, hydrolysis, biotransformation, biodegradation and mineralization in the aquatic system lead to the transformation of PAHs to other products. Mostly, high molecular weight PAHs in the aquatic systems will be degraded by photo-degradation [20]. Most PAHs are classified as semi-volatile organic compounds because of their low volatility [9].

PAHs Toxicity

PAHs easily can be found in the air, soil and water, it is ubiquitous and recalcitrance. PAHs bioaccumulate and lead to carcinogenic activity when humans consume marine foods containing high of PAHs or eat vegetables grown in contaminated soil. Some PAHs may evaporate from contaminated soil and some PAHs were released into the air, thus inhalation of these contaminated air for long term exposure may cause carcinogenic effects such as high

bladder cancer and lung cancer, and some PAHs may have anti-estrogenic or weak estrogenic impacts [21, 22].

Toxicity of PAHs in Water

The pollution of PAHs in the water are pollutants of concern due to their persistence in the marine ecosystem, thus could cause long-term adverse effects to marine life. PAHs may enter marine systems through petroleum spills, urban and suburban stormwater runoff, chemical refineries, recreational and commercial boats, volcanoes and atmospheric fallout of vehicle exhaust, and treated industrial and municipal wastewater discharges [17,23,24].

There are two major concerns on PAHs in the marine environment. First, low-molecular-weight PAHs can bioaccumulate into fish and shellfish making them not

suitable to market for consumption [25]. Second, metabolites of some of the high-molecular PAHs are potent animal and human carcinogens, for example. benzo[a]pyrene. Carcinogenic activity is closely related to structure [26], for example, benzo[e]pyrene and the four benzofluoranthene isomers all have a molecular weight of 252 Da, but are much less potent carcinogens than benzo[a]pyrene (Figure 3).

Lower- molecular-weight PAHs are readily taken up by marine animals, across gill surfaces, and through their diet [27]. They may bioaccumulate, particularly in shellfish,

Figure 3. Two isomers of benzopyrene

filter-feeding organisms, such as bivalve molluscs. Even though fish, marine vertebrates and marine mammals are also exposed to PAHs, they do not generally accumulate high concentrations of PAHs, they metabolize PAHs efficiently because they possess an effective mixed-function oxygenase (MFO) system that allows them to metabolize PAHs and to excrete them in bile [28, 29, 30].

Toxicity of PAHs in Contaminated Air

Emissions from motor vehicle exhaust were considered a major source of airborne PAHs in urban environments [31-33]. In ambient air, PAHs with molecular weight exceeding 228 Da are mostly associated with particles, whereas PAHs with lower molecular weights, such as phenanthrene (178 Da) and pyrene (202 Da), exist partitioned between its condensed state associated with particles and the gaseous phase [34,35]. Although most of the airborne PAH mass is partitioned to the gaseous phase [36], the relative carcinogenic potency of PAHs differs widely between different derivatives [33]. PAHs with a higher molecular weight and ultimately those associated with particulate matter (PM) give higher carcinogenic potency. Particulate matter is a generic term to classify air pollutants comprising of suspended particles in air, varying in composition and size, resulting from various anthropogenic activities. The particle size ranges between 2.5 μm (PM2.5) and 10 μm (PM10). Usually, the human respiratory system will be affected by PM depends upon the size of the particle, for example, the upper respiratory tract is affected by PM10 while lung alveoli is affected by ultrafine particles (0.1 μm in diameter) [37].

The majority of the PAHs bound to air particles are associated with the PM1 fraction, (fraction of particles with an aerodynamic diameter smaller than 1 μm) [36,38]. Indoor sources of PAHs include cooking and heating, cigarette smoking, and candle, incense burning and outdoor source [39]. PAHs originating from outdoor sources through infiltration and cigarette smoking be the major sources of PAHs in the indoor environments in Krakow, Poland [40]. To reduce and limits the particles entering the interior from the outdoor air, mechanical ventilation with air filtration was a good method. Mechanical ventilation with filtration has been shown to significantly reduce indoor particle levels of PM2.5 [41], submicron particles [42] and both total suspended particulates (TSPs) and PM10 [43], suggesting that there would be some possible health benefits associated with air filtration that may reduce the exposure to PAHs.

Toxicity of PAHs in Soil

Other than natural released of PAHs into environments, urbanization is one the major factor that contributes to the release of PAHs into soil and lead to its toxicity. Urbanization has magnified the current land exploitation globally since the last three decades due to population aggregation, landfills, road construction, and industrial exploitation [121,122]. Anthropogenic activities have accelerated the PAHs release to environments, following by its emissions into the urban rivers, through wastewater discharge, surface runoff, oil spillage, and atmospheric deposition [124]. Wu et al. (2019) recent studies about the impact and potential risks of rapid urbanization on sediment and soil from ditch wetlands, riverine wetlands, and agricultural lands along the lower reaches of the Shiwuli River feeding Chaohu Lake, China. They have concluded that the correlation between the distance from the built-up urban areas and pollutant concentration showed that the closer the distance, the greater the concentration of PAHs [123]. Generally, sediments or soils are the final deposition sinks of PAHs regardless its emission source from air or water. Following PAHs deposition on surface soil, PAHs may further accumulate in vegetables and other biota and finally transferred to humans via the food chain [126,127].

PAHs tend to persist in the soil due to their lipophilicity [125] and tend to strongly attach to soil, making it harder to degrade by remediation technologies [128]. PAHs enter the urban river system through water runoff, adsorbed in particulate matter and finally deposited in the sediment. Sedimentary PAHs can be released into the overlying water and continue to intoxicate the river ecosystems and risk to human health (128,129). Most PAHs are toxic, mutagenic and/or carcinogenic. PAHs are highly lipid-soluble, thus, in humans and other mammals, PAHs are readily absorbed from the gastrointestinal tract and rapidly distributed in a wide variety of tissues with a marked tendency for localization in body fat. PAHs metabolism occurs via the cytochrome P450-mediated mixed-function oxidase system with oxidation or hydroxylation as the first step [130].

BACTERIA BIODEGRADATION OF PAHs IN SOIL

Most PAHs will be sedimented into marine and soil. In soils, PAHs have low mobility and high durability depends on different factors such as temperature, pH, and soil organic matter content [44, 45] and ageing of the history of contamination in soils [46]. Although PAHs can be degraded through photolysis, volatilization, adsorption and chemical degradation process, a biodegradation method named bioremediation utilizing bacteria as PAHs degraders is the most well-known degradation process [47]. Bioremediation involves the use of microorganisms to degrade hazardous organic constituents to produce harmless substances such as carbon dioxide and water. PAH degradation depends on the environmental conditions, number and type of the microorganisms, nature and chemical structure of the chemical compound being degraded. The reported microbes such as *Pseudomonas aeruginosa, Pseudomons fluoresens*, *Mycobacterium* spp., *Haemophilus* spp., *Paenibacillus* spp. and *Rhodococcus* spp. are known for their catabolic activity in bioremediation, and changes in microbial communities are still unpredictable and the microbial community is still termed as a 'black box' [48].

Bioremediation can be naturally occurred by the use of bioaugmentation (whole-cell introduction) or biostimulation approaches by the use of nutrients or conditions to stimulate the native microbial community. The application of whole-cell bioremediation is somehow limited, enzymatic bioremediation may offer better benefits to the environment, avoiding the conditions that are required for whole-cell applications, especially in extreme environments where normally bacteria could not survive so long [49]. To overcome the limitations, enzymatic effectiveness can be improved in vitro by using molecular tools, such as DNA engineering, to generate super bioremediators. As an example of enzymatic bioremediation, PAH detoxification can be achieved by the use of laccases [50] as the enzymes are capable of catalyzing the oxidation of phenols, polyphenols, and anilines, coupled to the 4-electron reduction of molecular oxygen to water [51].

Bacteria Catabolism Degrading PAHs

Microorganisms, such as bacteria, green algae, cyanobacteria and fungi, are capable of degrading different components of petroleum under different environmental conditions (aerobic and anaerobic conditions at varied salinities and pHs) utilizing enzymatic mechanisms [49]. The bacteria, for instance, *Pseudomonas* sp. was preferred among the microorganisms [52] because of their rapid growth and metabolic rates and their capability to perform numerous degradation pathways that can be genetically manipulated to improve their bioremediation capabilities. Numerous bacteria have been found that capable to degrade PAHs, and some can utilize low-MW PAHs as their sole carbon source, for instance, *Pseudomonas, Mycobacterium, Haemophilus, Rhodococcus, Paenibacillus*, and *Ralstonia* are some of the most extensively studied bacteria for the bioremediation of organic compounds (Haritash and Kaushik, 2009).

PAHs degradation occurs gradually by the sequential metabolism of its compounds. The most common biochemical pathways studied for the bacterial degradation of PAHs such as naphthalene [53, 54], phenanthrene [55-58], anthracene and acenaphthene [59,60] have been well investigated. As we know, hydrocarbons have consisted of carbon and hydrogen only, thus they are lacking of functional groups, and making hydrocarbons largely apolar and exhibit low chemical reactivity at room temperature. Differences in their reactivities are primarily determined by the occurrence, type and arrangement of unsaturated bonds [61].

Aerobic Degradation

Biodegradation of hydrocarbons may occur under anaerobic or aerobic conditions. Generally, under aerobic conditions, oxygenase will introduce oxygen atoms into hydrocarbons (monooxygenases introduce one oxygen atom to a substrate while dioxygenases introduce two) [62]. The anaerobic degradation is catalyzed by anaerobic bacteria, such as sulphate-reducing bacteria, by utilizing different terminal electron acceptors. Aerobic catabolism of hydrocarbons is carried out in a much faster pace, due to the metabolic advantage of having the availability of O_2 as an electron acceptor compared to anaerobe [63].

The presence of molecular oxygen will initiate the enzymatic attack of PAH rings. Initially, dioxygenase will catalyze oxidation of arenes generally takes place in aerobic bacterial systems to yield vicinal cis-dihydrodiols as the first bioproducts by a multicomponent enzyme system. These dihydroxylated intermediates may then be cleaved by intradiol or extradiol ring-cleaving dioxygenases through either an ortho-cleavage pathway or a meta-cleavage pathway, leading to the production of central intermediates such as protocatechuates and catechols, that subsequently

further converted into tricarboxylic acid (TCA) cycle intermediates [64-67]. Aromatic hydrocarbons, such as benzene and naphthalene, can also be degraded in aerobic conditions. The degradation of aromatic hydrocarbons usually serves as an initial step in the formation of catechol or a structurally related compound. The catechol can be degraded, resulting in compounds such as acetyl-CoA and succinyl-CoA that later can be introduced into the citric acid cycle together with the production of electrons in the electron transport chain, and subsequently degrades the hydrocarbons to form CO_2 and water, the most safe end products [63,68]. The proposed

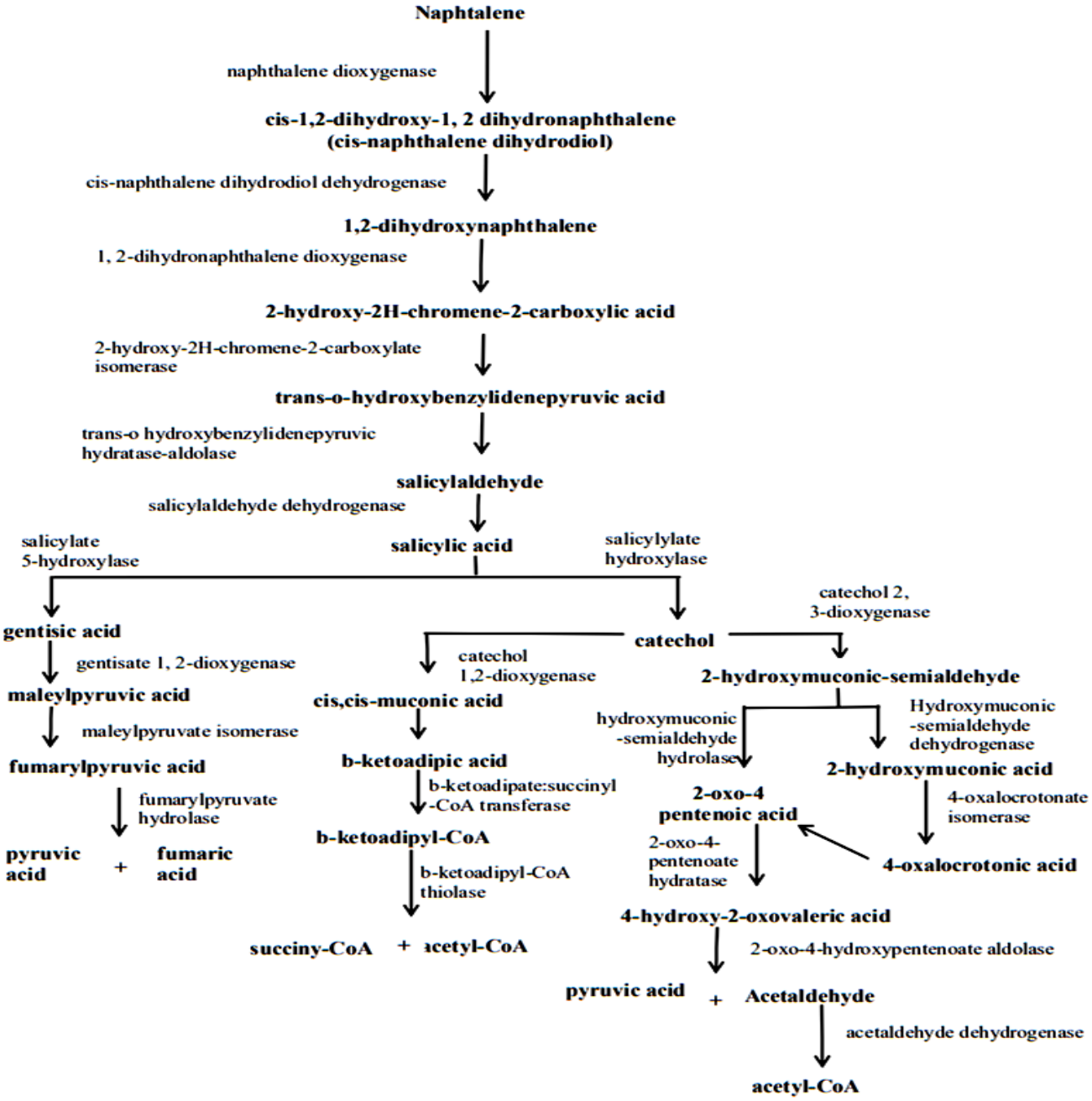

Figure 4. Naphthalene catalytic degradation in producing its intermediates products before Krebs Cycle [134].

catabolic pathways of naphthalene by aerobic bacteria are shown in Figure 4.

Anaerobic Biodegradation

Under anaerobic and reducing conditions, the biodegradation process of hydrocarbons can be divided into three major steps. Firstly, aromatic hydrocarbons are partly degraded under nitrate and sulfate-reducing conditions to form low molecular weight organic acids as metabolic intermediates. Secondly, organic acids act as ligands complexing insoluble Fe (III) oxides in the aquifer and mobilizing Fe (III). Lastly, the mobilized Fe (III) is available for iron-reducing bacteria and intensifies the degradation of aromatic hydrocarbons [69].

The sulfate-reducing bacteria were found to be involved in the degradation of phenanthrene and two- to four-ring PAH under anaerobic conditions with the involvement of methanogen and vancomycin microbial populations [70]. The proposed anaerobic biodegradation pathways of fluorene and phenanthrene by sulfate-reducing bacteria (SRB) as shown in (Figure 5). Tsai and friends found that the enriched SRB from anaerobic swine wastewater sludge could degrade 88% of fluorene and 65% of phenanthrene within 21 days period of incubation. It was observed that sulfate reduction was coupled with the biotransformation of fluorene and phenanthrene. Fluorene and phenanthrene were biotransformed through a sequence of hydration and hydrolysis reactions followed by decarboxylation with the formation of p-cresol (only in the phenanthrene system) and phenol [71].

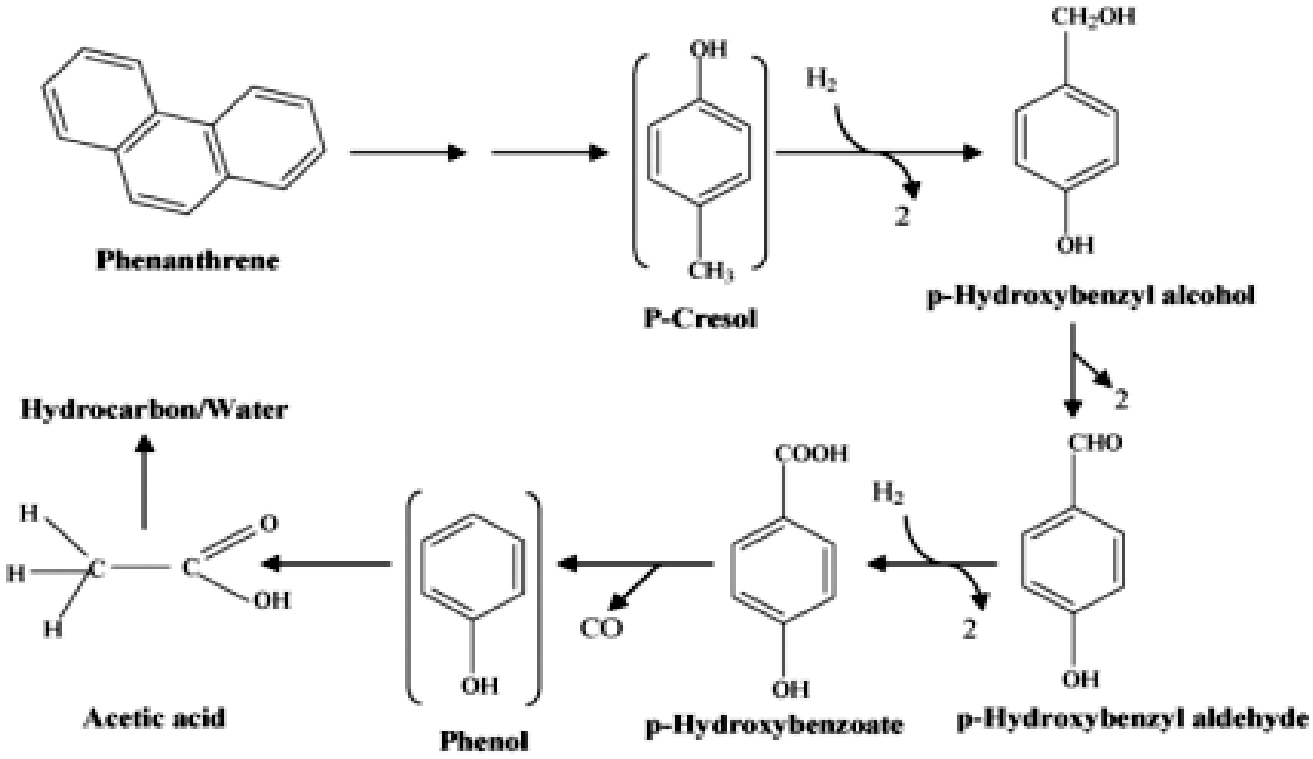

Figure 5. Proposed anaerobic biotransformation pathway of phenanthrene by sulfate-reducing bacteria (SRB) [71].

Most of anaerobic nitrate-reducing [72,73] and sulfate-reducing [74] bacteria, that capable to degrade PAHs, have been identified from the genus Pseudomonas. Under anaerobic conditions, the major intermediates are benzoate (or benzoyl-CoA) and, to a lesser extent, resorcinol and phloroglucinol [75,76]. Reactions involved in the channelling processes that lead to the central intermediates include carboxylations, decarboxylations, hydroxylations, reductions, reductive dehydroxylations, deaminations, dechlorinations, aryl ether cleavages, and lyase reactions. The aromatic central intermediates are reductively attacked, and cleaved by hydrolysis [77]. The resulting non-cyclic products are transformed by p-oxidation to central metabolites.

ENZYMATIC BIOREMEDIATION

The bioremediation of PAHs contaminated site is generally very slow because there are several biotic and abiotic factors responsible for successful bioremediation. Talking about

bacteria bioremediation and its producing enzymes, generally, the degradation process of PAHs may involve more than two enzymes to produce early functional products. As presented in Figure 4, degradation of naphthalene to catechol may consist of seven enzymes which are naphthalene dihydrodiol dehydrogenase, 1, 2-dihydronaphthalene dioxygenase, 2-hydroxy-2H-chromene-2-carboxylate isomerase, trans-o hydroxybenzylidenepyruvic hydratase-aldolase, salicylaldehyde dehydrogenase, and salicylate hydroxylase. Degradation steps and enzymes involved will be determined by the type of PAHs and their environmental conditions for instance aerobic or anaerobic conditions. The PCR primers that target genes related to petroleum-degrading enzymes for aerobic conditions are shown in Table 2.

Table 2: The modifying primers to amplify genes involved in petroleum degradation

Genes	Primer sequence	References
Catechol 2,3-dioxygenase genes	5'-CGACCTGATCTCCATGACCGA-3' 5'-TCAGGTCAGCACGGTCA-3'	[78, 79]
ALKA and/or ALKB genes (Alkane monooxygenase)	5'-AAYCANGCNCAYGARCTNGGVCAYAA-3′ 5′-GCRTGRTGRTCHGARTGNCGYTG-3′	[80,81]
Cytochrome P450 (CYP 153)	5'-TGTCGGTTGAAATGTTCATYGCNMTGGAYCC-3′ 5′-TGCAGTTCGGCAAGGCGGTTDCCSRYRCAVCKR TG-3′	[80,82]
Laccase	5'-ATGAGTGRCCTGRCBCAG-3' 5'-GCGGNTCCAGCCASACCARSGA-3'	[83]

Catechol dioxygenase

PAH biodegradation is mostly involved with these two key enzymes, PAH dioxygenase (PDO) and catechol 2,3-dioxygenase (C23O). Catechol and its derivatives are key metabolic intermediates in the catabolic pathway for aerobic degradation of monocyclic and polycyclic aromatic compounds [131]. The catechol dioxygenase is an example of an iron-containing enzyme class involved in the degradation of aerobic aromatic hydrocarbons. These enzymes can catalyze the addition of molecular oxygen atoms to 1,2-dihydroxybenzene (catechol) and its derivatives, with subsequent cleavage of the aromatic ring [84]. These enzymes can be found in a variety of biochemical processes, for example, chromosomally encoded pathways in *Pseudomonas* strains for degradation of benzoate and hydroxybenzoate, called the J3-ketoadipate pathway, and also from the plasmid-encoded pathway for the degradation of chlorobenzoate (Figure 6) [132].

Organisms that contain the benzoate or hydroxybenzoate degradative pathways can utilize these molecules as their sole source of carbon and energy. Likewise, the plasmid-encoded haloaromatic-degrading pathways enable soil bacteria to utilize halogenated organic compounds as sole sources of carbon and energy. These plasmids are, in fact, part of the machinery that allows certain bacteria not only to survive in soils polluted with halogenated organic compounds but, in doing so, to decontaminate the soils [132].

Alkane hydroxylases

Alkane hydroxylases are alkane-degrading enzymes that are distributed among many different species of bacteria, algae, fungi and yeast. van Beilen and Funhoff [108] had proposed three categories of alkane-degrading enzyme systems which are: C1–C4 (methane to butane, oxidized by methane-monooxygenase-like enzymes), C5–C16 (pentane to hexadecane, oxidized by integral membrane nonheme iron or cytochrome P450 enzymes), and C17+ (longer alkanes, oxidized by essentially unknown enzyme systems).

Then, van Beilen and Funhoff [108] listed the compositions, cofactors, substrate ranges, and presence of the main groups of alkane hydroxylases, for instance, soluble methane monooxygenase (sMMO), particulate methane monooxygenase (pMMO), AlkB-related alkane hydroxylases, eukaryotic P450 (CYP52, class II), Bacterial P450 oxygenase system and dioxygenase (CYP153, class I). Additionally, microorganisms that capable to degrade alkanes may contain multiple alkane hydroxylases and thus capable to consume a wide range of substrates [108]. As cited by van Hamme and colleagues [85], to date, one of the most studied alkane degradation pathways is from *Pseudomonas putida* Gpo1, encoded by the OCT plasmid [86,87]. In this case, the conversion of an alkane into an alcohol is first mediated by a membrane monooxygenase, soluble rubredoxin and rubredoxin reductase [85].

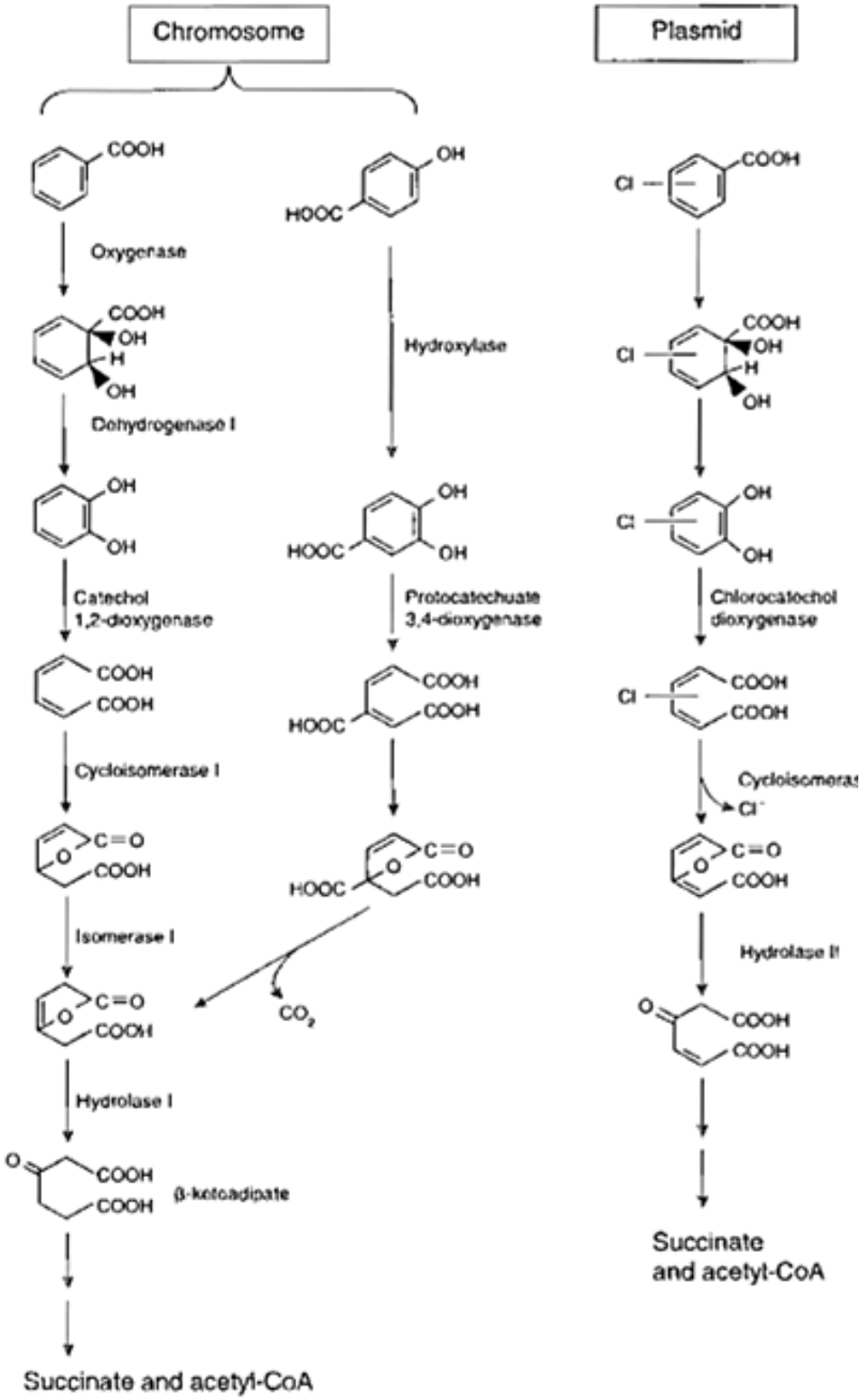

Figure 6. Bacterial degradative pathways for benzoic acids. The pathways for degradation of benzoate and p-hydroxybenzoate in *Pseudomonas* sp. are chromosomally encoded, whereas the chlorobenzoate pathway, which requires only three additional enzymes, is plasmid-encoded. Reactions in the chlorobenzoate pathway that are not labelled with enzyme names utilize the corresponding chromosomally encoded enzyme [132]

Laccases

Laccases are blue multicopper enzymes (EC 1.10.3.2) that functions to oxidize a broad range of both phenolic and non-phenolic substrates, via reduction of four-electron of oxygen to water [88,89]. Although laccases are heterogeneous in different species, with a wide variety of functions, four copper-binding motifs are conserved in most laccases, especially from bacterial forms [90]. The laccase activity can be affected by different metal ions either inducing or suppressing it. Metal ions that were known to accelerate laccase activity at a remarkable level are Cu^{2+}, Mn^{2+}, Ni^{2+}, Ca^{2+}, and Co^{2+} [91]. Laccases are important to biotechnological and industrial sectors such as organic synthesis, lignin degradation, food, textile, pharmaceutical industries. Laccases were used in bioremediation of contaminated environments, as well as the construction of biosensors and biofuel cells due to their capability to react at a broad spectrum of substrate [92-94].

The majority of laccases are produced from fungi. Different species of laccase producing fungi are *Basidiomycetes* such as *Phanerochaete chrysosporium, Theiophora terrestris,* and *Lenzites betulina* [95], and white-rot fungi [96,97] such as *Phlebia radiate* [98] *Pleurotus ostreatus* [99], and *Trametes versicolour* [100]. Laccase producers also derived from *Trichoderma* species such as *T. atroviride, T. harzianum* [101], and *T. longibrachiatum* [102].

To date, bacterial laccases are being studied extensively as their advantage in terms of growth rates and better suitability for modification of enzyme activity and gene expression compared to fungal laccase [103,104]. A few bacterial laccases have been identified and studied up to the molecular level. The first studied bacterial laccase is CotA from *Bacillus subtilis* followed by laccases from *B. coagulans, B. clausii* [105], and *B. licheniformis*. The other signifificant group of bacterial laccases are from *Streptomyces* species, such as *S. coelicolor* [109], *S. cyaneus* [110], *S. bikiniensis* [111], and *S. ipomoea* [112]. To date, three laccases from *Pseudomonas* species were expressed and characterized, one identified by Granja-Travez and collegues is laccase from *P. putida* KT2440 [113], and two identified by Mandic and collegues are *P. putida* CA-3 and *P.putida* F6 [83].

CONCLUSIONS

Despite all the advantages related to enzymatic bioremediation and its effectiveness, some problems must be overcome such as high production costs and low yields. DNA engineering can considerably reduce the problems, as Wong et al. [114] reported that studies of protein engineering, proteomics and metagenomics, are effectively contributing to cost reduction, minimizing chemical use and also improving cost-benefit ratios. The use of molecular tools for biocatalysis applications may solve the problem of GMO (bioaugmentation) applications into the environment. Molecular tools may increase the expression levels of enzymes by manipulating not only physiochemical conditions but also their genetic level. For instance, elastase overexpression was reported by Wong et al. (2010) from several genetic tools such as KRX/pUCP19/HindIII1500PstI of *E. coli* and PA01/pUCP19/HindIII1500PstI of *P. aeruginosa*, with increases in elastolytic activity to 13.83- and 5.04- fold, respectively, in relative to their controls [133]. Thus, this overexpression idea could improve enzyme production, efficiency and speed up petroleum degradation. Genetic manipulation could help to improve petroleum degradation in extreme environments, such as cold or hypersaline sites. The use of extremozymes would be advantageous in these extreme environments, since it could overcome several limitations, for example, bioremediation using whole cells (bioaugmentation) in extreme conditions that may lead to microbial competitiveness.

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