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Review

Bioremediation of Hydrocarbon Pollutants: Recent Promising Sustainable Approaches, Scope, and Challenges

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Abstract: The increasing population density and industrialization are adversely affecting the environment globally. The contamination of the soil, agricultural lands, and water bodies with petroleum wastes and other hydrocarbon pollutants has become a serious environmental concern as perceived by the impacts on the aquatic and marine ecosystem. Various investigations have provided novel insights into the significant roles of microbial activities in the cleanup of hydrocarbon contaminants. However, the burden of these pollutants is expected to increase many folds in the next decade. Therefore, it is necessary to investigate and develop low-cost technologies rapidly, focusing on eco-sustainable development. An understanding of the details of biodegradation mechanisms paves the way for enhancing the efficiency of bioremediation technology. The current article reviews the applicability of various bioremediation processes, biodegradation pathways, and treatments, and the role of microbial activities in achieving efficient eco-sustainable bioremediation of hydrocarbon pollutants. It is envisaged that an integrated bioremediation approach, including biostimulation and bioaugmentation is preferably advocated for the cost-effective removal of toxic petroleum hydrocarbons and their derivatives.

Keywords: bioremediation; eco-sustainable biotechnology; environmental cleanup; metabolic pathways; polycyclic aromatic hydrocarbons

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1. Introduction

Bioremediation is an eco-sustainable and efficient treatment method to degrade various hydrocarbon pollutants. The microbial activities can, directly and indirectly, lead to the degradation of hydrocarbon pollutants to simpler molecules. However, it is a complex process involving multiple steps and non-symmetric routes. The notable pollutants reaching the soil environment are waste sludge from petroleum refineries and processing industries. Petroleum waste sludge (PS) primarily consists of hydrocarbon (HC), ammonia, sulphide, etc. The physiochemical methods (incineration, pyrolysis, and solvent extraction) are incompetent, not feasible, and costly. In addition, the unpredictable alteration to the ecosystem by the spent chemicals (including its intermediate products and by-products)

can cause potential threats during their implementation. In this scenario, bioremediation offers a seemingly sustainable solution for the removal of hydrocarbon pollutants and fulfil the supplement pre-requisite for sustainable development [1–5].

There has been severe damage to the ecosystem due to the contamination of petroleum hydrocarbons in the last few decades. The dissemination of contaminants generally occurs through oil spills from oil tankers, drilling activities, the offshore release of petroleum by-products, and other anthropogenic activities. Petroleum hydrocarbons are stable and persist in the ecosystem for a longer period [6–9]. Oil contaminants degrade the water quality and thus affect aquatic lives. The ingestion of hydrocarbon contaminants can have serious effects and can lead to various diseases [10]. The olive mill waste (OMW), which is stored in evaporation ponds due to a lack of economic treatment, is one such example need attention for eco-friendly treatment [11]. Living organisms are directly or indirectly affected due to oil contamination. To overcome such problems, bioremediation that uses microorganisms to degrade oil or hydrocarbon contaminants can be used as an eco-friendly and cost-effective technology. The site location is important for the feasibility of in situ bioremediation. Biostimulation and bioaugmentation are the most important types of bioremediation methods. Bio-stimulation is a process of enhancing the site with nutrients, aerobic conditions, optimum pH, and temperature to increase the microbial population for enhanced biodegradation. In contrast to the above, bioaugmentation is the process of inoculating foreign microorganisms in the field to enhance the biodegradation rate [12]. Another approach is the integrated (bio-stimulation and bio-augmentation) treatment approach, an ex situ treatment method with enhanced functionality and applicability.

Bioremediation is a promising technology to remediate polycyclic aromatic hydrocarbons (PAHs) contaminated soil [13,14]. The treatment method proved to be versatile for the degradation of various organic hydrocarbon pollutants, including petroleum contaminants, explosives, pesticides, chlorophenols, and PAHs. Though there are many bio-inspired treatment methods for various organic compounds, a comprehensive overview of advanced technologies for the most toxic group of petroleum hydrocarbons is not available in the literature. Most of the field studies have reported limited evidence of any effective bioremediation for a long-term scenario, such as superfund sites. Hence, it is aimed to investigate the functional and eco-sustainable aspects of various bioremediation treatment methodologies. The study provides a comparative mechanistic insight into the effectiveness of such remediation strategies to recommend a suitable treatment combination (in other words, an integrated remediation approach) for in situ and ex situ conditions. The current review also highlights the significance of optimizing the microbial conditions for an effective and sustainable bioremediation implementation plan.

2. An overview of Bioremediation of Petroleum Pollutants

Petroleum contaminants are the most important pollutants worldwide, and they should be handled effectively to preserve marine lives and the ecosystem. The primary anticipation has been for evaluating the degradability of the toxic chemicals in the presence of the native microbial environment [15–18]. The hydrocarbon-contaminated drill mud waste from different tanks and petroleum waste sludge from refineries depicts the seriousness of the problem [19,20]. The bioremediation trials were made for the OMW sludge collected from seven long-term evaporation ponds polluted by abundant complex organic compounds [11]. The understanding of the associated mechanisms and the courses of action using microbes can guide better approaches for the bioremediation of contaminants. The treatment method proved to be versatile for the degradation of various organic hydrocarbon pollutants, including explosives, pesticides, chlorophenols, and PAHs (Figure 1). Recent works based on the PAH-contaminated aged field soil samples collected from a producer gas manufacturing plant and soil samples from an old diamond mining field proved the feasibility of bioremediation [11,21–27]. Many researchers made several trials to remediate generic hydrocarbon-contaminated soil and performed experiments on oily sludge collected from refineries [26], using amendment techniques for the pollutant sul-

famethoxazole during wetland remediation [28]. These efforts are important to understand the impact of bioremediation in the treatment of hydrocarbon pollutants.

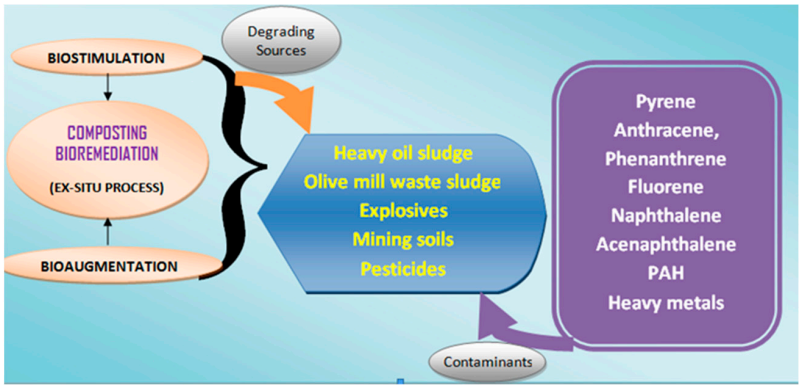

Figure 1. Schematic representation of the bioremediation used to treat hydrocarbon pollutants.

2.1. Role of Microorganisms in Hydrocarbon Biodegradation

Hydrocarbon degradation can occur by complex mechanisms involving microbial activities associated with the conversion of the complex hydrocarbons to simpler forms (Figure 2). The major pathways by aerobic and anaerobic microorganisms follow enzyme activation and then catalysis to simpler forms in optimized experimental conditions. The *Acinetobacter radioresistens* strain KA2 was isolated from oily waste sludge and performed two-stage methods. The experiment resulted in removing total petroleum hydrocarbon (TPH) up to 80% in 16 weeks. The technique successfully remediated the crude oil [8,9]. In another study, *A. radioresistens* strain KA5 and *Enterobacter hormaechei* strain KA6 were isolated from petroleum waste sludge (PWS) and two-stage bioremediations conducted for three months have been reported to remove the TPHs by 84% in 16 weeks. Oily sludge (OS) contaminant degraded using a culture-based medium consisting of *E. hormaechei* strain KA6. The in vessel experiment was conducted for a period of four months, and the rate of TPH removal was found to be up to 80% [26].

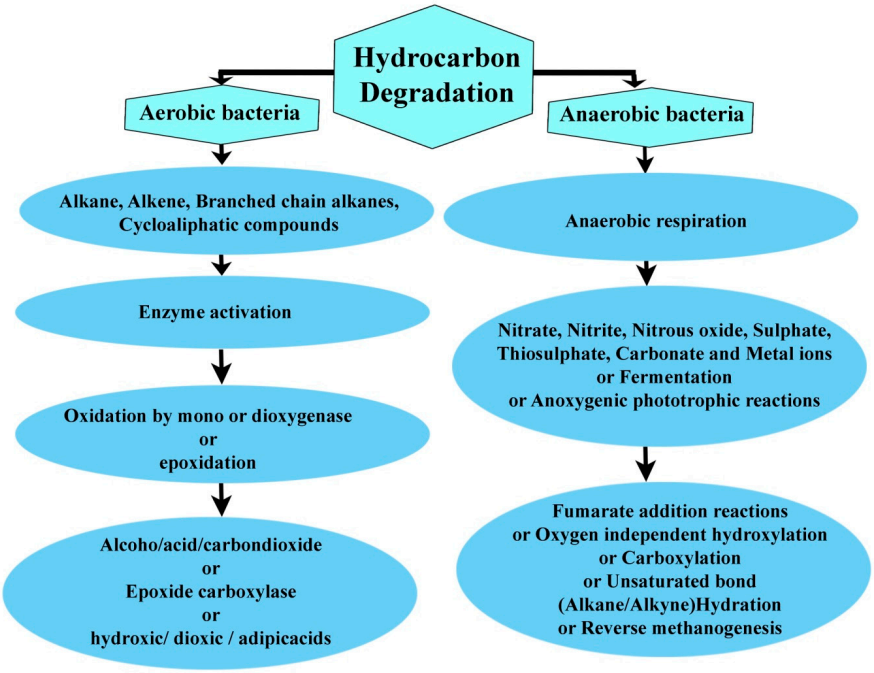

Figure 2. The schematic representation of aerobic and anaerobic biodegradation mechanisms.

The rapidly growing bacteria were isolated from heavy oil sludge, including *Staphylococcus equorum* strain KA4 and *E. hormaechei* strain KA3. The experiment was performed in a bioreactor for eight + eight weeks to degrade the mineral-based medium, and the TPH removal efficiency was up to 89% [7]. The fungal species *Fomitopsispinicola*, *Daedalea dickinsii*, and *Gloeophyllum trabeum* reduced the DDT contamination in the soil through bioremediation significantly. *A. radioresistens* strain KA5 and *E. hormaechei* strain KA6 were isolated from petroleum waste sludge (PWS) using 1% crude oil and mineral Bushnell-Haas (BH) medium. The rate of growth of the cells at various intervals was evaluated by measuring the optical density using a spectrophotometer. The strains were identified using the tests, such as catalase, citrate, oxidase, urease, triple sugar iron, nitrate reduction, H_2S production, indole production, and gram staining [20]. The fungal species *Aspergillus ochraceus H2* and *Scedosporium apiospermum H16* were isolated from OMW for the in situ method analysis, and microorganisms, such as *Proteobacteria (α, β, γ)*, *Actinobacter*, *Thermobifida*, and *Streptomyces* for effective biodegradation of pyrene, anthracene, phenanthrene, fluorene, naphthalene, acenaphthalene, and PAH contamination [11,13].

An experimental study using a hydrocarbon-contaminated drill mud waste along with cow bile and bacterial species *Brevibacterium casei* and *Bacillus zhangzhouensi* (as indigenous and combined experiments) resulted in TPH removal of up to 90% [19]. Similar observations have been summarized in Tables 1 and 2. These experimental observations and results are important for planning and designing large-scale studies for the bioremediation of hydrocarbons. However, there is a need for physical parameter optimization as well as scale-up analysis.

Table 1. Petroleum degrading microorganisms isolated from various contaminated sites.

Microorganisms Degrading Xenobiotics	Isolation Sites	References
Micrococcus and *Pseudomonas*	Soil samples contaminated with spent engine oil; from a workshop in Ado-Ekiti	[29]
Proteus vulgaris SR1	Freshly killed fish samples close to the point of oil spill in the Niger Delta, Nigeria	[30]
Pseudomonas sp., *Achromobacter* sp., *Bacillus* sp. and *Flavobacterium* sp.	Soil sample; obtained from a diesel spill region in north-central Alberta, British Columbia	[31]
Flavobacterium sp., and *Acinetobacterium calcoaceticum*	Soil sample; collected from Amanzimtoti, South Africa	[32]
Bacillus coagulans CR31, *Klebsiella pneumonia* CR23, *Klebsiella aerogenes* CR21 and *Pseudomonas putrefacience* CR33	Rhizosphere soil contaminated with spent engine oil in Sokoto, Nigeria	[33]
Pseudomonas sp., *Acinetobacter* sp., *Bacillus* sp., *Corynebacterium* sp. and *Flavobacterium* sp.	Soil samples from auto-mechanic workshops at Mgbukankpor, Nigeria	[34]
Pseudomonas putida, (Strain G1) and *Pseudomonas aeruginosa* (Strain K1)	Soil samples from abandoned coal power plant (PHC) at Ijora-Olapa, Lagos	[25]
Bacillus sp. S6 and S35	Soil samples from storage centre of oil products in Tehran refinery and Siri Island	[35]

Table 2. Summary of the efficiency of the removal of hydrocarbons according to potential microbes, substrate (s), and duration details.

Substrate	Microbes	Duration	Removal Efficiency (%)	References
Oily sludge	*Acinetobacter radioresistens* KA2	Two stage (8 + 8 weeks)	90	[9]
Petroleum waste sludge	*Acinetobacter radioresistens* KA5, *Enterobacter hormaechei* KA6	Two stage composting, 12 weeks	84	[20]
Olive mill wastewater	-	Two stage composting	84	[29]
Petroleum sludge	*Acinetobacter radioresistens* KA2	In vessel reactor, two phase composting (8 + 8 weeks)	88	[9]
Oily sludge	*Enterobacter hormaechei* KA6	In vessel experiment, 16 weeks	81	[26]
Contaminated soil	-	Soil inoculated sewage sludge, wood chips and incubated for 19 months	99	[23]
Heavy oily sludge	*Staphylococcus equorum* KA4, *Enterobacter hormaechei* KA3	Composting bioreactor (2 phase composting process 8 + 8 weeks)	89	[19]
Hydrocarbon contaminated drill mud waste	*Brevibacterium casei*, *Bacillus* sp.	Composting bioreactor, 6 weeks process	99	[7]

2.2. Optimization of Bioremediation Conditions

The performance criteria depend on various biotic and abiotic factors, such as microbial populations, aeration status, moisture content, temperature, etc. [36]. Further, the selection of a suitable method is significant for efficient bioremediation. There are various sequencing approaches now available to easily identify novel microbes from unique extreme environments [30,37]. The advancements in genome sequencing have paved the way for rapid microbial identifications and characterization of microbial strains [38,39].

The right microbial population determines the efficiency of the process. The optimum moisture conditions to be maintained are in the range of 50–55%. The pH value should not be too acidic or too basic. The microbial population is sensitive to these changes. The pH near neutrality is preferable, and a minimum of 40% organic content must be present, while the C/N ratio is also important and should exist below 50 for rapid biodegradation. The temperature should be in the range of 65–70 °C [40]. It is to be noted that the use of chemometrics methods can help optimize the conditions for bioremediation and improve the efficiency of the degradation process [41,42]. By analysing and modelling the relationship between the input variables and the output variables, chemometrics methods can help identify the key factors, such as temperature, pH, and nutrient concentration, that affect the efficiency of bioremediation and optimize the conditions accordingly. This is conducted by monitoring the progress of the biodegradation process by analysing the complex data sets generated, such as the changes in microbial populations and production of the metabolites [43,44]. Some of the chemometric methods commonly used in the optimization of bioremediation conditions include the design of experiments (DoE), response surface methodology (RSM), artificial neural networks (ANN), principal component analysis (PCA), and genetic algorithms (GA) [45,46]. Based on the current trends in bioinformatics and data analytics, the applications of chemometrics in bioremediation may give more efficient and cost-effective solutions for the sustainable implementation of bioremediation plans.

The biodegradation process is said to be of two stages, the maturation stage {including the mesophilic phase (25–45 °C) and the thermophilic phase (>45 °C)} and the curing stage (second mesophilic phase). The process also mainly depends upon the mixing ratio because inappropriate mixing leads to the inhibition of target microorganisms [13]. These two-stage methods are widely used for petroleum contaminants. For post-diamond mining soil, open-state biodegradation was preferred to remediate the contaminated soil [22]. In another approach, in vessel reactors for the bioremediation of petroleum sludge were widely preferred for laboratory experiments [7,9,26]. A lab-scale bioreactor was used for treating the PWS obtained from a petroleum refinery with finished compost of around three kilograms and pre-inoculum as the bulking material [20,26]. The findings revealed that maximum degradation can be achieved by near neutral pH and the maximum degrading ability possessed by isolated species from PWS compared to indigenous microbes. It was reported that the optimum moisture range is 12–25%, and the biodegradation rate is directly proportional to temperature and pH [12]. Another in situ bioremediation process was carried out to degrade the contaminated olive mill waste (OMW) using biowaste and animal waste, along with vermicomposting techniques [29]. Their finding reveals that trapezoidal pile methods of vermicomposting are versatile enough to degrade phenol compounds. Similar observations were found from a bioremediation experiment in an evaporation pond using a novel microbial-fungal consortium isolated from OMW [11]. For the pyrene-contaminated soil, an additional 14 days in vessel method remediated was required apart from 60 days under the mesophilic and thermophilic conditions. The process degraded various emerging petroleum contaminations, including PAHs, anthracene, phenanthrene, fluorene, naphthalene, and acenaphthalene [13]. For a 30-day study, an open vessel method was employed by using cow manure and diamond mining soil and was found to remove up to 78% of contaminants [22]. Similarly, a static pile method for the substrate petroleum hydrocarbon and sewage sludge was also performed, and efficient results were obtained [23].

An in vessel method using matured compost as bulking material along with oily sludge in a bioreactor was found to degrade the TPHs successfully [26]. A bioremediation experiment using a cylindrical bioreactor with heavy oil sludge was reported where finished compost was made of food waste and green waste for four months [7]. Since the isolated micro-organisms or microbial consortiums must grow properly to inoculate in the bioreactors or piles or windrows, the method of inoculation depends upon the substrates, contaminants, and prevailing biogeochemical conditions [9]. Researchers also inoculated 0.5 Mcfarland isolate solution to the cylindrical bioreactor initially and continued the same bacterial inoculation after eight weeks [7]. Abtahi et al. (2020) [20] selected two bioreactors for petroleum biodegradation using 1.5×10^8 CFU/g dry mixture inoculum in it. Another study reported the usage of 40 L of produced inoculums (7×10^7 CFU/vol. of material) for the olive mill waste sludge biodegradation [11]. Petroleum hydrocarbon-contaminated soil, when inoculated with a mix ratio of microbial consortium, has four species: *Pseudomonas poae*, *Actinobacter bouvetii*, *Stenotrophomonas rhizophila*, and *P. rhizosphaerae* has resulted in significant biodegradation of hydrocarbons, indicating the significance of microbial consortia in place of single population type [31]. The inoculation medium details and culture conditions have been summarized in Table 3.

Table 3. This table summarizes medium and conditions for bioremediation assays.

Substrate (s)	Medium	Conditions	References
Oil sludge	Bushnell-Haas, 1% Kerosene	150 rpm shaking, 1 week at 35 °C	[26]
Heavy oil sludge	Bushnell-Haas, 1% Crude Oil	160 rpm shaking, 1 week at 30 °C	[7]
Oily waste sludge	Bushnell-Haas, 1% Crude Oil	160 rpm shaking, 1 week at 30 °C	[7]
Petroleum sludge	Bushnell-Haas, 1% Crude Oil	120 rpm shaking, 12 days at 30 °C	[9]
Petroleum sludge	Bushnell-Haas, 1% Crude Oil	120 rpm shaking, 12 days at 30 °C	[20]
Olive mill sludge	Remazol brilliant blue R (RBBR) plate count agar-tannic acid or potato dextrose agar-tannic acid	Incubation at 30 °C for 48 h (bacteria) and 96 h fungi	[11]

3. Metabolic Pathways for Hydrocarbon Degradation

Hydrocarbon degradation mechanism and metabolism by microbial population follow diversified pathways and thus make it a complex process. The major challenges in the degradation of petroleum hydrocarbons and PAHs are attributed to high hydrophobicity. The presence of both polar molecules, such as phosphates and alcohol derivatives etc. and non-polar residues, such as fatty acids, on biosurfactants, leads to enhanced molecular interactions with PAHs and hydrocarbons. Thus, the amphiphilic nature of biosurfactants and its surface moieties provides better interaction by reducing the surface tension and interfacial tensions [47,48]. The biosurfactant from the bacterial strain, *Bacillus methylotrophicus* decrease the surface tension of water by approximately 40% and was found to degrade 92% of crude oil [49]. The biosurfactant from another *Bacillus* strain has shown improved solubilization and emulsification of oil sludge and enhanced bioavailability and biodegradation [50]. Thus, increasing the solubility and bioavailability of PAHs contribute to enhanced degradation of the compounds. The efficacy of a biosurfactant depends on multiple factors, such as bioavailability, reduced surface tensions, oxygen content or availability, nutrient availability, etc. [51–53]. Further, the major pathways by aerobic and anaerobic microbial activities include enzyme activation followed by catalysis. An implicit understanding of these mechanisms is a prerequisite for designing strategies for an efficient bioremediation process. A schematic representation of probable paths of degradation of major hydrocarbon contaminants is provided in Figures 3–8 (summarized from Refs. [35,37–40,54–56]). The important metabolic points and the sequence of the release of biproducts and endproducts of metabolism as also mentioned.

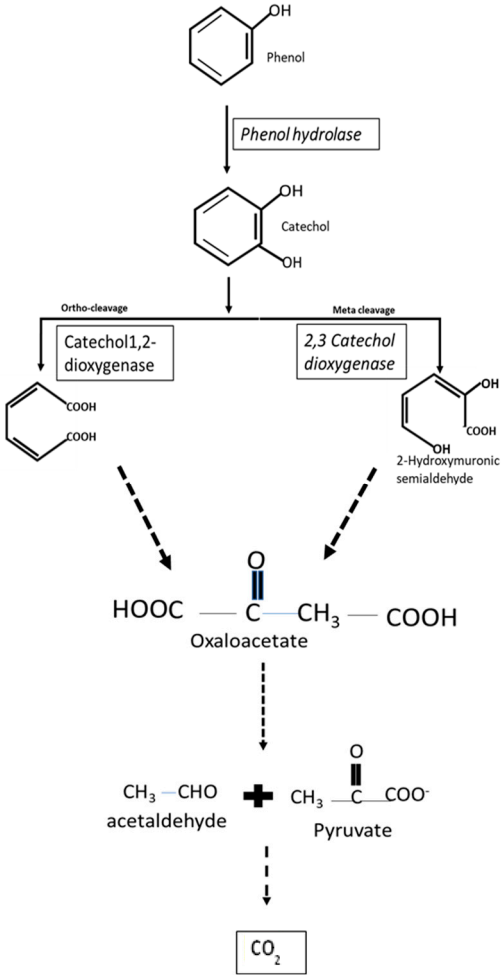

Figure 3. Degradation pathway of phenol.

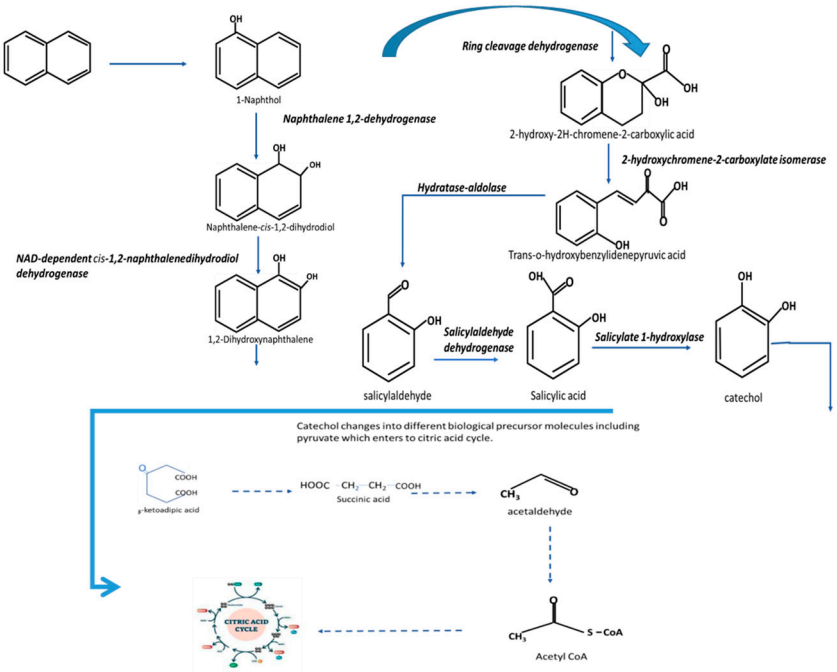

Figure 4. Degradation pathway of naphthalene.

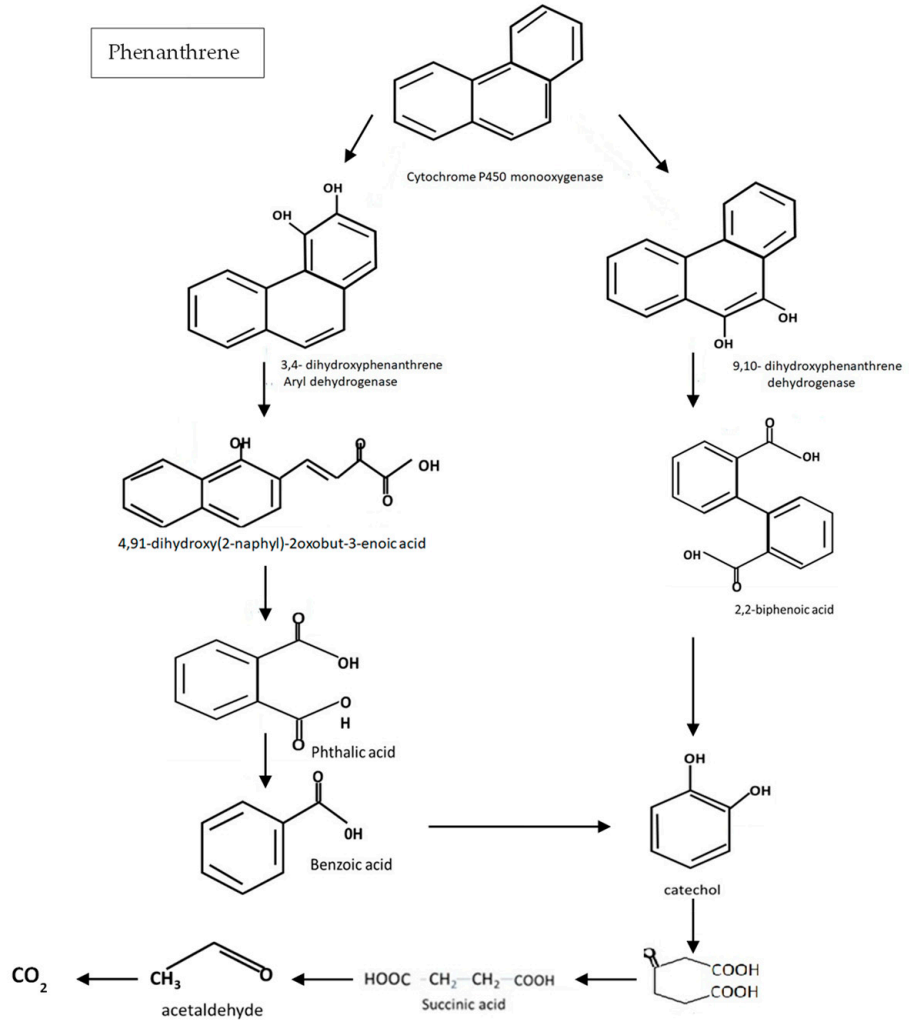

Figure 5. Degradation pathway of phenanthrene.

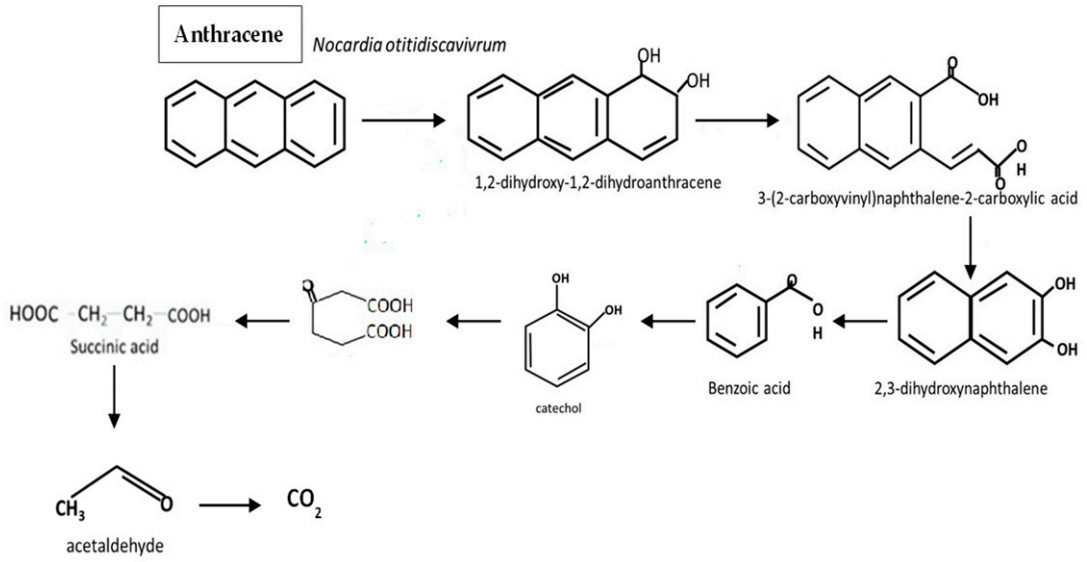

Figure 6. Degradation pathway of Anthracene.

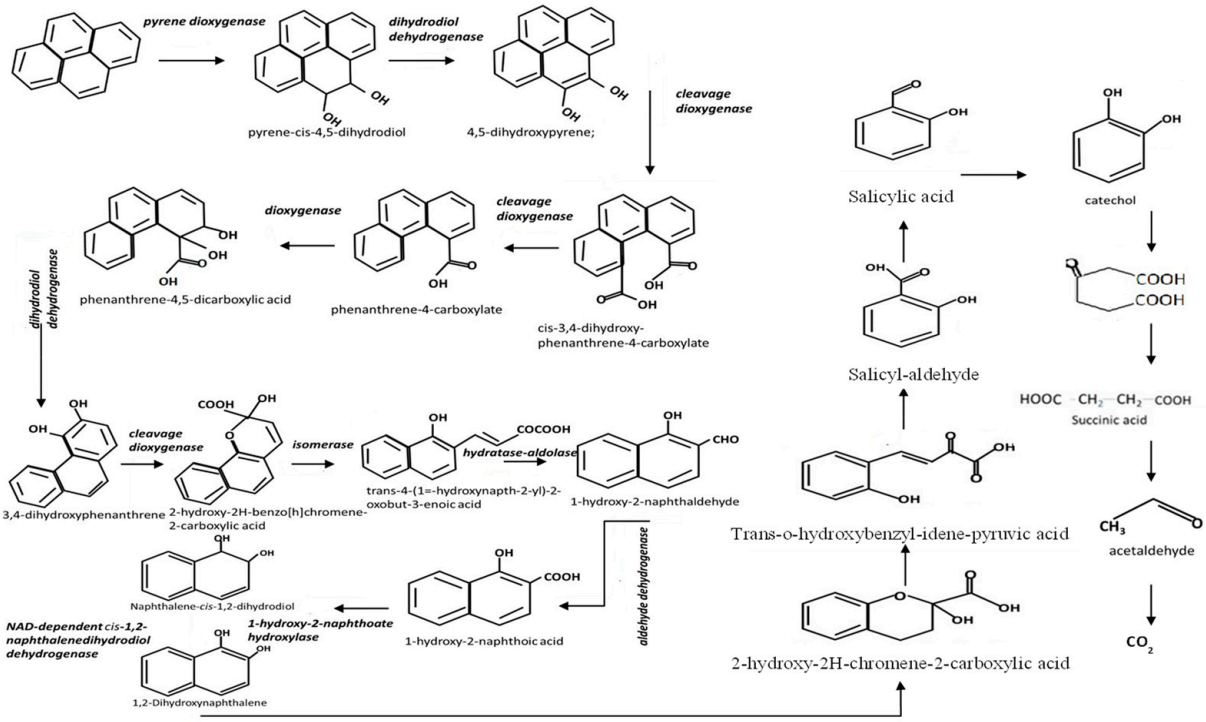

Figure 7. Degradation pathway of pyrene.

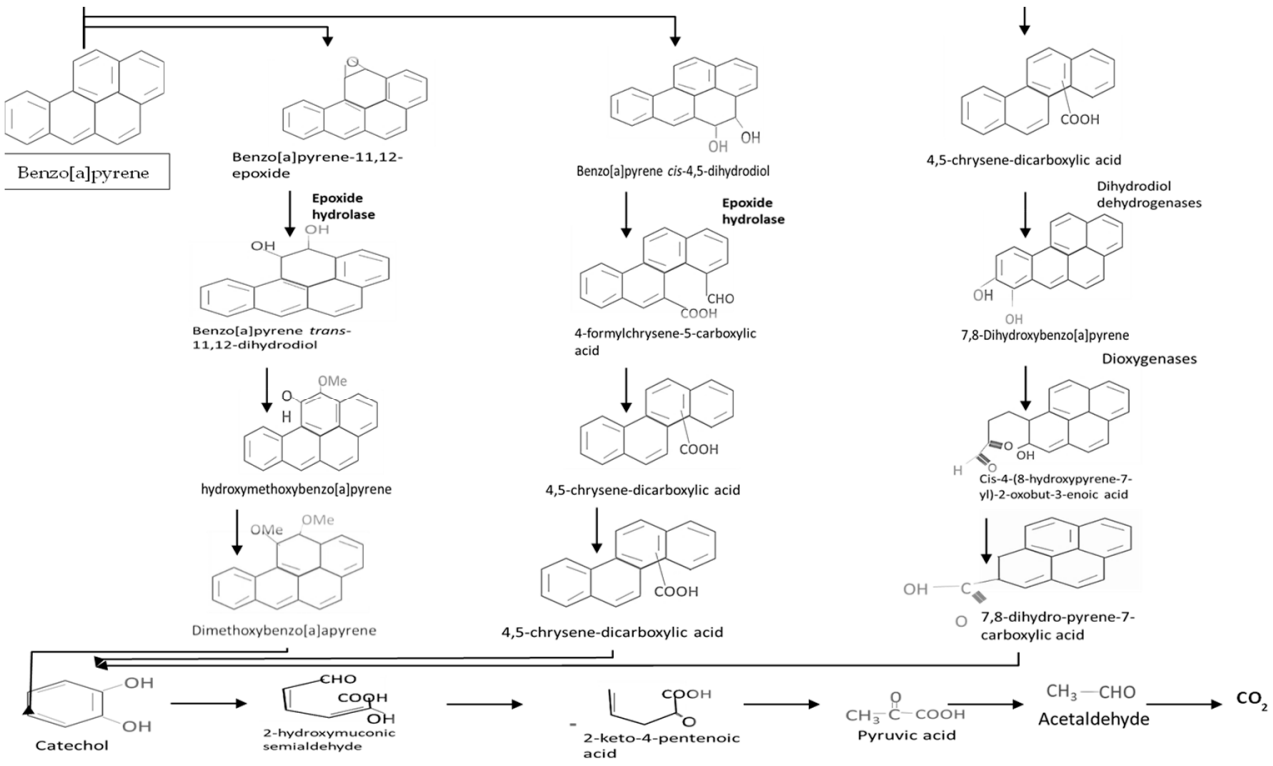

Figure 8. Degradation pathway of benzopyrene.

3.1. Major Intermediates and Biproducts

In general, the degradation of alkane compounds by bacteria follows three categories based on aliphatic hydrocarbon: low molecular weight (C8–C16), medium molecular weight (C17–C28), and high molecular weight (C29–C35) [24,32]. These alkane compounds

are initially activated by enzymes, and an oxidation process is carried out by monooxygenase and dioxygenase, which finally broken down into alcohol, acid or carbon dioxide as end products. The double bond compounds alkenes are more sensitive and higher reactive. Oxygen is accessed by bacteria using the monooxygenase process and the probable product is epoxide. In branched chain alkanes, the oxidation resulted in hydroxy acids and dioic acids, and the possible final product is a mono or dicarboxylic acid. Adipic acid might be the expected degraded product due to the oxidation of cycloaliphatic compounds by bacteria. On the other hand, the anaerobic bacteria degrade the hydrocarbon compounds using anaerobic respiration by nitrate compounds, nitrite and nitrous oxide, sulphate, thiosulphate, carbonate, and metal ions or through fermentation process or anoxygenic phototrophic reactions. By using a fumarate addition reaction, possible anaerobic hydrocarbon biodegradation takes place. It can be observed that the major biodegradation processes associated with hydrocarbon metabolism are the oxygen-independent hydroxylation process, carboxylation process, saturated bond hydration process, reverse methanogenesis process, and a few of the anaerobic fermentation processes [3,43,57,58].

The degradation of hydrocarbons by microbes involves a range of enzymes capable of breaking down complex hydrocarbons into simpler compounds that can be metabolized by the microbial cells. They are essential biological catalysts that accelerate biochemical reactions by reducing the activation energy required for the reaction to occur. Enzymes play a vital role in the degradation of biomolecules, such as carbohydrates, lipids, and proteins. The degradation of these biomolecules is necessary to provide the cell with energy, recycle cellular components, and eliminate waste products. Based on the characteristic structure of the substrates, they can be divided as (i) carbohydrate degrading enzymes (e.g., amylase, cellulase, and pectinase), (ii) lipid degrading enzymes (e.g., lipase and phospholipase), and (iii) protein-degrading enzymes (e.g., protease and peptidase). Based on the functional features, they are classified as oxygenase, hydrolase, dehydrogenase, decarboxylase, isomerase, and esterase [58–63]. In essence, the specific enzymes involved in hydrocarbon degradation will vary depending on the type of hydrocarbon and the specific microbial community involved in the process.

3.2. Mechanisms Used by Microorganisms to Enhance Degradation of Hydrocarbons

Most of the hydrocarbon-degrading microbes produce surfactant compounds to emulsify the hydrocarbon molecules to droplets or micelles, and that is again taken back by microorganisms. The most common role of such biosurfactants is to enhance the scattering of contaminants in the aqueous phase and intensification of the bioavailability of the hydrophobic substrate to microorganisms, with subsequent removal of contaminants through biodegradation. It is reported that *Candida sphaerica* (75% to 92% hydrocarbon removal rate) [7], *Candida tropicalis* (78% to 97% hydrocarbon removal rate) [8], and *Candida glabrata* UCP1002 (up to 92.6% hydrocarbon removal rate) [9] can remove oil spills, hydrocarbon from contaminated land or seawater by using a biosurfactant, such as a protein-carbohydrate-lipid complex or sophorolipids. Other microorganism-based biosurfactants, such as glucolipid, trehalose lipid, rhamnolipid, lipopeptide, glycolipid, etc. are also capable of removing the organic contaminants, as mentioned in Table 4. This table offers a list of diverse types of biosurfactants and their producing microorganisms with potential applications in the bioremediation of oil-polluted environments. The role of microbes is very important for understanding the mechanisms of action during a metabolic process [54,64,65]. These correlations help to decipher the metabolic linkages and the possible target sites to control the rate of reaction [14,55,56]. The hydrocarbon biodegradation process by microbial activities with the aid of non-biological agents is more complicated in nature and needs more intensive investigations to decode the details of the complete mechanism [47–51,66].

Table 4. Comparative evaluation of commercial microbial products for bioremediation.

Product Name	Function	Source	Significance of Microbes	Cost of Remediation ($ per acre)	Reference
BioSpill	Biodegradation of hydrocarbons	*Bacillus* sp.	*Bacillus* sp. can degrade a wide range of hydrocarbons, including crude oil, gasoline, and diesel.	100–500	[67]
Bio-Solve Pink	Biodegradation of petroleum hydrocarbons	*Pseudomonas* sp. and other bacterial strains	*Pseudomonas* sp. is known to degrade petroleum hydrocarbons efficiently and has been widely used in bioremediation.	200–1000	[68]
Petrox	Biodegradation of hydrocarbons and other pollutants	Mixed culture of microorganisms	The mixed culture of microorganisms can degrade a wide range of pollutants, including crude oil, gasoline, and diesel.	1000–5000	[69]
Nualgi	Biostimulation of indigenous microbes	Diatomaceous earth and micronutrients	Nualgi provides micronutrients to the indigenous microbial population to enhance their hydrocarbon-degrading abilities.	100–500	[70]
Oilgone	Biodegradation of hydrocarbons	*Bacillus* sp. and other bacterial strains	*Bacillus* sp. is known to degrade hydrocarbons efficiently and has been widely used in bioremediation. Oilgone contains a blend of bacterial strains.	500–2000	[71]

4. Mechanistic Insights of Biodegradation of Phenolic Compounds PAH Pollutants

4.1. Biodegradation of Phenolic Compounds

Biodegradation of phenolic compounds is an effective method to protect the global environment as they are widely present in industrial effluents and cause adverse effects on animal lives, marine lives, and humans. Phenol is also the end product of the degradation of various benzene conjugate compounds. Phenol hydrolase breaks down or converts phenol to catechol, which is acted upon by dioxygenase (catechol 1, 2 dioxygenases and 2, 3 catechol dioxygenases) to form semialdehyde forms. This is associated with ortho- and meta-cleavage of the catechol. These forms are further oxidized to oxaloacetate, which is hydrolyzed to acetaldehyde and pyruvate. These end-products can be metabolized to degrade it to the simplest form, thus completing the path of the degradation of the phenol (Figure 3).

4.2. Biodegradation of Naphthalene

Naphthalene is biodegraded to 1-Naphthol, which is the substrate for naphthalene 1,2 dehydrogenase. This enzyme does hydroxylation of 1-Naphthol to Naphthalene-cis-1,2-dihydrodiol, which is then acted upon by another dehydrogenase to form 1,2-Dihydroxynaphthalene. Then ring cleavage occurs with the help of an enzyme called 2-Hydroxychromene-2-carboxylate isomerase to form Trans-o-hydroxy-benzylidene pyruvic acid, which is acted upon by hydratase aldolase. This aldolase action yields salicylaldehyde. Another enzyme is expected to oxidize salicylaldehyde to salicylic acid. Salicylic acid can act as a substrate for Salicylate 1-hydroxylase, which does hydroxylation and release carbon from the molecule to form catechol. Catechol is an important point of the metabolism of hydrocarbons, and it gets converted into different biological precursors, including ketoadipic acid and pyruvate, which can be processed to simplest forms via the citric acid cycle (Figure 4).

4.3. Biodegradation of Phenanthrene

Phenanthrene is a complex conjugated ring compound, which is acted upon by cytochrome P450 monooxygenase to produce two products 3,4-dihydroxyphenanthrene and 9,10-dihydroxyphenanthrene. Out of these, the 3,4-dihydroxyphenanthrene is utilized by an enzyme called aryl dehydrogenase to form 4-9-dihydroxy(2-naphyhyl))-2-oxobut-3-enoic acid, which can then be acted upon by dioxygenase or hydratase or aldolase to produce phthalic acid, and further oxidized to benzoic acid. The benzoic acid can then be converted into catechol. In the second path of the metabolism of 9,10-dihydroxyphenanthrene, dehydrogenase has a major role which acts on 9,10-dihydroxyphenanthrene to produce 2-biphenoic acid to produce catechol. Hence, it can be observed that the generation of the catechol is unique in the degradation of phenanthrene compared to the other phenolic compounds. After catechol formation in the degradation process, there is the formation of cis-muconic acid, which can be converted to ß-Ketoadipic acid. This further can be transformed to succinic acid and then to Acetyl CoA for final assimilation and CO_2 release, thus completing the biodegradation of the phenanthrene (Figure 5).

4.4. Biodegradation of Anthracene

Anthracene degradation is very similar to that of phenanthrene in terms of the production of intermediate compounds. First, Anthracene is acted upon by cytochrome P-450 monooxygenase to form 1,2-dihydroxy-1,2-dihydroanthracene, which can be modified to 3-(2-carboxyvinyl) naphthalene-2-carboxylic acid. This is further oxidized to 2,3-dihydroxynaphthalene and further forms benzoic acid. The benzoic acid is then converted to produce catechol. After this, there is the formation of cis-muconic acid, which can be converted to ß-Ketoadipic acid. This further can be transformed to succinic acid and then to Acetyl CoA for final assimilation and CO_2 release, thus competing with the biodegradation of the anthracene (Figure 6).

4.5. Biodegradation of Pyrene

Pyrene is a complex ringed structure, which is oxidized by pyrene dioxygenase to form pyrene-cis-4,5-dihydrodiol. Then, an enzyme dihydrodiol dehydrogenase can act on the product to form 4,5-dihydroxypyrene, which undergoes cleavage to yield cis-3,4-dihydroxy-phenanthrene-4-carboxylate, which subsequently can undergo cleavage step wise step via phenanthrene-4-carboxylate, and phenanthrene-4,5-dicarboxylic acid. The phenanthrene-4,5-dicarboxylic acid can be acted upon by dihydrodiol dehydrogenase to form 3,4-dihydroxyphenanthrene. Then further ring cleavage happens to form 2-hydroxy-2H-benzo[h]chromene-2-carboxylic acid. Then isomerase acts and forms trans-4-(1=-hydroxynapth-2-yl)-2-oxobut-3-enoic acid. Then hydratase-aldolase acts to form 1-hydroxy-2-naphthaldehyde. Further, 1-hydroxy-2-naphthoic acid is formed by oxidation with the help of the enzyme aldehyde dehydrogenase. The enzyme 1-hydroxy-2-naphthoate hydroxylase acts to form naphthalene-cis-1,2-dihydrodiol, which becomes a substrate for NAD-dependent cis-1,2-naphthalenedihydrodiol dehydrogenase. This enzyme can form 1,2-dihydroxynaphthalene, which can be degraded to simplest forms via 2-hydroxy-2H-chromene-2-carboxylic acid and Trans-o-hydroxy benzylidene pyruvic acid. Then during this oxidation process there occurs the formation of salicylaldehyde which can be oxidized by salicylaldehyde dehydrogenase to salicylic acid, further leading to the formation of catechol by the activity of enzyme salicylate 1-hydroxylase. Then catechol formation can happen, which can be degraded to acetyl CoA via succinic acid (Figure 7).

4.6. Biodegradation of Benzopyrene

Biodegradation of Benzopyrene can occur in different ways. Benzopyrene can be broken down into Benzo[a]pyrene-11,12-epoxide, Benzo[a]pyrene trans-11,12-dihydrodiol by the activity of epoxide hydrolases and dihydrodiol dehydrogenases which acts on different conjugate rings to make it open and subsequently degrading it to simpler forms, such as hydroxymethoxybenzo[a]pyrene and dimethoxybenzo[a]apyrene. All these pathways

lead to the formation of intermediate molecule catechol which is further broken down into 2-hydroxymuconic semialdehyde, then to 2-keto-4-pentenoic acid, and finally forming pyruvic acid by citric acid cycle enzymes or related mechanisms (Figure 8).

5. Challenges in Bioremediation Process and Future Perspectives

The petroleum hydrocarbon pollutants are very stable and are not easy to degrade, persist for longer periods, thus damaging the ecosystem and associated lives. The biodegradability of hydrocarbons is also challenging because of their non-bioavailability to microbes owing to their hydrophobicity and insolubility in water. Bioremediation is one of the useful, cost-effective, and sustainable techniques to degrade these contaminants. Various research outputs have validated this perspective. The significant micro-organisms responsible for efficient degradation include *Pseudomonas* sp. *Micrococcus*, *Nocardiopsis*, *Bacillus* sp., *Acinetobacter radioresistens*, *Enterobacter hor-maechei* strain KA6, *Aspergillus ochraceus*, *Scedosporium apiospermum*, etc. [56,72–75]. As the type of contaminants may determine the biodegradation period, the bioremediation can be performed either as in situ, ex situ, or in vessel method. Many researchers reported experiments using small-scale bioreactors. These results should be corroborated with the large-scale or industrial-scale experiment. The most widely used medium to isolate petroleum-degrading microbes is Bushnell-Haas (BH) with 1% crude oil or kerosene, and the most widely used inoculation process is 0.5 McFarland isolate solution. The experimental design is very significant as the best combination of substrates and micro-organisms can remove TPHs up to 90% [7–9,22].

Further, the efficiency of the bioremediation highly depends upon the selected substrates, mix-ratio, prevailing biogeochemical transformation in the field, microbial type, population, and other physical parameters. It is reported that the bioremediation approach needs improvements for all emerging pollutants [21]. Therefore, along with pre-treatment, chemical or engineering treatment is also required. However, this must be negotiated with the cost of the implementation plan. In addition, bioremediation experiments performed in small lab-scale volumes with limited capacity need to be scaled up to larger volumes and should be validated in the field. The researchers must use numerical and other simulations to identify the potential efficiency of the process [74].

The cost of remediation can vary based on factors such as the site location, the extent of contamination, and the type of treatment method used. In general, bioaugmentation tends to be more expensive than biostimulation, but it may be necessary in cases where indigenous microbes are unable to degrade the hydrocarbons on their own. It is important to note that there are many commercial microbial products available for hydrocarbon bioremediation, and their efficacy and cost can vary depending on the specific product and site (Table 4). Additionally, the use of microbial products should be accompanied by a thorough understanding of the site conditions, the potential risks, and benefits of using the products.

In the context of bioremediation, immobilized enzymes on iron oxide surfaces can be used to catalyze the degradation of hydrocarbons and other pollutants. The iron oxide surface can act as a support material for the enzyme, providing a stable environment and improving the efficiency of the reaction [76]. Additionally, the immobilized enzyme can be easily separated from the reaction mixture, allowing for easy removal of the pollutant as well as for recycling of the enzyme [77]. Overall, the immobilization of enzymes on inorganic materials, such as iron oxides, is a promising approach for improving enzyme stability, activity, selectivity, and reducing costs in biotechnology and bioremediation applications [78,79]. The advanced approach includes modified enzymes and microbial adsorption methods to enhance the oxidation potential of the bioremediation approach.

The prospective integrated approach is useful to specifically increase the rate of bioremediation by various modifications, including site-specific mutations [66,80–82]. The functionality of the enzymes is dependent on their binding and accessibility to the molecules. The rate of hydrolysis is also influenced by molecular interactions between catalytic amino acid residues and the ligand molecules in the active sites [15,39,83]. The process and the oxi-

dation conditions can be modified, as discussed in previous sections. The monooxygenases and hydrolases can improve the oxidation potential of the process in the bioremediation of hydrocarbons, such as phenols, pyrenes, benzopyrene, phenanthrene, and naphthalene and its derivatives by site-specific mutations at active site residues [35,84–87]. For example, the targeted mutation in the 258th residue of the dioxygenase, associated with nitrotoluene degradation, has increased the rate of biodegradation [1]. The modification of Benzyl Succinate Synthase (bssA) gene has been reported to enhance the degradation of toluene and xylene [57,88]. The change in the binding process and introduction of the immobilized enzymes have been reported to enhance the rate of biodegradation [5,74,89,90]. Each modification can alter the course of the enzymatic reaction and, hence, can influence the rate of bioremediation and the effectiveness of the approach adopted [72,73]. This low-cost bioremediation approach would be the better solution to conserve the natural resources and ecosystem for sustainable development.

6. Conclusions

The eco-sustainable bioremediation approach is important for the treatment of petroleum pollutants, hydrocarbon wastes, and spills. The next-generation approaches, including the modification of enzymes and microbes, and microbial adsorption methods to enhance the bioremediation potential need to be scaled up for field implementation. The proposed integrated approach is intended to specifically increase the rate of bioremediation, including site-specific modifications in the active site of the enzyme(s) or recombinant microbial strain(s). An understanding of the mechanistic details paves the way for modification of the metabolic pathway for enhancing the reaction rates. The changes in the binding process and introduction of the immobilized enzymes are expected to enhance biodegradation in many folds. The rate of bioremediation can, thus, be enhanced by using advanced recombinant tools and strategies. Further, a deep understanding of the modes of action by microbial activities provides novel insights about the target sites and mechanistic enzymatic steps, which can be explored for enhancement of the rate of biodegradation. The amalgamation of the biological and non-biological approaches for the treatment of hydrocarbon pollutants should also be translated with cost-effective considerations. Therefore, there is a constant need for investigation and improvements of bioremediation methods for the cleanup of sites contaminated by hydrocarbon pollutants towards an efficient eco-sustainable development.

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