

The biochemical mechanisms of petroleum degradation by bacteria

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Abstract - Certain bacteria plays a key biochemical role in ecosystem functioning through organic matter transformation, decomposition, nutrient cycling, soil structure maintenance and the vast majority of biogeochemical processes in both aquatic and terrestrial habitat. Petroleum hydrocarbons are complex organic compounds made up of carbon and hydrogen and are known to be the most persistent organic pollutants affecting the ecosystems. In petroleum polluted areas, bacteria such as the hydrocarbon utilizing bacteria (HUB) has an intrinsic biochemical mechanisms of degrading petroleum hydrocarbons as their source of carbon and for other cellular activities. Fermentation bacteria, sulphate reducing bacteria, methanogenic achaea and Iron III reducing bacteria are the common groups of HUB. The intrinsic biochemical mechanisms exploit by bacteria to degrade petroleum hydrocarbons are by carrying out an enzymatic activation/functionalization reactions on the petroleum hydrocarbons to produce metabolites that are beneficial to their existence or by producing biosurfactants to enhance petroleum hydrocarbon degradation. Aerobic and anaerobic pathways are the major metabolic pathways utilize by HUB to degrade petroleum hydrocarbons. The intrinsic biomechanistic steps are sequenced first by an enzymatic activation/functionalization reactions to initiate an intracellular attack on the petroleum hydrocarbon by oxygenation, addition, hydroxylation or carboxylation reaction depending whether it is carried out under oxic or anoxic condition; next is the peripheral degradation pathway converting the activated organic pollutants into a limited number of carbon intermediates that are further biotransformed by a few central pathways to an intermediates of a central metabolism of a cell; and terminal oxidation of the intermediate to molecules such as CO₂ that are used as their source of carbon and for other cellular activities. The biochemical changes on the physiochemical properties of petroleum hydrocarbons due to bacterial degradation produces metabolites that are less toxic and can be used by bacteria and other living organisms in the environment for biological activities. A keen insight on the degradation mechanisms of petroleum hydrocarbons by bacteria is essential for effective development of bioremediation strategy in salvaging petroleum polluted areas, hence, the focus of this review.

Keywords: Biodegradation, Bioremediation, Biosurfactants, Hydrocarbon utilizing bacteria, Microorganism, Monooxygenase, Petroleum hydrocarbon.

Introduction

The world has never before seen or experience increase in the exploration and usage of petroleum products during the last century as it is of today. Pollution with petroleum and its products are considered a major problem worldwide due to their impact on the ecosystem [1]. Petroleum hydrocarbons are considered to be the most common group of persistent organic pollutants and are known to be toxic to many living organisms and the environment [2]. Moreover, it has been shown that petroleum hydrocarbons causes significant losses in soil quality due to their toxicity towards biological processes catalyzed by soil microorganisms [3]. The increase in demand for petroleum as a source of energy and a primary raw material for chemical industries in recent years has resulted in an increase in world production. This dramatic increase in production, refining and distribution of crude oil has brought with it an ever increasing problem such as terrestrial and aquatic pollution [1]. Petroleum contamination resulting from leaking above ground and underground storage tanks, spillage during transport of petroleum products, abandoned manufactured gas sites and various industrial processes is hazardous to soil and water ecosystems, and is expensive to remediate [4]. These together are threatening the lives of animals and native microbiological population in land, air, water surfaces and sub surfaces [5].

In the past decades, it is not a rare instance that a regional disease is associated with local soil or underground water contamination, such as the famous "itai-itai disease" as a result of long term cadmium intake from excessive cadmium accumulated rice and "cancer villages" located near industrial areas in developing countries [6], [7]. Human activities are the main source of significant petroleum hydrocarbon release to the environment [8].

Microbes are responsible for more than 80-90% of all the soil processes such as nutrient cycling, organic matter transformation, and maintenance of soil structure, with more than 90% of the energy flow in soil passing through microbial decomposers [9], [10]. Soil as a major component of the environment is inhabited by a wide range of microorganisms, including bacteria, fungi, algae, viruses and protozoa. Bacteria in the soil are the primary digestive system of the soil and their activity is responsible for almost 90% of all biological and chemical actions [11].

Biodegradation of petroleum hydrocarbons by natural population of microorganisms (e.g. bacteria) represents one of the primary mechanisms of eliminating petroleum pollution from the environment [12]. Petroleum hydrocarbons can be degraded by microorganisms such as bacteria, fungi, yeast and microalgae [13], [14]. However, the ability to degrade and/or utilize petroleum hydrocarbons substrates is exhibited by a wide range of bacteria [4], [15]. A greater degradation of petroleum hydrocarbon pollutants is carried out in situ by consortium of microorganism, that is, the use of multiple microbial species together rather than relying on catabolic capacity of any single species [16], [17] and more than 200 species of bacteria can biodegrade petroleum hydrocarbons [18]. The various genera that have been reported to contain hydrocarbon degrading species include; *Pseudomonas*, *Nocardia*, *Vibrio*, *Corynebacterium*, *Candida*, *Arthrobacter*, *Rhodotorula*, *Brevibacterium*, *Flavobacterium*, *Sporobolomyces*, *Achromobacter*, *Bacillus*, *Aeromonas*, *Thiobacillus*, *Acinetobacter*, *Lactobacter*, *Staphylococcus*, *Penicillium*, *Articulosporium*, *Halomonas*, *Klebsiella*, *Proteus*, *Aspergillus*, *Micrococcus*, *Neurospora*, *Rhizopus*, *Mucor*, *Trichoderma*. These organisms have been isolated in large numbers from many oil polluted waters and soils, but are found in less numbers in uncontaminated environment [19], [20]. Microbial species differ in their degradation capabilities and mechanisms. Biochemically, HUB utilize aerobic and anaerobic degradation pathway in transforming the petroleum organic pollutants into their source of carbon and energy for cellular activities.

Overview on Petroleum Products

Petroleum products such as gasoline, kerosene, diesel/fuel oil, crude oil are complex mixture of organic compounds basically of paraffinic, olefinic and aromatic hydrocarbons. Petroleum hydrocarbons can be divided into four classes; (1) The Saturates (aliphatics); (2) Aromatics; (3) Asphaltenes (phenols, fatty acids, ketones, esters, and porphyrins); (4) The resins (pyridines, quinolines, carbazoles, sulfoxides, and amides) [21], [22]. Petroleum in its natural state is called crude oil. It is used as raw material in numerous industries, including the refinery-petrochemical industry, where crude oil is refined through various technological processes into consumer products such as gasoline, oils, paraffin oils, lubricants, asphalt, domestic fuel oil, vaseline, and polymers [23].

Crude oil is formed by biological, chemical, and geochemical transformations of organic matter accumulated in favorable locations. In the first stage crude oil is transformed during sediment diagenesis at moderate temperatures up to 50°C. Due to defunctionalization and condensation, kerogen which is immature crude oil is formed. Kerogen accumulations are considered to be the richest coal accumulation on earth [24]. Based on geochemical studies, immature crude oil contains higher volume of hydrocarbons with an odd number of carbon atoms. This fact has a practical meaning in determining the Carbon Preference Index (CPI) [23]. The composition of oil varies both between sources and in source itself during years. Therefore, it is not possible to give an exact composition of oil in general. One can say that, Crude oil is a mixture of thousands of various compounds, organic and inorganic including aliphatic and aromatic hydrocarbons which in average reaches 75 % of its content, as well as resins and asphalts [25].

Hydrocarbons (HCs) are organic compounds which are composed of two main elements - carbon and hydrogen. They also contain small quantities of molecules containing sulphur, nitrogen, metals and oxygen. Because of the lack of functional groups, hydrocarbons are largely apolar and exhibit low chemical reactivity at room temperature [5], [26]. HCs in crude petroleum can be classified as alkanes, cycloalkanes, aromatics, polycyclic aromatics, asphaltines, and resins. Among the petroleum HCs, n-alkanes are the most amenable to biodegradation. Normally alkanes in the range of C5 to C10 are inhibitory to majority of the HC degraders at higher concentration as they disrupt lipid membrane when present as solvent. During degradation, alkanes are converted to alcohol by the action of oxygenase enzymes that attack the terminal methyl group. The alcohol is further oxidized to aldehyde and then to fatty acids. Further utilization of fatty acid occurs by β -oxidation of aliphatic chain [27].

The cycloalkanes or alicyclic HCs are less degradable than alkanes. Here the biodegradability decreases with increase in number of ring structures. Alkyl substituted cycloalkanes can be degraded more easily as compared to non-substituted HCs. Cycloalkanes are degraded to cyclic alcohol by the action of oxidases which is further dehydrogenated to ketone. The primary products of metabolism of cycloalkanes are cycloketones and cycloalkane-carboxylic acids [27].

Aromatic hydrocarbons molecules have benzene-based structure. As compared to most other cyclic compounds, aromatic compounds are more stable because of sharing of delocalized electrons by pi bonds. BTEX (benzene, toluene, ethylbenzene, xylene) compounds are comparatively more mobile and water miscible [27]. Polycyclic aromatic hydrocarbon (PAH) or polynuclear aromatic hydrocarbon (PNA) are group of compounds consisting of two or more benzene rings. Some of these compounds are suspected to be carcinogens. Increase in molecular weight and number of ring structures of PAHs decreases their solubility and volatility while increasing adsorption capacity. PAHs are degraded by mechanism similar to that of mono-aromatic compounds [28].

Non-hydrocarbon compounds include Sulphur compounds (0.01–8 %), mainly as hydrogen sulfide (H₂S), mercaptans (compounds containing the –SH group), sulfides and disulfides, thiophenes as well as benzothiophenes and naphthothiophenes that prevail in oil fractions. These compounds are unfavorable due to their chemical recalcitrance, therefore their presence is considered in evaluating crude oil quality [23], [29].

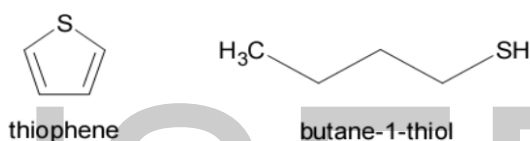


Fig. 1: Chemical structures of thiophene and 1-butanethiol (methyl mercaptan).

Nitrogen compounds occurs in crude oil at the level of 0.01–2 % weight, although over 10 % concentrations have been noted. The first group includes pyridines, acridines, and quinolines, and the second group comprises pyrroles, carbazoles, indoles, and heterocyclic compounds. Similarly, nitrogen also occurs as sulphur bonds, compounds from this group are concentrated in high-boiling fractions [29].

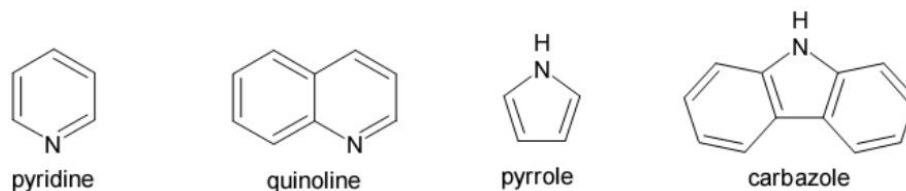


Fig. 2: Chemical structures of pyridine, qinoline, pyrrol, and carbazol.

Oxygen compounds include phenols, carboxylic acids (having the COOH functional groups), furans, and alcohols occur in the heavy fractions of crude oil [29].



Fig. 3: Structures of phenol, furan, and cyclohexanol.

Porphyryns often occur in crude oil. They are products of degradation of dyes produced by living organisms. They are composed of pyrrole rings connected by methine bridges. They often form chelate compounds of nickel, vanadium, and other metals. There

is a relationship between the maturity of crude oil and the concentration of porphyrins, which decreases with oil maturity. Oil formed from marine organisms contains more vanadium than nickel porphyrins [29].

Resin-asphalt substances are present in crude oil, particularly with a low degree of maturity. They have a very complex chemical structure and include most heteroatoms, trace elements, and polycyclic aromatic hydrocarbons. They contain in average up to 80% C, 10% H, and 14% heteroatoms in which 1-2% correspond to metal-organic compounds [23]. Asphaltines and resins are high molecular weight compounds containing nitrogen, sulphur, and oxygen in their structure. These compounds are recalcitrant to biodegradation as they are highly insoluble and consist of functional groups that are shielded from microbial attack by extensive aromatic ring structure [27].

Petroleum Hydrocarbon Degrading Bacteria

Biochemically, bacteria are the most active microbes in petroleum degradation operating as the primary degraders of spilled oil in environment. ZoBell [30] reported that nearly 100 species of bacteria representing 30 microbial genera had hydrocarbon oxidizing properties. Many of these organisms are more effective in form of consortium. A combination of bacterial strain with broad enzymatic capabilities are more effective in biodegradation of crude oil [31].

Hydrocarbon utilizing bacteria (HUB) are groups of bacteria with inherent potentials of degrading petroleum hydrocarbons. They carry out these processes by utilizing the petroleum hydrocarbon as their source of carbon and energy for cellular activities. The petroleum degrading bacteria can be grouped based on their biological activities into fermentation bacteria; sulphate reducing bacteria; methanogenic archaea; and Iron III reducing bacterial.

1. Fermentation Bacteria: Numerous species of fermentation bacteria have been detected in crude oil. Electron donors for these microorganisms may be sugars, proteins, H₂, CO₂, and hydrocarbons. The products of metabolic reactions are organic acids and gases such as H₂ and CO₂, which may cause increase of reservoir pressure [32].

Mesophilic fermentation bacteria are more uncommon than the thermophilic ones. Mesophilic fermentation bacteria are made up of haloanaerobes as Haloanaerobium acetoehtylicum, H. congolense, and H. salsugo that produce acetate or ethanol in the process of carbohydrate fermentation. These microorganisms differ also in the type of substrates used and their tolerance to salt content (up to 10%). For example, Spirochaeta smaragdinae isolated from a Congo oil field prefers salt contents of up to 5%. Thermophilic microorganisms contain thermostable enzymes that are capable of enduring temperatures exceeding even 100°C. This group of microorganisms belong species of Thermotoga: T. subterranean, T. elfii, and T. hypogea, which are capable of reducing thiosulfate to sulfides, as well as bacteria resembling Thermotoga that reduce elemental sulfur [23].

2. Sulfate Reducing Bacteria (SRB): SRB are heterotrophic organisms, an absolute anaerobes that uses sulfates as well as other oxygenated sulfur compounds (sulfites, thiosulfites, trithionate, tetrathionate, and elemental sulfur) as final electron acceptors in respiration processes [33], [34]. All SRB are gram negative with the exception of the species of Desulfonema. This group of bacteria is very diverse and depending on the soil and water composition, different kinds of bacteria can be found within this group such as psychro-, meso- and thermophilic, halo- and barophilic. The most common mesophilic SRB causing detrimental effects on drilling equipment and oil storage vessels include Desulfovibrio longus, D. vietnamensis, and D. gabonensis. These species incompletely oxidize organic compounds to acetate and use energy from the oxidation of hydrogen, lactate, and pyruvic acid. Thermophilic SRB are mainly responsible for processes of in-situ oil transformation. An important genus from this group is the Desulfotomaculum bacteria [23]. Pure strains of SRB capable of complete oxygenation of some hydrocarbons such as alkanes, xylenes, toluene, and naphthalene to CO₂ were isolated in the 1990s [35], [36].

3. Methanogenic Archaea (MA): Methanogenic archaea bacteria are important group of microorganisms occurring in crude oil reservoir settings. The product of their activity is methane, therefore the biological activity of these microorganisms is measured

by the methane production rate or by the volume of methane produced [32], [37]. MA bacterial development and activity is influenced by physical and chemical factors such as temperature, salt content, and pH. Most methanogenes are mesophilic organisms, although extremophiles are also present. Methanopyrus kandleri can occur at temperature of 110°C [38], [39].

4. Iron III reducing bacteria: Shewanella putrefaciens is an iron reducing bacteria that also has the ability to reduce elemental sulfur, sulfites, and thiosulfates to sulfides. This bacteria is capable of withstanding the harsh conditions of oil reservoirs. The electron donor may be H₂ or formate, and the acceptors are iron oxides and hydroxides. Deferribacter thermophilus is a bacterium that besides iron reduction also reduces manganese and nitrates using yeast extract or peptone. The source of energy is hydrogen and numerous organic acids [23].

Other Petroleum Degrading Organisms

Fungi: The mechanisms of degrading petroleum hydrocarbons by fungi are different from bacteria. However, fungi do not degrade petroleum hydrocarbons completely to CO₂ as bacteria often do. Fungi secrete extracellular oxidizing enzymes for degradation of lignin. These enzymes are able to make reactive peroxide from oxygen [40], [41]. Lignin is a complex random molecule containing a lot of aromatic groups. The degrading enzymes lignin peroxidase and manganese peroxidase are able to degrade some model lignin compounds. Peroxidase has been reported to be involved in the degradation of PAH to quinones [41], [42].

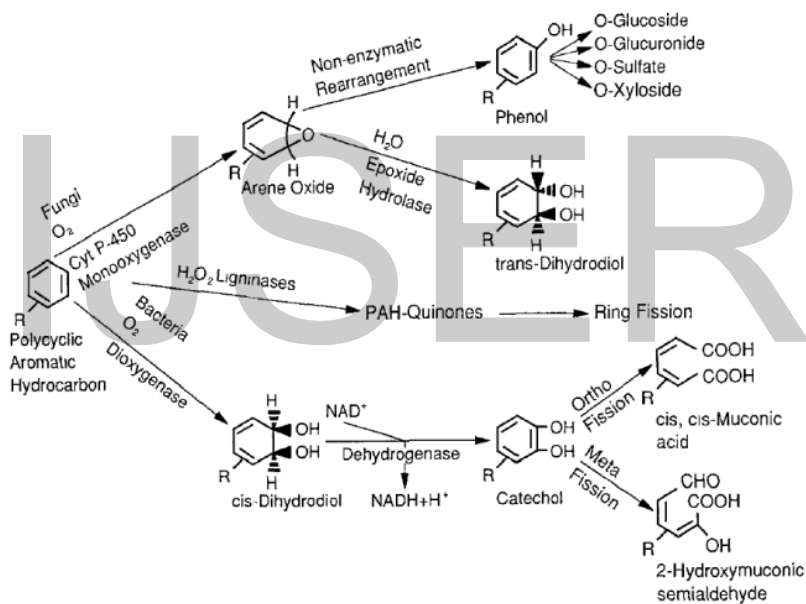


Fig. 4: Bacteria and fungi degradation of petroleum hydrocarbons [25].

Algae: They use the eukaryotic mechanism similar to fungi. This mechanism make use of a dioxygenase enzyme which leads to *cis-trans* hydroxyl groups. Algae are dependent on light for being able to degrade PAHs. The metabolites depend on the kind of light radiation the algae are irradiated with [43].

Plants: Plants uses peroxidase P450 enzyme used by fungi to degrade PAHs. These enzymes have the same concentration or a higher concentration in presence of PAHs. Plants with lower peroxidase activity also have lower metabolizing rates. It was shown also that, oxidative enzymes were important, and both oxidative and peroxidative enzymes were needed for PAHs degradation in plants [44]. However, plants are still able to stimulate biodegradation of PAHs by stimulation of bacteria associated with the root system. Plants secrete a useful solution for bacteria around the roots, also known as exudates. This solution contains amino acids, sugars and organic acids and some enzymes. A lot of degrading bacteria need this solution for survival, because they degrade PAHs only to prevent toxic effects and this process uses energy [45].

Habitual Degradation Rates of Petroleum Hydrocarbon

The highest habitual degradation rates of petroleum hydrocarbon occurs in soil environment followed by fresh water and marine water environment.

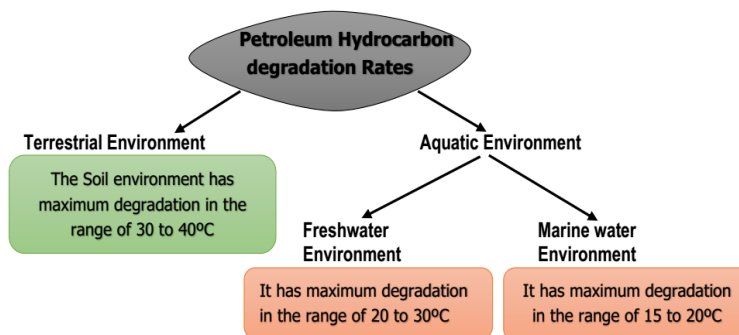


Fig. 5: Rates of petroleum hydrocarbon degradation in terrestrial and aquatic environments [46].

Bacterial Mechanisms of Degrading Petroleum Hydrocarbons

Bacterial degradation of petroleum hydrocarbons is one of the major and ultimate natural mechanism by which one can clean up the petroleum hydrocarbon pollutants from the environment [47]. The general overview of the intrinsic mechanisms of bacteria degrading petroleum hydrocarbons can be elucidated from two biochemical perspectives; 1.) Bacterial activation/functionalization reaction carried out by specific bacterial enzyme system that initiates an intracellular attack on the organic pollutants through oxygenation, addition, hydration or carboxylation reaction to generate a substrate/metabolite that are further biotransformed into a central metabolite that can be utilized as their source of carbon and other cellular activities; 2.) Bacteria are capable of producing biosurfactants solubilizing the organic pollutant following emulsification and the formation of micelles to enhance the uptake of the organic pollutants by bacteria for degradation.

1. Activation/functionalization mechanism: Mechanisms for hydrocarbon activation are basically different in aerobic and anaerobic microorganisms. Under oxic (aerobic) conditions, hydrocarbon metabolism is always initiated using molecular oxygen as a co-substrate in mono or dioxygenase reactions that enables the terminal or sub-terminal hydroxylation of aliphatic alkane chains or the mono or dihydroxylation of aromatic rings [48]. In the hydrocarbon activation under anoxic (anaerobic) conditions, some proposed reactions comprise: 1) addition to fumarate by glycyl-radical enzymes; 2) methylation of unsubstituted aromatics; 3) hydroxylation with water by molybdenum cofactor containing enzymes of an alkyl substituent via dehydrogenase; 4) carboxylation catalyzed by yet uncharacterized enzymes which may actually represent a combination of reaction 2 followed by reaction 1 [48].

Table 1: Overview of aerobic and anaerobic mechanisms for hydrocarbon activation by bacteria [26].

Hydrocarbons	Aerobic	Anaerobic
Short-Chain non-methane alkanes C2- C10	<ul style="list-style-type: none"> •Non-heme iron monooxygenase similar to sMMO (C2-C9). •Copper-containing monooxygenase similar to pMMO (C2-C9). •Heme-iron monooxygenases (also referred as soluble Cytochrome P450 (C5-C12). 	<ul style="list-style-type: none"> • Fumarate addition
Long-Chain alkanes >C10	<ul style="list-style-type: none"> • Heme-Monooxygenase (P450 type). • [Fe2] Monooxygenase. • Non-heme iron monooxygenase (AlkB-related) (C3-C13 or C10-C20). • Flavin binding monooxygenase (AlmA) (C20-C36). • Thermophilic Flavin-dependent monooxygenase (LadA) (C10-C30). 	<ul style="list-style-type: none"> • Fumarate addition • Carboxylation
Aromatic hydrocarbons	<ul style="list-style-type: none"> • [Fe]-Dioxygenase • [Fe2]-Monooxygenase • [Flavin]-Monooxygenase 	<ul style="list-style-type: none"> • Fumarate addition • Hydroxylation • Carboxylation

Enzymes participating in the degradation of hydrocarbons: Cytochrome P450 alkane hydroxylases constitute a super family of ubiquitous Heme-thiolate Monooxygenases which play an important role in the microbial degradation of oil, chlorinated hydrocarbons, fuel additives, and many other compounds [49]. The diversity of alkane oxygenase systems in prokaryotes and eukaryotes that actively participate in the degradation of alkanes under aerobic conditions like cytochrome P450 enzymes, integral membrane di-iron alkane hydroxylases (e.g. *alkB*), soluble di-iron methane monooxygenases, and membrane-bound copper containing methane monooxygenases has been reported [50]. The initial attack on the petroleum hydrocarcon by oxygenase is illustrated in Fig. 6.

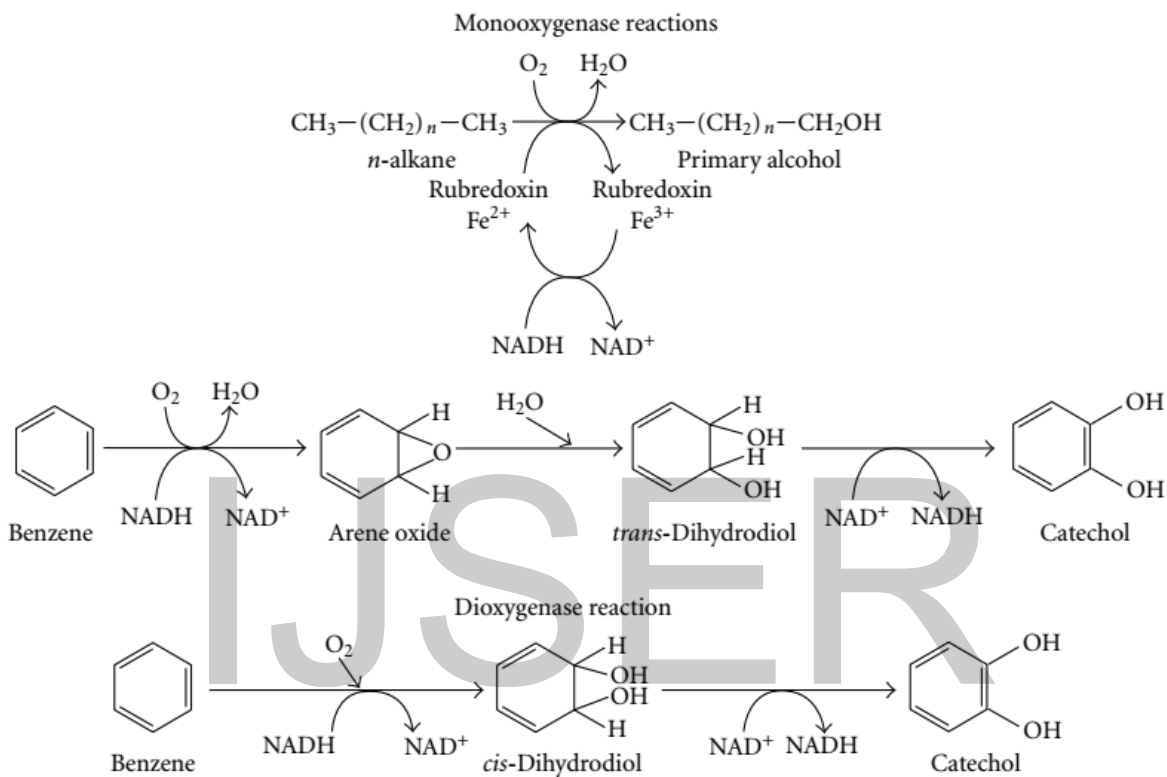


Fig. 6: Illustrate the enzymatic reactions involved in the processes of hydrocarbons degradation [46].

Table 2: Enzymes involved in biodegradation of petroleum hydrocarbons [46].

Enzymes		Substrates	Microorganisms
Soluble Monooxygenases	Methane	C1–C8 alkanes and cycloalkanes	<i>Methylococcus</i> , <i>Methylosinus</i> , <i>Methylocystis</i> , <i>Methylomonas</i> , <i>Methylocella</i> .
Particulate Monooxygenases	Methane	C1–C5 (halogenated) alkanes and cycloalkanes	<i>Methylobacter</i> , <i>Methylococcus</i> , <i>Methylocystis</i> .
AlkB related Hydroxylases	Alkane	C5–C16 alkanes, fatty acids, alkyl benzenes, cycloalkanes and so forth	<i>Pseudomonas</i> , <i>Burkholderia</i> , <i>Rhodococcus</i> , <i>Mycobacterium</i> .
Eukaryotic P450		C10–C16 alkanes, fatty acids	<i>Candida maltose</i> , <i>Candida tropicalis</i> , <i>Yarrowia lipolytica</i> .
Bacterial oxygenase system	P450	C5–C16 alkanes, cycloalkanes	<i>Acinetobacter</i> , <i>Caulobacter</i> , <i>Mycobacterium</i> .
Dioxygenases		C10–C30 alkanes	<i>Acinetobacter sp.</i>

HUB are able to degrade petroleum hydrocarbons to CO₂ and CH₄ as their source of carbon and other cellular activities. The mechanistic events can be elucidated from the sequence of reactions that begins with an initial intracellular attack on the organic pollutants (petroleum hydrocarbons) catabolically to produce monomeric units. The monomeric units undergoes bacterial fermentation by sulphate reducing bacteria (SRB) to yield a substrate that are futher metabolised to generate a central intermediary

metabolites such as acetate. Acetate is further metabolised to methane by methanogenic bacteria or CO₂ by sulphate reducing bacteria which can be used as their source of carbon and other cellular activities.

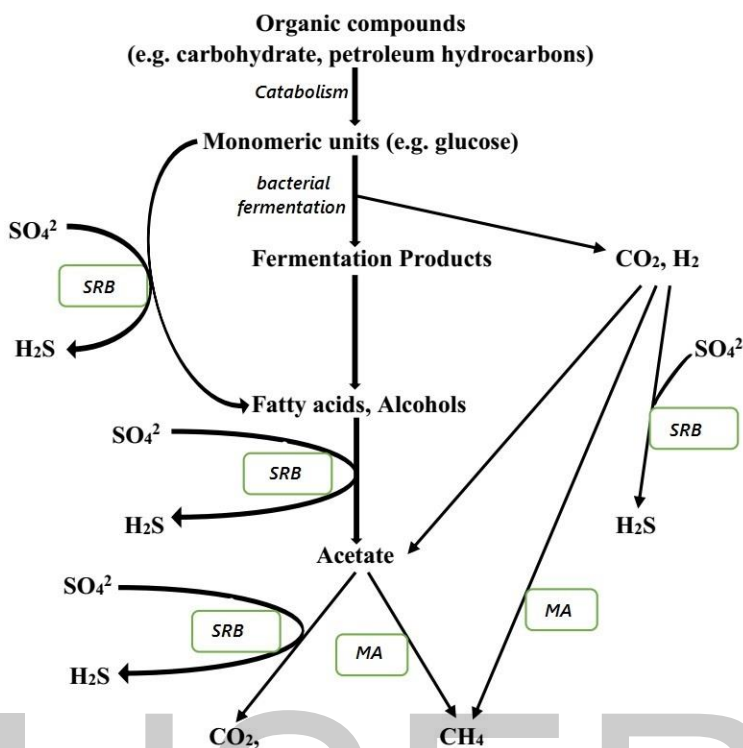


Fig. 7: Mechanism of bacterial degradation of petroleum hydrocarbon with the participation of SRB – sulfate reducing bacteria, MA – methanogenic archaea. [51].

2. Bacteria uptake of hydrocarbons through biosurfactants production mechanism: Biosurfactants are heterogeneous group of surface active chemical compounds produced by a wide variety of microorganisms. They enhance solubilization and removal of organic pollutants [52], [53], [54]. Microbial consortium consisting of two isolates of *Pseudomonas aeruginosa* and one isolate of *Rhodococcus erythropolis* from soil contaminated with oily sludge was able to degrade 90% of hydrocarbons in 6 weeks in liquid culture. This data substantiated the use of a crude biosurfactant for hydrocarbon remediation [46].

Pseudomonads, one the best known bacteria capable of utilizing petroleum hydrocarbons as carbon and energy sources by producing biosurfactants. Among *Pseudomonads*, *P. aeruginosa* is widely studied for the production of glycolipid type biosurfactants [55]. Biosurfactants increase the oil surface area for bacteria to utilize it. Biosurfactants can act as emulsifying agents by decreasing the surface tension and forming micelles. The micro droplets encapsulated in the hydrophobic microbial cell surface are taken inside and degraded [46], [56].

Table 3: Summary of the recent reports on biosurfactant production by different microorganisms [46].

Biosurfactants	Microorganisms
Sophorolipids	<i>Candida bombicola</i>
Rhamnolipids	<i>Pseudomonas aeruginosa</i>
Lipomannan	<i>Candida tropicalis</i>
Rhamnolipids	<i>Pseudomonas fluorescens</i>
Surfactin	<i>Bacillus subtilis</i>
Glycolipid	<i>Aeromonas sp.</i>
Glycolipid	<i>Bacillus sp.</i>

Fig. 8 demonstrates the involvement of biosurfactant (rhamnolipids) produced by *Pseudomonas sp.* and the mechanism of formation of micelles in the uptake of hydrocarbons.

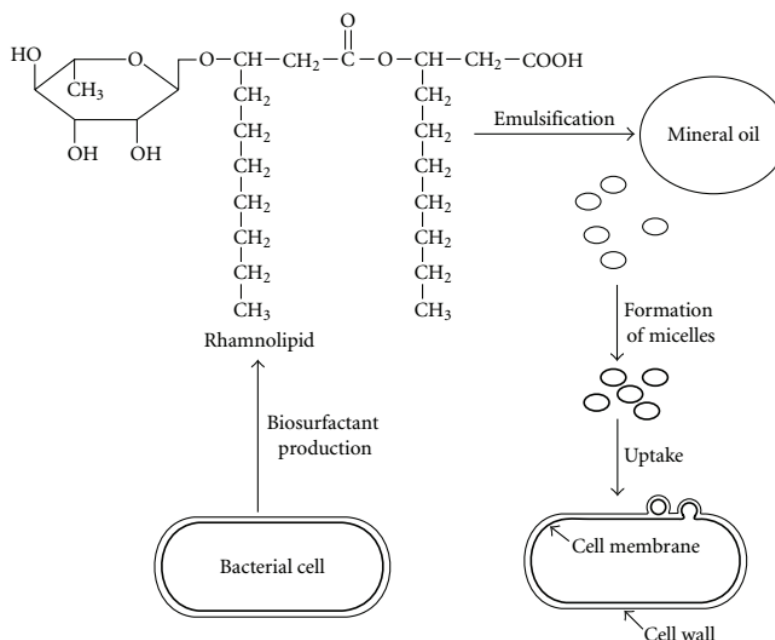


Fig. 8: Involvement of biosurfactant (rhamnolipid) produced by *Pseudomonas sp* in the uptake of hydrocarbons [46].

Pseudomonas p are capable of producing a biosurfactant such as rhamnolipid which undergoes emulsification to form mineral oil leading to the formation of micelles with the organic pollutant which are taken up by bacteria.

Metabolic Pathways of Petroleum Hydrocarbon Degradation

Biochemically, the major intrinsic metabolic pathways utilized by bacteria in degrading petroleum hydrocarbons can occur aerobically with oxygen or anaerobically with nitrate, ferric iron, sulfate or other electron acceptors [57].

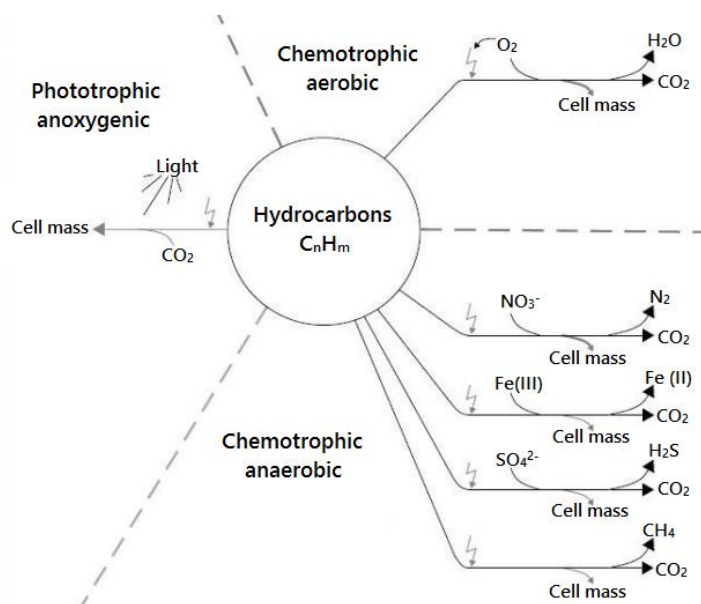


Fig. 9: An overview of the possible degradation mechanisms for hydrocarbons [25].

1. Aerobic metabolic pathway of petroleum hydrocarbon degradation

The most rapid and complete degradation of the majority of organic pollutants are carried out under aerobic condition [46].

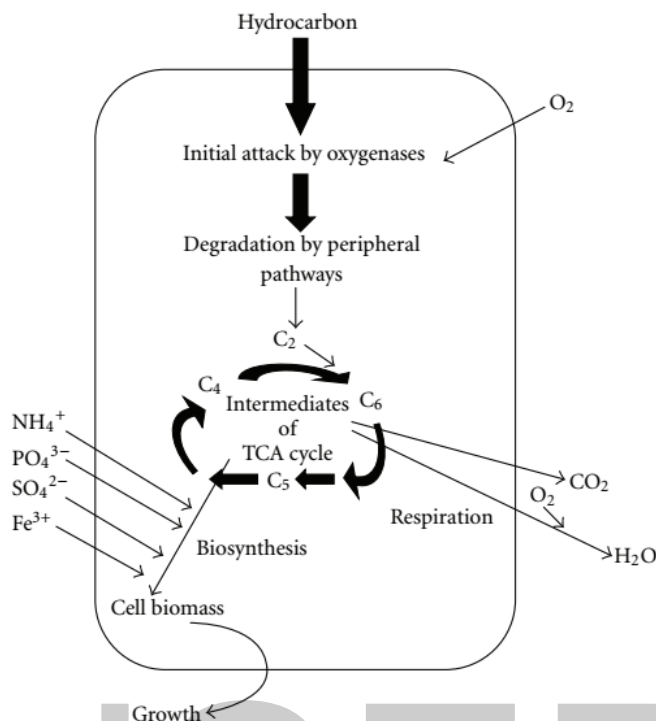


Fig. 10: The principle of aerobic degradation of hydrocarbons by bacteria [46].

Biochemically, in aerobic biodegradation process, the initial intracellular attack of organic pollutants is an oxidative process and the activation as well as incorporation of oxygen is an enzymatic key reaction catalyzed by oxygenases and peroxidases. Peripheral degradation pathways convert organic pollutants step by step into intermediates of the central intermediary metabolism, for example, the tricarboxylic acid cycle. Biosynthesis of cell biomass occurs from the central precursor metabolites, for example, acetyl-CoA, succinate, pyruvate [46].

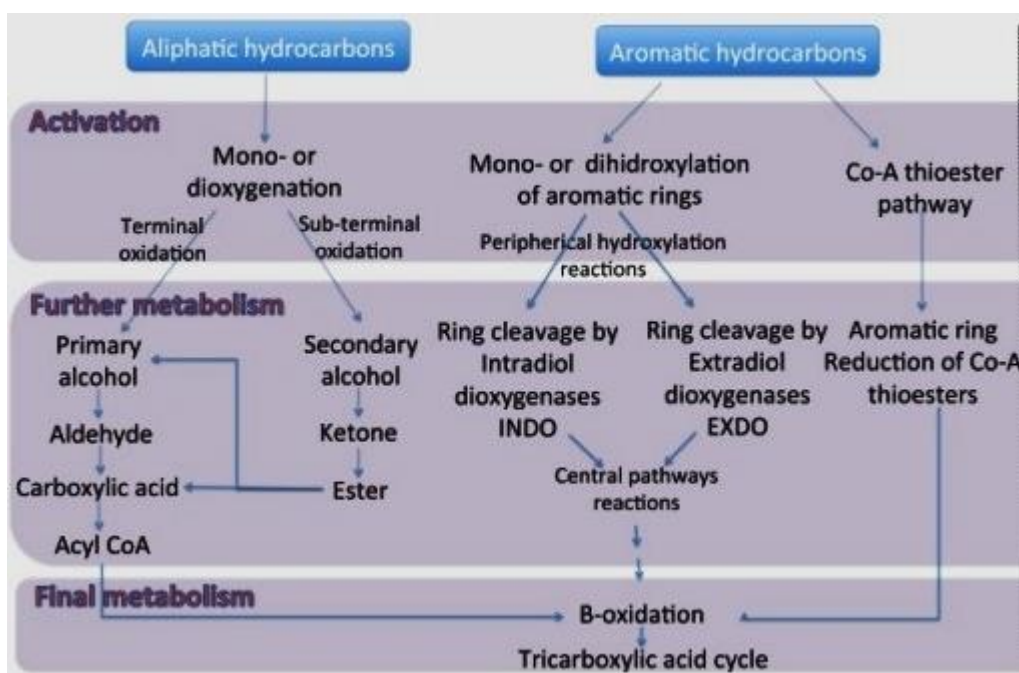


Fig. 11: Aerobic metabolic pathways of petroleum hydrocarbons degradation [26].

Aliphatic hydrocarbons: In most degradation pathways described, the substrate n-alkane is oxidized to the corresponding alcohol by substrate specific terminal monooxygenases/hydroxylases. The alcohol is then oxidized to the corresponding aldehyde, and finally converted into a fatty acid. Fatty acids are conjugated to CoA and subsequently processed by β – oxidation to generate acetylCoA. Sub-terminal oxidation has also been described for both short and long-chain alkanes. Both terminal and sub-terminal oxidation can coexist in some microorganisms [58], [59].

Initial terminal hydroxylation of n-alkanes in bacteria can be carried out by enzymes belonging to different classes, named: (1) propane monooxygenase (C3); (2) different classes of butane monooxygenase (C2-C9); (3) CYP153 monooxygenases (C5-C12); (4) AlkB-related non-heme iron monooxygenase (C3-C10 or C10-C20); (5) flavin-binding monooxygenase AlmA (C20-C36); (6) flavin-dependent monooxygenase LadA (C10-C30); (7) copper flavin-dependent dioxygenase (C10-C30) (Rojo, 2010). Among all the alkane activating enzymes, the integral membrane non-heme iron monooxy- genase (AlkB) is the best characterized one [58].

Aromatic hydrocarbons: The aerobic bacterial catabolism of aromatic compounds involves a wide variety of peripheral pathways that activate structurally diverse substrates into a limited number of common intermediates that are further cleaved and processed by a few central pathways to the central metabolism of the cell [60]. Metabolic pathways and encoding genes responsible for the degradation of specific members of a highly diverse range of aromatic compounds have been characterized for many isolated bacterial strains, predominantly from the Proteobacteria and Actinobacteria phyla [61]. Degradation by such isolates is typically initiated by members of one of the three superfamilies: the Rieske non-heme iron oxygenases (RNHO), the flavoprotein monooxygenases (FPM) and the soluble diiron multicomponent monooxygenases (SDM) [62].

In the case of hydrophobic pollutants, such as benzene, toluene, naphthalene, biphenyl or polycyclic aromatics, aerobic degradation is usually initiated by activation of the aromatic ring through oxygenation reactions catalyzed by RNHO enzymes or, as intensively described for toluene degradation, through members of SDM enzymes [61]. Aromatic hydrocarbons molecules have benzene-based structure. As compared to most other cyclic compounds, aromatic compounds are more stable because of sharing of delocalized electrons by pi bonds. BTEX (benzene, toluene, ethylbenzene, xylene) compounds are comparatively more mobile and water- miscible [27].

Two major steps involved in biodegradation of aromatic hydrocarbons are; i) Activation of the ring; ii) Ring cleavage.

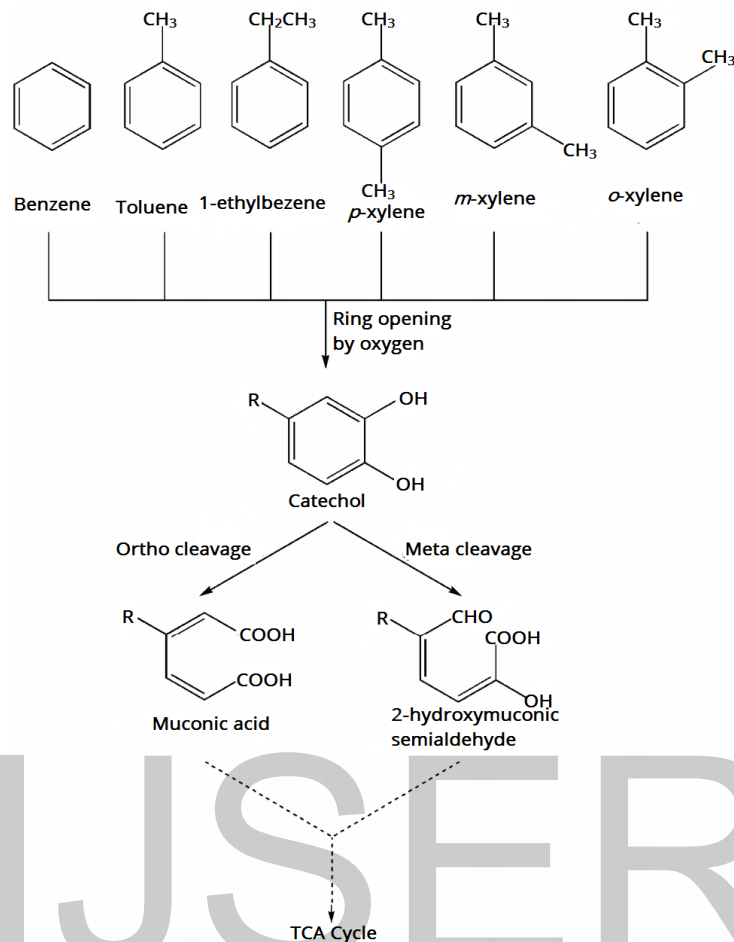


Fig. 12: General pathway for aromatic petroleum hydrocarbon degradation [63].

Activation of the ring is achieved by the incorporation of molecular oxygen into the aromatic ring leading to dihydroxylation of aromatic nucleus, and the enzymes responsible for this are oxygenases. Monooxygenases catalyze the incorporation of a single atom of oxygen to form an epoxide which can then undergo hydration to yield transdihydrodiols [64]. Dioxygenases catalyze simultaneous incorporation of two atoms of oxygen to form a dihydrodiol. These dioxygenase reactions have been shown to occur for benzene, halogenated benzenes, toluene, para-chlorotoluene, xylenes, biphenyls, naphthalene, anthracene, etc [63], [64]. These dihydrodiols are further oxidized to catechols which are precursors to ring cleavage. Catechol can be oxidized either via ortho-cleavage pathway which involves cleavage of the bond between carbon atoms of the two hydroxyl groups to yield muconic acid, or via the meta-cleavage pathway which involves cleavage of the bond between a carbon atom with a hydroxyl group and the adjacent carbon atom to yield 2-hydroxymuconic semi aldehyde [65]. These compounds are further degraded to form organic acids which are then utilized by microorganisms for their cell synthesis and energy generation [27].

2. Anaerobic metabolic pathway of petroleum hydrocarbon degradation

Anaerobic hydrocarbon degradation has gained more attention as it is considered to be the predominant mechanism occurring in several polluted environments and oil reservoirs.

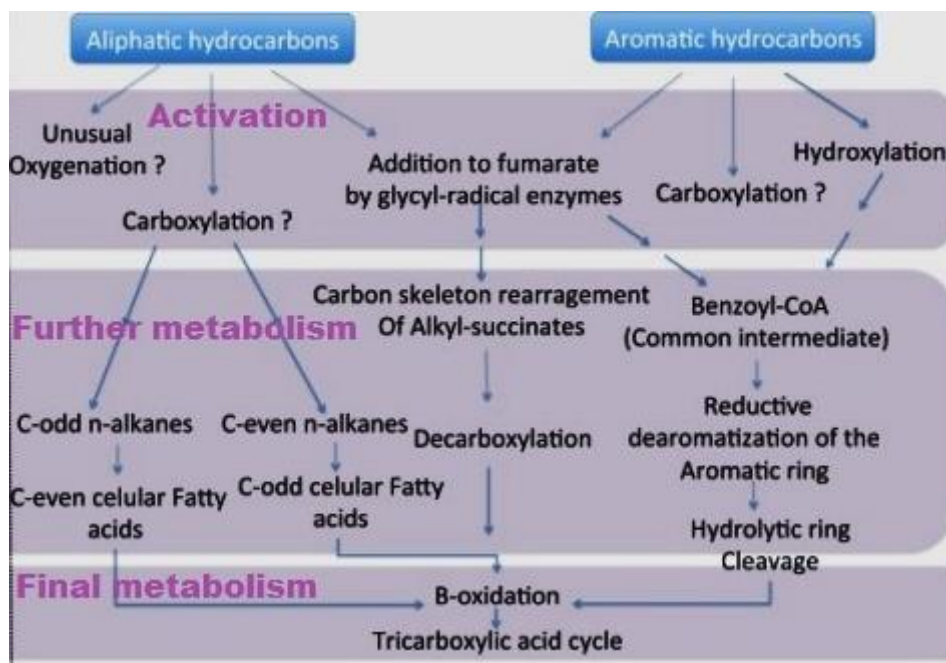


Fig. 13: Anaerobic metabolic pathways of petroleum hydrocarbons degradation [26].

Aliphatic hydrocarbons: Several anaerobic bacteria capable of degrading n-alkanes with six or more carbons in length, particularly hexadecane (C16), using sulfate or nitrate as electron acceptors have been isolated [66]. The two main mechanisms of anaerobic degradation of n-alkanes involves biochemical reactions that differ completely from those employed in aerobic hydrocarbon metabolism [67], the first involves activation at the subterminal carbon of the alkane by the addition of fumarate, analogously to the formation of benzyl succinate during anaerobic degradation of toluene. However, further reactions are completely different involving dehydrogenation and hydration [66], the second mechanism for alkane anaerobic degradation is the carboxylation, mainly developed from the growth pattern of the sulfate-reducing strain Hxd3, tentatively named as *Desulfococcus oleovorans* [68]. This strain differs from other alkane degraders for converting C-even alkanes into C-odd cellular fatty acids whereas growth on C-odd alkanes resulted in C-even cellular fatty acids [66], [68].

Other alternative activation mechanisms are proposed for the anaerobic degradation of alkanes. For instance, the mechanism referred as “unusual oxygenation” is used by the strain *Pseudomonas chloritidismutans* AW-1T, that is assumed to produce its own oxygen via chlorate respiration used for subsequent metabolism of alkanes [69]. Other alternative mechanism considers that activation in the anaerobic methanogenic system may be initiated by an anaerobic hydroxylation reaction [70].

Aromatic hydrocarbons: In the absence of oxygen, bacteria uses a pathway based in reductive reactions to attack the aromatic ring [71]. The biochemistry of some anaerobic degradation pathways of aromatic compounds has been studied to some extent; however, the genetic determinants of all these processes and the mechanisms involved in their regulation are much less studied [60]. Recent advances in genome sequencing have led to the complete genetic information for six bacterial strains that are able to anaerobically degrade aromatic compounds using different electron acceptors and that belong to different taxonomic groups of bacteria: denitrifying betaproteobacteria, *Thauera aromatica* and *Azoarcus* sp. EbN1, two alphaproteobacteria, the phototroph *Rhodospseudomonas palustris* strain CGA009 and the denitrifying *Magnetospirillum magneticum* strain AMB-1, and two obligate anaerobic deltaproteobacteria, the iron reducer *Geobacillus metallireducens* GS-15 and the fermenter *Syntrophus aciditrophicus* strain SB [60].

The best understood and apparently the most widespread of these anaerobic mechanisms is the radical-catalyzed addition of fumarate to hydrocarbons, yielding substituted succinate derivatives. This reaction has been recognized for the activation of

several alkyl-substituted benzenes as well for n-alkanes [72]. The key enzyme in this process is the enzyme benzylsuccinate synthase. All enzymes required for β -oxidation of benzylsuccinate are encoded by the *bbs* operon. Subsequent degradation of benzoyl-CoA proceeds via reductive dearomatization, hydrolytic ring cleavage, β -oxidation to acetyl-CoA units and terminal oxidation to CO_2 [73].

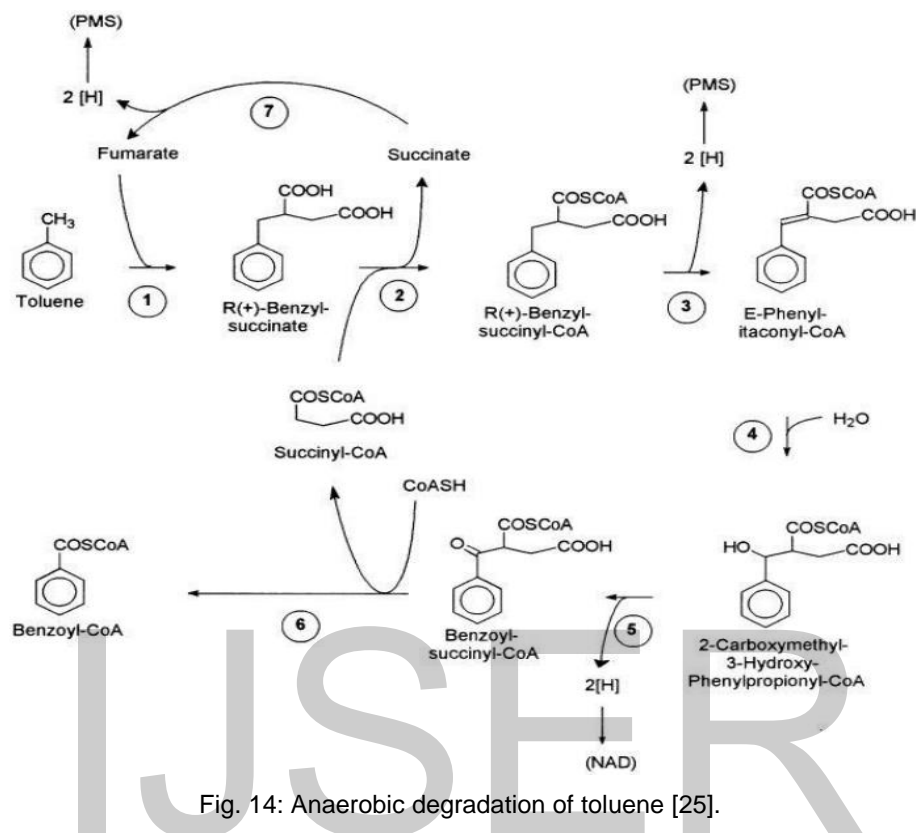


Fig. 14: Anaerobic degradation of toluene [25].

The degradation of toluene and some related aromatics is better understood and starts with the formation of benzylsuccinate molecule. This molecule reacts with coenzyme A to form Benzoyl-CoA and acetyl-CoA [74]. Benzoyl-CoA is further degraded by β -oxidation.

Factors Affecting Petroleum hydrocarbon Biodegradation

Bacterial degradation of petroleum hydrocarbons are influenced by several physiochemical factors in the environment such as temperature, pH, nutrient and oxygen availability, salinity, pressure, and light influence biodegradation of petroleum hydrocarbons [75].

1. **Oxygen availability:** hydrocarbon degradation takes place both in presence and absence of oxygen. However aerobic conditions are more favourable as oxygenases are the primary enzymes needed for degradation to occur. Oxygenases function in presence of oxygen, so degradation rates are higher in aerobic conditions as compared to those under anaerobic conditions.
2. **Nutrient availability:** The process of biodegradation can be enhanced by addition of essential nutrients such as nitrogen and phosphorous. In case of petroleum oil spills where nitrogen shortages can be acute; carbon, nitrogen and phosphorous are added in the ratio of approximately 100: 10: 1 (C: N: P).
3. **Temperature:** petroleum hydrocarbon degradation occurs over a wide temperature range (psychrophilic to mesophilic) from close to zero degrees to up to more than 30°C . Bacteria can adapt to temperature fluctuations in order to maintain metabolic activity, however seasonal temperature fluctuations in the natural environment have been shown to affect the rate at which degradation occurs [76].

4. *pH*: Highest rates of degradation are generally observed at neutral pH. However microorganisms growing on HC have been isolated from historically contaminated sites even at pH 2-3.
5. *Salinity*: Salt concentration has varied effect on degradation depending on the type of environment and the type of organisms involved. Higher salt concentrations tend to inhibit degradation. The ability of variation in salinity to affect the rate of HC degradation appears to be dependent on the natural variation in salinity regime of the sample source [77].
6. *Light*: Availability of light can have a positive impact on hydrocarbon degradation by photosynthetic microbes such as algae [78]. Light can also degrade petroleum compounds by direct photochemical action. Physical properties of petroleum compounds are affected due to the photochemical reactions. Emulsion formation and the solubility of petroleum fractions may get altered by light [25].

Effects of Petroleum Hydrocarbon Pollution

Anwasha et al., [5] reported that the major effects of hydrocarbon pollutions are; Global Warming; Loss of delicate species of animals that are on the verge of extinction or endangered, due to toxic contamination of oceans, land, and air; Reduced productivity of agricultural land if the soil is contaminated with spilled oil; The death of fishes in oceans, lakes, ponds, rivers due to toxicity and lack of oxygen when the water bodies are contaminated with petroleum contaminants, thus causing economic loss; Most hydrocarbons are carcinogenic; Inhalation of hydrocarbon can cause irritation in the respiratory tract, and they can cause the onset of asthma and other allergies; Polycyclic aromatic hydrocarbons (PAHs) can cause prenatal defects, reduced birth weight, developmental defects.

Conclusion

Degradation of petroleum hydrocarbon by certain bacteria is one of the major and ultimate natural mechanism by which one can clean up petroleum hydrocarbon pollutants from the environments. HUB has an inherent biochemical mechanisms of degrading petroleum hydrocarbons to a central intermediary metabolites that are further metabolized to produce molecules such as CO₂ or CH₄ that can be used as their source of carbon and for other cellular activities. Bacteria utilizes an enzymatic activation/functionalization mechanism or through the mechanism of bacterial production of biosurfactant to degrade petroleum hydrocarbons. Aerobic and anaerobic pathways are the two major biochemical pathway utilized by bacteria to degrade petroleum hydrocarbons. HUB enzymatically causes myriads of biochemical changes on the physiochemical properties of petroleum hydrocarbons, producing central intermediary metabolites that are less toxic and can be used by bacteria and other living organisms within the environment for biological activities. Understanding the biochemical events exploit by microbes to degrade petroleum pollutants will offer an insight in microbial biochemistry geared towards effective bioremediation strategies and applications.

Conflict of Interest

The authors declare no conflict of interest, financial or otherwise.

Authors Contributions

Idongesit A. Victor and Ebuana O. Iwok initiated the project. All the authors were actively involved in the literature review and drafted the initial manuscript. Idongesit A. Victor and Andem B. Andem proof-read and finalised the manuscript.

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