



Microbial Degradation of Petroleum Hydrocarbons: Realities, Challenges and Prospects

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Authors' contributions

This work was carried out in collaboration between all authors. Author AAU designed the study and wrote the first draft of the manuscript. All authors managed the literature searches and approved the final manuscript.

Article Information

DOI: 10.9734/BJI/2018/43957

Editor(s):

(1) Dr. Ghousia Begum, Toxicology Unit, Biology Division, Indian Institute of Chemical Technology, Hyderabad, India.

Reviewers:

(1) Anonymous, Nnamdi Azikiwe University, Nigeria.
(2) Graciela Pucci, Universidad Nacional de la Patagonia San Juan Bosco, Argentina.

(3) L. P. Peekate, Rivers State University, Nigeria.

Complete Peer review History: <http://www.sciencedomain.org/review-history/27190>

Review Article

Received 16 August 2018
Accepted 04 October 2018
Published 14 November 2018

ABSTRACT

One of the emerging environmental challenges today is hydrocarbon pollution arising from activities of petrochemical industries, natural sources of crude oil pollution and other anthropogenic activities. These petroleum products contain harmful, carcinogenic and mutagenic compounds which could have severe consequences on biotic and abiotic components of the ecosystem. Physicochemical and biological methods are employed for the remediation of hydrocarbon contaminated systems; however, the negative impacts of the physicochemical approach are presently directing greater attention to the exploitation of the biological alternatives. This article reviews basic concepts of petroleum hydrocarbons, hydrocarbon degrading microorganisms, factors that influence the biodegradation of contaminants and innovative technologies for the effective removal of these pollutants. This article also discusses the applications of relative advances in molecular biological techniques, such as the isolation of plasmid DNA of microbial communities and the use of genetically engineered microorganisms to increase the rates of biodegradation of hydrocarbon pollutants in the environment.

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Keywords: Recent advances; microbial; degradation; petroleum; hydrocarbon.

1. INTRODUCTION

Petroleum is indeed the world's most important derived energy and carbon source. However, the growth and activities of petroleum and petroleum associated industries in the world has led to increased oil pollution in the environment [1-2]. Crude oil, due to its attributes is one of the most dangerous pollutants in the environment as it has the potential of causing serious damages to the ecosystem including humans. Oil producing areas of the world have experienced devastating consequences of crude oil spills from natural or anthropogenic sources to both terrestrial and aquatic environments in the past 50 years [2].

Several physicochemical approaches employed in remediating oil contaminated environment such as incineration or underground disposal in secure landfills have become extremely expensive when the amounts of pollutants are large. This often results in cleanup delays while the contaminated soil continues to pollute groundwater resources if on land and death of aquatic life if on waterways [3], thus necessitating speedy removal of the

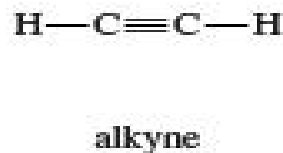
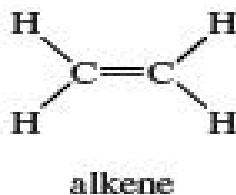
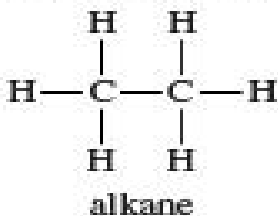
contaminants. Biological methods such as biodegradation involving bioremediation and biotransformation have acquired a new significance as an increasingly effective and potentially inexpensive cleanup technology [4-5].

Bioremediation which employs the biodegradative potentials of organisms or their attributes is an effective technology that can be used to accomplish both effective detoxification and volume reduction. Biodegradation is the mineralisation of organic chemicals which ultimately leads to the formation of CO₂, H₂O and biomass. These processes are environmental friendly and economical approaches for soil and water cleanup [4-5].

In 1946, Claude E. Zobell [6] recognised that many microorganisms have the ability to utilise hydrocarbons as the sole source of carbon and energy for metabolic activities. However, the microbial degradation of hydrocarbons depends on the nature of the compounds within the petroleum and on environmental determinants. Structures of the representative hydrocarbons are presented in Fig. 1.

Structures of representative hydrocarbons

aliphatic hydrocarbons



aromatic hydrocarbons

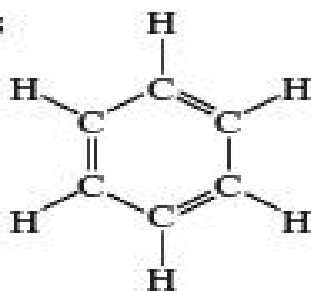


Fig. 1. Molecular structure of representative hydrocarbons

Source: [7]

2. MECHANISM OF PETROLEUM BIO-DEGRADATION

Biodegradation is the decomposition of organic substances by microorganisms into simpler substances such as carbon dioxide, water and ammonia. When oil is spilled in an environment, the most rapid and complete degradation of the majority of organic contaminants is brought about under aerobic conditions. Widdel and Rabus [8] however presented some detailed and comprehensive reviews on anaerobic biodegradation of hydrocarbons and the mechanisms involved (Fig. 2). Under aerobic conditions, the initial intracellular breakdown of organic pollutants is an oxidative process and the activation as well as injection of oxygen is the enzymatic key reaction catalysed by oxygenases and peroxidases [8]. Initial degradation pathways convert organic contaminants gradually into intermediates of the central intermediary metabolism, for example, the tricarboxylic cycle. Biosynthesis of cell biomass occurs from the central precursor metabolites, for example, acetyl-CoA, succinate and pyruvate. Sugars needed for various biosynthesis and growths are synthesised by Gluconeogenesis. The biodegradation of petroleum hydrocarbons can be mediated by a specific enzyme system.

2.1 Hydrocarbon Degrading Micro-organisms

Microbial biodegradation is a major and highly natural process by which one can utilise to clean up petroleum hydrocarbon contaminants from the environment. The ability to degrade these hydrocarbons is not restricted to a few microbial genera; a diverse group of bacteria, fungi and yeast have been shown to have this ability. Microbial degradation of petroleum hydrocarbons in a polluted tropical stream in Lagos, Nigeria reported by Adebusoye et al. [4] indicates that nine bacterial strains namely *Pseudomonas fluorescens*, *P. aeruginosa*, *Bacillus sp.*, *Alcaligenes sp.*, *Acinetobacter lwoffii*, *Flavobacterium sp.*, *Micrococcus roseus* and *Corynebacterium sp.*, were isolated from a crude oil polluted stream. These microorganisms have the tendency to thrive in this environment and degrade crude oil [4]. Other important genera of hydrocarbon degraders in aquatic and soil environment are *Achromobacter*, *Arthrobacter*, *Nocardia*, *Vibrio*, *Brevibacterium*, *Candida*, *Rhodotorula*, *Sporobolomyces*, *Penicillium*, *Burkholderia*, *Sphingomonas* and *Rhodococcus*.

Bacteria and yeasts are the most predominant hydrocarbon degrading microorganisms in

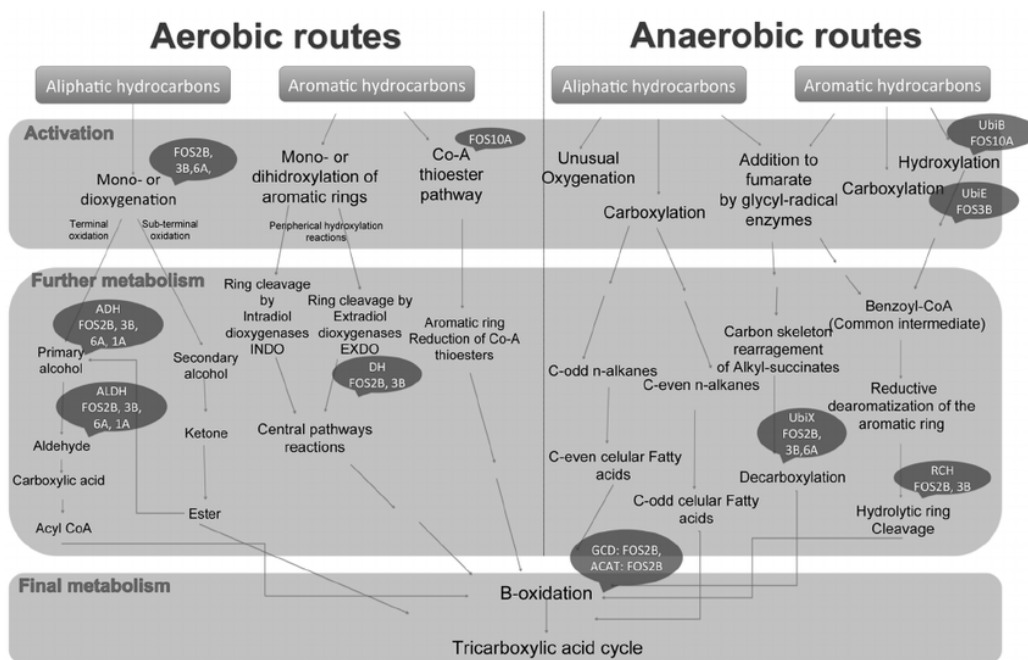


Fig. 2. Pathways for aerobic and anaerobic bacteria degradation of hydrocarbon compounds. Two arrows represent more than one reaction

Source: [8]

aquatic ecosystems. In contaminated freshwater ecosystems, bacteria, yeasts and filamentous fungi are all important hydrocarbon degraders. Fungi play an important role in the hydrocarbon-oxidising activities in soil samples. The reported efficiency of biodegradation ranged from 6% to 82% for soil fungi, 0.13% to 50% for soil bacteria and 0.03% to 100% for marine bacteria [4, 9]. Recent studies continue to expand the list of microbial species which have been demonstrated to degrade petroleum hydrocarbons. Many scientists report that mixed populations with overall broad enzymatic capacities are required to degrade complex mixtures of hydrocarbon in the environment [10].

3. PHYSICOCHEMICAL FACTORS INFLUENCING PETROLEUM HYDRO-CARBON DEGRADATION

3.1 Chemical Composition of Oil

The level of microbial degradation of petroleum hydrocarbons varies and is dependent on their chemical composition. N-alkanes of intermediate length (C₁₀-C₂₅) are the favourite substrates for microbes and tend to be the most readily degradable, whereas shorter chain compounds are rather more toxic [3]. Longer chain alkanes (C₂₅-C₄₀) are hydrophobic solids and are difficult to degrade due to their poor water solubility and bioavailability; and branched chain alkanes and cycloalkanes are also degraded more slowly than the corresponding normal alkanes [4]. Extremely packed aromatic and cycloparaffinic structures such as tars, bitumen and asphaltic materials exhibit the highest resistance to biodegradation.

3.2 Physical State of Oil

Oil spilled in the marine ecosystem tends to spread and form emulsions. This is referred to 'Pseudosolubilization' of the oil. The water-in-oil emulsion which occurs in seawater after oil spill is referred to as "Mousse". Dispersion of petroleum hydrocarbons in water column in the form of mousse increases the surface area of the oil and thus its availability for microbial attack [11-12]. Not only is the oil made more readily available to microorganisms, but movement of emulsion droplets makes oxygen and nutrients more readily available to microorganisms. However, large masses of mousse establish unfavourably low surface-to-volume ratios inhibiting biodegradation. Formation of tar balls or asphalt layers when oil is deposited on the beach and subsequently buried also restricts

access by microorganisms because of their limited surface area [13].

3.3 Concentration of the Oil

High concentrations of petroleum hydrocarbons can be related with heavy, undispersed oil slicks, formation of thick rafts, blankets or pools of oil which can lead to the inhibition of microbial biodegradation by nutrient or oxygen limitation or through toxic effects exerted by volatile hydrocarbons [14]. Thus, at high concentrations, those compounds most readily degraded will be attacked leaving the more resistant components behind. In the soil ecosystem, it can be observed that decrease in activity at high oil loading concentration is ascribed to inhibition of microbial activity by toxic components of oil sludge [15].

3.4 Temperature

Microbial biodegradation of petroleum hydrocarbons can occur over a wide temperature range. Temperature influences biodegradation with its effect on the physical nature and chemical composition of the oil, rate of hydrocarbon metabolism by microorganisms and composition of the microbial community [11,15]. Low temperature retards the rate of volatilisation of low-molecular-weight hydrocarbons which may be toxic to microorganisms leading to a delay of oil biodegradation [16-17].

Hydrocarbonoclastic microorganisms are most active in specified temperature ranges that guide the production of enzymes. The optimum temperature ranges for hydrocarbon degrading microorganisms are: psychrophiles (below 20°C), mesophiles (15°C - 45°C) and thermophiles (above 50°C). Most oil degrading microorganisms are active in the mesophilic range of 20°C to 35°C and provide optimum degradation rates at these temperatures. Generally, biodegradation rates are slower in cold environments than in warm climates [18]. At the mesophilic and thermophilic range of temperatures, the enzyme activity of microorganisms increases leading to an increased rate of hydrocarbon biodegradation. Diesel has however been noted to degrade at lower temperatures of 0°C to 10°C. The viscosity of oil increases at low temperatures suppressing the spread of oil on the surface making biodegradation difficult [18]. Therefore, mesophilic and thermophilic temperature ranges are the best option for biodegradation of petroleum hydrocarbons.

3.5 Water Activity

The water activity or water potential (a_w) of soils can range from 0.0 to 0.99, in contrast to the marine environment in which water activity is stable at a value near 0.98 [5]. All biological processes need moisture for effective transport of nutrients, food and waste materials in and out of the microbial cells. Hydrocarbon biodegradation in terrestrial ecosystems may be limited by the available water for microbial growth and metabolism. The optimum ratio of moisture will depend on the climate and soil type. Ratios range from 30%-90% of soil water holding capacity [18].

3.6 Oxygen

The early steps in the catabolism of aliphatic, cyclic and aromatic hydrocarbons by microorganisms such as bacteria and fungi involve the oxidation of the substrate by enzymes such as oxygenases for which molecular oxygen is required. Aerobic conditions are therefore necessary for this route of microbial oxidation of hydrocarbons in the environment [19]. Oxygen is not always limited in the upper levels of the water column in aquatic environments. The availability of oxygen in soils is dependent on rates of microbial oxygen consumption, the type of soil, the soil texture and structure and the pressure of utilisable substrates which can lead to oxygen depletion [20]. Anaerobic degradation of petroleum hydrocarbons by microorganisms have been shown in some studies to occur only at negligible rates, and its ecological significance has been generally considered to be minor [8].

3.7 Nutrient Availability

Microorganisms require nutrients such as nitrogen, phosphate and potassium (N, P and K) for cellular metabolism and successful growth. In contaminated sites, where organic carbon levels are often high due to the nature of the pollutant, available nutrients can become rapidly depleted during microbial metabolism [18]. It is however recommended to supplement polluted environments with nutrients such as nitrogen and phosphates to stimulate the *in situ* microbial community for effective microbial biodegradation.

3.8 Salinity

Microorganisms are typically well adapted to cope with the wide range of salinities common to the world's ocean [11]. There is little or no evidence to suggest that these microorganisms

are affected by other hyper saline environments, such as saltwater from oil wells. Estuarine ecosystem may present a special case because salinity values will vary in levels as compare to ocean. Thus, if microorganisms are to be added to the environment through bioaugmentation, it must be known if they are compatible with the saline levels present in the system [11,21].

3.9 Pressure

The importance of pressure as a variable in the biodegradation of hydrocarbons is most probably confined to the deep-sea environment [22,4]. The degradation of hydrocarbons at ambient and *in situ* pressure for deep-sea bacteria was examined by Schwarz et al. [22]. The rate and ambient temperatures was found to be significantly less than rates found under conditions of ambient temperatures and atmospheric pressure. For example, 94% of hexadecane was degraded within 8 weeks at 1 atm, and at 500 atm it took 40 weeks for similar degradation to occur. Therefore, oil which reaches the deep-ocean ecosystem will be degraded at a very slow rate by hydrocarbon degrading microorganisms and consequently will persist for a long period of time.

3.10 Biological Determinants of Petroleum Hydrocarbon Bio-degradation

3.10.1 Adaptation due to prior exposure

In microbial degradation of petroleum hydrocarbons, previously exposed species are important determinants to the level in which subsequent hydrocarbons contamination will be degraded. This phenomenon which results from an increase in the hydrocarbon-oxidising potential of the microbial community is known as adaptation. Adaptation can occur by three interrelated mechanisms: induction or depression of specific enzymes, genetic changes resulting in new metabolic capabilities and selective enrichment of organisms [11].

Studies by scientists have shown that the number of microorganisms increases upon exposure to petroleum or other hydrocarbon pollutants and the level of hydrocarbon degrading microorganisms generally reflect the degree of contamination of the ecosystem [9]. Generally, an increase in hydrocarbonoclastic microorganisms in marine, fresh water and soil ecosystems is seen as a response to acute

pollution index of petroleum hydrocarbons and prior exposure of pollutant of these microbial species.

3.10.2 Adaptation due to alteration of genetic composition

The primary genetic mechanism for the adaptation of the microbial community is the amplification by means of selective enrichment, gene transfer and mutation of genes involved in the metabolism of the chemical contaminant [23, 24]. Direct assessment of this phenomenon has recently been made easy by the development and application of DNA probes specific for the genes encoding hydrocarbon-catabolic pathways. Dot blot hybridisation, and metagenomic analyses in which DNA is extracted from environmental samples and then probed, can be used to detect specific DNA sequences in the environment without the need for isolation and culture of microorganisms [2, 25].

Polymerase chain reaction (PCR) technique can improve the sensitivity of the dot blot method [26]. The use of these molecular-based methods and other "omic" techniques in analysing genes involved in hydrocarbon metabolism will allow measurement of the frequency of those genes within the microbial community. This will permit assessment of the relative degree of adaptation of the community as well as a more detailed analysis of the dynamics of gene amplification associated with adaptation [27].

3.10.3 Seeding

The introduction of allochthonous microbial species into the natural ecosystem for the purpose of enhancing the rate of biodegradation of pollutants is referred to as seeding. The objective of the process is generally based on the fact that autochthonous microbial communities may not be buoyant enough to degrade a wide range of potential substrates present in complex mixtures such as petroleum products pollutants [28]. Several criteria are involved for effective seeding. These included: the ability to degrade most petroleum components, genetic stability, and viability during storage, rapid growth, a high degree of enzymatic activity, ability to compete with indigenous microorganisms, non-pathogenicity and inability to produce toxic metabolites [18]. Seeding of petroleum-contaminated aquatic environments has been attempted with mixed results, unlike the terrestrial ecosystem where

the soils contain higher concentrations of organic and inorganic matter and significant hydrocarbon degrading components which readily increases in response to hydrocarbon contamination [2, 18].

4. THE USED OF GENETICALLY ENGINEERED MICROORGANISMS (GEMS)

Through the process of genetic engineering, an artificial combination of genes capable of degrading specific contaminants such as petroleum hydrocarbons can be created. The development of genetic engineering techniques and intensive studying of metabolic potential of microorganisms allowed designing genetically modified microorganisms (GMMs) [25]. The GMMs is an alternative solution for wild strains of microorganisms which degrade contaminants slowly or not at all. In 1987, in the USA, the first two genetically modified strains of *Pseudomonas aeruginosa* and *Pseudomonas putida* were patented [23]. Produced by Charkrabarty in early 70s, the organisms contained genes for naphthalene, salicylate and camphor degradation.

According to Davison [23], ubiquitous organisms that are efficient degraders of many toxic compounds which both their chromosomes and plasmids may carry genes for metabolism of these compounds are the main source of catabolic genes for genetic engineering. The common cloning vectors applied in genetic engineering are plasmids which multiply or express particular genes.

Filonov et al. [29] constructed a genetically modified strain of *Pseudomonas putida* able to degrade naphthalene in the soil. For its construction, three strains of bacteria were used. They included *Escherichia coli* with plasmid (carrying tetracycline resistance gene), *Pseudomonas sp.* able to degrade naphthalene and *Pseudomonas putida* with *gfp* gene localised in chromosome. The results from this study confirmed that recombinant bacteria could degrade naphthalene and transfer plasmid to autochthonous microorganisms. Genetic engineering as a new technology therefore provides an excellent and great option for the application of natural ability of bacteria in construction of GMMs. The combination of microbiological and ecological knowledge, "omic" techniques, biochemical mechanisms and field engineering designs are essential elements for successful *in situ* bioremediation using GMMs [25].

4.1 Application of Biosurfactants

The recent advances in sustainable technologies have driven the quest for natural biodegradable compounds for the abatement of contaminated sites by petroleum hydrocarbons. This development has led to the discovery of natural agents called biosurfactants synthesised by living organisms such as saponins produced by plants and glycolipids produced by microorganisms. Biosurfactants are structurally divergent group of surface active compounds synthesised mainly by aerobic microorganisms facilitates the translocation of insoluble substrates across cell membranes [30-31]. These compounds have amphipathic molecules with hydrophobic and hydrophilic portions that act between fluids of different polarities allowing access by hydrophobic substrates and causing a reduction in surface tension, an increase in the area of contact of insoluble compounds and the enhancement of the mobility, bioavailability and biodegradation of such compounds [32].

Biosurfactants have great advantages over chemical surfactants due to their simple chemical structure, environmental compatibility, low toxicity, high selectivity due to presence of specific functional groups and activity under conditions of extreme temperatures, pH and salinity [31]. Biosurfactants play a significant physiological role in microorganisms in mediating the contact of microbial cells with hydrophobic substrates. Surfaces of most microorganisms are hydrophilic and permit effective interaction with the water-soluble compounds and ensure the normal operation of membrane-bound enzyme systems. The versatility and efficiency demonstrated in the application of biosurfactants in oil production chain and removal of hydrophobic contaminants make these compounds promising biomolecules [30-33]. The mechanisms of hydrocarbon removal by biosurfactants depending on their molecular

mass and concentration as well as the involvement of biosurfactant (rhamnolipid) produced by *Pseudomonas sp* in the uptake of hydrocarbons are presented in Figs. 3 and 4 respectively.

4.2 Application of Immobilised Cells

The application of immobilised cells has been of great impacts in the manufacturing of useful chemicals, abatement of waste waters and bioremediation of contaminated sites such as petroleum hydrocarbons due to its longer operating period and extended stability and survival of the cells. Diaz et al. [21] reported that immobilisation of bacterial cells significantly enhanced the biodegradation rate of crude oil compared to free-living cells. The use of immobilised cells has been investigated as an alternative technology for environmental applications [21]. The many advantages of immobilized cells systems are providing high biomass, providing cell reuse and reducing the costly processes of cell recovery and cell recycle, high resistance to toxic chemicals, pH, temperature, solvents and heavy metals, elimination of cell washout problems at high dilution rates, high flow rates allow high volumetric productivities, improving genetic stability [12,21,34].

Immobilisation is the restriction of movement of a molecule or microbial cells in space either completely or to a small limited region by attachment to a solid structure. Immobilisation is a naturally occurring process that refers to the act of restricted movement due to little degree of freedom. Radwan et al. [12] provided evidence that the immobilisation principle was already found in nature as microalgal samples collected along the Gulf coast were covered by biofilms of oil utilising bacteria that help degrade hydrocarbons found in seawater.

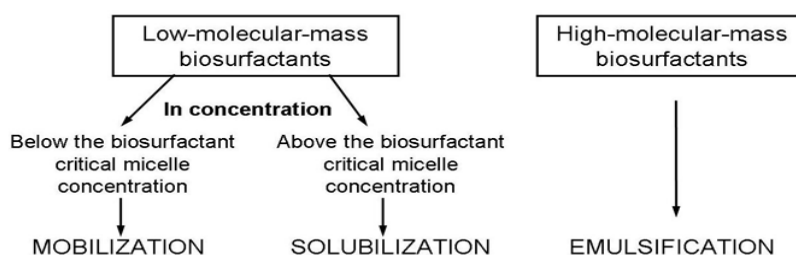


Fig. 3. Mechanisms of hydrocarbon removal by biosurfactants depending on their molecular mass and concentration

Source: [30]

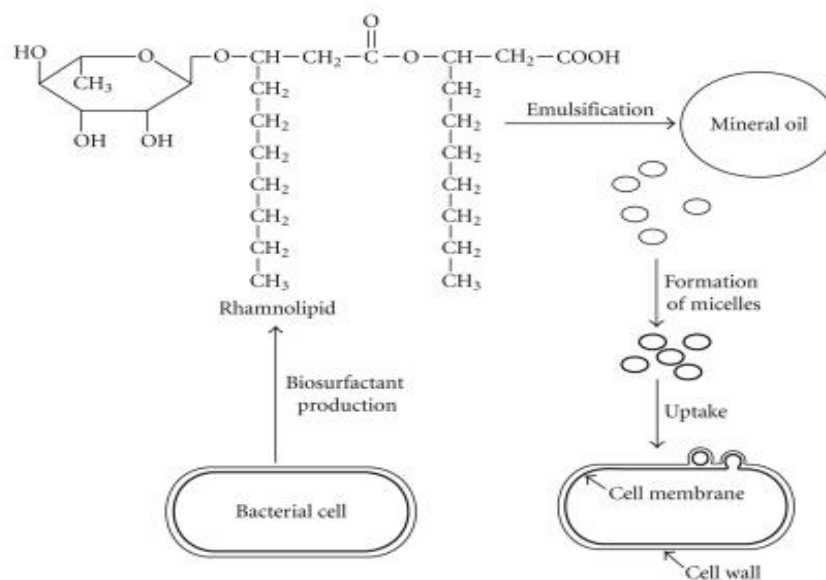


Fig. 4. Involvement of biosurfactant (rhamnolipid) produced by *Pseudomonas sp* in the uptake of hydrocarbons

Source: [32]

4.3 The role Enzymes in Biodegradation

Depending on the presence of certain metabolic pathways, microbial cells can biodegrade hydrocarbons, specific to each function in the environmental conditions. The availability of high enzymatic capacity allows microbial communities to degrade complex hydrocarbons. Biodegradation of petroleum hydrocarbons both aliphatic and aromatic compounds may occur under anaerobic or aerobic conditions [20,34-35].

The super enzyme cytochrome P450 alkane hydroxylases constitutes a 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 [20]. In microorganisms, P450 multiplicity can only be found in few species. These enzymes have been isolated from yeast species such as *Candida maltose*, *Candida tropicalis* and *Candida apicola* [5,20].

5. CONCLUSION

Microbial degradation of petroleum hydrocarbons and other petroleum product pollutants in the environment is a complex one. Several factors such as the nature and concentration of the hydrocarbons present, the ambient and seasonal environmental conditions and the composition of the indigenous microbial community plays a key role in this process [35-37].

Biodegradation, especially in aquatic ecosystems depend greatly on factors such as dispersion and emulsification [38,39]. In terrestrial ecosystems, the spilled oil is absorbed, thereby reducing its toxicity [40,41]. The growth of hydrocarbon-oclastic microorganisms in the environment can greatly influence the process of biodegradation. Bacteria and fungi are the predominant microbial communities that aid the process of hydrocarbon biodegradation, with bacteria playing the dominant role in marine ecosystems, and fungi the most dominant determinant in terrestrial and freshwater environments. Biotic determinants such as seeding of hydrocarbon polluted ecosystems with hydrocarbon degrading microorganisms and the application of previously exposed microbial consortia are successful approaches in bioremediation of hydrocarbons contaminated ecosystems [9,16].

Recently, technological advances such as metagenomics analyses and other "omic" techniques that are environmental friendly have been introduced to analyse the genomic make up of environmental microorganisms towards assessing their capabilities and rate of biodegradation of petroleum hydrocarbons.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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