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Review Paper

Microbial Enzyme Remediation of Poly-Aromatic Hydrocarbon (PAH's) A Review

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Abstract: Pollution of soil by petroleum hydrocarbon (HC) has continued to draw serious concern due to their recalcitrant nature. The HC pollutant are majorly aliphatic and aromatic complexes of incomplete combustion of waste products from automobiles. This HC pollutant can survive in the soil for long time, causing deleterious effect to plant, animals, and humans. Microbial break down or utilization of HC by bacteria and fungi population within the polluted environment can be achieved through biostimulation or bioaugumentation technology by enzymes embedded in the microbial cells. Density, viscosity, pour-point, and solubility are some of the physicochemical parameters that may influence microbial response to HCs. Lack of nutrients, temperature, pH, oxygen are major factors that slows down HC remediation. The degradation of aliphatics by monooxygenase, attacks the terminal methyl group responsible for primary alcohol formation, which is further broken down, to aldehyde and fatty acid. The degradation of aromatics follows dioxygenase-catalyzed oxidation of arenes in aerobic microbial population to yield Vicinal and Vicinal Cis-dihydrodiols Cis, cis-muconic acid (ortho-cleavage) and 2-hydroxymuconic semialdehyde (meta-cleavage) is the final product of enzyme catechol 1,2-dioxygenase (C12O) and catechol 2,3-dioxygenase (C23O) catalyzed degradation of HC in the Tricarboxylic Acid Cycle (TCA). The ability of microbial isolates to produce significant enzymes such as C12O, C23O highlights their future remediation significance.

Keywords: *Hydrocarbons (HCs), Biostimulation, Bioaugumentation, Enzymes, Remediation, Ortho-cleavage, Meta-cleavage,Tricarboxylic Acid Cycle (TCA)*

Introduction

Pollution is the introduction of components such as elements, compounds, or energy into the environment at concentrations that impairs biological functioning or that present an unacceptable risk to humans or other targets that use or are linked to that environment (Fernández-Luqueño et al., 2011). The major component of petroleum hydrocarbon pollution is the aliphatic and aromatic complexes formed after combustion of petroleum products (Okolafor and Ekhaise, 2021). Several researchers have reported the deleterious effect of aromatic compounds such as Polyromantic hydrocarbons (PAHs) to human and animals. PAHs are implicated to be carcinogenic, mutagenic and teratogenic to human and animal health (Dugay et al., 2002; Juhasz and Naidu, 2000; Meudec et al, 2006; Wilcke, 2000); thus PAHs have been listed as priority pollutants by United State Environmental Protection Agency (Thavamani et al., 2012).

Most hydrocarbon degrading bacterial population are known to metabolize or mineralize aliphatic and aromatic by the action of enzymes embedded in the bacterial cells. These enzymes serve two main purposes such as peripheral recognition of aliphatic and aromatic compounds and conversion of degradable aliphatic and aromatic molecules by fission, thereby allowing free entry as route for carbon sequestration and energy generation (Mishra et al., 2011). However, one major challenge in the remediation of aliphatic and aromatic is the lack of sufficient nutrient to the microbial community present in the artificially or accidental polluted soil. Lack of essential nutrients such as carbon, phosphorus, nitrogen and oxygen are major factors that could affect biodegradation of hydrocarbon by microorganisms in soil and water environment (Abioye et al., 2012). The addition of inorganic or organic rich nutrients is an effective approach to improve the bioremediation processes (Hollender et al., 2003; Semple et al., 2006; Walworth et al., 2007).

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Petroleum Hydrocarbon

Petroleum are naturally occurring valuable world resources derived from the Latin word petra (rock) and oleum (oil) (Kumar et al., 2018). Petroleum HCs are made from refining and processes of crude oil. Crude oil is made up of about 50–98% HC components and non-HC component (nitrogen, Sulphur, oxygen and other trace metals) in a complex or broad group.

Report on comprehensive oil property as stated by Lee et al. (2011) revealed information on over 400 oils, posted by Environment Canada. Crude oil is classified into three types based on the origin (terrestrial or geological location), American Petroleum Institute (API) gravity (density) and sulphur content. Low density crude are known as light crude, high density crude are called heavy crude oil and Sulphur containing crude are called sour crude (Kumar et al., 2018). Petroleum HC are classified into four main groups (Table 1) based on their solubility in organic solvents. Four physical characteristics of oil that have adverse effects on the environment and cleanup responses of microorganisms are discussed below:

Density

API reported that gravity and specific gravity are commonly used to determine the density of oil. Specific gravity is the measured of the ratio of mass of oil to mass of corresponding water at known temperature. The API gravity suggests a value of 10^0 to pure water at 10 ^{0}C (60^0F). Extremely high API gravity is an indication of oils with low densities or low specific gravities. Kumar et al. (2018) reported the specific gravities of crude oil ranged from 0.79 to 1.00, which is equivalent to API gravities of 10 - 48. Density is an important index that may be used to predict the composition of oil fate in water.

Viscosity

Viscosity is the physical property of a fluid that allows it to withstands changes in shape or motion (Xu et al., 2017). The decrease in viscosity result in corresponding increase in fluid flow. The viscosity of petroleum products is associated with the oil compositions and the surrounding temperature (Saeed et al., 2016). Viscosity is an essential index of spilled HC oil spreading rate.

Pour point

Temperature in which oil stops flowing or converted to semi-solid is known as pour point. Temperature range of −57 to 32 °C is the pour point of oil (Yasin et al., 2013). Pour point is the major characteristic of oil fate and cleanup plan action of HCs.

Solubility in water

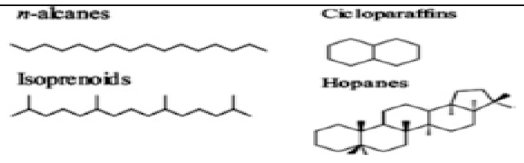
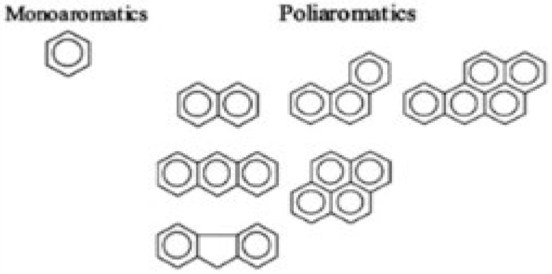
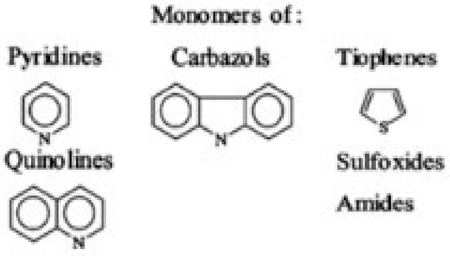
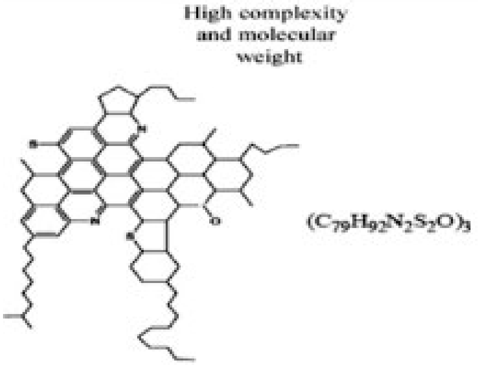
Petroleum HCs solubility in water is determined by temperature and chemical compositions of the HCs. The deficient in the solubility of common crude oil is reported to be around 30 mg/L (Adeniji et al., 2017). The solubility in water property of oil is critical in determining the oil toxicity, oil fate and appropriate bioremediation approach to follow. The most soluble oil constituents are low molecular weight aromatics compounds such as toluene, benzene and xylene (Kumar et al., 2018).

Hydrocarbon as Pollutants

Hydrocarbon (HC) pollutants in the environment can be classified as organic or inorganic (Chikere, 2013). The organic pollutants are mainly HCs in different forms. The most common are petroleum HCs which include n-alkanes and other aliphatics, aromatic compounds and other components in trace amounts (Atlas and Philp, 2005; Sarkar et al., 2005).

Spillage from crude oil and refined fuel have damaged the natural ecosystems in the Niger Delta region, South-South Nigeria and many other places worldwide. The quantity of oil spilled during accidental release of oil to the environment ranged from a few hundred tons to several hundred thousand tons (Deepwater Horizon Oil Spill, Atlantic Empress, Amoco Cadiz), but it is a limited measurement of damage or impact (Godleads et al., 2015; Stephen and Temola, 2014). Oil spills in soil reduces the ability of the soil to support the growth of plants, leaches into the ground to pollute groundwater, and increases the presence of heavy metals which bioaccumulate and biomagnify thereby causing adverse health effects to the receiving environment. Major constrains facing scientists and industrialists today is how to tackle the problem arising from pollution of soil and water by HCs; using environmental friendly, safe approach and cost implications of degrading these contaminants. Bioremediation technology is a veritable tool for HCs degradation that is intensively studied today (Godleads et al., 2015).

Table 1: Various categories of hydrocarbons

Categories	Carbon Atoms	Components	HC compound (s)
Saturated	1-45	Normal alkanes	
		Branched alkanes	
Aromatic	4-45	Two or more fused aromatic rings	
Resins	>40	Polar compounds	
Asphaltenes	>40	NSO(s)	
		Poorly characterized HCs	
		High-molecular- weight compounds	
		Heavy metals	

Adapted from (Kumar et al., 2018)

Hydrocarbon soil interactions

HCs and soil interaction at the surfaces (clay, silt and sand) are only substantial when percentage organic matter is 0.1 (Chikere et al., 2011). Thus, the importance of organic matter in the fate and behavior of organic contaminants is far-reaching. Organic matter is divided into two major phases; soft carbon (elastic) which is the expanded, flexible arrangements having fulvic, and humic acids as key components with sorption described as irreversible hard carbon (glassy) defined as rigid, condensed structures with humin, kerogen and pyrogenic carbons as commonly identified components (Chikere et al., 2011). Uptake of hydrocarbons materials within the glassy region is characterized by irreversible sequestration (Stroud et al., 2007; Van Elsas et al., 2007). HCs sequestration within the soil through sorption to organic matter and mineral elements or diffuse into the three-dimensional structure of the soil as showed in Figure 1.

The rate to which these physical interactions occur increases with time and is termed ‘aging’. For aromatics fractions, their fate in the soil is dependent on their molecular size, i.e., the number of aromatic rings they possess. An increase in the size and angularity of aromatics results in a related increase in electrochemical stability, hydrophobicity, high sorption capacity and their persistence in the soil (Kanaly and Harayama, 2000; Van Hamme et al., 2003).

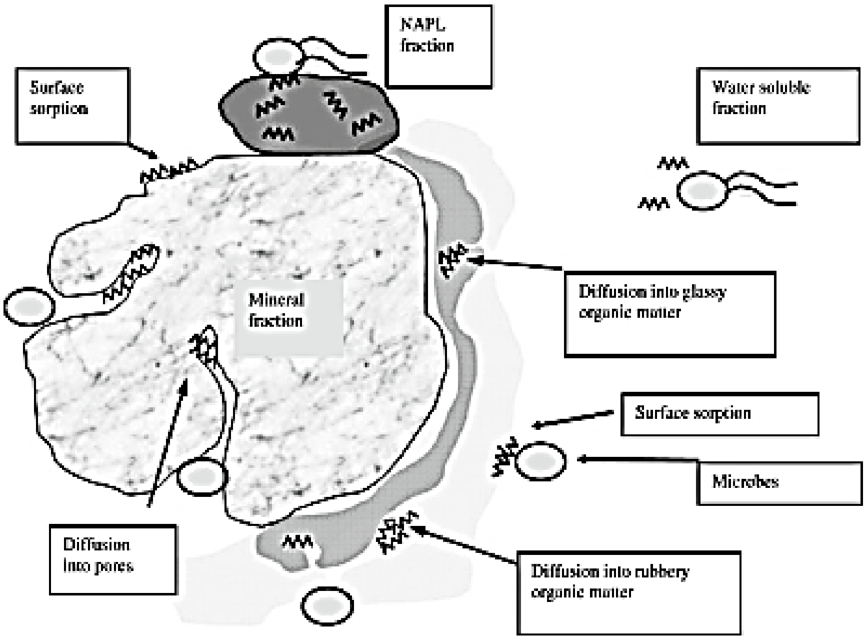


Figure 1. Interactions between soil matrices and aliphatic hydrocarbons; NAPL, non-aqueous liquid phase (Stroud et al., 2007).

Microbial hydrocarbon interactions in soil

Two major characteristics that define HCs-oxidizing microorganisms in the soil are; membrane-bound, group-specific oxygenases; and mechanisms for optimizing contact with microorganisms and the water-insoluble HCs (Chikere et al., 2011). HCs are known substrate for microbial oxidation by bioavailability and bioaccessibility (Semple et al., 2003). A compound is said to be bioavailable if it is freely available to cross an organism's membrane from the medium the organism inhabits at a given time. Bioaccessible compound is a compound available to cross an organism's cell membrane from the environment it inhabits if the organism can access the polluted environment.

Microbial diversity of HCs degradation in the soil

The degradation of HCs is mediated by diverse groups of microorganisms. it is widely reported that bacteria and fungi are primary mediators in HCs degradation. Bacteria are reported to be more versatile than fungi and therefore may play a greater role during biodegradation of HCs compounds. The most important HC-degrading bacterial genera in soil environments include *Alcaligenes*, *Bacillus*, *Achromobacter*, *Acinetobacter*, *Arthrobacter*, *Burkholderia*, *Collimonas*, *Corynebacterium*, *Dietzia*, *Flavobacterium*, *Gordonia*, *Micrococcus*, *Pseudomonas*, *Mycobacterium*, *Nocardia*, *Nocardioides*, *Ralstonia*, *Rhodococcus*, *Sphingomonas*, *Variovorax* and other unculturable bacteria (Chikere, et al., 2009; Hamamura et al., 2006; Obayori and Salam, 2010). Among fungi genera that can degrade complex HCs are *Mucor*, *Aspergillus*, *Candida*, *Cunninghamella*, *Fusarium*, *Penicillium*, *Rhodotorula*, *Phanerochaete*, *Sporobolomyces* and *Trichoderma* (Chaillan et al., 2004; Das and Chandran, 2011; Singh, 2006).

Bioremediation of HC contaminated soils

Bioremediation is the removal, destruction, or conversion of contaminants to less harmful substance (Agamuthu et al., 2013). Bioremediation remains a highly potential approach since many studies have reported its effectiveness in removing numerous pollutants from contaminated sites (Nie et al., 2009; Rimmer et al., 2006; Li et al., 2013). Generally, bioremediation technologies can be classified as *in situ* or *ex situ*. *In situ* bioremediation involves treating the contaminated material at the site while *ex situ* involves the removal of the contaminated material to be treated elsewhere (Gavrilescu, 2010).

Studies on petroleum HC biodegradation adopted various methodologies (Bidoia et al., 2010; Cerqueira et al., 2014; Zhang et al., 2016), although they all indicated that degradation can occur in exact fractions of the compound to be degraded. There is no rule of thumb in petroleum HC biodegradation as most compound showed preferential remediation of lighter HC compounds, while others directed towards heavier HCs (Huang et al., 2004).

Biodegradation is the breakdown of HCs through enzymatic activity of microorganisms (fungi and bacteria) depending on the ability of the microbes to emulsify the undissolved carbon component in the culture medium (Chrzanowski et al., 2005; Mancera-López et al., 2008). There exist three main types of bioremediations, they include biostimulation, bioaugumentation and phytoremediation.

Bioaugmentation: Bioaugmentation is a major type of bioremediation technologies today (Simarro et al., 2013). This process involves the introduction of nonindigenous organisms that possesses high degradation potentials to the soil in order to promote the bioremediation process (Wu et al., 2013). Microorganisms used in bioaugmentation must meet the requirements for effective degradation of the target pollutant, possess the ability to adapt to the environment and they must be nonpathogenic in nature (Szulc et al., 2014).

Various studies showed that bioaugmentation surges the biodegradation of HC compounds in wet land soils polluted with petroleum compounds (Suja et al., 2014; Taccari et al., 2012). The effectiveness of bioaugmentation depends on the type of inoculum and nutrient requirements (Abed et al., 2014; Suja et al., 2014).

Biostimulation: Increase in biodegradation of indigenous bacteria resulting to increase in bacterial population due to increased nutrients is referred to biostimulation. Microorganisms will proliferate and degrade compounds when nutrients and electron acceptors are sufficient. Available nitrogen compounds such as NO_3 or NH_4^+ are electron acceptor and nutrient requirements for bioremediation process (Kumar et al., 2018). Since HC biodegradation is limited by absence of physicochemical factors such as temperature, pH, moisture, oxygen, other soil properties (Al-Sulaimani et al., 2010; Atagana et al., 2008; Bundy et al., 2002), biostimulation during bioremediation process is inevitable. The advantage of biostimulation is that it will be carry out by already present indigenous microorganisms that are suitable to the subsurface soil, while the transfer of additives in a manner that permits the additives to be made available to the subsurface of the microorganisms is the major challenge (Godleads and Prekeyi, 2015).

Effect of Biological Factors in HCs Degradation

Major factors affecting microbial HCs degradation are the utilization of pollutants by microorganisms with the catabolic capability to degrade them, the activity of degrading microorganisms, limited number of degrading microbes in the soil and complex molecular structure of the organic pollutant (Semple et al., 2003). About 10^4 to 10^7 colony forming unit are present in soil, to accomplish biodegradation process; the above figure should not reduce below 10^3 per gram of soil, since it is an indication organic or inorganic contaminants in the soil (Margesin et al., 2003). Other factors that influence biodegradation are temperature, nutrient availability, moisture level, and oxygen demand of soil.

Temperature

Organic matter decay and degradation is directly influence by temperature. Biochemical activities which is the metabolic action of the organisms are hindered by temperature fluctuation. Hydrocarbon degraders (bacteria) grow best in optimized temperature conditions for a given species of the organisms. Temperature ranges of 20 - 33 ^{0}C is reported as the optimum temperature for microbial decomposition of HCs in the environment (Iranzo et al., 2001) . Temperature can slow down or increase biodegradation activity but may not affect responses to biochemical action. Temperature also affects the moisture of soil and retention potential of the soil. The rate of biodegradation increases with increase in temperature and decreases when temperature is decreases (ESTCP, 2007).

Nutrients

In chemical perspective, nutrients are grouped into macro, micro, and trace elements based on the quality and the vital requirements of HC degrading microorganisms. Carbon, phosphorus and nitrogen are mainly macronutrients comprising up to 14% of dry weight of microbial cells. Micronutrient group include sulfur, magnesium and calcium containing up to 0.5 % dry weight of a cells. Trace elements such as iron, cobalt, manganese, copper, and zinc are not required by HC degrading organisms. Graj et al. (2013) reported that oil HCs reduces the accessibility of plant nutrients in the soil. Hydrocarbon degraders in the soil consume available nutrients or fix nitrogen and phosphorus, thereby reducing the nutrient availability. Nutrients are always available for microbial growth in an HC polluted site, although nutrients can be introduced in a utilized form as organic substrate amendment (Godleads et al., 2015), which acts as electron donor to fuel bioremediation.

pH

Hydrocarbon degradation occurs more at pH range 6 to 8 (Iranzo et al., 2001; Mammitzsch et al., 2014). Bacteria and microbial growth decreases with decrease in pH less than the optimum pH level required for microbial species. The pH affects availability of carbon and nutrients and the solubility of heavy metals in the soil (Mousavi et al., 2017). Therefore, pH is an important factor for microbial community (Wang et al., 2017). Biodegradation of HCs compounds depends solely on the presence of enzymes that are dependent on pH value (Wang et al., 2017). Hydrocarbon biodegradation occurs faster at pH 7 particular fungi species compared to bacteria that can allow up to pH 5 (Das and Chandran, 2010).

Oxygen

Hydrocarbon degradation undergoes aerobic and anaerobic conditions. Aerobic respiration involves the utilization O_2 by microorganisms as final electron acceptor during metabolic reactions and oxidation (reduction) process. Microbes use nitrate-iron, sulfate and CO_2 as electron acceptor in anaerobic respiration (Ladino-Orjuela et al., 2016). Aerobic respiration process results in the breakdown of toxic matters/compounds non-toxic substance such as CO_2 and water (Rockne and Reddy, 2003). Degradation of HC compounds occurs faster during aerobic reaction than anaerobic conditions, and then HCs are broken down to H_2O and CO_2. Therefore, the rate of bioremediation increases with increase in oxygen level.

Degradation of Aliphatic and Aromatic HCs

It is reported that aromatic component of HCs is highly toxic compared to aliphatic HCs components (Uche and Dadrasnia, 2017). The aromatic compounds possesses antagonistic effects on the native soil microflora. Compounds such as isobornyl, camphene, acetate, limonene, and α-pinene were inhibitors of many microorganisms (Kumar et al., 2018). Phenolic and quinonic naphthalene derivatives demonstrated antagonistic activity against microbial cell growth (Qingren et al., 2011).

Enzyme Activity in the Degradation of Aliphatic and Aromatics

Aliphatic HCs degradation

Aliphatic HC Degradation results in the formation of CO_2 and H_2O. Two major biodegradation pathways are involved in the degradation of alkanes. The initial step is the aerobic degradation of saturated, aliphatic HC (n-alkanes) involves the enzymes that possesses a strict prerequisite for molecular oxygen; which are monooxygenases (mixed function oxidases) or dioxygenases (Chikere et al., 2011). The common pathway depends on the activity of monooxygenase enzymes which are specific for n-alkanes (Stroud et al., 2007). Monooxygenase attacks the terminal methyl group responsible for primary alcohol formation (Van Hamme et al., 2003). The alcohol is oxidized to aldehyde and fatty acid (Fig. 2).

Secondly, a dioxygenase enzyme acts on the terminal methyl group of an n-alkane leading to the addition of two oxygen atoms, resulting in the formation of a peroxide which is converted to a fatty acid (Figure 2). The carboxylic acid groups in the fatty acids are further metabolized via the b-oxidation pathway (Figure 2), which is a common catabolic pathway in living cells, to form acetyl CoA or propionyl CoA depending on the number of carbon atoms present in the n-alkane. These compounds are further metabolized via the tricarboxylic acid cycle (TCA cycle) to CO_2 and H_2O (Turley et al., 1981; Van Elsas et al., 2007; Van Hamme et al., 2003).

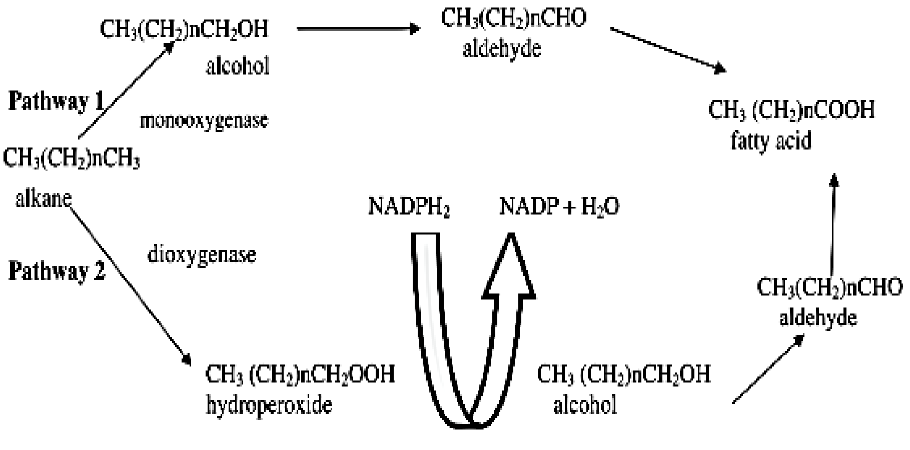


Figure 2. Pathway for the biodegradation of aliphatic HCs (alkanes) (Van Elsas *et al.*, 2007)

Aromatic HCs degradation

The major reason for the low rate of biodegradation of aromatic HCs are low solubility, metabolite repression, production of toxic dead-end metabolites, presence of preferred substrates and lack of co-metabolic substrates (Kanaly and Harayama, 2000; Van Elsas et al., 2007; Van Hamme et al., 2003). While other aromatic HCs such as BTEX are present in petroleum, naphthalene, with two aromatic rings, represents the simplest form of polycyclic aromatic hydrocarbon (PAH).

No single microorganism has been able to utilize high molecular weight aromatic compounds such as benzo(a)pyrene as the sole source of energy, even though the transformations through co-metabolic activities have been reported (Van Elsas et al., 2007). The first step of aromatic HC degradation, dioxygenase-catalyzed the of oxidation of arenes in aerobic bacteria to yield vicinal vicinal cis-dihydrodiols as early intermediates by a multicomponent enzyme system (Chikere *et al.*, 2011). The byproducts dihydroxylated cleave by intradiol or extradiol ring cleaving dioxygenases through either an ortho-cleavage pathway (Figure 3) or meta-cleavage pathway (Figure 4), resulting to intermediates such as protocatechuate and catechols (Fig. 5) using benzene as an example (Peng *et al.*, 2008)

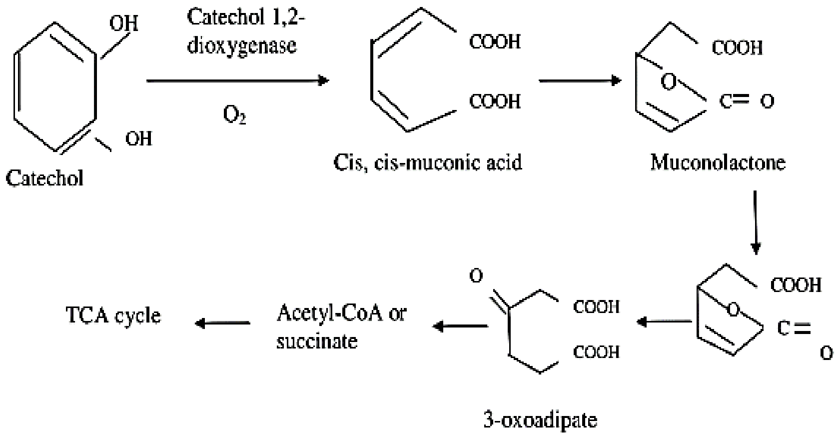


Figure 3. Ortho-cleavage of catechol in the TCA (Tricarboxylic acid) cycle (Van Elsas *et al.*, 2007)

Complete breakdown of metabolites such as succinate, acetate, pyruvate or acetaldehyde from the above pathways enters the TCA cycle and are thus available as carbon or energy sources to the cell (Turley et al., 1981Turley et al., 1981; Kanaly and Harayama, 2000). Degradation of crude oil by Microbes results in the attack on aliphatic or aromatic fractions of HCs. Studies have reported removal of HCs at high rates under optimal conditions (Turley et al., 1981Turley et al., 1981; Hamamura et al., 2006; Leahy and Colwell, 1990; Maila et al., 2006; Margesin et al., 2003; Rosenberg et al., 1992). High molecular weight aromatics HCs such as resins and asphaltenes are considered recalcitrant or exhibit only slow biodegradation rates (Turley et al., 1981; Stroud et al., 2007; Van Hamme et al., 2003).

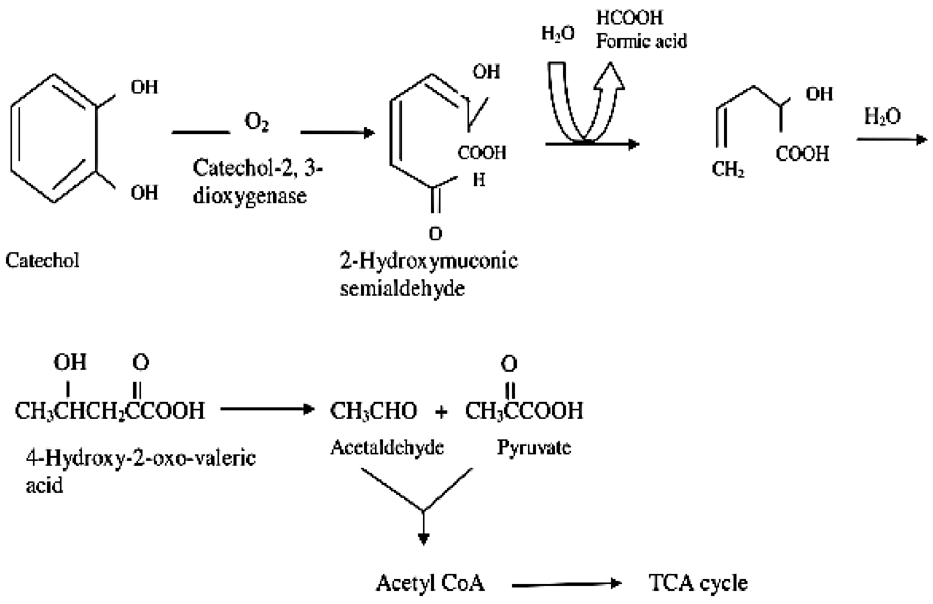


Figure 4. Meta-cleavage of catechol in the TCA (Tricarboxylic acid) cycle (Van Elsas *et al.*, 2007)

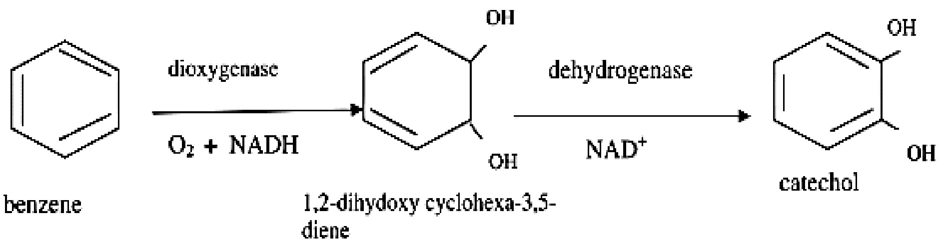


Figure 5. Biotransformation of aromatic benzene to catechol (Van Elsas *et al.*, 2007)

Conclusion

The damages incurred from aliphatic and aromatic petroleum hydrocarbon pollution remain a challenge to the environment. Since recycling of byproducts of petroleum hydrocarbon containing pollutants is not visible for the now, alternative means of remediating this waste can be explored using enzyme (monooxygenase and dioxygenase) catalyzed microbial remediation. The ability of microbial isolates to produce significant enzymes such as C12O, C23O highlights the future remediation significance of microbial population of HC polluted soils.

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