

Standard Review

Biodegradation alternative in the cleanup of petroleum hydrocarbon pollutants

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Extensive petroleum hydrocarbon exploration activities often result in the pollution of the environment, which could lead to disastrous consequences for the biotic and abiotic components of the ecosystem if not restored. Remediation of petroleum-contaminated system could be achieved by either physicochemical or biological methods. However, the attendant negative consequences of the physicochemical approach are currently directing greater attention to the exploitation of the biological alternatives. This paper provides a review of the menace of petroleum hydrocarbon pollution and its biodegradation in the environment with the view of understanding the biodegradation processes for better exploitation in bioremediation challenges.

Key words: Petroleum hydrocarbon, pollution, environment and biodegradation.

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1.0 Introduction

Claude U. Sable had as far back as 1946, recognised that many microorganisms have the ability to utilise hydrocarbons as the sole source of carbon and energy, and that such microorganisms are widely distributed in nature. He further recognised that the microbial utilisation of hydrocarbons was highly dependent on the chemical nature of the components within the petroleum mixture, and environmental determinants (Atlas 1981).

However, it was only after the sinking of the super tanker *Torrey Canyon* in the English Channel that the attention of the scientific community was drawn towards the problems of oil pollution. Thereafter, several studies have examined the fate of petroleum in various ecosystems (Boehm et al., 1995; Whittaker et al., 1999). The development of petroleum industry into new frontiers, the apparent inevitable spillages that occur during routine operations, and records of acute accidents during

transportation has called for more studies into oil pollution problems (Timmis et al., 1998), which has been recognized as the most significant contamination problem on the continent (Snape et al., 2001). Also, the extensive use of petroleum products leads to the contamination of almost all compartments of the environment, and biodegradation of the hydrocarbons by natural populations of microorganisms has been reported to be the main process acting in the depuration of hydrocarbon-polluted environments (Challain et al., 2004), the mechanism of which has been extensively studied and reviewed (van Hamme et al., 2003).

Crude oil can be accidentally or deliberately released into the environment leading to serious pollution problems (Thouand et al., 1999). Even small releases of petroleum hydrocarbons into aquifers can lead to concentrations of dissolved hydrocarbons far in excess of

regulatory limits (Spence et al., 2005). These pollution problems often result in huge disturbances of both the biotic and abiotic components of the ecosystems (Mueller et al., 1992), more so that some hydrocarbon components have been known to belong to a family of carcinogenic and neurotoxic organopollutants (Hallier-Soulier et al., 1999). The processes leading to the eventual removal of hydrocarbon pollutants from the environment has been extensively documented and involves the trio of physical, chemical and biological alternatives.

The currently accepted disposal methods of incineration or burial in secure landfills (USEPA 2001; ITOF 2006) can become prohibitively expensive when the amounts of contaminants 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 (Pye and Patrick 1983), thus necessitating speedy removal of the contaminants.

The biodegradation of oil pollutants is not a new concept as it has been intensively studied in controlled conditions (Sugiura et al., 1997; Chaillan et al., 2004) and in open field experiments (Chaîneau et al., 2003; Gogoi et al., 2003), but it has acquired a new significance as an increasingly effective and potentially inexpensive clean-up technology. Its potential contribution as a countermeasure biotechnology for decontamination of oil-polluted systems could be enormous. The initial interest was in the fate and persistence of pesticides in soils (Prince 1993). However, the field has expanded in recent years to encompass a wide variety of chemicals and a broad array of issues. Some technologies are being developed that markedly enhance microbial destruction or degradation of organic pollutants that otherwise would have persisted at the cleanup of many polluted groundwater and soils using the orthodox physical and chemical methods (Alexander 1994).

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. It is useful in the recovery of sites contaminated with oil and hazardous wastes (Caplan 1993). Besides, bioremediation technology is believed to be non-invasive and relatively cost effective (April et al., 2000). In some cases it may not require more than the addition of some degradation enhancers to the polluted system. It could end up being the most reliable and probably least expensive option for exploitation in solving some chemical pollution problems (Mesarch et al., 2000).

Westlake (1982) noted that no single microbial species has the enzymatic ability to metabolise more than two or three classes of compounds typically found in crude oil. A consortium composed of many different bacterial species is thus required to degrade crude oil significantly. The use of a bacterial consortium provides certain advantages over biostimulation in cases where pollutant

toxicity or a lack of appropriate microorganisms (both quantity and quality) is important. Determination of the potential success of application of bacterial consortium requires an understanding of the survival and activity of the added microorganism(s) or their genetic materials, and the general environmental conditions that control the degradation rates such as the peculiarity of the contaminated site, for example, water or soil systems (Vogel 1996). These factors may very well vary from place to place and from organism to organism.

It is a common stance that many farmers in the oil exploration areas in developing countries are experiencing tremendous difficulties in restoring the fertility of pollution devastated farmlands due to lack of knowledge on appropriate remediation procedures. This problem could be attended to if adequate attention is given to the need for baseline data for the evaluation of the application of bioremediation technology in the peculiar localities, using indigenous isolates of microorganisms such as reported by Ebuehi et al. (2005). The non-chalant attitude to the problem of oil pollution is particularly of serious concern for food safety in such neglected areas as the Niger delta regions of Nigeria as persistence of the pollution could result in the release of toxic pollutants into the food chain and water products (Bradley et al., 1997).

The success of the bioremediation efforts in the cleanup of the oil tanker *Exxon Valdez* oil spill of 1989 (Atlas and Bartha, 1998) in Prince William Sound and the Gulf of Alaska has created tremendous interest in the potential of biodegradation and bioremediation technology. Many countries are currently convinced of the full potential of this technology and are, therefore, encouraging research into improving and optimising the procedures.

2.0 Chemistry and Biodegradability of petroleum hydrocarbon

Petroleum has been known for several years to occur in the surface seepage and was first obtained in pre-Christian times by the Chinese. The modern petroleum industry had its beginning in Romania and in a well-sunk in Pennsylvania by Colonel E. A. Drake in 1859 (Alloway and Ayres 1993). The principal early use of the product of the petroleum industry was for the replacement of expensive whale oil for lighting. Today, its consumption as a fuel and its dominance in the world market as a source of chemicals has diversified tremendously.

Petroleum is defined as any mixture of natural gas, condensate, and crude oil. Crude oil which is a heterogeneous liquid consisting of hydrocarbons comprised almost entirely of the elements hydrogen and carbon in the ratio of about 2 hydrogen atoms to 1 carbon atom. It also contains elements such as nitrogen, sulphur and oxygen, all of which constitute less than 3% (v/v). There are also trace constituents, comprising less than

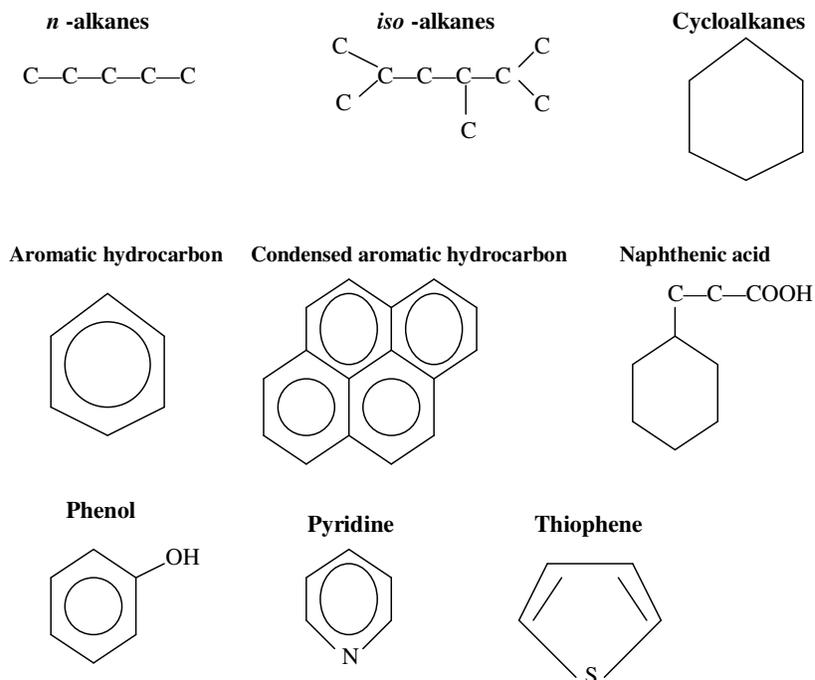


Figure 1. Structural classification of some crude oil components (Alloway and Ayres 1993).

1% (v/v), including phosphorus and heavy metals such as vanadium and nickel. Crude oils could be classified according to their respective distillation residues as paraffins, naphthenes or aromatics and based on the relative proportions of the heavy molecular weight constituents as light, medium or heavy. Also, the composition of crudes may vary with the location and age of an oil field, and may even be depth dependent within an individual well. About 85% of the components of all types of crude oil can be classified as either asphalt base, paraffin base or mixed base. Asphalt base contain little paraffin wax and an asphaltic residue (Atlas 1981). The sulphur, oxygen and nitrogen contents are often relatively higher in comparison with paraffin base crudes, which contain little or no asphaltic materials. Mixed crude oil contains considerable amount of oxides of nitrogen and asphalt.

Petroleum oil biodegradation by bacteria can occur under both oxic and anoxic conditions (Zengler et al., 1999), albeit by the action of different consortia of organisms. In the subsurface, oil biodegradation occurs primarily under anoxic conditions, mediated by sulfate reducing bacteria (e.g., Holba et al., 1996) or other anaerobes using a variety of other electron acceptors as the oxidant. On a structural basis, the hydrocarbons in crude oil are classified as alkanes (*normal* or *iso*), cycloalkanes, and aromatics (Figure 1). Alkenes, which are the unsaturated analogs of alkanes, are rare in crude oil but occur in many refined petroleum products as a consequence of the cracking process. Increasing carbon numbers of alkanes (homology), variations in carbon

chain branching (*iso*-alkanes), ring condensations, and interclass combinations e.g., phenylalkanes, account for the high numbers of hydrocarbons that occur in crude oil. In addition, smaller amounts of oxygen – (phenols, naphthenic acids), nitrogen- (pyridine, pyrrole, indole), and sulfur- (alkylthiol, thiophene) containing compounds, collectively designated as “resins” and partially oxygenated, highly condensed asphaltic fraction occur also in crude but not in refined petroleum (Atlas and Bartha 1973).

The inherent biodegradability of these individual components is a reflection of their chemical structure, but is also strongly influenced by the physical state and toxicity of the compounds. As an example, while *n*-alkanes as a structural group are the most biodegradable petroleum hydrocarbons, the $C_5 - C_{10}$ homologues have been shown to be inhibitory to the majority of hydrocarbon degraders. As solvents, these homologues tend to disrupt lipid membrane structures of microorganisms. Similarly, alkanes in the $C_{20} - C_{40}$ range, often referred to as “waxes”, are hydrophobic solids at physiological temperatures. Apparently, it is this physical state that strongly influences their biodegradation (Bartha and Atlas 1977).

Primary attack on intact hydrocarbons always requires the action of oxygenases and, therefore, requires the presence of free oxygen. In the case of alkanes, monooxygenase attack results in the production of alcohol. Most microorganisms attack alkanes terminally whereas some perform sub-terminal oxidation (Figure 2). The alcohol product is oxidised finally into an aldehyde

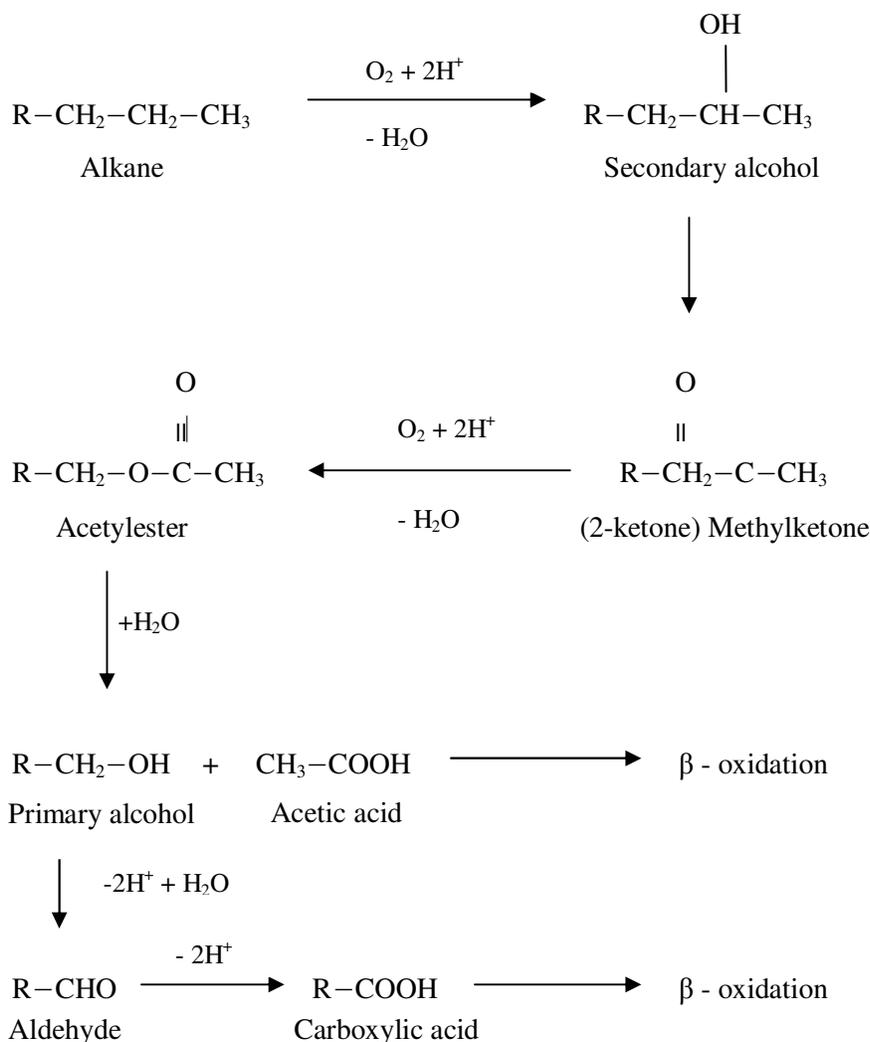


Figure 2. Pathways, through which subterminal oxidation of alkanes yield two fatty acid moieties, which are metabolized further by beta-oxidation (Atlas and Bartha 1998).

and finally, to a fatty acid. The latter is degraded further by *beta*-oxidation.

Extensive methyl branching interferes with the beta-oxidation process and necessitates diterminal attack (Figure 3) or other bypass mechanisms. Therefore, *n*-alkanes are degraded more readily than iso alkanes. Cycloalkanes are transformed by a not fully characterized oxidase system to a corresponding cyclic alcohol, which is dehydrated to ketone. Then, a monooxygenase system lactonises the ring, which is subsequently opened by a lactone hydrolase. These two oxygenase systems usually never occur in the same organisms and hence, the frustrated attempts to isolate pure cultures that grow on cycloalkanes (Bartha 1986b). However, synergistic actions of microbial communities are capable of dealing with degradation of various cycloalkanes quite effectively.

As in the case of alkanes, the monocyclic compounds, cyclopentane, cyclohexane, and cycloheptane have a

strong solvent effect on lipid membranes, and are toxic to the majority of hydrocarbon degrading microorganisms. Highly condensed cycloalkane compounds resist biodegradation due to their structure and physical state (Bartha 1986a).

Prokaryotes convert aromatic hydrocarbons by an initial dioxygenase attack, to *trans*-dihydrodiols that are further oxidised to dihydroxy products, e.g., catechol in the case of benzene (Atlas and Bartha, 1998). Eucaryotic microorganisms use monooxygenases, producing benzene 1, 2-oxide from benzene, followed by the addition of water, yielding dihydroxydihydrobenzene (*cis*-dihydrodiol). This is oxidised in turn to catechol, a key intermediate in biodegradation of aromatics, which is then opened by *ortho*- or *meta*-cleavage, yielding muconic acid or 2-hydroxymuconic semialdehyde, respectively.

Condensed polycyclic aromatics are degraded, one ring at a time, by a similar mechanism, but biodegrada-

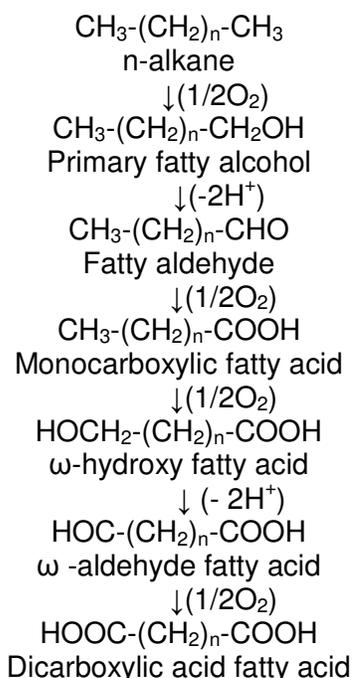


Figure 3. Pathway of diterminal alkane oxidation (Atlas 1984).

bility tend to decline with the increasing number of rings and degree of condensation (Atlas and Bartha 1998). Aromatics with more than four condensed rings are generally not suitable as substrates for microbial growth, though, they may undergo metabolic transformations. Biodegradation process also declines with the increasing number of alkyl substituents on the aromatic nucleus. Asphaltics tend to increase during biodegradation in relative and sometimes absolute amounts. This would suggest that they not only tend to resist biodegradation but may also be formed *de novo* by condensation reactions of biodegradation and photodegradation intermediates.

In crude petroleum as well as in refined products, petroleum hydrocarbons occur in complex mixtures and influence each other's biodegradation. The effects may go in negative as well as positive directions. Some *iso*-alkanes are apparently spared as long as *n*-alkanes are available as substrates, while some condensed aromatics are metabolised only in the presence of more easily utilisable petroleum hydrocarbons, a process referred to as co-metabolism (Wackett 1996).

3.0 Factors influencing petroleum hydrocarbon biodegradation

Successful application of bioremediation technology to contaminated systems requires knowledge of the characteristics of the site and the parameters that affect

the microbial biodegradation of pollutants (Sabate et al., 2004). However, a number of limiting factors have been recognised to affect the biodegradation of petroleum hydrocarbons. Typical examples of these factors are as listed in Table 1. Biodegradability is inherently influenced by the composition of the oil pollutant. For example, kerosene, which consists almost exclusively of medium chain alkanes is, under suitable conditions, totally biodegradable. Similarly, crude oil is biodegradable quantitatively, but for heavy asphaltic-naphthenic crude oils, only about 11% may be biodegradable within a reasonable time period, even if the conditions are favourable (Bartha, 1986a). Okoh et al. (2002) reported that between 8.8 and 29% of the heavy crude oil *Maya* was biodegraded in soil microcosm by mixed bacterial consortium in 15 days, although major peak components of the oil was reduced by between 6.5 and 70% (Okoh et al., 2003). Also, about 89% of the same crude oil was biodegraded by axenic culture of *Burkholderia cepacia* RQ1 in shake flask (Okoh et al., 2001) within similar time frame, although petroleum biodegradation has been reported to be mostly enhanced in presence of a consortium of bacteria species compared to monospecies activities (Ghazali et al., 2004).

The composition and inherent biodegradability of the petroleum hydrocarbon pollutant, therefore, is the first and most important consideration when the suitability of a cleanup approach is to be evaluated. We have reported elsewhere (Okoh, 2002) that heavier crude oils are generally much more difficult to biodegrade than lighter ones, just as heavier crude oils could be suitable for inducing increased selection pressure for the isolation of petroleum hydrocarbon degraders with enhanced efficiency. Also, Okoh et al. (2002) noted that the amount of heavy crude oil metabolised by some bacterial species increased with increasing concentration of starter oil up to 0.6% (w/v), while degradation rates appeared to be more pronounced between the concentrations of 0.4 and 0.6% (w/v) oil. In another report (Rahman et al., 2002), the percentage of degradation by the mixed bacterial consortium decreased from 78% to 52% as the concentration of crude oil was increased from 1 to 10%.

Although, there has been a reported case of lack of correlation between degradation rates, specific growth rates and concentration of the starter oil (Thouand et al., 1999), in such a case, it would appear that biomass was required only to a particular threshold enough to produce the appropriate enzyme system that carry through the degradation process even when biomass production had ceased (Okoh 2002), a phenomenon completely at systems (Pitter and Chudoba 1990), where production of variance with the theory of microbial growth in batch cells is totally dependent on the consumed carbon source. However, such scenario is relevant especially in the light of public clamour for the use of cell free systems in bioremediation works against the scepticism that has befallen the application of live organisms, especially

Table 1. Some common factors affecting petroleum hydrocarbon biodegradation.

Limiting factor	Explanation or examples
Petroleum hydrocarbon- composition (PHC).	Structure, amount, toxicity.
Physical state	Aggregation, spreading, dispersion, adsorption.
Weathering	Evaporation, photooxidation.
Water potential	Osmotic and matrix forces, exclusion of water from hydrophobic aggregates
Temperature	Influence on evaporation and degradation rates.
Oxidant	O ₂ required to initiate oxidation, NO ₃ ⁻ or SO ₄ ²⁻ to sustain, PHC biodegradation.
Mineral nutrients	N, P, Fe may be limiting
Reaction	Low pH may be limiting
Microorganisms	PHC degraders may be absent or low in numbers

Source: Bartha 1986a.

GMOs in bioremediation of polluted environment. Also, although many reports on the effect of sunlight irradiation so far published have focused on the physicochemical changes on intact crude oil other than to biodegraded crude oil (Jacquot et al., 1996; Nicodem et al., 1998), recent studies have reported that photooxidation increases the biodegradability of petroleum hydrocarbon by increasing its bioavailability and thus enhancing microbial activities (Maki et al., 2005). In a related study assessing the bioremediation efficiency of a weathered and recently contaminated soil in Brazil (Trindale et al., 2005), the authors reported low biodegradation efficiencies in the weathered soil contaminated with a high crude oil concentration compared to recently contaminated soil. Also, both soils (weathered and recently contaminated) submitted to bioaugmentation and biostimulation techniques presented biodegradation efficiencies approximately twice higher than the ones without natural attenuation.

Temperature plays very important roles in biodegradation of petroleum hydrocarbons, firstly by its direct effect on the chemistry of the pollutants, and secondly on its effect on the physiology and diversity of the microbial milieu. Ambient temperature of an environment affects both the properties of spilled oil and the activity or population of microorganisms (Venosa and Zhu, 2003). At low temperatures, the viscosity of the oil increases, while the volatility of toxic low-molecular-weight hydrocarbons is reduced, delaying the onset of biodegradation (Atlas 1981). Temperature also variously affects the solubility of hydrocarbons (Foght et al., 1996). Although hydrocarbon biodegradation can occur over a wide range of temperatures, the rate of biodegradation generally decreases with decreasing temperature. Highest degradation rates generally occur in the range of 30–40 °C in soil environments, 20–30 °C in some freshwater environments, and 15–20 °C in marine environments (Bossert and Bartha 1984; Cooney 1984). However, the bulk of information on hydrocarbon degradation bothers on activities of mesophiles, although significant biodegradations of hydrocarbons have been

reported in psychrophilic environments in temperate regions (Yumoto et al., 2002; Pelletier et al., 2004; Delille et al., 2004). Nevertheless, documented research on the environmental consequences of terrestrial spills in cold regions is still scarce, even though petroleum contamination is recognized as a significant threat to polar environments. Full-scale in situ remediation of petroleum contaminated soils has not yet been used in Antarctica for example, partly because it has long been assumed that air and soil temperatures are too low for an effective biodegradation (Delille et al., 2004).

Microbial utilisation of hydrocarbons (being fully reduced substrates) requires an exogenous electron sink. In the initial attack, this electron sink has to be molecular oxygen. In the subsequent steps too, oxygen is the most common electron sink. In the absence of molecular oxygen, further biodegradation of partially oxygenated intermediates may be supported by nitrate or sulphate reduction. Little or no hydrocarbon metabolism occurs in strictly anoxic sediments (Dibble and Bartha 1976). Inadequate mineral nutrient, especially nitrogen, and phosphorus, often limits the growth of hydrocarbon utilisers in water and soils. Iron was reported to be limiting only in clean, offshore seawater (Swannell et al., 1996). Sulphur, in form of sulphate ions, is plentiful in seawater, but could be limiting in some freshwater environments. The slight alkaline pH of seawater seems to be quite favourable for petroleum hydrocarbon degradation, but in acidic soils liming to pH 7.8 – 8.0 had a definite stimulatory effect. The diversity of petroleum hydrocarbon degraders in most natural environments is usually high, but in the absence of a previous pollution history, their numbers may be low (Swanell et al., 1996).

Nutrients are very important ingredients for successful biodegradation of hydrocarbon pollutants, especially nitrogen, phosphorus and in some cases iron (Cooney 1984). Depending on the nature of the impacted environment, some of these nutrients could become limiting thus affecting the biodegradation processes. When a major oil spill occurs in marine and freshwater environments, the supply of carbon is dramatically increa-

sed and the availability of nitrogen and phosphorus generally becomes the limiting factor for oil degradation (Atlas 1984). This is more pronounced in marine environments, due to the low background levels of nitrogen and phosphorus in seawater (Floodgate 1984), unlike in freshwater systems that regularly fluctuate in nutrient status as result of perturbations and receipt of industrial and domestic effluents and agricultural runoff (Cooney 1984). Freshwater wetlands are typically considered to be nutrient limited, due to heavy demand for nutrients by the plants, and they could also be nutrient traps, as a substantial amount of nutrients may be bound in biomass (Mitsch and Gosselink 1993). Hence the additions of nutrients are necessary to enhance the biodegradation of oil pollutants (Choi et al., 2002; Kim et al., 2005). Pelletier et al. (2004) assessed the effectiveness of fertilizers for crude oil bioremediation in sub-Antarctic intertidal sediments over a one-year and observed that chemical, microbial and toxicological parameters demonstrated the effectiveness of various fertilizers in a pristine environment. In another study using poultry manure as organic fertilizer in contaminated soil, biodegradation was reported to be enhanced in the presence of poultry manure alone, but the extent of biodegradation was influenced by the incorporation of alternate carbon substrates or surfactants (Okolo et al., 2005). However, excessive nutrient concentrations can inhibit the biodegradation activity (Challain et al., 2006), and several authors have reported the negative effect of a high NPK levels on the biodegradation of hydrocarbons (Oudot et al., 1998; Chaineau et al., 2005) and more especially on the aromatics (Carmichael and Pfaender, 1997).

Uptake and utilisation of water insoluble substrates, such as petroleum alkanes in crude oil, require specific physiological adaptations of the microorganisms. Synthesis of specific amphiphilic molecules, i.e., biosurfactants, has often been taken as a prerequisite for either specific adhesion mechanisms to large oil drops or emulsification of oil, followed by uptake of submicron oil droplets. Various species of bacteria have been observed to develop different strategies to deal with water insoluble substrates, such as hydrocarbons (Rosenberg 1991). Hence, to facilitate hydrocarbon uptake through the hydrophilic outer membrane, many oil-utilising microorganisms produce cell wall-associated or extracellular surface-active agents (Haferburg et al., 1986). This includes such low molecular weight compounds such as fatty acids, triacylglycerols and phospholipids, as well as the heavier glycolipids, examples of which include *emulsan* and *liposan* (Cirigliano and Carman 1984).

It is clear that the introduction of external nonionic surfactants, e.g., the main components of oil spill dispersants, will influence the alkane degradation rate (Bruheim and Eimhjelle, 1998; Rahman et al., 2003). Experience so far indicates that the use of surfactants in situations of oil contamination may have a stimulatory,

inhibitory, or neutral effect on the bacterial degradation of the oil components (Liu et al., 1995). The need to accurately characterise the roles of chemical and biological surfactants has been proposed in order that performance in biological systems may be predicted (Rocha and Infante 1997; Lindstrom and Braddock 2002). However, in contrast to chemical dispersants, which caused ecological damage after application for abatement of spilled oil in marine ecosystems (Smith 1968), biosurfactants from soil or freshwater microorganisms are less toxic and partially biodegradable (Poremba et al., 1991).

4.0 Distribution of petroleum hydrocarbon utilising microorganisms

Hydrocarbon degrading bacteria and fungi are widely distributed in marine, freshwater, and soil habitats. Similarly, hydrocarbon degrading cyanobacteria have been reported (Challana et al., 2004; Lliros et al., 2003), although contrasting reports indicated that growth of mats built by cyanobacteria in the Saudi coast led to preservation of oil residues (Barth 2003). Typical bacterial groups already known for their capacity to degrade hydrocarbons include *Pseudomonas*, *Marinobacter*, *Alcanivorax*, *Microbulbifer*, *Sphingomonas*, *Micrococcus*, *Cellulomonas*, *Dietzia*, and *Gordonia* groups (Brito et al., 2006). Molds belonging to the genera *Aspergillus*, *Penicillium*, *Fusarium*, *Amorphoteca*, *Neosartorya*, *Paecilomyces*, *Talaromyces*, *Graphium* and the yeasts *Candida*, *Yarrowia* and *Pichia* have been implicated in hydrocarbon degradation (Chaillan et al., 2004). However, reports in literature on the actual numbers of hydrocarbon utilisers are at variance with one another because of the methodological differences used to enumerate petroleum-degrading microorganisms. The initial method involved the use of hydrocarbons incorporated into agar-based medium (Horowitz et al., 1978). This approach has its problems. In some cases, a high correlation has been found between growth on agar and media containing hydrocarbons as the sole carbon source, and the ability to rigorously demonstrate hydrocarbon utilisation by isolates from these media in liquid culture. In other studies, only a low percentage of isolates from agar-based media could be demonstrated to be capable of hydrocarbon utilisation.

The use of silica gel-oil medium for the enumeration of petroleum degrading microorganisms has been recommended (Walker and Colwell 1976), suggesting that counts of petroleum degraders be expressed as a percentage of the total population rather than as total numbers of petroleum degraders *per se*. Also, the ability to utilise hydrocarbons is widespread, even in environments not subjected to high levels of hydrocarbon pollution. Atlas (1978) reported that quantitative differences in the distribution of hydrocarbon utilizers were rela-

tively unimportant over large geographic distances.

The Most Probable Number (MPN) procedure has also been tried as a substitute for the plate count technique for the estimation of hydrocarbon utilising microorganisms, since it eliminates the need for a solidifying agent and permits direct assessment of the ability to actually utilise hydrocarbons. The use of liquid media for MPN also permits removal of trace organic contaminants and allows for the chemical definition of a medium with a hydrocarbon as a sole source of carbon. This technique thus incorporates the specificity for counting only hydrocarbon utilizers and eliminates the problem of counting organisms growing on other trace organic contaminants (Braddock and Catterall 1999).

The problems of culture techniques arise from the fact that most (90–99%) of the species making up competent degrading communities do not form colonies when current laboratory-based culture techniques are used (MacNaughton et al., 1999). However, the application of molecular techniques for the analysis of the microbial communities that take part in *in situ* hydrocarbon biodegradation activities is helping to address these problems. The measurement of lipid biomarkers, specifically, phospholipid fatty acids (PLFA), together with nucleic acid-based molecular techniques for fingerprinting the 16S ribosomal DNA (rDNA) component of microbial cells is a powerful combination of techniques for elucidating the microbial ecology of actively bioremediating communities (Stephen et al., 1999). Lipid biomarker-based techniques measure the lipid profiles of microbes in the environment irrespective of culturability, thereby avoiding culture bias (White et al., 1998). These methods provide insight into several important characteristics of microbial communities, especially the viable biomass, community structure, and nutritional status or physiological stress responses of the gram-negative bacteria (White et al. 1998).

Molecular genetic techniques allow researchers to examine microbial communities without cultivation using universal primers 16S rRNA gene primers (Borneman et al., 1996). PCR has been particularly useful for detecting genes involved in the degradation of xenobiotic compounds (Joshi and Walia 1996). Despite the shortcomings of cultivation-based techniques, standard culture methods are still adequate for site evaluation to determine whether indigenous bacteria are capable of degrading the contaminants.

5.0 Genetic aspects of petroleum hydrocarbon degradation

Several new methodologies have enabled recent studies on the microbial biodegradation mechanisms of organic pollutants. Culture-independent techniques for analysis of the genetic and metabolic potential of natural and model microbial communities that degrade organic pollutants have identified new metabolic pathways and enzymes for aerobic and anaerobic degradation (Pieper et al., 2004).

The study of the genetics of hydrocarbon utilising micro-organisms has essentially been stepped up within the last decades. Before now, our insight into the genetics of simple aromatic hydrocarbon utilising microorganisms surpassed our knowledge and understanding of the genetics of alkane utilising microorganism. This is due to the availability of precise and definite database for aromatics metabolism compared to their *n*-alkane counterpart (Singer and Finnerty 1984).

Genetic studies have focused mainly on aerobic pathways, and many details of these metabolic routes have been documented (van der Meer et al., 1992), although Widdel and Rabus (2001), Heider et al. (1998) and Sporman and Widdel (2000) presented some detailed and comprehensive reviews on anaerobic biodegradation of hydrocarbons and the mechanisms involved. A general comparison of the major pathways for catabolism of aromatic compounds in bacteria has revealed that the initial conversion steps are carried out by different enzymes but that the compounds are transformed to a limited number of central intermediates, such as protocatechuate and (substituted) catechols (Chaudry and Chapalamadugu 1991). These dihydroxylated intermediates are channelled into one of two possible pathways, either a *meta*-cleavage-type pathway or an *ortho*-cleavage type pathway (Figure 4). Both types of pathways lead to intermediates of central metabolic routes, such as the tricarboxylic acid cycle. This generalised scheme of catabolic pathways for aromatic compounds suggests that microorganisms have extended their substrate range by developing peripheral enzymes, which are able to transform initial substrates into one of the central intermediates (van der Meer et al., 1992).

Genetic factors play important roles in conferring biodegradation potentials on microorganisms. Plasmids probably play leading role in this aspect. The ability to degrade more recalcitrant components of petroleum such, as the aromatic fractions are generally plasmid mediated (Cerniglia 1984).

Exposures of a microbial community to hydrocarbons have been shown to result in an increase in the number of bacterial plasmids types (Leahy et al., 1996). Catabolic plasmids are non-essential genetic elements in so far as viability and reproduction of an organism is concerned, but they do provide a metabolic versatility not normally present in the cell. Such genetic potential allows for the evolution of integrated and regulated pathways for the degradation of hydrocarbons. The observed increase in the study of the genetics of such systems has closely paralleled the development of advances in molecular biology, particularly the application of recombinant DNA technology (Singer and Finnerty 1984), gene probes (Barriault and Sylvestre 1993), and polymerase chain reaction (PCR) technology. Many bacterial catabolic pathways are specified by conjugative plasmids (Frantz

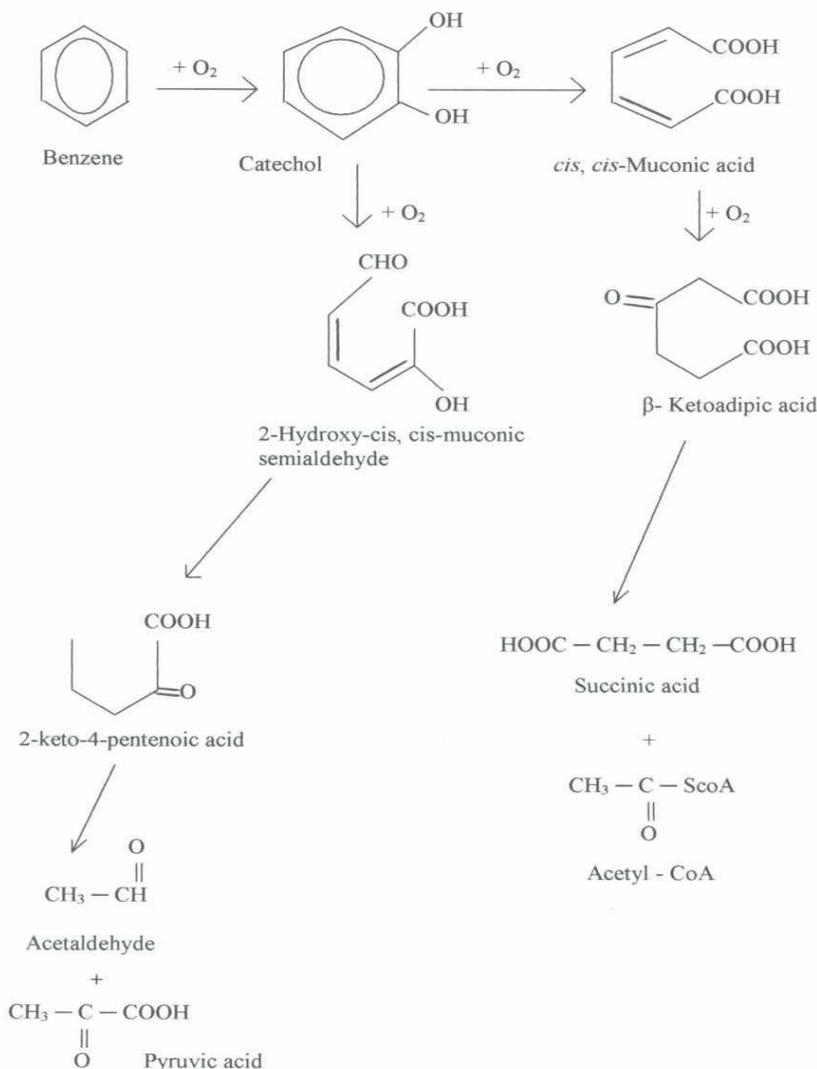


Figure 4. Microbial metabolism of the aromatic ring by *meta* or *ortho* cleavage as shown for benzene (Atlas and Bartha 1998).

and Chakrabarty 1986; Table 2). These plasmids are readily transferred laterally into new host bacteria, thereby enhancing the metabolic potential of other members of an ecosystem. Conjugative plasmids are thus important agents of genetic changes and evolution in bacteria, and could be picked up from or brought together in different organisms as groups of genes, which through mutations and recombination can specify new metabolic functions (Lessie and Gaffney 1986). For example, five aerobic toluene degradative pathways are characterized in pseudomonads. The best characterized of these pathways is encoded by the TOL plasmid (pWW0) of *P. putida* PaW1 (Worsey and Williams 1975), which converts toluene to benzyl alcohol, benzaldehyde, benzoate, and catechol, which further undergoes *meta* cleavage by an extradiol dioxygenase, or catechol 2,3-dioxygenase (C230). *Pseudomonas putida* F1

metabolizes toluene to 3-methylcatechol, which undergoes *meta*-cleavage by a C230 (Gibson et al., 1970).

Three other pathways for the catabolism of toluene were observed in *Burkholderia cepacia* G4 (Shields et al., 1989), *P. pickettii* PK01 (Kukor and Olsen 1990), and *P. mendocina* KR1 (Whited and Gibson 1991), in which toluene is respectively converted to *ortho*-, *meta*-, and *para*-cresol. Peripheral oxygenases of these five toluene degradative pathways possessed well-distinguished substrate specificity (Duetz et al., 1994). Particularly, xylenes can only be degraded by the *xyl* pathway encoded by TOL plasmid.

In a study of environmental *P. aeruginosa* strains isolated in Morocco, Belhaj et al. (2002) reported the presence of genetic information for several alkane monooxygenases. Most strains do carry genes very simi-

Table 2. Plasmids encoding catabolic functions.

Plasmid	Host	Component(s) catabolised
TOL	<i>Pseudomonas putida</i>	Toluene, <i>p</i> - and <i>m</i> -xylene
NAH	<i>Pseudomonas putida</i>	Naphthalene
SAL	<i>Pseudomonas putida</i> .	Salicylate
pND50	<i>Pseudomonas putida</i>	<i>p</i> -cresol
pWW31	<i>Pseudomonas putida</i> .	Phenylacetate
pJP1	<i>Alcaligenes paradoxa</i>	2,3- dichlorophenoxyacetic acid
pJP4	<i>Alcaligenes eutrophus</i>	2,4- dichlorophenoxyacetate and 3- chlorobenzoate
pKF1	<i>Acinetobacter</i> sp.	4- chlorobiphenol
pAC21	<i>Pseudomonas</i> sp.	4- chlorobiphenol
pRE1	<i>Pseudomonas putida</i>	Isopropyl benzene
pCIT1	<i>Pseudomonas</i> sp.	Aniline
pAC25	<i>Pseudomonas putida</i>	3-chlorobenzoate
pWR1	<i>Pseudomonas</i> sp.	3-chlorobenzoate
pCS1	<i>Pseudomonas diminuta</i>	Parathion

Source: Frant and Chakrabarty 1986.

lar to *alkB1* and *alkB2* of strain PAO1, suggesting that two alkane hydroxylase complexes are commonly involved in the degradation of long chain alkanes in *P. aeruginosa*. A few strains carry genetic information for a third alkane hydroxylase complex, with a monooxygenase that is very similar to AlkB of strain GPO1. These latter strains have an extended spectrum of alkane utilization, from C₁₂-C₂₂ to C₆-C₂₂ alkanes, and can degrade toxic water-soluble alkanes. Whilst saturated alkanes with C₁₂ to C₁₈ chains or longer are readily attacked by a large variety of bacteria. The degradation of water-soluble short chain alkanes such as pentane, hexane, heptane and octane, which are toxic for the environment, is less frequent (Bouchez-Naïtali et al., 1999). The first step of alkane degradation is the oxidation of the methyl group to alcohol by the "alkane hydroxylase system" that is composed of a membrane-bound mono-oxygenase, the alkane hydroxylase per se (45.8 kDa) encoded by *alkB*, and a soluble electron transport system composed of two rubredoxins and an NADH-dependent rubredoxin reductase encoded by *alkG*, *alkF* and *alkT* respectively (Belhaj et al., 2002).

In an environment rich in a particular organic compound, a selective pressure may lead to acquisition and maintenance of a plasmid that specifies a corresponding catabolic pathway. Many degraders of exotic compounds have been isolated from soil or water contaminated with such compounds. In some cases, the same catabolic genes may be located on a plasmid in one organism and on the chromosome in another, and these catabolic genes may influence the expression of other set of catabolic genes present in the same cell (Haramaya and Timmis 1989). In at least one instance of the TOL plasmid, transposition of catabolic genes from

the plasmid to the chromosome has been demonstrated in the laboratory (Tsuda and Iino 1987).

6.0 CONCLUSION

Petroleum hydrocarbon especially in the form of crude oil has been a veritable source of economic growth to society from the point of view of its energy and industrial importance. These realizations, which have become more pronounced in the last decade, have resulted in extensive exploration for more oil reserves. The resultant effects of these exploratory activities have been the extensive pollution of the environment. Bioremediation, which exploits the biodegradative abilities of live organisms and their attributes have proven to be the preferred alternative in the long-term restoration of petroleum hydrocarbon polluted systems, with the added advantage of cost efficiency and environmental friendliness. Although extensive investigations have been carried out regarding hydrocarbon biodegradation, these studies have been exhaustive, not exhausted. Nevertheless, the effectiveness of this technology has only rarely been convincingly demonstrated, and in the case of commercial bioremediation products, the literature is virtually completely lacking in supportive evidence of success (Zhu et al., 2004). Most existing studies have concentrated on evaluating the factors affecting oil bioremediation or testing favored products and methods through laboratory studies (Mearns, 1997). Only limited numbers of pilot-scale and field trials, which may provide the most convincing demonstrations of this technology, have been reported in the peer-reviewed literature (Venosa et al., 2002). The scope of current understanding of oil bioremediation is also limited because

se the emphasis of most of these field studies and reviews has been on the evaluation of bioremediation technology for dealing with large-scale oil spills on marine shorelines (Zhu et al., 2004). Some shortcomings are evident in petroleum hydrocarbons degradation studies. The identification of active strains is not always ascertained to a sufficient degree, and misidentifications or incomplete identifications are sometimes reported. Molecular techniques for the identification of hydrocarbon-degrading bacteria have been only rarely used in environmental studies (Röling et al., 2002), and the biodegradation activities are not always confirmed by chemical analyses of the degraded Hydrocarbon (Challain et al., 2004). Much need still exist for the optimization of the process conditions for more efficient application of biological degradation of oil pollutants under different climatic conditions and other diverse environmental milieu.

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