Chapter 1

General introduction and review of literature

1.1. Introduction

Every year spillage of tones of fuel and lubricating oil has become a major environmental hazard. In October 2014, a single oil spillage of 546 tones was reported at Mid-Valley Pipeline of Louisiana, United States. A recent incident highlighted how time consuming a cleanup operation of oil spillage can be. According to CNN International, in Nov 2013, oil-spillage occurred in Phillipines due to a typhoon and till date the cleaning up process is going on. There are also long-term effects on ecosystems related to the release of toxic components over a prolonged period as the oil breaks up and the concentration of toxicants in organisms towards the top of the food chain increases (Lin *et al.,* 2008).

These contaminations of the environment with petroleum hydrocarbons provides serious problems for many countries including India. Release of petroleum hydrocarbons into the environment whether accidentally or due to human activities, is a main cause of water and soil pollution. Soil contamination with hydrocarbons cause extensive damage to local system as accumulation of pollutants in animals and plant tissues results in death or mutations (Mehrasbi *et al.,*2003 and Head *et al.,* 2006). Several physical, chemical and thermal methods have commonly been employed to clean up the oil-contaminated sites (Frick *et al.,* 1999). However, these techniques are relatively expensive and also require site restoration (Lundstedt *et al.,* 2003).

Biodegradation of hydrocarbon-contaminated soils exploits the ability of microorganisms to degrade or detoxify organic contamination. It has been established as an efficient, economic, versatile and environmentally sound treatment (Bundy *et al.,* 2004).Microorganisms that biodegrade the components of petroleum hydrocarbons are isolated from various environments, particularly from petroleum-contaminated sites (Ghanavati *et al.,* 2010). Bacterial community composition can be correlated with ability to degrade target pollutants for the evaluations of indigenous microorganisms (Bestetti *et al.,* 2005).

Microorganisms have the capacity to degrade the majority of natural hydrocarbon components, especially the dominant saturated and unsaturated alkanes. The poly-aromatic hydrocarbons (PAHs), resins and asphaltenes are more recalcitrant to biodegradation and require a keen attention due to their

inherent mutagenic properties (Johnsen *et al.*, 2007). Hydrocarbon-degrading microbes must come into contact with their substrate for hydrocarbon uptake to occur and the insoluble nature of the majority of petroleum hydrocarbon molecules limits this contact (Hamme, 2003). The most widely recognized modes of hydrocarbon accession are direct microbial adherence to large oil droplets and interaction with pseudo solubilized (emulsified) oil. Hence, attempts to optimize or accelerate processes of hydrocarbons degradation include strategies for improving hydrocarbon accession by microorganisms.

Floodgate (1984) listed 25 genera of hydrocarbon-degrading bacteria and 27 genera of hydrocarbon-degrading fungi, which have been isolated from the marine environment; a similar compilation by Bossert and Bartha (1984) for soil isolates includes 22 genera of bacteria and 31 genera of fungi. Based on the reports the most common hydrocarbon degrading bacteria and fungi that are widely distributed in marine, freshwater, and soil habitats include *Pseudomonas, Marinobacter, Alcanivorax, Microbulbifer, Sphingomonas,Micrococcus, Cellulomonas, Dietzia,* and *Gordonia* groups *Aspergillus, Penicillium, Fusarium, Amorphoteca, Neosartorya, Paecilomyces, Talaromyces, Graphium* and the *yeasts Candida, Yarrowia* and *Pichia* have also been implicated in hydrocarbon degradation (Chaillan *et al.*, 2004; Brito *et al.*, 2006).). Reports of gram positive bacteria in hydrocarbon degradation are less common compared to gram negative bacteria. The underrepresentation of gram positive bacteria in hydrocarbon degradation may be due to the composition of cell envelope (Vermue *et al.*, 1999).

Microorganisms have adapted several mechanisms to survive and grow in the presence of toxic solvents. This includes modification of the membrane and cell surface properties, changes in the overall energy status or the activation of active transport systems for excluding solvents from membranes into the environment.

Since, the crude oil production in North east, India is a nage old routine process, therefore, the environmental contamination due to crude oil exploration is highly alarming. As a consequence, it is important to assess the native bacterial strains for bioremediation of crude oil contaminated site.

1.2 Objectives

Thus, this study was undertaken to achieve the following objectives:

- Isolation of petroleum hydrocarbon degrading bacteria from soil sample.
- Screening of the best petroleum hydrocarbon degrading bacterial strain
- Biochemical characterization and molecular identification of the particular bacterial strain.
- Determination of petroleum hydrocarbon degradation capability of the isolated strain
- Characterization of the gene responsible for petroleum hydrocarbon degradation.
- Optimization of lipase production from the isolated petroleum hydrocarbon degrading bacterial strain for biodiesel production
- Purification and characterization of lipase
- Application of the isolated petroleum degrading bacteria for biodiesel production.

1.3. Review of the literature

1.3.1 Structure and classification of petroleum

Petroleum is defined as any mixture of natural gas, condensate and crude oil. The crude oil in petroleum is a heterogeneous liquid consisting of hydrocarbons comprised almost entirely of hydrogen and carbon in the ratio of about 2 hydrogen atoms to 1 carbon atom (Ghanavati *et al.*, 2010). 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 1% (v/v), including phosphorus and heavy metals such as vanadium and nickel. The hydrocarbons in crude oil can be divided into four classes on the basis of the relative proportions of the heavy molecular weight constituents as light, medium or heavy (Okerentugba and Ezeronye, 2003):

- Saturated hydrocarbon
- Aromatics [benzene, toluene, ethylbenzene and xylenes; (BTEX) and polyaromatic hydrocarbons (PAHs)]
- Asphaltenes (phenols, fatty acids, ketones, esters, and porphyrins) and
- Resins (pyridines, quinolines, carbazoles, sulfoxides, and amides)

On a structural basis, the hydrocarbons in crude oil are classified as alkanes (*normal* or *iso*), cycloalkanes, and aromatics (Fig 1). Alkenes, which are the unsaturated analogs of alkanes, occur inmany refined petroleum products. 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 petroleum.(Johnson *et al.*, 2011)

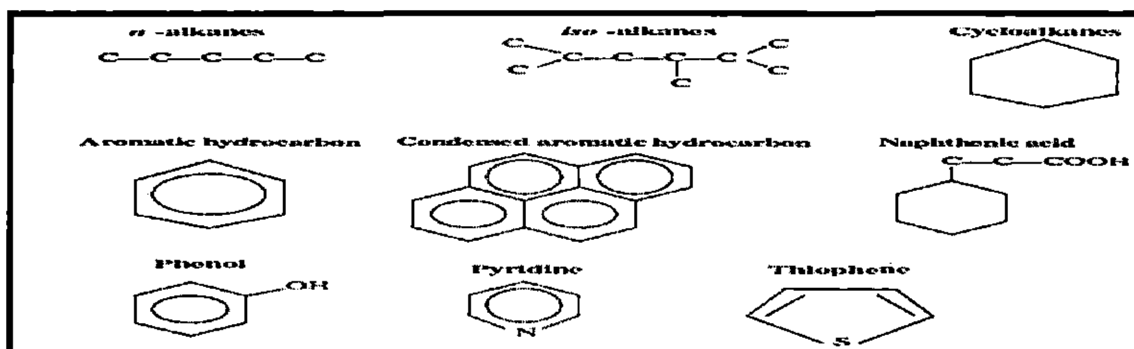

Fig 1. Structural classification of petroleum crude oil components. (Basha *et al*, 2010)

1.3.2 Recent scenario of petroleum spills and its hazardous effect

International scenario

In this current 2014, in different areas of USA alone almost 800 metric tones of oil was spilled due to bursting of pipelines. Some of the oil spill incidents that happened in last 5 years across the globe are listed in the table 1.

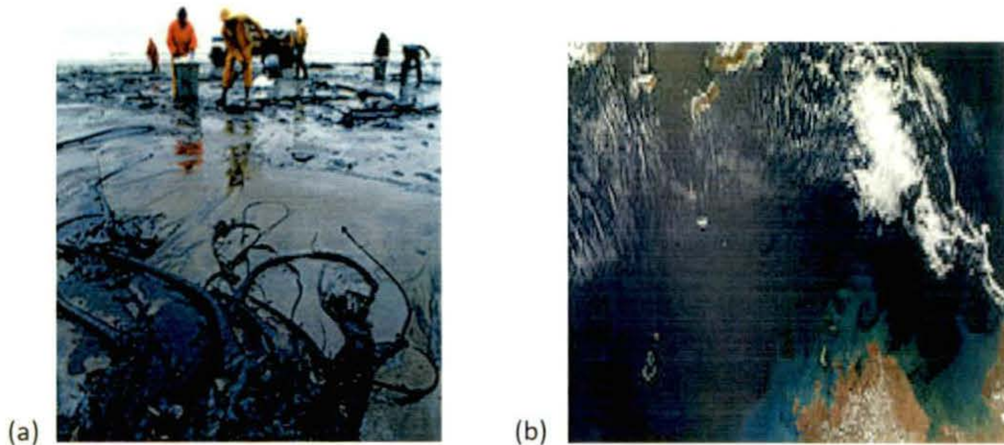

Fig 2. Spread of oil slicks over the water surface (a), Satellite view of the wide spead oil slick on ocean due to oil spillage (b).

Table 1. World wide major oil spillage events in last 5 years

Spill / Vessel	Location	Dates	Tonnes
LA pipeline	California, USA	15 May 2014	160
MV *Miss Susan*/MV *Summer Wind*	Texas, USA	22 Mar 2014	546
North Dakota pipeline	North Dakota, USA	21 Mar 2014	110
North Dakota train collision	North Dakota,USA	30 Dec 2013	1,300
Rayong oil spill	Gulf of Thailand	27 Jul 2013	163
Lac-Mégantic derailment	Québec, Canada	6 Jul 2013	4,830
Sundre, Alberta	Canada, Sundre	8 Jun 2012	410
Guarapiche	Venezuela, Maturín,	4 Feb 2012	41,000
Nigeria	Nigeria, Bonga Field	21Dec2011	5,500
TK Bremen	France, Brittany,	16 Dec2011	220
Campos Basin	Brazil, Campos Basin,	7 Nov 2011	400
Rena oil spill	New Zealand	5 Oct 2011	350
North Sea oil	North Sea, UK	10 Aug2011	216
Yellowstone River	United States,	1 Jul 2011	140
Bohai Bay oil spill	China, Bohai Bay	4 June 2011	204
Little Buffalo oil spill	Canada, Alberta	29 Apr 2011	3,800
Fiume Santo power station	Italy, Sardinia,	11 Jan 2011	15

Barataria Bay oil spill	United States, Mexico	27 Jul 2010	45
Kalamazoo River oil spill	United States, Michigan	26 Jul 2010	3,250
Xingang Port oil spill	China, Yellow Sea	16 Jul 2010	90,000
Trans-Alaska Pipeline	United States, Alaska	25 May 2010	1,200
MT *Bunga Kelana 3*	Singapore,	25 May 2010	2,500
ExxonMobil	Nigeria,	1 May 2010	95,500
Deepwater Horizon	United States, Gulf of Mexico	Apr 2010	627,000
Great Barrier Reef / MV *Shen Neng 1*	Australia, Keppel Island	Apr 2010	4
Port Arthur oil spill	United States, Texas	Jan 2010	1,500
Yellow River oil spill	China, Chishui River	Jan 2010	130

Source: Wikipedia

National scenario

A number of incidents of oil spillage have been reported happening every year in the Indian shore line (Fig 2). Recently, on 21st January 2011, ONGC trunk oil pipeline burst at Uran, Mumbai. About40 to 45 metric tons of oil was spilled and spread around 4 sq km area. On 7thAugust, 2010 a foreign cargo ship, collided with another vessel about 10 km off Mumbai harbor, that spilled the oil in spite of the hectic effect made by Navy and Coast Guard to contain the leak, around 700 tons of fuel oil and 28 tons of diesel oil was spilled (*Times of India, 22nd Jan, 2011*).

Hazardous effect

These oil spill damage the waterways of birds and animals, interrupt breeding and fouling of breeding grounds. Besides, they thin bird and turtle egg shells and also damage the fish larvae, causing deformities. Soil ecosystem is disturbed and it effects the plant growth.

The primary effects of exposure to petroleum hydrocarbons are central nervous system, depression and polyneuropathy. The International Agency for Research on Cancer (IARC) has listed gasoline as possibly carcinogenic (2B) to humans (IARC 2000). Among the components of gasoline, N-heptane and cyclohexane are known to cause narcosis and irritation of the eyes and mucous membranes. Cyclohexane has been reported to cause liver and kidney changes in rabbits, and n-heptane has been reported to cause polyneuritis following prolonged exposure. The IARC, the National Toxicology program (NTP) and the Occupational Safety and Health Adminstration (OSHA) all classify benzene as a

human carcinogen. Other compounds of interest in petroleum are benzene toluene, ethyl benzene, and xylenes (BTEX) causes impairment of coordination and momentary loss of memory at exposures of 200–500ppm, and causes palpations at 500–1,500 ppm.

1.3.3 Remediation processes

1.3.3.1. Physico - chemical process

The various physical and chemical processes commonly used in the remediation of contaminated areas include recovery, dispersion, dilution, sorption, volatilization and abiotic transformations of hydrocarbon for its elimination. However, these types of treatment systems require heavy machinery and the environmental consequences of removing pollutants with these techniques may result in massive air pollution (Matsumiya and Kubo 2007). The rate of removal of petroleum hydrocarbon depend on the media in which the molecules are exposed including soil, water, presence or absence of light (Koch, 2011). Different petroleum sludge treatment technologies are categorized in table 2.

Photo-Oxidation:

Photo-oxidation is when sunlight in the presence of oxygen transforms hydrocarbons by photo oxygenation (increasing the oxygen content of a hydrocarbon) into new by-products. Oil can strongly absorb solar radiation and undergo transformation through photo degradation. In water and air, photo degradation of oil can occur rapidly when light is present; however in soil, photo degradation occurs to a very limited extent due to the low intensity of light. In water, this process can occur either directly by exposure to light at a wavelength less than 290 nm, or indirectly by exposure to oxidizing agents including OH radicals, O_3, and NO_3 (Douben 2003). Photo degradation plays an important role in enhancing the degradation of petroleum hydrocarbon by attacking the tertiary carbon atoms (a carbon atom bonded to three other carbon atoms with single bonds) in this. Atmospheric photochemical reactions transform hydrocarbons to less volatile and more polar derivatives, including nitrated, oxygenated and hydroxylated PAHs, which can lead to an increase in bioavailability and toxicity (Douben.2003).

Table 2. Evaluation of different petroleum sludge treatment technologies

Remediation	Technology	Comments
Bioremediation	Bioreactor	Application of natural and specialized microorganisms in controlled environmental and nutritional conditions, high biodegradation rates, accommodates variety of sludges, nonhazardous residues, on-site operation, cost-effective
	Landfarming	Uses natural microbial population and supplements of mineral nutrients, slow degradation rates, year-round operation difficult, potential to contaminate ground and surface water, cost-effective
	Biopiling	Uses natural microbial population and supplemented nutrients and air, slow degradation rates, year-round operation difficult, potential to contaminate ground and surface water
	Bioventing	A combination of advective soil venting and biodegradation method for in situ treatment of soils, most of the lighter hydrocarbons are volatilized
	Biostimulation/bioaugmentation	Application of mineral nutrients/surfactants and/or microorganisms to stimulate or supplement natural microbial population at contaminated site
	Phytoremediation	Uses plants and rhizospheric microorganisms for the treatment of contaminated soil, potential for removal of petroleum contaminants being evaluated, presumably cost-effective
Physicochemical	Incineration	High-temperature treatment, air pollution risks, expensive control equipment, high capital cost
	Thermal desorbtion	High-temperature oil removal and recovery method from oily solids, high capital and material preparation costs, nonhazardous residues
	Coker	Complicated sludge preparation for coker feed, some oil recovery, high capital and transportation costs
	Cement kiln	Complicated sludge preparation for use of fuel, high material preparation, transportation, and disposal costs
	Solvent extraction	Uses solvents and centrifugation or filtration for the separation of oil from sludges, safety hazard with solvent use. high capital cost

Chemical oxidation

Petroleum hydrocarbon can be chemically altered in soils by chemical oxidants including Fe and Mn metal ions, oxides and oxyhydroxides of Al, Mn, Si and Fe and by unsaturated fatty acids of plant residues (Koch, 2011).Common chemical oxidants that are used for biodegradation include ozone, hydrogen peroxide and permanganate. The addition of an oxidative agent such as hydrogen peroxide leads to the production of very strong non-selective oxidizing agents (hydroxyl radicals) that can react with hydrocarbons and induce transformation (Ferrarese *et al.*, 2008).

1.3.3.2. Biological process

Biological cleaning method involves natural processes in order to meet today's demands for cleaning and waste elimination without the use of potentially harmful chemicals. The biodegradation of these compounds is often a complex series of biochemical reactions and is often different when different microorganisms are involved. Biodegradation of organic wastes is a useful side effect of microbial metabolism, thus the fundamental principles of biodegradation are integrally linked to microbial physiology. Hydrocarbons differ in their

susceptibility to microbial attack. The susceptibility of hydrocarbons to microbial degradation can be generally ranked as follows: linear alkanes >small aromatics >branched alkanes >cyclic alkanes (Turki, 2009). Some compounds, such as the high molecular weight polycyclic aromatic hydrocarbons (PAHs) may not be degraded at all. The most rapid and complete degradation of the majority of organic pollutants is brought about under aerobic conditions. On the basis of mode of application bioremediation is classified in to two types namely In-situ bioremediation and ex-situ bioremediation.

In situ and ex situ methods

Bioremediation is broadly classified as ex situ and in situ methods. *Ex situ* methods are those treatments that involve the physical removal of the contaminated material for treatment process. In contrast, *in situ* techniques involve treatment of the contaminated material in place (Boopathy, 2000). Some of the examples of *in situ* and *ex situ* bioremediation are given below:

- Land farming: Solid-phase treatment system for contaminated soils: may be done in situ or ex situ.
- Composting: Aerobic, thermophilic treatment process in which contaminated material is mixed with a bulking agent; can be done using static piles or aerated piles.
- Bioreactors: Biodegradation in a container or reactor; may be used to treat liquids or slurries.
- Bioventing: Method of treating contaminated soils by drawing oxygen through the soil to stimulate microbial activity.
- Biofilters: Use of microbial stripping columns to treat air emissions.
- Bioaugmentation: Addition of bacterial cultures to a contaminated medium; frequently used in both in situ and ex situ systems.
- Biostimulation: Stimulation of indigenous microbial populations in soils or ground water by providing necessary nutrients.
- Intrinsic bioremediation: Unassisted bioremediation of contaminant; only regular monitoring is done.
- Pump and treat: Pumping ground water to the surface, treating, and reinjecting.

1.3.4.Factors affecting hydrocarbon degradation

A number of limiting factors have been recognized to affect the biodegradation of petroleum hydrocarbons. Typical examples of these factors are as listed in Table 3.

Table 3. Factors affecting petroleum hydrocarbon biodegradation.

Limiting factors	Explanation or examples
Petroleum hydrocarbon composition	Structure, amount, toxicity
Physical state	Aggregation, spreading, dispertion, adsorption
Weathering	Evaporation, photooxidation
Water potential	Osmotic forces
Temperature	Influence on evaporation and degradation rates
Oxidants	O_2 required to initiate oxidation,
Micronutrients	N, P, Fe may be limiting
Reactions	Low pH may be limiting
Microorganisms	PCB degraders may be low in number or absent

(Okoh, 2006)

Concentration of the Oil or Hydrocarbons:

The composition of the petroleum hydrocarbon pollutant is the first and most important consideration when the suitability of a remediation approach is to be evaluated. The 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 metabolized by some bacterial species increased with increasing concentration of starter oil up to0.6% (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%.

Chemical composition of the Oil

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 are reasonable time period, even if the conditions are favorable (Okoh,2006). Maki *et al.*,(2005) 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 65 - 70% . Also, about 89% of the same crude oil was biodegraded by axenic culture of *Burkholderia cepacia* RQ1 in shake flask 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).

Temperature

Temperature plays very important roles in biodegradation of petroleum hydrocarbons, firstly by its direct effect on the chemistry of the pollutants, and secondly its effect on the physiology and diversity of the microbial habitat (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 (Yumoto *et al.*, 2002). Temperature also variously affects the solubility of hydrocarbons (Okoh, 2006). Hydrocarbon biodegradation can occur over a wide range of temperatures, the rate of biodegradation generally decreases with decreasing temperature. Hydrocarbon biodegradation can occur over a wide range of temperatures, the rate of biodegradation generally decreases with the decreasing temperature (Delille *et al.*, 2004). Figure 3 shows that highest degradation rates that generally occur in the range 30–40 °C in soil environments, 20–30°C in some fresh water environments and 15–20°C in marine environments (Pelletier *et al.*, 2004). Researchers have also reported that ambient temperature of the environment affect both the properties of spilled oil and the activity of the microorganisms. Significant

biodegradation of hydrocarbons have been reported in psychrophilic environments in temperate regions (Mrozik *et al.*,2010).

Oxygen

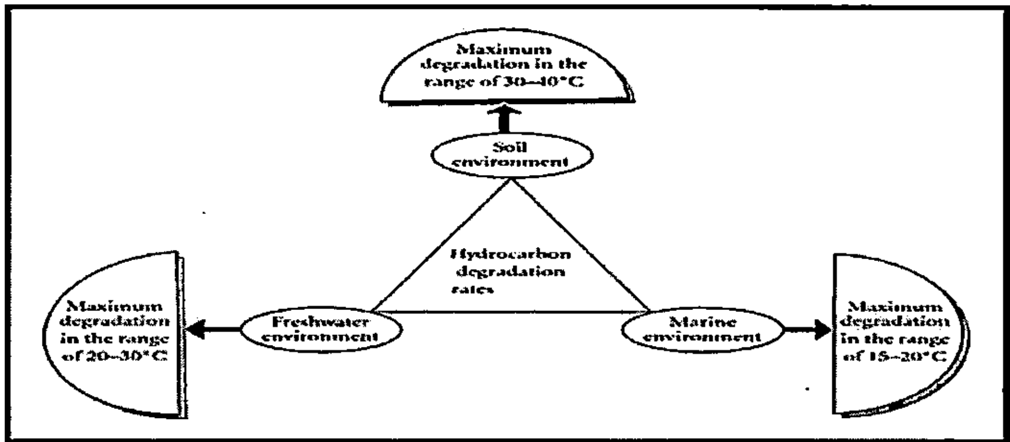

Fig 3. Hydrocarbon degradation rates in soil, fresh water, and marine environments. (Das and Chandran, 2011)

Microbial biodegradation of hydrocarbon requires an exogenous electron acceptor, with oxygen being the most common one. Aerobic conditions are therefore necessary for the microbial oxidation of hydrocarbons in the environment. The concentration of oxygen has been identified as the rate-limiting variable in the biodegradation of petroleum in soil. The availability of oxygen in soils is dependent upon rates of microbial oxygen consumption, the type of soil, whether the soil is waterlogged, and the presence of utilizable substrates which can lead to oxygen depletion (Bossert and Bartha, 1984).

Anaerobic degradation of petroleum hydrocarbons by microorganisms has been shown in some studies. In the absence of molecular oxygen, nitrate, iron, bicarbonate, nitrous oxide and sulfate have been shown to act as alternate electron acceptors during hydrocarbon degradation. The microbial degradation of oxidized aromatic compounds such as benzoate and of halogenated aromatic compounds such as the halo benzoates, chloro phenols and polychlorinated biphenyls has been shown to occur under anaerobic conditions (Chen *et al.,* 1988). Report indicates that microbial consortia from soil and sludge are capable of metabolizing unsubstituted and alkyl-substituted aromatics, including benzene,

toluene, xylene, 1, 3-dimethylbenzene, acenaphthene and naphthalene in the absence of molecular oxygen(Wilkes *et al.*, 2002).

Nutrients

Nutrients are very important ingredients for successful biodegradation of hydrocarbon pollutants especially nitrogen, phosphorus, and in some cases iron (Okoh, 2006). Some of these nutrients could become limiting factor thus affecting the biodegradation processes. Leo *et al.*(2011) reported that when a major oil spill occurred in marine and freshwater environments, the supply of carbon was significantly increased and the availability of nitrogen and phosphorus generally became the limiting factor for oil degradation. In marine environments, it was found to be more pronounced due to low levels of nitrogen and phosphorous in seawater. Freshwater wetlands are typically considered to be nutrient deficient due to heavy demands of nutrients by the plants. Therefore, additions of nutrients were necessary to enhance the biodegradation of oil pollutant. In nutrient limited waste waters, a BOD:N:P ratio of 100:5:1 is often used as a bench mark for nutrient addition.(Choi *et al.*, 2002; Kim *et al.*, 2005).On the other hand, excessive nutrient concentrations can also inhibit the biodegradation activity. Several authors have reported the negative effects of high NPK levels on the biodegradation of hydrocarbons especially on aromatics (Chaillan *et al.*, 2004).

Salinity

There is an inverse relationship between salinity and hydrocarbon solubility (Mailem *et al.*,2010). The substrate availability to microorganisms should be lower in saline than non saline environments. Some earlier reports indicated that even in the presence of high NaCl concentrations, actinomycetes (Al-Mueini *et al.*, 2007) and archaea (Tapilatu *et al.*,2010) could oxidize petroleum hydrocarbons. Other reports indicate that salinity did not affect microbial hydrocarbon biodegradation (Al-Mailem *et al.*,2010, 2013).

Pressure

Pressure is another important factor in the biodegradation of hydrocarbons. The degradation of tetradecane, hexadecaneand a mixed hydrocarbon substrate by a mixed culture of deep-sea sediment bacteria was monitored at 1 atm (ca.101 kPa) and 495 or 500 atm (ca. 50,140 or 50,650 kPa)

by Schwarz *et al.* (1974; 1975). Colwell and Walker (1976) have suggested that oil which reaches the deep ocean environment will be degraded very slowly by microbial populations and consequently, that certain recalcitrant fractions of the oil could persist for years or decades.

pH

Environmental parameters such as pH influence biodegradation of petroleum hydrocarbon. Extreme pH conditions are expected to have a negative influence on the ability of microbial populations to degrade the hydrocarbons. Microbial growth is higher in pH 6 and 7 but the biodegradation rate was higher at pH 7.0. Most heterotrophic bacteria and fungi favor a neutral pH (Venosa and Zhu, 2003). The pH may affect the solubility, bioavailability, and the chemical form of the pollutants and of macro-and micronutrients. Degradation of petroleum hydrocarbon increases with increasing pH, and that optimum degradation occur under slightly alkaline conditions (Guo *et al.*, 2005).

Production of biosurfactants by microorganisms

Biosurfactants are heterogeneous group of surface active chemical compounds produced by a wide variety of microorganisms (Ilori *et al.*, 2005, Kiran, 2009). It enhances solubilization and removal of contaminants due to increased bioavailability of pollutants. Biosurfactants actually can act as emulsifying agents by decreasing the surface tension and forming micelles. The microdroplets encapsulated in the hydrophobic microbial cell surface are taken inside and degraded. Table 4 summarizes the recent reports on biosurfactant production by different microorganisms. Bioremediation of oil sludge using biosurfactants has been reported.

Table 4. Biosurfactants produced by microorganisms

Biosurfactants	Microorganisms
Sophorolipids	*Candida bombicola*
Rhamnolipids	*Pseudomonas aeruginosa*
	Pseudomonas fluorescens
Lipomannan	*Candida tropicalis*
Surfactin	*Bacillus subtilis*
glycolipid	*Aeromonas sp.*
	Bacillus sp.

(Thavasi *et al.*, 2011)

by Cameotra and Singh (2008) in which a microbial consortium consisting of two isolates of *Pseudomonas aeruginosa* and one isolate *Rhodococcus erythropolis* was able to degrade 90% of hydrocarbons in 6 weeks in liquid culture. The ability of the consortium to degrade sludge hydrocarbons was tested in two separate field trials. The utilization of biosurfactants by *Pseudomonas* sp. for uptake of petroleum hydrocarbon is shown in Fig 4.

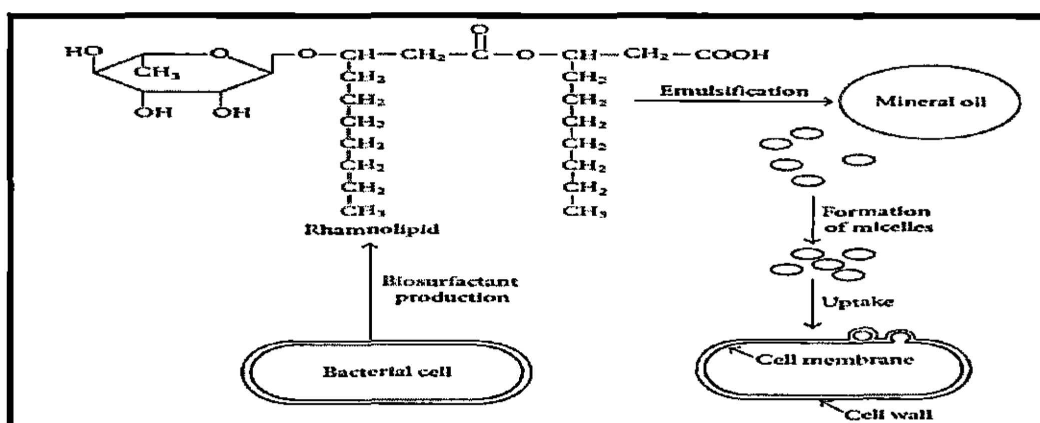

Fig 4. Involvement of biosurfactant (rhamnolipids) produced by *Pseudomonas* sp. in the uptake of hydrocarbons (Fritsche and Hofrichter, 2000).

The consortium degraded 91% of the hydrocarbon content of soil contaminated with 1% (v/v) crude oil sludge in 5 weeks. Separate use of any one additive along with the consortium brought about a 91–95% depletion of the hydrocarbon content in 4 weeks, with the crude biosurfactant preparation being a more effective enhancer of degradation. However, more than 98% hydrocarbon depletion was obtained when both additives were added together with the consortium.

1.3.5. Microorganisms involved in petroleum hydrocarbon degradation

Degradation of hydrocarbons by environmental micro flora involves microorganisms having specialized metabolic capacities. Bacterial strains isolated from petroleum contaminated waste water and soil can be good petrol and diesel degraders (Kashyap *et al.*, 2012).

These petroleum hydrocarbons degrading microorganisms share the following characteristics (Obire *et al.*, 2008):

- ➢ Efficient hydrocarbon uptake via special receptor sites for binding hydrocarbons and/or unique compounds that assist in the emulsification and transport of hydrocarbons into the cell.
- ➢ Enzymes that introduce molecular oxygen into the hydrocarbon and generate intermediates that subsequently enter common energy-yielding catabolic pathways.
- ➢ Inducer specificity: Exposure to petroleum and its constituents activate the two systems above.

Floodgate (1984) listed 25 genera of hydrocarbon-degrading bacteria and27 genera of hydrocarbon-degrading fungi which have been isolated from the marine environment; a similar compilation by Bossert and Bartha (1984) for soil isolates includes 22 genera of bacteria and 31 genera of fungi. Based on the reports the most common hydrocarbon degrading bacteria and fungi that are widely distributed in marine, freshwater, and soil habitats include *Pseudomonas, Marinobacter, Alcanivorax, Microbulbifer, Sphingomonas,Micrococcus, Cellulomonas, Dietzia,* and *Gordonia* groups (Brito *et al.*, 2006), *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, the actual numbers of hydrocarbon degraders are at variance with one another because of the methodological differences used to enumerate petroleum-degrading microorganisms. A list of microorganisms involved in degradation of petroleum hydrocarbon is summarized in Table 5.

Most of the microorganisms in the environment are alkene-degraders (Ting *et al.*, 2009), but it is the isolates that can degrade both aliphatic and aromatic hydrocarbons that are highly desirable, monocyclic hydrocarbons (Scragg, 2001), aromatic hydrocarbons (Ting *et al.*, 2009), resins and asphaltenes. One of the most extensively studied species of hydrocarbon degraders is the *Pseudomonas* species, known to degrade petroleum and a variety of polycyclic aromatic hydrocarbons crude oil (Bento *et al.*, 2003). Among the many *Pseudomonas* species, *P. aeruginosa* (Ijah and Antai, 2003), *P. alcaligenes* (Mahony *et al.*, 2006) and *P. putida* are more thoroughly investigated and their effectiveness as

biodegradative agents for hydrocarbon bioremediation is well-established. A number of studies have reported the ability of *Arthrobacter* sp. to degrade hydrocarbon. GC-MS analysis of metabolites produced during petroleum degradation by RKJ4 suggested that degradation occurs via the production of the conventional lower pathway intermediates *o*-phthalic acid and protocatechuic acid (Seo *et al.*, 2006). The ability of *Arthrobacter* sp. F101 isolated from sludge at an oil refinery wastewater treatment plant to utilize fluorene as a sole source of carbon and energy has also been reported (Koch, 2011).Several studies have reported on the roles of *Bacillus* sp. in hydrocarbon biodegradation. It was postulated that *Bacillus* sp. are more tolerant to high levels of hydrocarbons in soil due to the irresistant endospores (Ghazali *et al.,* 2004). The ability of *Bacillus* to accelerate the petroleum degradation significantly in various conditions indicates the potentials of this microorganism in clearing oil spills (Lin *et al.*, 2009).*Bacillus* has demonstrated the capacity of degrading petroleum effectively in contaminated sea water and sand with a significant higher degradation rate (Mandri and Lin, 2007). *Alkaligenes sp.* which are found within the vicinity of produce water discharge zone are capable of degrading produce water hydrocarbons and most especially the sparingly soluble components such as petroleum hydrocarbons that are very difficult to remove with the conventional mechanical treatment presently in use. Reports suggest that *Alkaligenes sp.* could degrade petroleum hydrocarbons from its original concentration of 1407 mg/l to 19.58mg/l (Amund *et al.*, 2010).This is a sure indication that the *Alkaligenes* sp. that dominates microbial activities at the discharge zone of produce water effluents can be beneficial to the environment because of its excellent hydrocarbon degradation potential. Different strains of *Acinetobacter* have adopted varying strategies to adhere to petroleum drops. In *Acinetobacter* the interaction between the petroleum droplets and the cell envelope are a complex process during which n-alkanes induces glycosylation of membrane proteins involved in oil uptake (Baldi *et al.*, 2003) and in biofilm formation due to cell-to-cell contact and synthesis of a composite material constituted by exopolysaccharides (EPSs) and n-alkanes. The cell-to-cell aggregation is parallel to an increase in cell envelope hydrophobicity and is then followed by internalization of petroleum droplets (Mara *et al.*, 2012). A completely different strategy is adopted by in-depth-studied *Acinobacter,* whose cells produce a strong

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biosurfactant, the lipopolysaccharide emulsion that interfaces between cell membranes and oil (Mercaldi *et al.,* 2008). They solubilize hydrocarbons in vesicles composed of proteins, phospholipids and lipopolysaccharides (Leahy *et al.,*1990). Enzymatic degradation of hydrocarbons, is usually catalyzed by the alkene mono oxygenase complex formed by three different subunits: i) alkene hydroxylase (encoded by alkM); ii) rubredoxin; and iii) rubredoxin reductase. This complex has been characterized in detail in *P. putida*GPo1, where the alk genes are operonically organized into the octane utilization (OCT) plasmid (Van Beilen *et al.,* 2007). A different type of organization was found in some *Acinetobacter* strains, such as *Acinetobacte*r baylyi ADP-1 (Hamme *et al*., 2003) and *A. venetianus* VE-C3, where genes responsible for degradation of n-alkanes are located in the bacterial chromosome. However, analysis of Alk mutants suggested that genes involved in hydrocarbon uptake were also present on the two plasmids pAV1 (10,820 bp) and pAV2 (15,135 bp) (Decorosi *et al.,* 2006).The petroleum hydrocarbon degrading *Mycobacterium* grew in nutrient-supplemented artificial seawater with a heavy fuel oil as the sole carbon source, causing the complete removal of all linear (C_{12} to C_{40}) and branched alkanes from the aliphatic fraction, as well as an extensive degradation of the three- and four-ring polycyclic aromatic hydrocarbons (PAHs) (Vila *et al*., 2010).*Rhodococcus* strain removed 50% of the aliphatic fraction of petroleum, completely depleting all linear alkanes (up to C_{20}) and pristane but showing limited action on higher-molecular-weight alkanes and phytane (Kim *et al*., 2010).

Among the fungi *Aspergillus,* one of the commonest and the most abundant fungal specie found river and it helps to degrade petroleum hydrocarbons (Amund *et al*., 2010). This ubiquitous fungal could degrade various fractions of petroleum hydrocarbons especially the recalcitrant PAHs and it help to reduce the problem of bioaccumulation of these organic compounds in the marine animals, and also the resultant risks of potential health hazards associated with the consumption of the contaminated sea foods. *Penicillium* species were the most efficient metabolizers of hydrocarbons (Obire *et al*., 2008).This strain has the ability to produce extracellular enzymes and degrade petroleum hydrocarbons (Leitao, 2009).

Penicillium is able to grow at high concentrations of salt as well as in its absence, while also processing high resistance to hydrocarbon degradation efficiency, could be used as agents for abatement of these pollutants in hypersaline conditions, as well as in non-saline environment. *Fusarium* sp. F092 produced extra and intra-cellular enzymes, especially in liquid medium which helps in the rapid degradation of hydrocarbons (Tachibana, 2011). *Trichoderma* preferably degrades longer chain hydrocarbons (20-40°C) after 9 days of incubation with optimal physical and nutrient parameters.

Table 5.Selected microorganisms used in biodegradation of petroleum hydrocarbon. (Mrozik and Seget, 2010).

Microorganisms	Contaminants treated	References
Single strain		
Comamonas testosteroni BR60	Crude oil, PAHs	Gentry et al. (2001)
Arthrobacter chlorophenolicus A6L	4-Chlorophenol	Jernberg and Jansson (2002)
Absidia cylindrospora	Fluorene	Garon et al. (2004)
Pseudomonas sp. ST41	Marine gas oil	Stallwood et al. (2005)
Pseudomonas aeruginosa WatG	Diesel oil	Ueno et al. (2006)
Sphingobium chlorophenolicum ATCC 39723	Pentachlorophenol	Dams et al. (2007)
Burkholderia sp. FDS-1	Fenitrothion	Hong et al. (2007)
Aspergillus sp. LEBM2	Phenol	dos Santos et al. (2008)
Aspergillus sp. LEBM1 and LEBM3	Chlorobenzene	dos Santos et al. (2008)
Gordonia sp. BS29	Aliphatic and aromatic hydrocarbons	Franzetti et al. (2009)
Pseudomonas putida ZWL73	4-Chloronitrobenzene	Niu et al. (2009)
Aspergillus sp. *Trichocladium canadense* Fusarium oxysporum	LMW-PAHs (2–3 rings)	Silva et al. (2009a)
Trichocladium canadense *Aspergillus* sp. *Verticillium* sp. *Achremonium* sp.	HMW-PAHs (4–7 rings)	Silva et al. (2009a)
Consortia		
Rhodococcus sp., *Acinetobacter* sp., *Pseudomonas* sp.	PAHs (fluorene, phenanthrene, pyrene)	Yu et al. (2005)
Bacillus subtilis DM-04, *Pseudomonas aeruginosa* M and NM	Crude petroleum-oil hydrocarbons	Das and Mukherjee (2007)
Mycobacterium fortuitum, *Bacillus cereus*, *Microbacterium* sp., *Gordonia polyisoprenivorans*, *Microbacteriaceae* bacterium, *Fusarium oxysporum*	PAHs (anthracene, phenanthrene, pyrene)	Jacques et al. (2008)
Rhizopus sp., *Penicillium funiculosum*, *Aspergillus sydowii*	Petroleum hydrocarbons	Mancera-López et al. (2008)
Bacillus strains B1F, B5A and B3G, *Chromobacterium* sp. 4015, *Enterobacter aglomerans* sp. B1A *Achremonium* sp., *Aspergillus* sp., *Verticillium* sp.	Mixture of PAHs (naphthalene, phenanthrene, anthracene, pyrene, dibenzo[a]anthracene, benzo[a]pyrene)	Silva et al. (2009b)

Trichoderma is able to grow in a relatively wide range of pH from 5.0 to 7.0, suggesting that this organism could degrade petroleum hydrocarbons under not only acidic but also neutral conditions (Hamzah*et al.*, 2012).*Candida* from soil samples contaminated with acidic oily sludge (pH 1-3) could degrade 73% of the total petroleum hydrocarbons present in the medium at pH 3 in a week. This type of yeast could efficiently degrade the aliphatic and aromatic fractions of the acidic oily sludge at pH 3 and this was confirmed by gas chromatography (Abraham *et al.*, 2011).

1.3.6. Metabolism of petroleum hydrocarbon

Aerobic Alkane Metabolism

Microorganisms are equipped with metabolic machinery to use petroleum as a carbon and energy source. The most rapid and complete degradation of the majority of organic pollutants is brought about under aerobic conditions

The degradation of petroleum hydrocarbons can be mediated by specific enzyme system. Figure 5 shows the initial attack on xenobiotics by oxygenases .Other mechanisms involved are (1) attachment of microbial cells to the substrates and (2) production of biosurfactants (Satpute *et al.*, 2010). The uptake mechanism linked to the attachment of cell to oil droplet is still unknown but production of biosurfactants has been well studied.

The mechanism of *n*-alkane metabolism and the genes involved in the metabolism has been reported by many researchers. From a regulatory genetic standpoint, the most extensively characterized alkane degradation pathway is encoded by the OCT plasmid carried by *Pseudomonas putida* Gpo1 (formerly *Pseudomonas oleovorans*) (Witholt *et al.*, 2001). The membrane-bound monooxygenase and soluble rubredoxin and rubredoxin reductase serve to shunt electrons through NADH to the hydroxylase for conversion of an alkane into an alcohol. The alcohol can be further oxidized to an aldehyde and acid prior to proceeding into the β-oxidation and tricarboxylic acid cycles. Beilen *et al.*, (2001) studied the OCTplasmid, while Canosa *et al.*, (2000) and Panake *et al.*, (1999) examined expression of the AlkS regulator, and Yuste *et al.* (2001) studied the catabolite repression system.

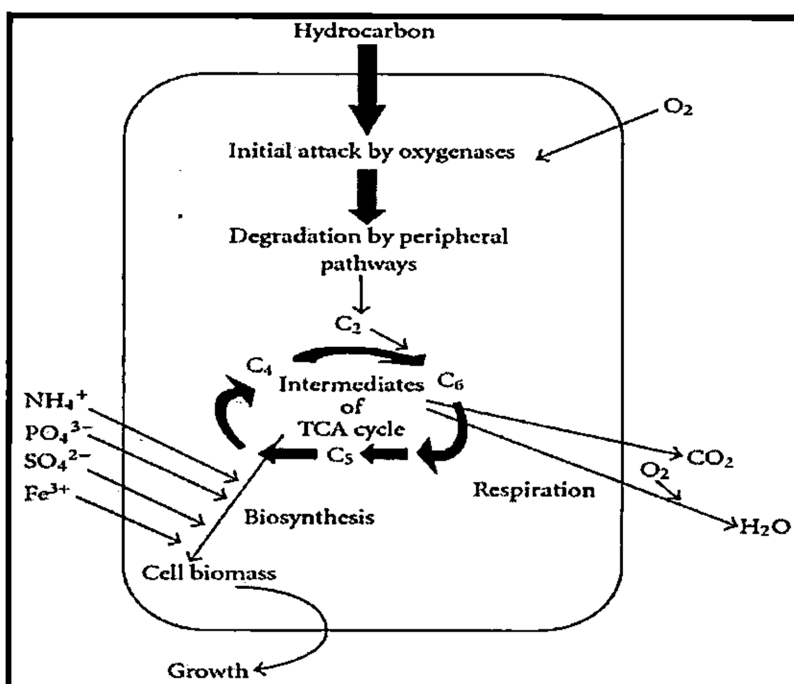

Fig 5 Principle of aerobic degradation of hydrocarbons by microorganisms.(Das and Chandran, 2011)

A model for alkane metabolism, including the locations of the Alk proteins and regulation of the *alk*genes, is shown in Fig. 6. Here, the *alkBFGHJKL* operon encodes the enzymes necessary for converting alkane into acetyl-coenzyme A (CoA), while *alkST* encode a rubredoxin reductase (AlkT) and the positive regulator for the *alkBFGHJKL* operon (AlkS). These two operons are located end to end, separated by 9.7 kb of DNA, within which lies *alkN*, a gene coding for a methyl-accepting transducer protein that may be involved in alkane chemotaxis. The function of *alkL* remains unknown, although it is suspected to be involved in transport. Comparative analysis of insertion sequences in*P. putida* P1 and the previous observation that the G+C content of the *alk* genes is lower than that of both the host strain and the OCT plasmid suggest that the genes are part of an integrated mobile element. Two other plasmid systems have been partially characterized: the OCT plasmid in *Pseudomonas maltophilia* has an *alkA* gene distinct from that of *P. putida*, and the unique pDEC plasmid in *Pseudomonas* sp. strain C12B (Ward *et al.*, 2003).The clustering and regulation of alkane degradation genes varies among the bacteria. *Burkholderia cepacia* has an *alkB* gene that is not linked to other alkane

degradation genes as it is in *P. putida*. The *PalkB* promoter in this organism is down regulated by catabolite repression more strongly than in *P. putida* GPO1 (Rojo *et al.*, 2001). Other differences include the repression of alkane degradation by citrate and the maintenance of repression during stationary phase in *B. cepacia*, two phenomena not observed in *P. putida* GPO1. In *Acinetobacter* sp. strain ADP1, *alkM*, the terminal alkane hydroxylase-encoding gene, is regulated by *alkR*, which shows no similarity to the LuxR-UhpA-like *alkS* regulator in *P. putida*. In addition, the genes in *Acinetobacter* sp.strain ADP1 are not found in a large operon or on a plasmid. Indeed, the genes are 396 kb from *rubA* and *rubB*, which encode rubredoxin and rubredoxin reductase (Ward *et al.*, 2003).

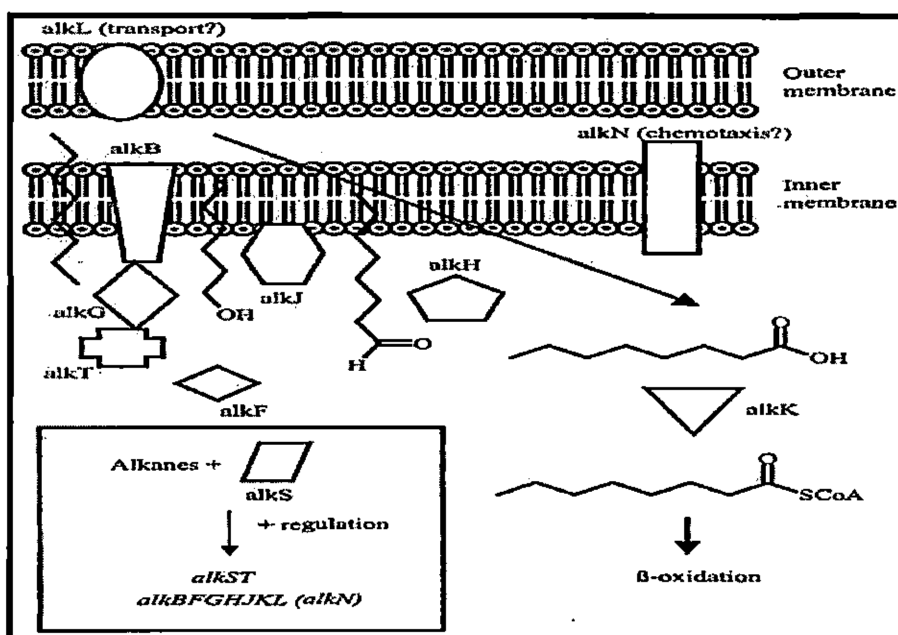

Fig 6. Schematic representation of alkane degradation in gram-negative bacteria, showing the locations and functions of the *alk* gene products(Witholt *et al.*, 2001).The products include AlkB (alkane hydroxylase), AlkF and AlkG (rubredoxins), AlkH (aldehyde dehydrogenase), AlkJ (alcohol dehydrogenase), AlkK (acyl-CoA synthetase), AlkL (outer membrane protein that may be involved in uptake), AlkN (a methyl-accepting transducer protein that may be involved in chemotaxis), AlkT (rubredoxin reductase), and AlkS (positive regulator of the *alkBFGHIJKL* operon and *alkST* genes).

Little information is available for pathways other than the aerobic monooxygenase-mediated pathway for alkane degradation. Evidence where a dioxygenase converts alkanes to aldehydes through *n*-alkyl hydroperoxides without an alcohol intermediate has been described for *Acinetobacter* sp.strain M1 (Koma et *al.*, 2003). The dioxygenase requires molecular oxygen to catalyze

the oxidation of *n*-alkanes (C10 toC30) and alkenes (C12 to C20) without the production of oxygenradicals. A flavin adenine dinucleotide chromophore was detected and the enzyme is thought to contain $Cu2^{+}$. Unlike the case for the 1-monooxygenase in *P. putida*, rubredoxin and NAD(P)H are not required.

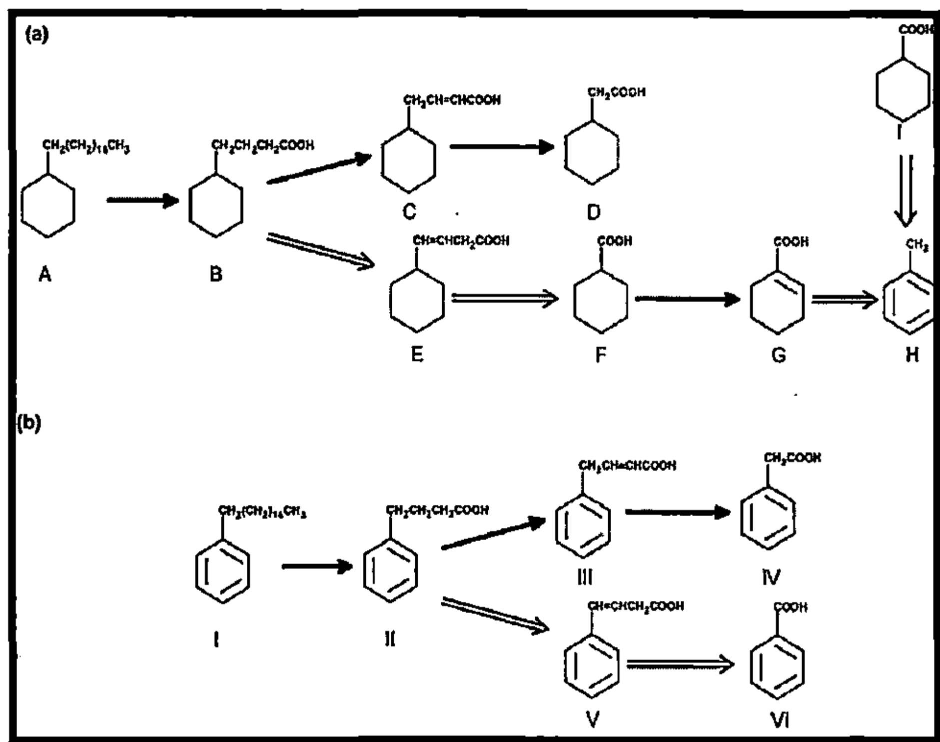

Fig 7. Metabolic pathway illustrating biodegradation of an *n*-alkylcyclohexane (a) and an *n*-alkylbenzene (b) by an *Alcanivorax* sp.strain MBIC4326 (Dutta and Harayama (2001).The major metabolic route of β oxidation is shown with bold arrows, while minor routes are indicated with open arrows and a novel metabolic route by large open arrows. Pathway a: A, *n*-octadecylcyclohexane; B, 4-cyclohexabutanoic acid; C, 4-cyclohexyl-2-butanoic acid; D, cyclohexane acetic acid; E, 4-cyclohexyl-2-butenoic acid; F, cyclohexane carboxylic acid; G, 1-cyclohexene-1- carboxylic acid; H, benzoic acid; I, 3-cyclohexene-1-carboxylic acid. Pathway b: I, *n*-hexadecylbenzene; II, 4-phenylbutanoic acid; III, 4-phenylbutenoic acid; IV, phenylacetic acid; V, 4-phenylbutenoic acid; VI, benzoic acid.

Another novel metabolic pathway has been observed in a *Rhodococcus* mutant. In this case, aliphatics are *cis* desaturated producing products with double bonds mainly atthe ninth carbon from the terminal methyl group. It is postulatedthat a coenzyme A-independent *cis*-desaturase may be involved in this activity. Dutta and Harayama (2001) noted that the degradation of the long side chains of *n*-alkylbenzenesand *n*-alkylcyclohexanes by *Alcanivorax* sp. strain MBIC 4326 proceeds mainly by β oxidation (Fig. 7). However, minor products suggest the possibility of other degradativeroutes.

Aerobic PAH Metabolism

A wide array of microbial species (bacteria, fungi, algae and cyanobacteria) have a diversity of tools to use both low(three rings or fewer) and high (four or more rings) molecular weight PAHs such as naphthalene, acenaphthene, anthracene, fluoranthene, pyrene, and chrysene as sole carbon and energy sources. While no strains have yet been found to utilize PAHs with more than four rings, such as benzo[*a*]pyrene as a sole carbon and energy source, co metabolic transformations have been characterized (Juhasz and Naidu, 2000).The low water solubility and high sorbtion capacity of PAHs are often found to greatly influence biodegradation, but other factors including production of toxic or dead-end metabolites, metabolite repression, the presence of preferred substrates and the lack of cometabolic or inducer substrates must be considered when PAH is persistent (Juhasz *et al.,* 2002). The majority of information on PAH metabolism has come from studying naphthalene catabolic plasmids such as NAH7 from *Pseudomonas putida* strain G7. The first operon (*nahAaAbAcAdBFCED*) encodes the pathway for naphthalene conversion to salicylate (upper pathway), and the second (*nahGTHINLOMKJ*) codes for the conversion of salicylate via catechol*meta*-cleavage to acetaldehyde and pyruvate (lower pathway)(Simmon *et al.,* 1992). The regulator for both operons is encoded by a third operon containing *nahR,* which is induced by salicylate (Schell and Wender, 1986). Molecular and biochemical evidence that the naphthalene plasmid degradative enzymesystem could mineralize other PAHs, such as phenanthrene and anthracene, was first provided by Menn *et al.,* (1993). A variety of new iso functional gene sequences have been also reported in different bacterial species, most notably in *Nocardia, Rhodococcus,* and *Mycobacterium* sp., some of which are capable of using high-molecular-weight PAHs such as pyrene as carbon and energy sources. In addition, with respect to PAH metabolism, novel gene sequences and gene orders have been observed in a variety of strains, including *Burkholderia* sp. strain RP007, *phnFECDAcAdB* (Laurie and Lloyd-Jones,1999); *Pseudomonas* sp. strain U2, *nagAaGHAbAcAdBF*(Fuenmayor *et al.,* 1998); *Rhodococcus* sp. strain I24, *nidABCD* (Treadway *et al.,* 1999); *Mycobacterium*sp. PYR1, *nidDBA* (Khan *et al.,* 2001); and *Nocardiodes* sp. strain KP7, *phdABCD* (Sato *et al.,* 1997). Sequence diversity, and the fact that naphthalene catabolic genes have now been found on the chromosome as well as on plasmids indicate that lateral gene

transfer.and genetic recombination may have played an important role in the development of these versatile metabolic pathways. A list of microorganisms involved in aerobic degradation of petroleum hydrocarbon is summarized in table 6.

High levels (90%) of homology and a conserved genearrangement are observed in the *nah, ndo, pah,* and *dox* sequences (Kroutil and Faber, 2000). The first example of two isofunctionalsalicylate hydroxylases in one strain is exhibited by *Sphingomonasyanoikuyae* B1, which has modified andreorganized genes to obtain catabolic pathways for naphthalene,phenanthrene, anthracene, biphenyl, toluene, and *m*- and*p*-xylene. In this case, *nah, bph,* and *xyl* genes are present butare not arranged in three distinct operons (Kim and Zylstra, 1999). This gene clustering may be typical of *Sphingomonas* sp. capable of degrading aromatic compounds. Romine et al. (1999) sequenced the pNL1 (184 kb) plasmid of *Sphingomonasaromaticivorans* F199, which is capable of degradingtoluene, xylenes, salicylate, biphenyl, dibenzothiophene,flourene, and benzoate. In this plasmid, at least 13 gene clustersare predicted to encode all of the necessary enzymes.

Table6. Microorganisms involved in aerobic degradation of petroleum hydrocarbon.

Strain	Location	Substrate	Gene	Encoded protein or function
Pseudomonas putida strains	Plasmid	Naphthalene (upper pathway)	*nahAa*	Reductase
			nahAb	Ferredoxin
			nahAc	Iron sulfur protein large subunit
			nahAd	Iron sulfur protein small subunit
			nahB	*cis*-Naphthalene dihydrodiol dehydrogenase
			nahF	Salicyaldehyde dehydrogenase
			nahC	1,2-Dihydroxynaphthalene oxygenase
			nahE	2-Hydroxybenzalpyruvate aldolase
			nahD	2-Hydroxychromene-2-carboxylate isomerase
		Salicylate (lower pathway)	*nahG*	Salicylate hydroxylase
			nahT	Chloroplast-type ferredoxin
			nahH	Catechol oxygenase
			nahI	2-Hydroxymuconic semialdehyde dehydrogenase
			nahN	2-Hydroxymuconic semialdehyde dehydrogenase
			nahL	2-Oxo-4-pentenoate hydratase
			nahO	4-Hydroxy-2-oxovalerate aldolase
			nahM	Acetaldehyde dehydrogenase
			nahK	4-Oxalocrotonate decarboxylase
			nahJ	2-Hydroxymuconate tautomerase
		Regulator for both operons	*nahR*	Induced by salicylate
Pseudomonas putida NCIB9816	Plasmid	Naphthalene	*ndoA*	Naphthalene-dioxygenase genes (these 3 genes correspond to NahAb,-c, and-d listed above)
			ndoB	
			ndoC	
Pseudomonas sp. strain C18	Plasmid	Dibenzothiophene Naphthalene phenanthrene	*doxA*	Naphthalene dioxygenase
			doxB	DoxA, -B, -D correspond to NahAb, -c, and-d listed above
			doxD	
			doxE	*cis*-Naphthalene dihydrodiol dioxygenase
			doxF	Salicylaldehyde dehydrogenase
			doxG	1,2-Dihydroxynaphthalene dioxygenase
			doxH	Isomerase (interchangeable with *doxJ*?)
			doxI	Hydratase-aldolase
			doxJ	Isomerase
Pseudomonas sp. strain U2	Plasmid	Naphthalene	*nagAa*	Ferredoxin reductase
			nagG	Subunit of salicylate 5-hydroxylase with Rieske-type iron-sulfur centre
			nagH	Subunit of salicylate 5-hydroxylase
			nagAb	Ferredoxin
			nagAc	Large dioxygenase subunit
			nagAd	Small dioxygenase subunit
			nagB	Naphthalene *cis*-dihydrodiol dehydrogenase
			nagF	Salicylaldehyde dehydrogenase
Burkholderia sp. strain RP007	Plasmid	Naphthalene phenanthrene	*phnR*	Regulatory
			phnS	Regulatory
			phnF	Aldehyde dehydrogenase
			phnE	Hydratase-aldolase
			phnC	Extradiol dioxygenase
			phnD	Isomerase

Organism	Location	Substrate	Gene	Product
			phnAc	Large dioxygenase subunit (Rieske-type [2Fe-2S]
			phnAd	Small dioxygenase subunit
			phnB	Dihydrodiol dehydrogenase
Pseudomonas putida OUS82	Chromosome	Naphthalene	*pahAa*	Ferredoxin reductase
		Phenanthrene	*pahAb*	Ferridoxin
		A variety of homo-hetero-, and monocyclics converted to phenols	*pahAc*	Large subunit of iron-sulfur protein
			pahAd	Small subunit of iron-sulfur protein
			pahB	*cis*-Dihydrodiol dehydrogenase
			pahC	Dioxygenase
			pahD	Isomerase
			pahE	Hydratase-aldolase
			pahF	Dehydrogenase
Pseudomonas stutzeri AN10	Chromosome	Naphthalene	*nahG*	Salicylate 1-hydroxylase
		2-Methylnaphthalene	*nahW*	Salicylate 1-hydroxylase (outside *meta*-cleavage transcriptional unit)
Nocardiodes sp. strain KP7	Chromosome	Phenanthrene	*phdA*	Alpha subunit of dioxygenase
			phdB	Beta subunit of dioxygenase
			phdC	Ferredoxin
			phdD	Ferredoxin reductase
			phdK	2-Carboxybenzaldehyde dehydrogenase
Rhodococcus sp. strain 124	Chromosome	Naphthalene	*nidA*	Naphthalene-inducible dioxygenase system
		Toluene	*nidB*	Dioxygenase small subunit
		Indene	*nidC*	*cis*-Dihydrodiol dehydrogenase
			nidD	Putative aldolase
Mycobacterium sp. strain PYR-1	Chromosome	Anthracene, Phenanthrene	*nidD*	Aldehyde dehydrogenase
		Fluoranthene	*nidB*	Small subunit of dioxygenase
		Pyrene, benzo [a]pyrene, 1-nitropyrene	*nidA*	Large subunit of dioxygenase
Sphingomonas paucimobilis var. EPA505		Phenanthrene	*pbhA*	Ring fission dioxygenase
		Anthracene, benzo[b]fluoranthene	*pbhB*	Rieske-type ferridoxin subunit of multicomponent dioxygenase
		Naphthalene	*pbhC*	Hydratase-aldolase
		Fluroanthene, pyrene	*pbhD*	Pyruvate phosphate dikinase
		Intermediate catabolites		

Anaerobic Hydrocarbon Metabolism

Anaerobic metabolism is a vital process with respect to petroleum hydrocarbon biodegradation. A list of microorganisms involved in anaerobic degradation of petroleum hydrocarbon is summarized in table 7. Microbial consortia has been reported to metabolize hydrocarbons such as toluene (Elshahed *et al.*, 2001), alkylbenzenes including *m*-, *o*-, and *p*-xylene and trimethylbenzenes (Phelps and Young, 2001), benzene, naphthalene and phenanthrene (Meckenstock *et al.*, 2000), methylnaphthalene and tetral in (Annweiler *et al.*, 2002), C6 *n*-alkanes (Ehrenrich *et al.*, 2001), branched alkanes (Canosa *et al.*, 2000), and hydrocarbon mixtures under anaerobic conditions. These reactions take place under Fe(III)-reducing, denitrifying, and sulfate-reducing conditions by anoxygenic photosynthetic bacteria or in syntrophic consortia of proton-reducing and methanogenic bacteria. Other terminal electron acceptors used during anaerobic hydrocarbon metabolism include manganese oxides (Langenhoff *et al.*, 1999), soil humic acids and the humic acid model compound anthraquinone-2,6-disulfonate (Cervantes *et al.*, 2001), and fumarate in a fermentative oxidation process (Meckenstock *et al.*, 2001). The diverse set of bacteria including members of α, β, γ and δ subclasses of proteobacteria form an excellent framework to understand the underlying biochemical and molecular mechanisms involved in anaerobic hydrocarbon metabolism. Toluene has been the most studied hydrocarbon with respect to enzymatic and genetic characterizations in the denitrifying bacteria *Azoarcus* sp. strain T, *Thauera aromatica* strain K172,and*Thauera*sp. strain T1 (Achong *et al.*, 2001). In the proposed pathway, fumarate addition totoluene is mediated by benzyl succinate synthase to form benzyl succinate. This unusual addition reaction results in a series of modified β oxidation reactions to convert benzylsuccinate to benzyl-CoA (Beller *et al.*, 2002), which is a central intermediate in the anaerobic degradation of aromatic compounds (Heider *et al.*, 2007). The *bbs* (beta-oxidation of benzylsuccinate) operon contains *bbsDCABE*, with *bbsCAB* encoding the γ, α, and β subunits of benzyl succinate synthase.

Table 7. Microorganisms involved in anaerobic degradation of petroleum hydrocarbon.

Organism	Hydrocarbon(s) used
Anoxygenic photoheterotrophic bacterium	
Blastochloris sulfoviridis ToP1	Toluene
Denitrifying bacteria	
Azoarcus sp. strain EB1	Ethylbenzene
Azoarcus sp. strain T	Toluene, *m*-xylene
Azoarcus tolulyticus Td15	Toluene, *m*-xylene
Azoarcus tolulyticus To14	Toluene
Dechloromonas sp. strain JJ	Benzene, toluene
Dechloromonas sp. strain RCB	Benzene, toluene
Pseudomonas sp. strain NAP-3	Naphthalene
Strain EbN1	Ethylbenzene, toluene
Strain HdN1	C_{12}–C_{20} alkanes
Strain HxN1	C_{6}–C_{8} alkanes
Strain M3	Toluene, *m*-xylene
Strain mXyN1	Toluene, *m*-xylene
Strain OcN1	C_{8}–C_{12} alkanes
Strain PbN1	Ethylbenzene, propylbenzene
Strain pCyN1	*p*-Cymene, toluene, *p*-ethyltoluene
Strain pCyN2	*p*-Cymene
Strain T3	Toluene
Strain ToN1	Toluene
Thauera aromatica K172	Toluene
Thauera aromatica T1	Toluene
Vibrio sp. strain NAP-4	Naphthalene
Fe(III)-reducing bacteria	
Geobacter grbiciae TACP-2[T]	Toluene
Geobacter grbiciae TACP-5	Toluene
Geobacter metallireducens GS15	Toluene
Sulfate-reducing bacteria	
Desulfobacula toluolica To12	Toluene
Desulfobacterium cetonicum	Toluene
Strain AK-01	C_{13}–C_{18} alkanes
Strain Hxd3	C_{12}–C_{20} alkanes, 1-hexadecene
Strain mXyS1	Toluene, *m*-xylene, *m*-ethyltoluene, *m*-cymene
Strain NaphS2	Naphthalene
Strain oXyS1	Toluene *o*-xylene, *o*-ethyltoluene
Strain Pnd3	C_{14}–C_{17} alkanes, 1-hexadecene
Strain PRTOL1	Toluene
Strain TD3	C_{6}–C_{16} alkanes

1.3.7. Microbial physiological response to petroleum hydrocarbon

Microbial physiological responses such as changes in membrane architecture, active uptake and efflux to hydrocarbons have a high degree of impact on bioremediation of petroleum.

The lipophilic molecules such as hydrocarbon partition between the monolayer of the cytoplasmic membrane and the outer membrane (Zahir *et al.*, 2006). Hydrocarbons tend to reside in the hydrophobic area between membrane mono-layers in the acyl chains of phospolipids, with partitioning being related to the octanol-water partition coefficient of the lipophilic compound. Hydrocarbon insertion alters membrane structure by changing fluidity and protein conformations and results in disruption of the barrier and energy transduction functions while affecting membrane-bound and embedded enzyme activity (Heipieper *et al.*, 2007).

In terms of general stress responses, bacteria may form biofilms, alter their cell surface hydrophobicity to regulate their partitioning with respect to

hydrocarbon-water interfaces. In addition, energy-dependent repair mechanisms may be used to compensate for losses in membrane integrity resulting from the partitioning of lipophilic compounds. For example, membrane fluidity can be decreased through increased membrane ordering by affecting *cis/trans* phospolipid isomerizations, by decreasing unsaturated fatty acid content, and by altering phospholipid head groups (Pepi *et al.,* 2009). These changes may be associated with an overall increase in phospholipid content and increased phospholipid biosynthesis in solvent-stressed cells.

These alterations serve to produce a physical barrier to the intercalation of hydrocarbons in membranes, thus offsetting the passive influx of hydrocarbons into the cell. It is generally believed that hydrocarbons interact with microorganisms nonspecifically and move passively into the cells. When microorganisms contact water-solubilized hydrocarbons, decreasing solubility with increasing molecular weight is restrictive. Two additional modes of hydrocarbon accession are direct adherence to large oil droplets and interaction with pseudo solubilized oil (Ward*et al.,* 2003). Hamme and Ward (2001) described a *Rhodococcus* strain that grew directly on crude oil droplets and could be removed with the addition of exogenous chemical surfactant, while a *Pseudomonas* strain required surfactant-solubilized oil to efficiently access hydrocarbons.

Phenanthrene uptake by *Pseudomonas fluorescens* LP6a was reported to be passive via energy dependent phenanthrene efflux (Bugg *et al.*, 2000). With respect to active transport, proton motive force uncouplers have been shown to apparently decrease both *n*-hexadecane (Beal *et al.,* 2000) and naphthalene uptake, which indicate that energy-dependent uptake is important in some strains.

Ramos *et al.* (1995) isolated *P. putida*DOT-T1E, which metabolized toluene and was capable of growing in the presence of 90% (v/v) toluene. DOT-T1E was found to increase membrane rigidity by converting cis-9,10-methylenehexadecanoic acid to 9-cis-hexadecanoic acid and subsequentlyto the corresponding transisomer in less than 1 min upon exposure to toluene.

In the long-term (15 to 20 min) exposure, DOT-T1E decreased the amount of phospatidylethanolamine in the phospolipid polar head groups and increased cardiolipid levels, again increasing membrane rigidity. These changes increase

lipid ordering to restore membrane integrity and reduce organic solvent partitioning in the membrane.

Kabelitz *et al.* (2009) discussed that the adaptive response towards alcohols is related to the physico-chemical properties of short-chain alcohols, which can only penetrate slightly into the hydrophobic center of the phospholipid bilayer, causing a swelling effect on the hydrophilic head groups. To counteract this effect, the insertion of unsaturated fatty acids seems to be a better reaction against those short-chain alcohols. In contrast, long-chain alcohols and aromatic solvents, which are more hydrophobic, penetrate deeply into the membrane, thus causing an increase in the degree of saturation.

1.3.8. Recent advances in petroleum degradation

Role of Plasmids in Adaptation

Genetic factors play important roles in biodegradation potentials on microorganisms. Plasmids probably play leading role in this aspect. The ability to degrade more components of petroleum such, as the aromatic fractions are generally plasmid mediated (Okoh 2006). Exposures of a microbial community to hydrocarbons help to increase in the number of bacterial plasmids types. Catabolic plasmids are non-essential genetic elements but they do provide a metabolic versatility. Such genetic potential of plasmid allow the evolution of integrated and regulated pathways for the degradation of hydrocarbons along with the development in molecular biology, particularly in the application of recombinant DNA technology, gene probes (Mrozik *et al.,* 2010) and polymerase chain reaction (PCR) technology.

Many bacterial catabolic pathways are specified by conjugative plasmids (Table 8). These plasmids are readily transferred laterally into new host bacteria, and enhancing the metabolic potential of other members of an ecosystem. Conjugative plasmids are 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 (Okoh 2006). The best

characterized of these pathways is encoded by the TOL plasmid (pWW0) of *P. putida* PaW1 (Salam and Obayori, 2014), 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 (Saghafi *et al.,* 2010).

Table 8. Plasmids encoding catabolic functions. (Okoh, 2006)

Plasmid	Host	Components catabolized
TOL	*P. putida*	BTEX
NAH	*P. putida*	Naphthalene
SAL	*P. putida*	Salicylate
pND50	*P. putida*	p-cresol
pWW31	*P. putida*	Phenylacetate
pJP1	*Alcaligenes paradoxa*	2,3- dichlorophenoxyacetic acid
pKF1	*Acinetobacter sp.*	4- chlorobiphenol
pAC21	*Pseudomonas sp.*	3-chlorobenzoate
pRE1	*P. putida*	3-chlorobenzoate

Genetically Modified Bacteria

Applications for genetically engineered microorganisms (GEMs) in bioremediation have received a great deal of attention to improve the degradation of hazardous wastes under laboratory conditions. There are reports on the degradation of environmental pollutants by different bacteria. Table 9 shows some examples of the relevant use of genetic engineering technology to improve bioremediation of hydrocarbon contaminants using bacteria. The genetically engineered bacteria showed higher degradative capacity. However, ecological and environmental concerns and regulatory constraints are major obstacles for testing GEM in the field. These problems must be solved before GEM can provide an effective clean-up process at lower cost. The use of genetically engineered bacteria was applied to bioremediation process monitoring, strain monitoring, stress response, end-point analysis, and toxicity assessment. Examples of these applications are listed in Table 10.

Table 9. Genetic engineering for biodegradation of contaminants.(Das and Chandran, 2011).

Microorganisms	**Modification**	**Contaminants**
P. putida	pathway	4- ethylbenzoate
P. putida KT2442	pathway	Toluene/ benzoate
Pseudomonas sp. FRI	pathway	Chloro-, methylbenzoate
Comamonas testosterone VP44	Substrate specificity	o-,p-MCB
P. pseudoalcaligens KF707-D2	Substrate specificity	TCE, BTEX
Pseudomonas sp. LB400	Substrate specificity	PCB

The range of tested contaminants included chlorinated compounds, aromatic hydrocarbons, and non polar toxicants. The combination of microbiological and ecological knowledge, biochemical mechanisms, and field engineering designs are essential elements for successful in situ bioremediation using genetically modified bacteria.

Table10. Application of genetically modified bacteria for assessing the biodegradation process efficiency.(Das and Chandran, 2011).

Microorganisms	**Applications**	**Contaminants**
A. eutrophus H850Lr	Process monitoring	PCB
P. putida TVA8	Process monitoring	TCE, BTEX
P. fluorescens HK44	Process monitoring	Naphthalene, anthracene
A. cepacia BRI6001L	Strain monitoring	2, 4D
P. fluorescens 10586s	Strain response	BTEX
Pseudomonas sp. Shk1	Toxicity assessment	2, 4 DNTP
A. eutrophus 2050	End point analysis	Non polar narcotics

Biodegradation of Petroleum Hydrocarbons by Immobilized Cells

Immobilized cells have been used and studied for the bioremediation of numerous toxic chemicals. Immobilization not only simplifies separation and recovery of immobilized cells but also makes the application reusable which reduces the overall cost. Luo *et al.* (2012) used free suspension and immobilized *Pseudomonas sp.* to degrade petrol in an aqueous system. The study indicated that immobilization resulted in a combination of increased contact between cell and hydrocarbon droplets and enhanced level of rhamnolipids production. The rhamnolipids caused greater dispersion of water-insoluble n-alkenes in the

aqueous phase due to their amphipathic properties and the molecules consist of hydrophilic and hydrophobic moieties reduced the interfacial tension of oil-water systems. This resulted in higher interaction of cells with solubilized hydrocarbon droplets much smaller than the cells and rapid uptake of hydrocarbon into the cells. Diaz *et al.* (2002) reported that immobilization of bacterial cells enhanced the biodegradation rate of crude oil compared to free living cells in a wide range of culture salinity. Immobilization can be done in batch mode as well as continuous mode. Packed bed reactors are commonly used in continuous mode to degrade hydrocarbons. Cunningham *et al.* (2004) used polyvinyl alcohol (PVA) cryogelation as an entrapment matrix and microorganisms indigenous to the site. They constructed laboratory biopiles to compare immobilised bioaugmentation with liquid culture bioaugmentation and biostimulation. Immobilised systems were found to be the most successful in terms of percentage removal of diesel after 32 days.

Rahman *et al.* (2006) conducted an experiment to study the capacity of immobilized bacteria in alginate beads to degrade hydrocarbons. The results showed that there was no decline in the biodegradation activity of the microbial consortium on the repeated use. It was concluded that immobilization of cells are a promising application in the bioremediation of hydrocarbon contaminated site.

Commercially available bioremediation agents

Bioremediation agents are classified as bioaugmentation agents and biostimulation agents based on the two main approaches to oil spill bioremediation (Nicholsrate, 2001).In 2002, the U.S. EPA compiled a list of 15 bioremediation agents as a part of the National Oil and Hazardous Substances Pollution Contingency Plan (NCP) Product Schedule, which was required by the Clean Water Act, the Oil Pollution Act of 1990, and the National Contingency Plan (NCP) as shown in Table 11. But the list was modified, and the number of bioremediation agents was reduced to nine.

Studies showed that bioremediation products may be effective in the laboratory but significantly less in the field (Venosa, 2003). This is because laboratory studies cannot always simulate complicated real world conditions such as spatial heterogeneity, biological interactions, climatic effects, and nutrient

mass transport limitations. Therefore, field studies and applications are the ultimate tests for the most convincing demonstration of the effectiveness of bioremediation products.

Compared to microbial products, very few nutrient additives have been developed and marketed specifically as commercial bioremediation agents for oil spill cleanup. It is probably because common fertilizers are inexpensive, readily available, and have been shown effective if used properly. However, due to the limitations of common fertilizers (e.g., being rapidly washed out due to tide and wave action), several organic nutrient products, such as oleophilic nutrient products, have recently been evaluated and marketed as bioremediation agents (Das and Chandran, 2011). Four agents, namely, Inipol EAP22, Oil Spill Eater II (OSE II), BIOREN 1, and BIOREN 2, listed on the NCP Product Schedule have also been put into this category. Inipol EAP22 (Societe, CECA S.A., France) is listed on the NCP Product Schedule as a nutrient additive and probably the most well-known bioremediation agent for oil spill cleanup due to its use in Prince William Sound, Alaska.

Oil Spill Eater II (Oil Spill Eater International, Corp.) is another nutrient product listed on the NCP Schedule (U.S. EPA, 2002). This product is listed as a nutrient/ enzyme additive and consists of nitrogen, phosphorus, readily available carbon, and vitamins for quick colonization of naturally occurring bacteria. A field demonstration was carried out at a bioventing site in a Marine Corps Air Ground Combat Center (MCAGCC) in California to investigate the efficacy of OSEII for enhancing hydrocarbon biodegradation in a fuel contaminated vadose zone (Das and Chandran, 2011).

Researchers from European EUREKA BIOREN program conducted a field trial in an estuary environment to evaluate the effectiveness of two bioremediation products (BIOREN 1 and 2). The two nutrient products were derived from fish meals in a granular form with urea and super phosphate as nitrogen and phosphorus sources and proteinaceous material as the carbon source.

Table 11. Bioremediation agents in NCP product schedule (Adapted from USEPA, 2002)

Bioremediation agents	Manufacturer
BET BIOPETRO	BioEnviro Tech, TX
BILGEPRO	International Environmental Products, LLC, PA
INIPOL EAP 22	Societe, France
RESTORATION MICRO BLAZE	Verde Environmental Inc, TX
OIL SPILL EATER II	Oil Spill Eater, Dallas, TX
STEP ONE	B & S Research Inc, MN
WMI-2000	WMI International Inc

The major difference between the two formulations was that BIOREN 1 contained a biosurfactant. The results showed that the presence of biosurfactant in BIOREN 1 was the most active ingredient which contributed to the increase in oil degradation rates whereas BIOREN 2 (without biosurfactant) was not effective in that respect. The biosurfactant could have contributed to greater bioavailability of hydrocarbons to microbial attack.

1.3.9. Application of petroleum degrading microorganism for biodiesel production

Biodiesel, an alternative diesel fuel, is defined as monoalkyl ester of fatty acids from vegetable oil and produced by enzyme mediated catalytical transesterification with petrochemically derived methanol. This process is also called alcoholysis (Youssef, 2007). Oleaginous microorganisms like microalgae, bacillus, fungi and yeast are now used for substituting conventional oil in biodiesel production. A potential future fuel completely produced by bacteria is Microdiesel (Kalscheuer et al. 2006). A highly solvent-tolerant *P. putida* DOTT1E, employed for the biotransformation of BTEX, also served as a potential candidate for biodiesel production (Ramos-Gonzalez *et al.,* 2003).

Table12. Comparison of transesterification activities of different lipases on different substarates (Bajaj *et al.*, 2010)

Source of oil	Source of enzyme	Alcohol	Optimum conditions	Immobilization medium	Maximum yield	Other details	Reference
Jatropha	*Chromobacterium viscosum*	Ethanol	40 °C, 10 h, 200 rpm	Celite-545	92%	Water content 0.5%	Shah et al. [2]
	Burkholderia cepacia	Ethanol	40 °C, 24 h, 200 rpm	–	79%	Ultrasonic pretreatment, 150 W, 2-3 h	Shah and Gupta [11]
	Enterobacter aerogenes	Methanol	55 °C, 48 h, 200 rpm	Silica	94%	Used t-butanol as solvent	Kumari et al. [64]
Tallow	*Mucor miehei*	Methanol	45 °C, 5 h, 200 rpm	–	94.8%	Hexane solvent	Nelson et al. [5]
		Ethanol			98.3%		
		Propanol			98.6%	Used hexane as solvent, 6 mol% water based on triglyceride was added	
		Butanol			98.5%	Used hexane as solvent	
		Isobutanol			98.4%	Used hexane as solvent, 6 mol% water based on triglyceride was added	
Soybean oil	*Thermomyces lanuginosa*	Methanol (stepwise addition)	40 °C, 50 h, 150 rpm	Silica gel	>90%	Molar ratio of methanol to oil 1:1, lipase 10%	Du et al. [4]
	Mucor miehei	Ethanol	45 °C, 5 h, 200 rpm	–	97.4%	Hexane solvent	Nelson et al. [5]
	Pseudomonas fluorescens (psychrophilic strain B68)	Methanol (stepwise addition)	35 °C, 12 h, 180 rpm	Cellulose fabric	92%	n-Heptane solvent	Luo et al. [12]
	Pseudomonas fluorescens	Ethanol (initial molar ratio to oil 3:1)	50 °C, 24 h, 180 rpm	–	71%	Iso-octane solvent, solubilized lipase AK	Zhao et al. [13]
		Methanol (molar ratio to oil 3:1)	40 °C, 72 h, 200 rpm	–	80.5%	5 mmol/l Ca^{2+}, 5% water	Yang et al. [15]
	Rhizomucor miehei (Lipozyme RM-IM)	Methanol	36.5 °C, 6.3 h, 200 rpm	Macroporous weak anionic resin beads	92.2%	n-Hexane solvent, water content 5.8%, molar ratio of methanol to oil 3.4:1	Shieh et al. [11]
Mixture of soybean and rapeseed oils	*Candida antarctica*	Methanol (stepwise addition)	30 °C, 48 h, 130 rpm	SR.10	98.4%	Water content <450 ppm	Shimada et al. [16]
Sunflower oil	*Candida antarctica* (Novozyme 435)	Methanol (continuous addition)	50 °C, 15 h, 130 rpm	Macroporous resin support, diameter 0.3-0.9 mm	97%	Water content 400 ppm	Bélafi-Bakó et al. [42]
Waste cooking oil	*B. subtilis* 1.158	Methanol (stepwise addition)	40 °C, 72 h, 220 rpm, pH 6.5	Hydrophobic carrier with magnetic particles (Fe_3O_4)	93%	–	Ying and Chen [43]

Linko *et al.* (1998) reported that lipase was used as the biocatalyst for the production of variety of biodegradable esters and polyesters. 97% conversions of esters were obtained using *Candida rugosa* lipase powder during tranesterification of rapeseed oil with 2-ethyl-I-hexanol. De *et al.* (1999) demonstrated the use of immobilized *Mucor miehei* lipase (Lipozyme IM-20) for the conversion of fatty alcoholesters (C_4-$C_{18:1}$) in a solvent-free system. The lipase from *M. miehei* was the most efficient for conversion of triglycerides to alkyl esters with primary alcohols whereas lipase from *C. antarctica* was most efficient for transesterifying triglycerides with secondary alcohols to give branched alkyl esters. 94.8-98.5% of maximum conversion was noted for the primary alcohols such as methanol,ethanol, propanol, butanol and isobutanol. 61. 2-83.8% conversion was noted for the secondary alcohols such as isopropanol and 2-butanolin the presence of hexane as a solvent (Nelson *et al.,* 1996).Several methods were used to increase the enzyme yield and to decrease the reaction time. Various parameters such as temperature, time duration and agitation speed vary greatly and are optimized with different combinations of lipase source, immobilization media, and alcohol. Table 12 summarizes the yields and acyl-

acceptors on enzymatic transesterification of oil for producing biodiesel using extracellular lipases.

The cost of the lipase is still the main obstacle for exploiting its potential in biodiesel industry. Therefore, reuse of lipase is essential which can be achieved by using immobilized lipases.

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