APPLIED MICROBIOLOGY

Petroleum and Hydrocarbon Microbiology

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Key words

Petroleum microbiology; hydrocarbon metabolism; bioremediation; hydrocarbon degradation.

Introduction

Petroleum is the oily, flammable liquid that occurs naturally in deposits, usually beneath the surface of the earth; it is also called crude oil. It consists principally of a mixture of hydrocarbons with traces of nonmetallic elements such as sulphur, oxygen and nitrogen. The physical properties and exact chemical composition of crude oil varies from one locality to another. Natural gas is one of the hydrocarbon components of petroleum. Liquefied natural gas**,** or LNG, is natural gas that has been pressurized and cooled so as to liquefy it for convenience in shipping and storage. The boiling point of natural gas is extremely low, and only in the 1970s did cryogenic technology advance enough to make the production and transport of LNG commercially feasible. Various fractions are obtained by subjecting the petroleum to fractional distillation. Light fractions of crude oil, gasoline or petrol, is volatile mixture of hydrocarbons for use in internal combustion engine and as an organic solvent.

Another is a black tarry substance that is rich in asphalt used commonly in road making, roofing and water proofing. The lighter fractions, especially gasoline, being in greatest demand, cracking processes have been developed using heat, pressure, and special catalysts to break up the large molecules of heavy hydrocarbons into small molecules of light hydrocarbons. Some of the heavier fractions like paraffin which is white, translucent, odorless and colorless waxy solid, find use as lubricating oils and used in candles, for coating papers etc.. Highly refined medicinal substances such as petrolatum: colorless to yellowish-white hydrocarbon mixture obtained by fractional distillation of petroleum, in its jellylike semisolid form (known as petroleum jelly and also by several trade names) is used in preparing medicinal ointments and for lubrication.

Petroleum microbiology is an interdisciplinary area involving microbiologists, biochemists, chemists, chemical engineers, physicists and geologists. A wide range of studies have dealt with processes like biotransformation, biodegradation and bioremediation of petroleum hydrocarbons. Studies pertaining to petroleum degradation by microorganisms and its exploitation in environmental clean up in particular bioremediation of oil spills has become central to petroleum microbiology. Additionally wetland based hydrocarbon treatment, biofilteration of volatile hydrocarbons; microbial enhanced oil recovery, oil and fuel up-gradation by desulphurization and denitrogenation are some other applied research areas concerning petroleum microbiology.

F. Gale in 1952 summarized the concept of microbial infallibility by suggesting that there existed in nature a microorganism capable of metabolizing any conceivable compound that the organic chemist might choose to synthesize. The opposing doctrine of molecular recalcitrance was advanced by M. Alexander subsequently, who noted that many synthetic chemicals continue to persist in soil for long duration. It is estimated that nearly 60,000 tons of hydrocarbon oil enters the environment each year. Man-made chemicals used as refrigerants, fire retardants, paints, solvents and herbicides are also known to cause considerable environmental pollution and human health problems as a result of their persistence, toxicity and transformation into hazardous metabolites. The ability of microorganisms to degrade hydrocarbons has been known since 1895 with the report on paraffin utilization. It is the consortium of microorganisms rather than a single organism, that is more appropriate to degrade a mixture of hydrocarbons like crude oil, kerosene, or a waste water from refinery or petrochemical industries.

Formation of Petroleum Hydrocarbons

The term hydrocarbon embraces all those organic substances solely composed of carbon and hydrogen. There are extensive deposits of complex mixtures of hydrocarbons on the earth, both below ground (74.5×10^9 tones) and on the surface (66×10^9 tones). These are thought to have produced originally by the combined effect of heat and pressure on organic material. Much of the sedimentary material in the marine environment is comprised of decayed plant and animal materials, which have remained buried during the past 600 million years under thick layers of rock. It is believed that petroleum consists of the remains of these organisms. Biochemical changes made over the period in these sedimentary deposits as a result of microbial activities lead to the formation of petroleum. Many microorganisms on their own are also known to contribute towards hydrocarbon formation. *Botryococcus braunii*, a green alga, excretes long chain hydrocarbons (C30 - C36) having the consistency of oil. About 30% of its dry cell weight is petroleum. Many photosynthetic bacteria, algae and higher plants synthesize carotenoid pigments, which are unsaturated hydrocarbons. Surface waxes found on leaves of many plants contains high molecular weight (C25 – C33) hydrocarbons. Hydrocarbons are also synthesized by animals and may occur as components of insect cuticle lipids.

Microorganisms Oxidizing Hydrocarbons

Occurrence

Hydrocarbon degrading microorganisms are ubiquitous in nature but are found at relatively higher densities in petroleum contaminated sites. Arctic environments, estuaries, oceans and marine sediments, deep sea, thermal rents, etc. are some of the sites explored for isolating hydrocarbon degrading microorganisms. Among those isolated from aquatic habitats, *Pseudomonas, Vibrio, Achromobacter, Arthrobacter, Micrococcus, Corynebacter, Acinetobacter, Nocardia* etc. were predominant hydrocarbon utilizers, while *Aureobasidium, Candida, Rhodotorula* and *Sporobolomyces* were the most common fungi and yeasts isolated from marine environments. Table 1 summarizes some hydrocarbon degrading microorganisms.

Table 1: Some hydrocarbon degrading microorganisms

Crude oil component	Microorganisms
Saturates	*Arthrobacter* sp., *Acinetobacter* sp., *Candida* sp., *Pseudomonas* sp., *Rhodococcus* sp., *Streptomyces* sp., *Bacillus* sp., *Aspergillus japonicus*
Monocyclic aromatic hydrocarbons	*Pseudomonas* sp., *Bacillus sp.* *B. stereothermophilus*, *Vibrio* sp., *Nocardia* sp., *Corynebacterium* sp., *Achromobacter* sp.
Polycyclic aromatic hydrocarbons	*Arthrobacter* sp, *Bacillus* sp., *Burkholderia cepacia*., *Pseudomonas* sp., *Mycobacterium* sp., *Xanthomonas* sp., *Phanerochaete chrysosporium, Anabena* sp., *Alcaligenes*
Resins	*Pseudomonas* sp., Members of Vibrionaceae., Enterobacteriaceea., *Moraxella* sp.

Approaches to catalogue microbial diversity are broadly divided into culture-dependent and culture independent methods. The culture dependent methods are most familiar and are based on differential morphological, biochemical and physiological traits. These include isolation and cultivation on solid media, most-probable-number liquid assays, and substrate utilization plates. Culture independent methods for community analysis began with direct examination of metabolically active microorganisms with differential stains such as 4', 6'-diamidino-2-phenylindole, and (INT)- formizan. Fluorescent *in situ* hybridization (FISH) technique is the method of choice for identifying a specific group of microorganism in the natural habitat having particular metabolic activity. Chromosomal painting involves generating fluorescent labeled oligonucleotide probes specific for a single gene or set of genes. One can identify the relative abundance of hydrocarbon catabolizing bacteria in a particular habitat. Phylogenetic stains used are fluorescing oligonucleotides complementary to signature sequences in 16S ribosomal RNA and hence used for tracking a particular organism or a group of organisms. With the advent in molecular genetic techniques, PCR- based approaches have emerged and are becoming extremely common to study specific microorganisms or groups of microorganisms and specific genes and to evaluate over all community profiles.

Screening and isolation of hydrocarbon degrading microorganisms

A number of techniques have been evolved for screening of hydrocarbon degrading bacteria including the use of liquid medium with hydrocarbons, oil containing mineral agar plates, measurement of turbidity in microtiter plates, O_2 consumption, the most probable number technique and sheen screen technique. However, all these methods are either time consuming, laborious, expensive or not reliable. A rapid, simple and reliable technique is developed based on the fact that hydrocarbons being highly reduced substrates require an electron acceptor for initial oxidation step. A screening technique using redox indicator, 2, 6-dichlorophenol indophenol (2, 6-DCPIP) has been developed to isolate and assess the potential of the various isolates to degrade crude oil. The rate and extent of color change of 2, 6-DCPIP from blue (oxidized) to colorless (reduced) is indicative of hydrocarbonoclastic activity of an isolate.

Factors affecting hydrocarbon degradation

Abiotic factors

Structure and physical state: The susceptibility of hydrocarbons to biodegradation is determined by the structure and molecular weight of the hydrocarbon molecule. Aliphatic hydrocarbons are degraded and assimilated by a wide range of microorganisms. n-alkanes of intermediate chain length (C_{10}-C_{24}) are degraded most rapidly. Short chain alkanes (less than C9) are toxic to many microorganisms but being volatile generally is lost rapidly in the atmosphere. Higher chain length alkanes are generally resistance to biodegradation. Branching in general reduces the rate of biodegradation. Aromatics may be partly oxidized but are assimilated by only a few bacteria. Aromatic compounds, especially polyaromatic hydrocarbons (PAHs) are degraded slowly. Alicyclic compounds can be degraded via a process known as co-metabolism.

The bioavailability of hydrocarbons which is largely a function of concentration and physical state, hydrophobicity, sorption onto soil particles, volatilization and solubility of hydrocarbons greatly affects the extent of biodegradation.

Oxygen: Hydrocarbons being highly reduced substrates, require an electron acceptor, with molecular oxygen being most common. Though most studies have shown biodegradation of hydrocarbon to be an aerobic process, anaerobic biodegradation of hydrocarbons has also been reported. In the absence of molecular oxygen, nitrate, iron, bicarbonate, nitrous oxide and sulfate, have been shown to act as an alternate electron acceptor during hydrocarbon degradation.

Temperature and pH: Temperature also plays an important role in the biodegradation process since it influences both, the substrate (physical and chemical composition) and the degrading organism. Biodegradation of hydrocarbon has been shown to occur over a wide range of temperature from 0°C to as high as 70° C, though in general optimum degradation occurs in the mesophilic temperature range.

pH is not of much significance in marine environments since it is well buffered at about pH 8.5, but soil pH varies widely and pH of 7 to 8 has been found to support optimum degradation.

Biotic factors

The type of microorganisms, their genetic make up and their prior exposure to hydrocarbons determines the efficacy with which the hydrocarbons can be degraded. Uptake and degradation of hydrocarbons is enhanced if they are in a form that is easily accessible to the degrading microorganisms. Emulsification aids in the true dissolution of hydrocarbons in water and the formation of emulsions through the production of surface active agents (biosurfactants / bioemulsifiers) has been shown to play significant role in hydrocarbon uptake by bacteria and fungi. The cell surface hydrophobicity has also been shown to contribute to the uptake and degradation of hydrocarbons by facilitating hydrophobic interactions between cell surface and hydrocarbon substrate.

The hydrocarbon oxidizing potential of microorganisms was shown to increase, if they had prior exposure to hydrocarbons and an increase in hydrocarbon degrading population was noted at the contaminated sites. The increase in degradation capability due to changes in the genetic make up is attributed to the bacterial plasmids encoding catabolic genes. This is called adaptation which favors an abundance of hydrocarbon degrading bacteria at hydrocarbon contaminated environment than from unpolluted sites. The involvement of plasmids in *Pseudomonas* sp. in hydrocarbon degradation has been widely reported. Number of plasmids that have also been reported, characterized and shown to encode pathways for degradation of hydrocarbons are listed in Table 2.

Table 2: Natural catabolic plasmids

Plasmid	Host	Substrate
CAM	*Pseudomonas putida*	Camphor
SAL	*Pseudomonas* sp.	Salicylate
NAH	*P. putida*	Naphthalene
OCT	*P. oleovorans*	Octane
TOL	*P. putida*	Toluene, m/p-Xylene
pAC25	*P. putida*	p-Cresol
pJP1	*Alcaligenes paradoxus*	2,4-D, Halopesticides

Hydrocarbon degradation

There are two principle routes by which complex mixtures of hydrocarbons are degraded in nature.

Non-biological

Many of the hydrocarbons undergo auto-oxidation by free-radical formation during exposure to UV light (< 400 nm), heat or metal ions. Free radical chain reactions lead to the formation of hydro peroxides that are most unstable and hence rapidly oxidized subsequently (Fig.1).

Initiation	$RH \rightarrow R^{\bullet} + (H^{\bullet})$
Propagation	$R^{\bullet} + O_2 \rightarrow RO^{\bullet}_2$
	$RO^{\bullet}_2 + RH \rightarrow RO_2H + R^{\bullet}$
Termination	$R^{\bullet} + R^{\bullet} \rightarrow RH + R\ (-H)$
	$RO^{\bullet}_2 + {}^{\bullet}OH \rightarrow ROH + O_2$
	$R^{\bullet} + R^{\bullet} \rightarrow R - R$

Fig. 1: Free radical mechanism of hydrocarbon decay

At ambient temperature, the tertiary –CH groups in alkanes are oxidized more easily than the primary groups. Alkenes are preferentially degraded over alkanes except at double bonds. Many of the phenolic and some of the heterocyclic components of petroleum inhibit the rate of decay whereas some of the metal ions and organ metallic compounds stimulate it.

Non-biological decay of many of the pesticides mediated by the inorganic soil fraction is well documented. Carbonates and sulfides of iron, manganese and cobalt are capable of catalyzing oxidation and reductions, whereas cupric ions are responsible for the hydrolysis of organophosphates. In addition, acidic and basic sites on clay particles can catalyze the isomerization of endrin and hydrolysis of atrazine and DDT.

Microbiological

There is a broad spectrum of distribution of microorganisms that have ability to degrade or transform hydrocarbons in nature.

Aliphatic hydrocarbons are preferentially utilized by many bacteria than aromatics. The short chain alkanes (i.e. <C9) are thought to be poor substrates because of their toxic effects on microbes exerted at the level of cell membrane integrity. Saturated hydrocarbon are more readily degraded than unsaturated ones. Also, straight chain hydrocarbons are preferentially degraded over the branched chain ones. Alicyclic hydrocarbon are least susceptible to microbial decay and many are quite recalcitrant.

Metabolic pathways for degradation of Alkanes, Alkenes and Alkynes

Methane

Large quantities of methane are continuously generated in the anoxic environments due to anaerobic decomposition by methanogenic archea. It is extensively found in nature in the anoxic environments such as muds, marshes, lake sediments, the rumen and the mammalian intestinal tracts etc. It is also present in coal formations. Variety of bacteria with diverse morphologies are capable of utilizing methane and few other most oxidized one carbon

compounds such as methanol, formic acid, formamide, and methylamine as electron donors for energy generation and as sole source of carbon. These bacteria are aerobes and are widespread in soil and water. They are called methanotrophs e.g. *Methylomonas, Methylobacter, Methylococcus* etc.

There are number of other one carbon compounds known to be utilized by microorganisms. Organisms that can grow using only one carbon organic compound are generally called methylotrophs. Many but not all methylotrophs are also methanotrophic. Examples of methylothrophs include *Hypomicrobium sp. Pseudomonas sp. Bacillus sp vibrio sp.* etc. Generally methylotrophs can be compared with autotrophs in that both groups of organism can utilize compounds with no carbon-carbon bonds, the difference being is that the former utilizes organic one carbon compound while the later utilizes inorganic one carbon compounds.

The biochemistry of aerobic methane oxidation by bacteria is summarized in fig 2 .^{18}O-incorporation experiments suggested involvement of an oxygenase. A methane monooxygenase enzyme catalyzing the oxidation of methane to methanol was first reported in *Methylococcus* sp., *Pseudomonas* sp. and *Methylosinus* sp.

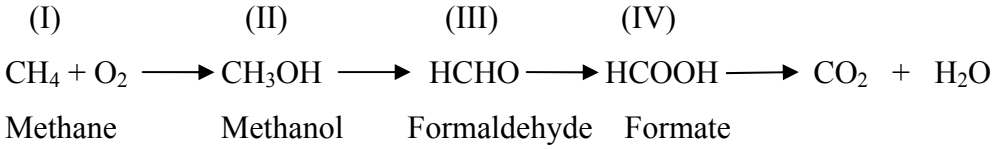

Fig. 2: The oxidation of methane by methanotrophic bacteria
I-Methane mono-oxygenase, II- Methanol dehydrogenase, III- Formaldehyde dehydrogeanse, IV- Formate dehydrogenase

The need of O_2 for initiating the oxidation makes it obligatory for all methanotrophs to be aerobes. Further studies showed mono-oxygenases to be NADH linked and analogous to higher alkane mono-oxygenases. The enzyme consists of three components; a copper protein, a CO-binding cytochrome C, and a low molecular weight component. The electrons needed to drive this step come from cytochrome C and hence no ATP synthesis occurs during oxidation of methane to methanol. This lack of ATP synthesis is consistent with the fact that growth yield (grams of cells produced per mole of substrate consumed) of methanotrophs are same whether methane or methanol is used as the growth substrate. The cytochrome also accepts electrons arising from further oxidation of methanol, formaldehyde and possibly formate. Methanol dehydrogenase enzyme oxidizing methanol to formaldehyde has a pteridine prosthetic group and it is thought that electrons derived from further oxidation of methanol supply the reducing power for mono-oxygenases by a recycling system. There is evidence that ATP is generated during this process and the system represents a mechanism to avoid the major loss of potential metabolic energy in the substrate resulting from the involvement of a conventional wasteful NADH linked mono-oxygense.

The ability of methane utilizers to co-oxidize higher hydrocarbons whilst growing on methane is note worthy. This is attributed to the wide substrate specificities of the methane mono-oxygense enzyme. Methane monooxygenase can oxygenate C_1-C_8 n- alkanes to the corresponding 1- and 2- alcohols, terminal alkanes to 1,2-epoxides, carbon monoxide to carbon dioxide and some cyclic alkanes to aromatic compounds. It could also co-metabolize highly chlorinated solvents such as trichloroethylene (TCE). The application of methanogens

for co-metabolic degradation of TCE is a strategy under development for bioremediation of ground water contaminated with TCE.

Methanotrophs unique among prokaryotes in possessing relatively large amounts of sterols, which are absent in most prokaryotes. A symbiotic association between methanotrophic bacteria and marine mussels and certain types of marine sponges are well established. Mussels live in the vicinity of hydrocarbon seeps where there is high prevalence of methane. Presence of methanotrophs as symbionts was established by detecting the stacks of intra-cytoplasmic membranes specific of type I methylotrophs. Methanotrophs are classified into two types on the basis of mechanism for C1 assimilation. Type I methanotrophs have ribulose monophosphate pathway and Type II have serine pathway.

Alkanes

Variety of methanotrophs have been reported to have methane mono-oxygenase and methanol dehydrogenase with relaxed substrate specificities, which confer upon them the ability to co-oxidize C_1-C_8 n-alkanes to their corresponding 1- and 2- alcohols. In addition to methanotrophs, several ethane utilizing bacteria, moulds and yeasts have been isolated e.g. *Acremonium* spp. oxidizes ethane to ethanol by NADPH dependent monooxygenase, which is subsequently oxidized to acetaldehyde and acetic acid. Acetate thus formed is assimilated into cellular carbon via reverse tricarboxylic acid cycle and glyoxalate bypass. Similarly a number of propane and butane utilizers have been reported that are also capable of growth on long chain alkanes such as n-dodecane and n-hexadecane.

The biochemical pathway of higher alkane metabolism can broadly be categorized into three basic routes on the basis of initial attack on the alkane molecule (Fig. 3).

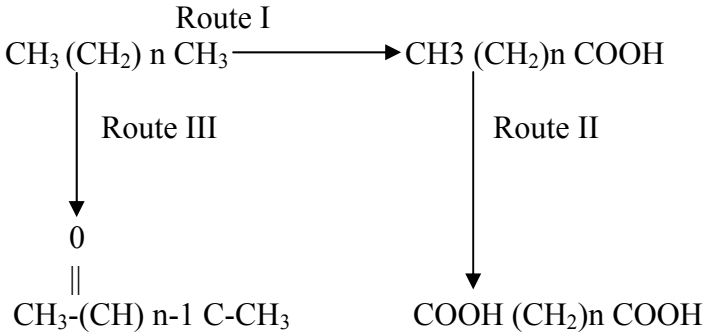

Fig. 3: Initial enzyme attack on alkanes

Route I involves the oxidation of one of the terminal methyl groups to carboxylic acid via primary alcohol and aldehyde. The second route (II) brings about the oxidation of both ends of the molecule to form an α, ω-dicarboxylic acid. Occasionally, type III route leads to the formation of ketone due to sub-terminal oxidation.

Of the three routes of alkane degradation by microorganisms, route I is most common. Enzymes involved in the initial terminal oxidation have been well characterized from *Pseudomonas oleovorans* and *Corynebacterium* species, former bacterium is carrying a Rubredoxin bearing mono-oxygenases whereas the later one is having cytochrome P-450 mono-oxygenases. The alcohol formed is subsequently oxidized to fatty acid by the action of two NAD (NADP) linked dehydrogenases. The fatty acid so produced is further metabolized to acetate by conventional β-oxidation.

Alkenes and Alkynes

Relative to alkane degradation, there are fewer studies that have been reported for microbial utilization of alkenes. *Candida lipolytica* has been found to produce monooxygenase that form epoxide from alkenes with double bond at 1,2 position, which is further oxidized to a diol. There are also reports of water addition across the double bond and yet in other cases the saturated end of the molecule is attacked first. The initial attack in most cases being at both ends of the molecule. Variety of products like 1,2-epoxide,1,2-diol, ω- unsaturated fatty acid, ω -unsaturated primary and secondary alcohols and 2-hydroxy- acid are reported to be formed depending up on the type of attack. Different ways in which alkenes are degraded is summarized in Fig. 4.

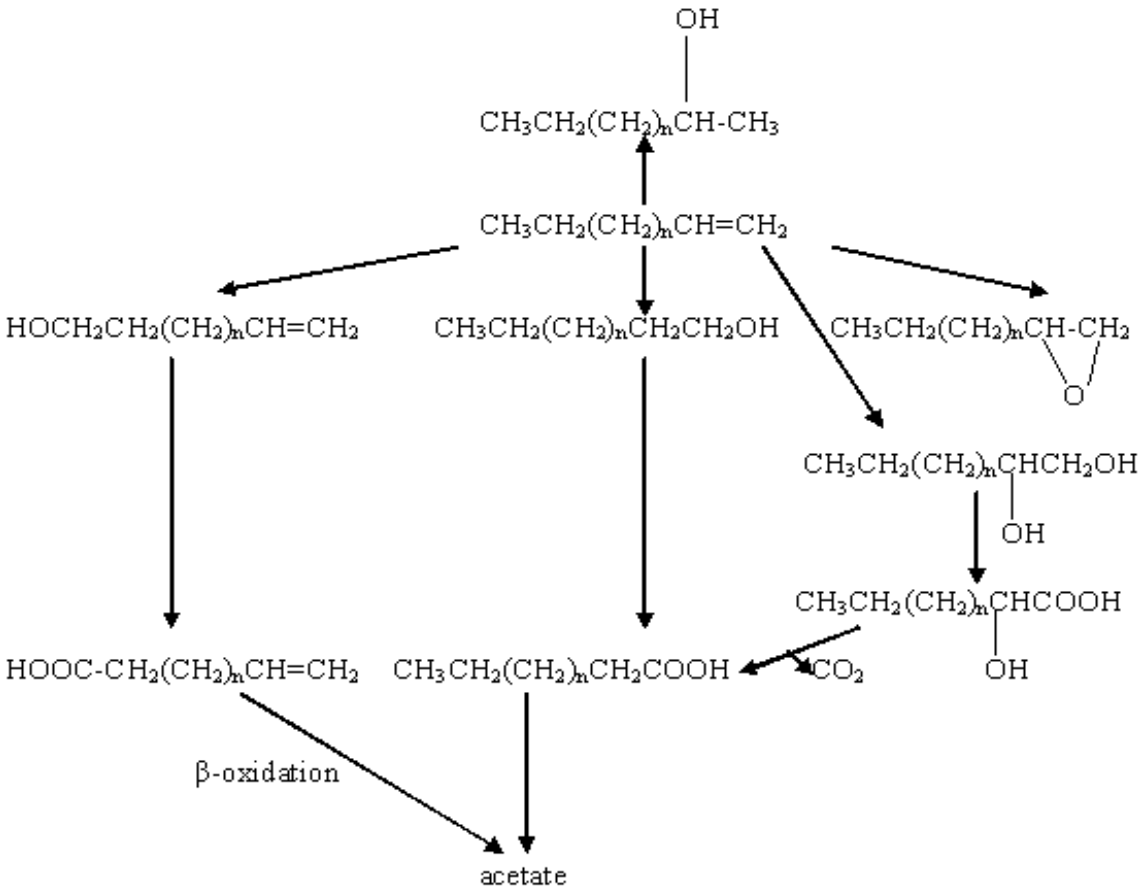

Fig. 4: Diversity of initial microbial oxidative metabolism of alkenes

Microbial degradation of gaseous alkenes and alkynes is observed in very few bacteria. *Corynebacterium* species having a broad substrate specificity could grow on most alkenes from C_3-C_{18} and several alk-1-enes, but failed to grow on ethylene, propylene or but-2-ene. Microbes having nitrogenase enzyme acquires the ability to reduce acetylene to ethylene, which can be co-metabolized in soil by some ethane and methane-utilizers.

Branched Chain Alkanes and Alkenes

Relatively very few reports are available on the branched chain hydrocarbon metabolism since most of the hydrocarbon degrading microorganisms do not have ability to degrade these compounds. *Brevibacterium erythrogenes* can use 2-methylundecane as substrate for growth by a combination of ω- and β-oxidation. *Arthrobacter* sp. has been reported to metabolize

squalene (C_{30}-multiple, methyl branched compound) to geranylacetone, which is accumulated in the medium as the organism cannot further metabolize this product. *Corynebacterium* sp. and *B. erythrogenes* have been shown to degrade pristane (2,6,10,14-tetramethyl pentadecane) involving ω-oxidation followed by β-oxidation yielding propionyl CoA and acetyl-CoA units alternately. Most hydrocarbon degrading microorganisms do not have the ability to degrade branched chain compounds since most of the enzymes involved in β-oxidation do not act on them.

Biodegradation of aromatic hydrocarbons

The benzene ring is the most abundant unit of chemical structure in the biosphere, next only to glucose and hence microbial infallibility in attacking this ring greatly facilitates the continuous operation of carbon-cycle in nature. Except for metabolism of phenylalanine, tyrosine and tryptophan, the ability of animals to degrade aromatic compounds is extremely restricted. The relative metabolic inertness of the benzene nucleus is due to its stable resonance structure. Microbial ingenuity of investing their resources for reducing the resonance energy barrier of the nucleus leads to fission, and this investment is repaid by subsequent reactions that release energy.

Two general reasons have been given for initiating the degradation of aromatic compounds by microbes: either as a source of nutrient for growth or because of the relaxed specificities of enzymes that recognizes structural features of a chemical resembling the natural substrates. *Cunninghamella elegans*, a filamentous fungus is reported to oxidize, benzopyrene, a carcinogen to a complex mixture of polar products as a means of its detoxication.
Under anaerobic conditions microorganisms overcome the resonance barrier due to conjugation of unsaturated bonds by reduction, whereas aerobic microorganisms make direct use of triplet oxygen to hydroxylate the benzene ring and so facilitate its fission by further reaction with molecular oxygen.

Anaerobic Fission

The anaerobic degradation of the benzene nucleus of aromatic compounds is initiated by reduction of benzoic acid (1) to cyclohexane carboxylic acid (2) (Fig. 5). This intermediate is metabolized by three different routes The sequence a) is used by certain denitrifiers that transfer hydrogen atoms to nitrate ions, the sequence b) by photosynthetic *Pseudomonas* sp which obtains cellular carbon by a process resembling β-oxidation of fatty acids and sequence c) by anaerobes that form short chain fatty acids which by accepting hydrogen atoms are converted to methane by methanotrophic bacteria present in the consortium. Compounds (3), (4), and (5) shown in the figure are formed as intermediate by organisms in the process of using nitrate as terminal electron acceptor. These intermediates ultimately give rise to free fatty acids (pathway c).

Anaerobic processes make an important contribution to carbon cycle in nature. It was observed that sulfate mediated respiration of organic matter in a salt marsh was twelve times higher than that mediated with oxygen. In addition to benzoic acid and phenol, the lignoaromatic compounds ferulic acid, vanillin, cinnamic acid, protocatechuic acid and catechol can be anaerobically transformed by soil microbial consortia into methane.

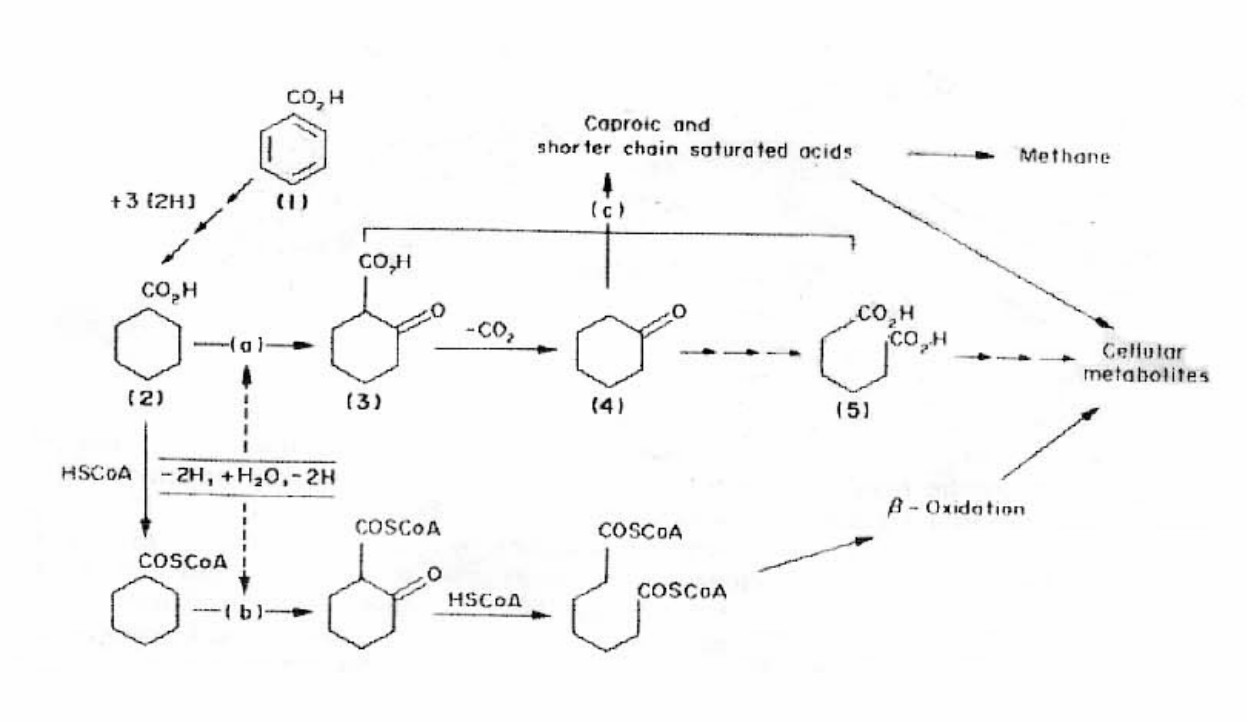

Fig. 5: Anaerobic degradation of benzoic acid

Aerobic Fission

Since all aromatic hydrocarbons that occur in petroleum are derivatives of benzene, initial catabolic reaction of aromatic substrates is cleavage of benzene ring. Molecular oxygen serves a reactant in the pathway and both atoms from molecular oxygen get incorporated into the substrate. The enzyme called dioxygenase catalyze this reaction leading to the formation of benzene dihydrodiol. It is three component dioxygenase comprised of two non-haem iron proteins and an FAD- containing protein. The oxidation of benzene leads to catechol as depicted in Fig.6.

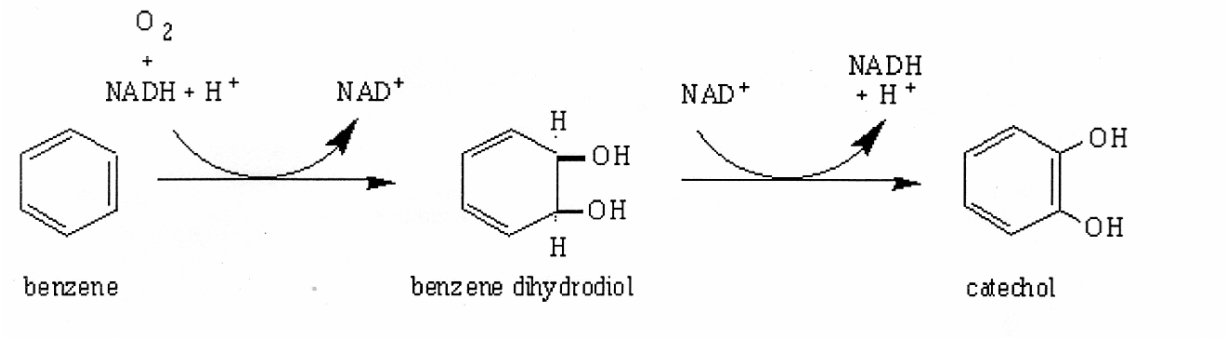

Fig. 6: Oxidation of benzene to catechol

Ring cleavage and subsequent bacterial metabolism of benzene ring requires that the aromatic ring be destabilized and made more reactive. Aerobic fission of the benzene ring can occur at different position as shown in Fig.7 where the dotted lines show the main points of cleavage when the ring opens and O_2 is fixed in the reaction product. There is no all purpose

dioxygenase present. One microorganism can open the benzene ring in several of the ways as shown in the figure using distinct dioxygenases for each occasion. The main substrates for these enzymes are: catechol (1) hydroxyquinol (2) protocatechuic acid (3) gentisic acid (4) homoprotocatechuic acid (5) homogentisic acid (6) Oxygen is incorporated into each substrate by (a) protocatehuate 4,5-dioxygenase (b) protocatehuate 3,4 dioxygenase.

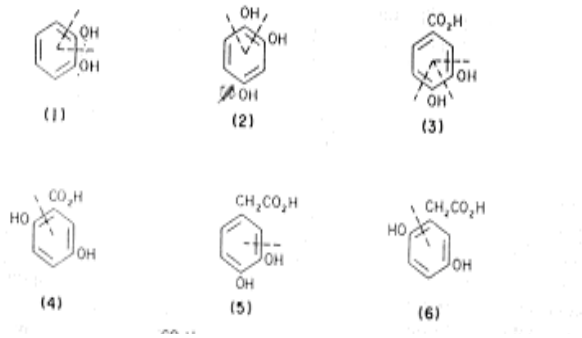

Fig. 7: Dioxygenases catalyzed fissions of the benzene nucleus

The product of initial metabolism of aromatic hydrocarbons are commonly catechol or substituted catechol which can subsequently be metabolized by either of the two pathways – the ortho-cleavage pathway (Fig.8), in which an aromatic ring is cleaved between two carbon atoms bearing hydroxyl groups or the meta cleavage pathway (Fig.9), in which the ring is split between a hydroxylated carbon atom and an adjacent unsubstituted carbon atom. Though the enzymes involved in their catabolism are different, both pathways lead to generation of TCA cycle intermediates (acetate and succinate) or to products that can be easily transformed to TCA cycle intermediates (pyruvate and acetaldehyde).

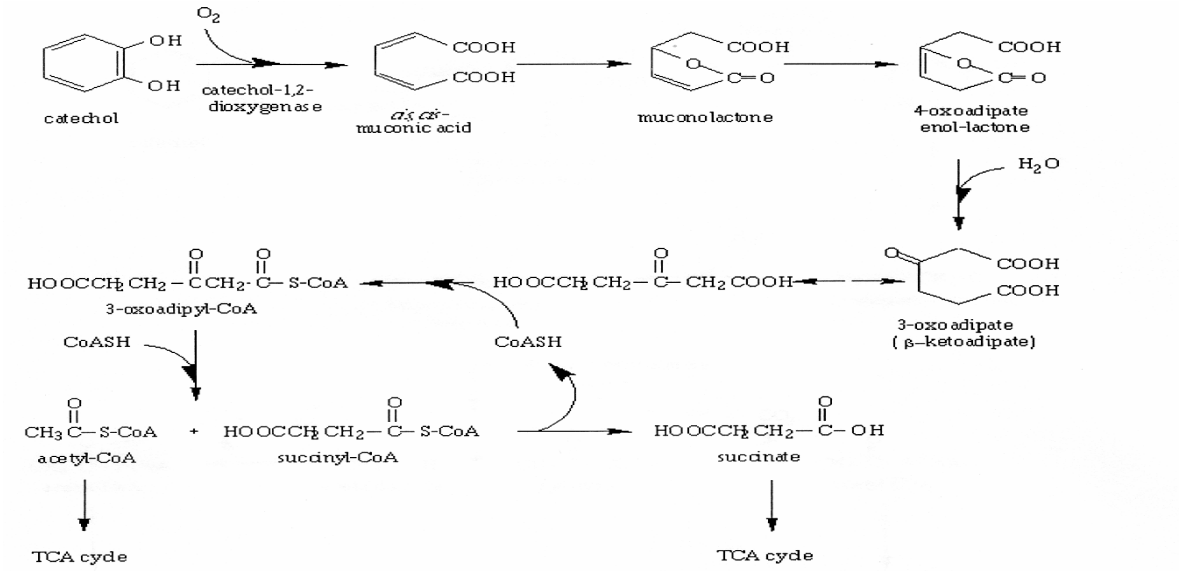

Fig. 8: Ortho-cleavage pathway for catabolism of catechol

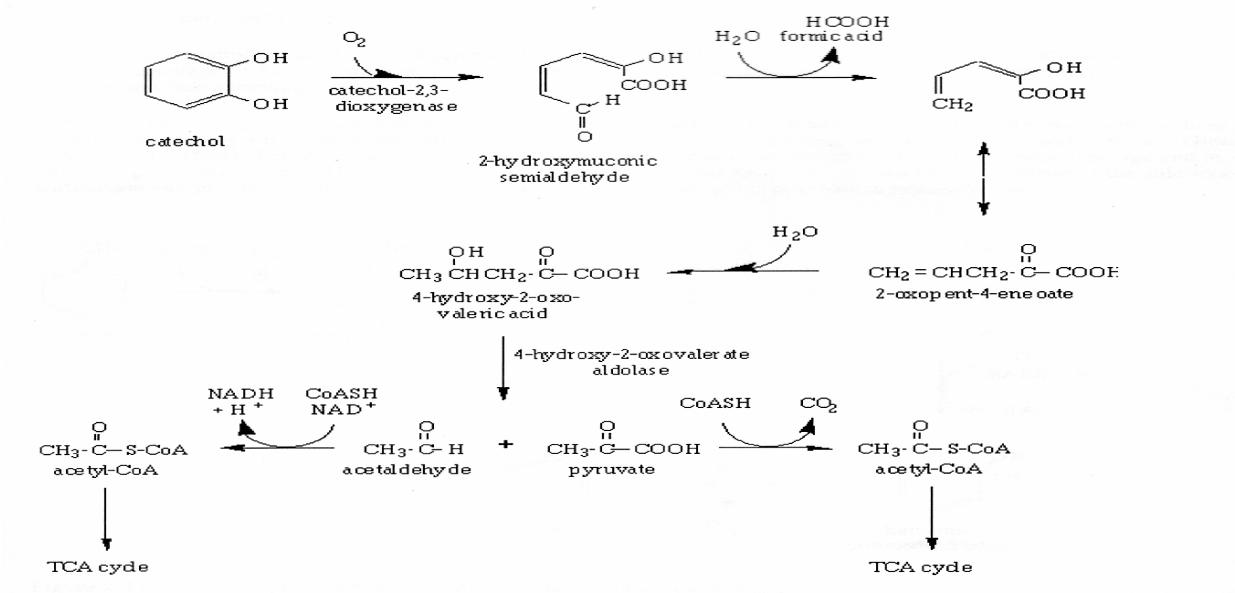

Fig. 9: Meta-cleavage pathway for catabolism of catechol

Biodegradation of halogenated hydrocarbons

Many environmentally important xenobiotics introduced for industrial use are halogenated, and halogenation often is responsible for recalcitrant nature of these compounds. Halogenated organic compounds are used as herbicides plastics, solvents, degreasers, pesticides, and numerous industrial solvents. Chlorinated compounds serve as indicators for the basis for the biotransformation of xenobiotics compounds.

The chlorinated hydrocarbons degraded by microorganisms are grouped into three classes:
i) aliphatic, ii) polycyclic and iii) aromatic.

i) Halogenated aliphatic hydrocarbons: These groups of compounds are prevalent ground water contaminants and form main components of hazardous waste and landfill leachates. Main amongst these are halogenated alkanoic acid, trichloroethane, ethylene dibromide. Chlorinated ethanes and ethers are commonly used refrigerants in manufacturing as solvents in dry-cleaning and semiconductor manufacturing.

Soil microorganisms like *Pseudomonas* sp. and *Alcaligenes* sp. utilizing halogenated alkanoic acid produce an enzyme dehalogenase which removes the halogen moiety from the substrate making it amenable to further degradation. The enzyme was found to be encoded on a plasmid pUU204.

The metabolism of trichloroethylene is relatively well worked out as this compound is frequently detected in drinking water aquifer and presence of these compound in drinking water is of public concern because of their toxicity and carcinogenicity. Under anaerobic conditions reductive dechlorination of TCE to 1,2-dichloroethyleneis well demonstrated. TCE can be metabolized under aerobic conditions as well. TCE was mineralized when soil micro flora were exposed to natural gas in air, implicating involvement of methanotrophs in the process. Another aerobic microorganism degraded TCE in the presence of phenol. A

toluene dioxygenase has been reported to involve in this process. Thus TCE degradation by these microorganisms occurs by a co-metabolic process.

ii) Chlorinated polycyclic compounds: There are several report on the biodegradation of chlorinated polycyclic hydrocarbons. Of these DDT, polychlorinated biphenyls (PCBs) and p-chlorobiphenyls (p-CBs) are of great interest because of their wide spread occurrence in the environment. Species of *Acinetobacter, Alcaligenes, Achromobacter, Klebsiella* have been shown to degrade p-CB to 4-chlorobanzoate (4CBA). The enzyme necessary for this degradation is shown to be plasmid encoded. Since 4CBA is major product of p-CB degradation, more rapid and complete degradation of p-CB has been shown to occur in the mixed culture where another strain degraded 4CBA.

PCBs with higher chlorination tend to be more environmentally persistent. Both aerobic and facultative anaerobic bacteria capable of utilizing PCBs have been isolated from the environment. *Pseudomonas cruciviae* could grow on more than 10 biphenyl related compounds and it was demonstrated that biphenyl ether was degraded through ortho cleavage pathway and that biphenyl was degraded through meta cleavage pathway. The degradation of PCB occurs through ring cleavage and dehalogenation and can take place both under aerobic and anaerobic conditions.

iii) Chlorinated aromatic compounds: Chlorinated aromatic compounds are major environmental pollutants because they are often released in substantial quantities, toxic and resistant to degradation therefore persists in nature. Many soil and aquatic microorganisms can metabolize compounds such as chlorobenzoates, chlorobenzenes, chlorophenol, chlorotoluene etc.

The chlorinated phenols used as wood preservatives, herbicides and general biocides are a large group of toxic xenobiotics that are serious environmental pollutants. The toxicity of chlorinated phenol tends to increase with the increase in their degree of chlorination. Microorganisms such as *Arthrobacter* sp. *Pseudomonas cepacia, Flavobacterium* sp. can degrade some of these chlorinated phenols. The degradation of pentachlophenol (PCP) by *Flavobacterium* sp. leads its conversion to tetrachloro-p-hydroquinone and then to trichloroquinone and dichlorohydroquinone. Under anaerobic conditions however, PCP is degraded into tri-,di-, and monochlorophenol. Reductive dechlorination of PCP has been observed in flooded soils and in anaerobic sewage sludges

The extensive use of chlorobenzoates as solvents, fumigants, and intermediates in the production of dyes and pesticides has lead to their widespread release into the environment. *Alcaligens* sp. and *Pseudomonas* sp. are implicated in the degradation of chlorobenzoates. The metabolic pathways for the biodegradation of o-dichlorobenzoate (DCB), m-DCB and p-DCB proposes that they all form a common intermediate, dichlorocatechol, and then the benzene ring is broken. Dechlorination of chlorobenzene has been shown to occur under anaerobic conditions as well.

Biodegradation of lignin compounds

The complex biopolymer, lignin contains numerous benzoid residues that carry methoxyl group substituents. Methoxylated aromatic acids such as ferulic, vanillic and syringic acids are produced by several lignin degrading fungi.

These compounds are efficiently utilized by several soil bacteria as growth substrate that releases formaldehyde as one of the by product. Strains of *Pseudomonas putida* capable of utilizing 3,4,5-trimethoxy cinnamic acids have been isolated. Methyl group of any methoxyl group is bound to the benzene ring by an ether link that is resistant to hydrolysis, however *Ps. putida* capable of utilizing it as growth substrate transforms it into an intermediate that have methoxyl group with an ester grouping, thereby facilitating the release of methanol by hydrolysis, which is not utilized by *Ps. putida*. Methanol released this way provide opportunity for methanotrophs to establish.

Ortho-fission pathways is the major route employed by *Trichosporon cutaneum* for the utilization of aromatic compounds. It is the only well characterized yeast that reflects the preference of eukaryotes for ortho-fission over meta-fission of benzene nucleus of aromatics.

Biodegradation of Polycyclic Aromatic Hydrocarbons

Polycyclic aromatic hydrocarbons (PAHs) constitute a large and diverse class of organic compounds consisting of three or more fused aromatic rings in various structural configurations. Their abundance in environment is primarily attributed to emissions from combustion processes, tar oil from coal gassification, vehicle emissions, heating and power-plants, industrial processes and open burning. They have been detected in air, soil, sediments surface water, ground water and road runoff.

Their persistence in the environment is primarily due to the hydrophobicity and molecular stability that increases with the increase in PAH molecular weight. Because of their lipophilic nature, they have a high potential for biomagnification through trophic transfers.

Microbial biodegradation involving bacterial and fungal isolates have been reported both aerobically and anaerobically of virtually all PAHs. The initial catabolic step in aerobic oxidation of a PAH molecule occurs via formation of a dihydrodiol by a multicomponent dioxygenase enzyme system, which is further metabolized by either an ortho- or a meta-pathway, leading to intermediates such as protocatechuates and catechols.

However, many *Mycobacterium* spp. are known to degrade PAHs by mono-oxygenase attack. Several nonlignolytic fungi metabolize PAHs via cytochrome P450 mono-oxygenase forming an arene oxide, trans-dihydrodiols, phenols, tetralones, quinines, dihydrodiol epoxides and various conjugates of hydroxylated intermediates, but a few have the ability to completely mineralize PAHs to CO_2.

Microbial processes explored for

Production of single cell protein from hydrocarbons

Microbial cells are generally produced for two main applications. 1) As a source for animal and human food supplement represented by single cell protein, SCP and 2) for use as commercial inoculants in food fermentations and for agricultural and waste treatment.

As a commodity, SCP must be competitive with animal and plant proteins, in terms of processes and nutritional value and must conform to human and animal food safety requirements.

The later part of the sixties saw the rise and fall of single cell protein production from oil and natural gas. Companies like BP and ICI entered into SCP production mainly to produce at low cost, high value SCP from petroleum, for addition to animal feed and to replace protein additives such as soybean meal. A large market for SCP was forecast as the population in the third world countries continued to increase despite a considerable shortfall in food supply. However the developments of SCP died in its infancy largely due to the sharp rise in the price of oil which made it economically non-viable. However some of the findings obtained with SCP production world over around sixties and seventies are summarized as under.

The development in those years of microbial protein from hydrocarbon was considered as a solution to the world food problem. This concept was appreciated then, based on the fact that a diversion of a mere 15-20% of the world's production of petroleum was expected to meet world's entire protein requirement.

In India the petroleum crude showed a 10 to 12 % wax content. These waxes had no ready market. The efforts therefore were concentrated in converting these to the much needed protein concentrates. Research was initiated in India and world over towards large scale production of SCP from petroleum hydrocarbons and further testing the nutritive value of the product for use as feed supplement for animal and human food.

Pseudomonas spp. were shown to assimilate n-paraffin fractions of crude oil and accumulated high content of protein. The protein was found to contain all essential amino acids and the product was tasteless and odorless so as to be acceptable as human food additive.

Some yeast strains like *Candida utilis* and *Candida lypolytica* were also explored for SCP production. The original alkane SCP fermentation process developed by British Petroleum (BP) in France used 10-20% wax contained in gas oil. Substrate costs were very low, however due to their crude nature exhaustive processing was required to recover the yeast free of a gas-oil flavor taint.

Other alkane based SCP processes were developed in Italy, Japan and Romania but many of them suffered from the problems of potential carcinogenic residues and most of the plants never ran on full capacity or had to be closed.

The factors which contributed to the failure of hydrocarbon SCP to make a major commercial impact was dramatic increase in oil prices in 1973. When one considers that crude oil prices increased by a factor of six in 1973 and that the cost of substrate for SCP processes represents 40-60% of the total manufacturing costs, the negative impact on hydrocarbon based SCP processes were evident.

Microbial processes for Recovering and Upgrading Petroleum

Microbial Enhanced Oil Recovery (MEOR)

This technique makes use of the ability of microorganisms either indigenous or injected to produce useful products such as gases, biosurfactants, polymers etc. to improve oil recovery. This technology requires consideration of the physicochemical properties of the reservoir in terms of salinity, pH, temperature, pressure and nutrient availability. Only bacteria amongst other microorganisms are considered as promising candidates for MEOR. Many petroleum reservoirs have high NaCl concentrations and bacteria capable of growing up to 8% NaCl and producing biosurfactant and polymers are potential candidates for MEOR.

Bacteria, producing a variety of fermentation products, e.g. carbon dioxide, methane, hydrogen, biosurfactants, and polysaccharides from crude oil, pure hydrocarbons and a variety of non- hydrocarbon substrates are most suitable candidates for the process. Xanthan gum, a microbial biopolymer is frequently used in microbial enhanced oil recovery field testing. Desirable properties of polymers for MEOR include shear stability, high solution viscosity, compatibility with reservoir brine, stable viscosity over a wide range of pH, temperature, and pressure and resistance to biodegradation in the reservoir environment. Organic acids produced through fermentation readily dissolve carbonates and can greatly enhance permeability in limestone reservoirs and attempts have been made to promote their anaerobic production. Organic solvents and dissolved carbon dioxide can decrease oil viscosity. Fermentation gases can depressurize wells leading to displacement of oil through a revitalized gas-driven mechanism.

Residual oil in reservoirs can be recovered when highly permeable watered-out regions of oil reservoirs are plugged with bacterial cells and biopolymers. Bacteria and nutrients are injected into the reservoir and the system is shut in to allow the biomass to plug the more permeable region as it grows. Water is then flooded to force oil trapped in less permeable regions of the reservoir to go into the recovery wells. Added or in-situ produced biosurfactants aid in emulsification and detachment of films from rocks and thus has considerable potential in MEOR. Biosurfactants from thermo-and halo- tolerant species, respectively those of *Bacillus licheniformis* and *Bacillus subtilis* strains have been tested for with various levels of success in reservoirs and in laboratory simulations. More than 400 microbial enhanced oil recovery field tests have been conducted in United States alone. Reservoir heterogeneity significantly affects oil recovery efficiency. Despite numerous MEOR tests considerable uncertainty remain regarding process performance. Ensuring success requires an ability to manipulate environmental condition to promote growth and / or product formation by participating microorganisms. In addition conditions vary from reservoir to reservoir which calls for reservoir specific customization of MEOR process and this can undermine microbial process economic viability.

Microbial Deemulsification

Oilfield water-in-oil emulsions, formed at various stages of exploration, production and oil recovery represent a major problem for petroleum industry. These emulsions are characterized according to their stability as tight or loose. To produce saleable oil, petroleum water-in-oil emulsions must be destabilized by costly physical and/or chemical methods.
Microbial species like *Nocardia* sp., *Corynebacterium* sp., *Rhodococcus* sp., *Micrococcus* sp., *Pseudomonas* sp, etc. are known to exhibit deemulsification capabilities. The microbial deemulsifying activity has generally been observed in water-in-oil emulsions and rate varies with differences in emulsion composition. Generally physicochemical deemulsification processes are capital intensive, and emulsions often generated at the well-head have to be transported to central processing facilities. Since microbial emulsifier can exert their effect at varied conditions of extremity, an effective microbial deemulsifier could be used directly to treat emulsions at the well head, thus saving on transport and high capital equipment costs. However due to great variability among the properties of crude oil emulsions, inconsistencies are experienced in the performance of all deemulsification processes, physical, chemical, and biological.

Microbial desulphurization

Sulphur is third most abundant element in crude oil ranging from 0.5 to 5% going upto 14% in heavier oil. Most of the sulphur in crude oil is organically bound and requires expensive physicochemical method like hydrodesulphurization to get rid of sulphur from crude oil. Developing a low cost biological based alternative without degrading the non-sulphur fuel components would be a good consideration. *Rhodococcus erythropolis* and related species have been shown to remove sulphur from compounds such as dibenzothiophene (DBT). However some of the strains exhibit little activity towards other compounds like thiophenes and benzothiophenes. Microbial processes for desulphurization of crude oil are limited by challenges of having to operate in a two- phase aqueous oil system. Recent research has resulted in a 200- fold increase in expression of key desulphurization genes in best strains. Much remains to be done now towards broadening the substrate specificity of this enzyme.

Microbial decomposition of petroleum and petroleum products in the environment

Environmental impacts from the petroleum industry is mainly contributed from recovery, transport, refining and product usage. Oil spill catastrophes resulting in shoreline contamination also largely contributes to the environmental impact. In various operations of oil production, processing, and storage, large volumes of waste are generated as oily sludge. Hydrocarbons bind strongly to solid surfaces, including soils, and remediation of these materials represent a significant challenge. Because petroleum is a rich source of organic matter and the hydrocarbons within it are readily attacked aerobically by a variety of microorganisms, petroleum or its products in nature when get exposed to air and moisture becomes amenable to microbial attack. The term bioremediation refers to the clean up of oil or other pollutants by microorganisms and in the recent years bioremediation of oil spills in the marine environments have been amply demonstrated. Compared to physicochemical methods, bioremediation offers an effective technology for the treatment of oil pollution because majority components of crude oil are biodegradable and oil-degrading microorganisms are ubiquitous.

Though contaminating oil is primarily degraded by a combination of environmental factors such as photo-oxidation, volatilization, leaching etc. its fate to a very large extent is determined by the indigenous microbial population and biodegradation thus constitutes the major route for the elimination of hydrocarbon pollutant from the environment.

As per the organization of Economic Cooperation and Development, the world wide market potential for bioremediation technology for pollution abatement increased, from $40 billion in 1990 to $ 75 billion in the year 2000. This is attributed to the enormous metabolic diversity of microorganisms to degrade numerous organic compounds.

Petroleum hydrocarbons are widespread environmental pollutants that are amenable to removal by bioremediation. Millions of underground storage tanks have leaked gasoline containing toxic benzene, toluene and xylene (BTX) into soils and groundwater. Major oil spills contaminated coastal marine environments with tons of crude oil. In most of these cases indigenous microorganisms degrade many of the petroleum hydrocarbons but at rates that are too low. This is because populations of hydrocarbon degraders are generally less than 1% of the total microorganisms, which increases to 10% in polluted habitats and also the degradation is limited by the unavailability of nutrients like nitrogen or phosphorous. Mixed cultures having varied metabolic potential are commonly proposed as inocula for seeding to bioremediate contaminated sites. A genetically engineered hydrocarbon degrading

Pseudomonas was the first organism patented in US. although that organism has never been used to treat a contaminated site.

Metabolic routes

Among microorganisms, bacteria play a key role in biodegradation of hazardous hydrocarbon compounds. They attack hazardous organic compounds in one of the following ways:

a) Mineralization: The compound is oxidized in CO_2, water and biomass.
b) Mineralization with co-metabolism: It is a gratuitous metabolic transformation of a substance by microorganism growing on another substrate. The organism derives energy and biomass from the substrate which gets mineralized and not from the co-metabolised substrate which gets transformed to non-toxic form in the process.
c) Bio-transformation: The pollutant is not mineralized but gets transformed to another compound which may or may not be toxic.

Bioremediation technologies

This is an attractive alternative strategy to physical removal and subsequent destruction of pollutant by incineration which is at least ten times costlier to bio-treatment. It can be applied to different types of sites like soil, sludge, ground water, marine habitat etc.

Technology development stages

Three main stages lead to successful development of bioremediation technology.

a) Site characterization: This requires site assessment to know whether bioremediation technology is appropriate for restoration of damaged ecosystem. This mainly involves chemical characterization of the contaminant and physico-chemical analysis of the damaged site.
b) Treatability study: This involves designing and drawing criteria and parameters based on the information obtained from study on kinetics of degradation of the contaminant.
c) Commercialization stage: Treatment approach is finalized. It also includes procurement, fabrication and pre-commissioning of required equipment for operation.

Bioremediation approaches

Two basic forms of bioremediation are currently being practiced:

1. Microbiological approach: Involves augmentation of a contaminated site with one or more species of indigenous specific microorganisms. The rationale is to increase the rate of pollutant degradation because of increased density of contaminant specific microorganisms. Two types of microorganisms can be used to augment the site.

One is use of pre-packaged contaminant specific microorganisms. Numbers of firms are known to market pre-packaged microorganisms having varied capabilities to degrade variety of organic contaminants.

The second method involves selection, culturing and application of site-specific strains exhibiting desirable degradative qualities. This requires isolation of contaminant specific microorganisms from the site, growing it in large quantity in the laboratory and applying it on the site.

Generally a site that is recently contaminated by a high concentration of a pollutant and requires rapid clean up is treated using microbiological approach as mentioned above.

2. The microbial ecology approach: This approach involves use of indigenous contaminant specific microbial community in the affected area. It requires identifying and adjusting certain physical and chemical factors that may be impeding the rate of degradation *in situ*.

A site that has been contaminated for some time and the concentration of contaminant is of moderate level is generally treated using this approach.

Bioremediation Treatment Technologies

Bioremediation treatment technologies have been broadly divided into two categories based on whether biodegradation is stimulated *in situ* or carried out *ex situ.*

1 In situ Bioremediation Techniques: Bioremediation of organic contaminants *in situ* requires stimulation of the degradative activities of endogenous microbial populations by augmenting the site of nutrients and/or external electron acceptors. Alternatively, it may involve the addition of competent, exogenous microbial inocula, with or without nutrient and electron acceptor supplementation.

The requirement of nutrient addition (N, P) has been extensively studied with respect to hydrocarbon contaminated marine environments and soil / aquifer system. Serious accidental oil spills in the marine environment have provided the impetus for large scale bioremediation experiments involving addition of nutrient cocktails to marine and foreshore environment. This approach has been practiced with varying degrees of success. In many soil bioremediation applications, nutrient addition has been combined with percolation of oxygen saturated water, air sparging or bioventing. Hydrogen peroxide has also been widely used as an alternative oxygen source in percolation trials

Bioventing is a technique used to add oxygen directly to a site of contamination in the Vadoa zone (unsaturated zone). To initiate bioventing, vacuum is applied on to the well to force accelerated air movement through the contamination zone. The main objective of bioventing is to stimulate *in situ* aerobic degradation. In cases like gasoline spills, due to forced aeration pollutant volatility becomes an issue. However, the air contaminated with volatile components can be treated biologically by passing the air through above ground soil beds in a process called biofilteration.

Anaerobic degradation processes may offer an effective alternative to aerobic *in situ* bioremediation of chlorinated compounds. Chlorinated aliphatic and aromatic compounds are some of the most troublesome groundwater contaminants. These compounds are toxic and low concentrations, carcinogenic and tend to resist aerobic degradation. They are reported to get transformed anaerobically by a process called reductive dehalogenation. The chlorinated compounds acts as the electron acceptor and in the process the chloride moiety is removed from the molecule and replaced by hydrogen.

Many chlorinated aliphatic solvents such as trichloroethane and trichloroethylene are also known to be biologically recalcitrant in the presence of oxygen. These compounds are transformed by co-metabolism using methane as a substrate. Methane provides both carbon and energy for microbial growth, while the chlorinated solvent gets coincidently metabolized due to broad substrate specific methane monooxygenase enzyme.

2 Ex situ bioremediation: Ex situ bioremediation techniques are usually aerobic and involve treatment of contaminated soils or sediments using solid or slurry phase systems.

a) Land farming: It is the most conventional bioremediation technology commercially used for several kinds of wastes and contaminated soils. A treatment bed lined by a high density polyethylene plastic sheet is constructed to collect the contaminated soil. Sand is placed on plastic sheet for protection of bed. Contaminated soil is spread over the sand generally 203 feet height. Degradation rate is increased by augmentation of nutrient, buffer or microbes. Soil is periodically tilled for mixing out oxygen contact. This technique has been used for remediation of heavily contaminated petroleum waste.

b) Composting: This technique is relatively cheaper and has higher degradation potential compared to land farming. The technique involves physical removal of contaminated soil or sediments to specially constructed platforms or compost sheds. It is mixed with composting material like straw or wooden chips to enhance water and air holding capacity and improve physical handling properties. Periodic mixing or turning is applied in order to ensure adequate aeration. Composting techniques are used for remediation of highly contaminated sites and have proved successful for military sites contaminated with explosives such as TNT, RDX and Tetryl.

c) Slurry bioremediation: This is a batch treatment technique in which excaved soils or sediments are mixed with water and treated in reactor vessels or in contained ponds or lagoons. It is adequately aerated, mixed and nutrients together with surfactants or dispersants as per requirement are added. Effective bioremediation has been achieved using this techniques for sediments contaminated with petroleum hydrocarbons, pentachlorophenols, polychlorinated biphenyls, creosote, coal tars etc.

Case study of using bioremediation technology for cleaning Exxon Valdez oil spill

Marine oil spills catch the public's attention, generating intense pressure on the parties responsible for the spill, backed by legislation, for a prompt and effective response. It is interesting to consider what happens when oil is released into the marine environments. Almost all commercially used oils, upon release into the marine environment, float and spread on the water leading to increase in surface area. This enhances evaporation of components containing up to about 12 carbons and dissolution of few compound with an appreciable aqueous solubility. The floating slick also adsorbs water and some of the compounds get photo-oxidized in the slick.

This is a natural phenomenon aided by wave energy. If the spill is near land it is likely that some oil will reach shoreline. Physical collection is the first choice of recovery. The oil is collected with skimmers and this was the method applied following Exxon Valdez spill. Bioremediation played major role in the clean up of the Exxon Valdez spill.

In March 41 million liters of Alaskan North Slope crude oil was pilled from the Exxon Valdez in the Prince William Sound Alaska. This resulted in contamination with oil of approximately 2000 km of rocky intertidal shorelines in that region. . Conventional clean up using physical methods failed to remove all the oil on the beaches and under the rocks in the beach sediments. Exxon together with Environmental Protection Agency (EPA) in May 18 decided to test Bioremediation technology as a clean up strategy.

Microbial Ecology approach was used as a strategy. Site analysis of polluted beaches showed presence of hydrocarbon degrading microorganisms. Plentiful carbon in the form of spilled oil and sufficient oxygen was available for biodegradation process. However the intrinsic degradation rates were limited mainly by low availability of nutrients such as nitrogen and phosphorous. The sites were amended by fertilizer nutrient formulations. A liquid oleophilc fertilizer (Inipol EAP-22) that could adhere to the oil covered surfaces and a slow release water soluble fertilizer (Customblen) were tested as nutrient sources to augment the process of bioremediation. Within about two weeks after application of the fertilizer, there was a visible decrease in the amount of oil on rock surfaces treated with fertilizer formulations. About three to eight fold increase in the oil removal over the intrinsic biodegradation rate observed.

Tata Energy Research Institute (TERI) India has developed a product designated as Oilzapper. It mainly is comprised of a consortium of crude oil and oily sludge degrading bacteria obtained from natural habitats and mixed with appropriate carrier. The product when applied in both oil contaminated marine and terrestrial sites could restore the site to its original condition within a period of four months. The consortium was found to be active at high temperature also. This is a good example of microbiological approach of bioremediation where contaminant specific organisms were augmented on the site.
Such well documented case studies have gained attention as the potential bioremediation technology for cleaning up of oil contaminated sites.

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