2.1 The paraffin deposition problem in oil well tubings

The oil and petrochemical industries have unquestionably been key drivers in the development of the modern world. Besides, powering our cities, transporting our goods and providing the raw materials for the production of innumerable pharmaceuticals, dyes, plastics and consumer products, the wealth generated by the oil industry has been used for the advancement of general health and improving living standards around the world (Rae et al., 2001). A major problem that the oil industry faces since its inception, is that of paraffin-wax deposition that occurs during the production, storage and transportation of crude oil. Paraffin related problems occur throughout the production process of nearly all kinds of crude oils all over the world (Dong et al., 2001). During production transit of crude oil from the reservoir to the oil terminal, the paraffins deposit on the wall of down hole tubulars and other production points thereby reducing the crude oil pumping rate as also presenting start up problems after pipeline shut down (Tung et al., 2001). The reason for the pandemic nature of this problem could be attributed to the fact that only a small amount of wax i.e. 5-10% is required to cause a crude oil to experience pour point problems (Matlach and Newberry, 1983). The paraffin-wax present in petroleum crudes primarily consists of paraffin (alkane) hydrocarbons (C$_{13}$-C$_{36}$) known as macrocrystalline waxes or naphthenic hydrocarbons (C$_{30}$-C$_{60}$) known as microcrystalline waxes (Koshel and Kapoor, 1999). These solid particles cause a change in the flow behaviour of crude oil and result in a marked increase in crude oil viscosity and hence pressure drop increasing the cost of pumping and decreasing pipeline and tubing efficiency (Elsharkawy et al., 2000). If the temperature of the pipe or conduit through which the paraffinic crude oil is flowing reaches the cloud point (the temperature at which paraffin crystals first precipitate from solution at atmospheric pressure) of the crude oil, crystals of wax begin to form on the wall. This process of nucleation, that causes the precipitation of the paraffin crystals from the crude oil, results in the formation of a stable nucleus to which molecular paraffin crystals are continuously added. These wax crystals then grow in size until the whole inner wall is covered. If this process is looked at more closely, the process of wax precipitation occurs in three stages, wax separation, the growing of wax crystals and the
deposition of wax. Controlling any of these three stages could lead to the inhibition of wax deposition (Dong et al., 2001). There are several methods currently in use to handle paraffin deposition. These can be divided into two categories: remedial which involves the removal of the deposits by mechanical, thermal and chemical means or preventive using dispersants or crystal modifiers (Garcia, 2001). While using preventive means has obvious economic advantages over removal procedures, the latter are more commonly followed.

Thermal treatment which is based on the correlation between temperature and cloud point of the paraffin, includes the use of electric heating cable to prevent wax separation (Dong et al., 2001) as also hot oil or steam passage (Biao and Lijian, 1995). In this case the hot crude is pumped down the tube-casing annulus and the heat transfer through the tube causes the wax deposit to melt down into the oil. The problem with this method, however, is that it has to be a continuous process since the paraffin build up may recur. Line heaters overcome the problem of frequent hot oiling but the energy and operational costs are considerably large.

Mechanical removal of the paraffin deposits include scraping or pigging of the deposits (Garcia and Chiaravallo, 2001). This is among the most commonly used techniques with the advantage that it positively cleans the deposit. However, the problem is alleviated only temporarily and frequent use of personnel, time and equipment adds to the cost compared to thermodynamic and chemical means of removal (Haq, 1999).

Xylene and toluene are the most commonly used aromatic solvents for removal of wax deposits (Rae et al., 2001), however solvents pose a potential fire hazard. Besides, the addition of solvents, lining/coating the pipelines have also been attempted to prevent the wax deposition. Chemical additives have proved useful since they alter the flow behaviour of the waxy crude oil. Among the commonly used ones are polyethylene, ethylene copolymers, naphthalene, surfactants etc. (Haq, 1999). However, chemicals can solve only small to moderate size paraffin problems but for larger problems huge amounts of chemicals are required which is usually prohibited (Haq, 1999). Besides, like many other industrial chemicals, some of these chemicals maybe toxic or recalcitrant (Rae et al, 2001).

Besides these, some physical methods like the application of magnetic fields have also been attempted (Biao and Lijian, 1995). However, most of the paraffin deposition treatments in use are either temporary, or merely shift the problem from one place to another or in the case of chemicals and solvents, they carry a toxicity or fire hazard. Most of these techniques require production down time leading to economic losses or the solutions are at best temporary besides being costly.
The use of microbes to address the problems of paraffin-wax deposition; provides a more economically feasible and environmentally benign approach. The microbial treatment of paraffin wax is aimed at solubilizing the deposited paraffins and to increase the percentage of volatiles by its activity. Bacteria remove paraffins from surface by two mechanisms (a) Dispersion: where the bacterial metabolic by-products such as fatty acids and alcohols loosen paraffin deposits. Under fluid flow, the paraffin deposits are removed from the surface and once in suspension, the paraffin can be broken down further or become part of the fluid flow (b) Solubilization: where the bacteria increase the solubilization of the oil by increasing the volatiles and thereby solubility of long chain paraffins in oil, hence preventing further deposition (Lazar\textsuperscript{a}, 1999).

The ability of microbes to degrade paraffins has been known for a long time (Haines and Alexander, 1974). Eyk and Bartels had studied the induction of paraffin oxidation by intact cells of \textit{Pseudomonas aeruginosa} as far back as 1968. Lazar\textsuperscript{a} \textit{et al.} (1999) have studied paraffin degradation by bacteria using the production of biosurfactants and biosolvents as the screening criteria at a growth temperature of 28°C. They had obtained 15 bacterial strains (mainly Pseudomonads) that showed a utilization varying from 61 to 88% of hydrocarbons (at 28°C) found in paraffinic crude oils of Romania. Koma \textit{et al.} (2001) studied the degradation of paraffins of waste oil of car engine using a facultative anaerobic gram-negative \textit{Acinetobacter} sp. strain. This strain did not produce extracellular biosurfactants but was capable of an efficient and rapid degradation of the \textit{n}-paraffins. Lu \textit{et al.} (2003), on the other hand studied the effect of biosurfactants on the degradation of hydrocarbons by growing the indigenous bacteria obtained from oil-contaminated site of China at 30°C. \textit{Acinetobacter} sp. strains are the best-known degraders of long chain paraffinic alkanes with alkane degradation being the rule rather than the exception among \textit{Acinetobacter}. Koma \textit{et al.}, (2001) had found an \textit{Acinetobacter} sp. that can degrade long chain paraffins capable of degrading alkanes at 30°C. \textit{Acinetobacter} has also shown potential as a degrader of long chain alkanes capable of accumulation of wax esters in intracellular inclusions (Ishige \textit{et al.}, 2000). Maeng \textit{et al.}, (1996) had isolated an \textit{Acinetobacter} strain M-1, capable of using long chain alkanes ranging from \textit{C}_{13} to \textit{C}_{44} (paraffins) as the sole carbon source. More recently, Mandri and Lin (2007) have shown the degradation of upto 80% of paraffins by \textit{A. calcoaceticus} in two weeks at 30-37°C. Etoumi (2007) on the other hand had used strains of \textit{Pseudomonas} sp. and \textit{Actinomyces} sp. for the degradation of paraffinic compounds at mesophillic temperatures. The \textit{P. aeruginosa} strain WatG, could degrade paraffinic alkanes hexatriacontane (\textit{C}_{36}) and tetracosane (\textit{C}_{40}) in a mineral salts medium containing crude oil (Hasanuzzaman \textit{et al.}, 2007). In China, microbes have been used that use the wax, gum and bitumen as their nutrition. Through their biological
decomposition, they degrade the long hydrocarbon chain to shorter chains; reduce the wax and asphaltum content thereby decreasing the pour point and viscosity.

Biodegradation by microorganisms modifies waxy crude oils in beneficial ways, but conditions for down-hole applications require the use of thermophiles, resistant to organic solvents, with heat stable enzymes and reduced oxygen environments (Van Hamme et al., 2003). While the degradation of paraffins at mesophilic temperatures has been widely reported there are fewer reports of degradation of paraffin-wax as the sole carbon source by thermophilic bacteria. Acinetobacters are known for their efficient degradation of paraffinic alkans but they work at mesophilic temperatures not suitable for application to oil wells. Kato et al., (2001) had isolated a thermophilic Bacillus thermoleovorans strain B23 from a subterranean petroleum reservoir, that is able to degrade long chain alkanes (C15<) at 70°C. They have advocated the merit of alkane degradation under high temperature conditions, because melting temperatures of long-chain alkanes are higher (eg 66°C for C30) than shorter chain alkanes. Feitkenhauer et al., (2003) have demonstrated the ability of thermophilic Bacillus sp. and Thermus brockii in the degradation of hexadecane and pyrene at 60 -70°C. Kato et al. (2001) on the other hand had found an alkane degrading thermophile, Bacillus thermoleovorans (now Geobacillus) but it was not grown on a medium containing the alkanes as the sole source of carbon and energy. Marchant et al., (2002) have found Geobacilli strains that can grow at temperatures of 40 -80 °C and utilize alkanes.

2.1.1 Mechanism of paraffin degradation

The degradation of medium and long chain alkanes that comprise paraffins proceed by similar mechanisms. Alkanes are chemically quite inert (Labinger and Bercaw, 2002) hence for degradation to occur, alkane molecules have to be activated by the addition of oxygen. In anaerobic microbes, this is achieved by the addition of a C1 (CO2) or a C4 (fumarate) compound (van Beilen et al., 2003). In aerobic microorganisms however, the oxygen activation step is carried out by oxygenase enzymes. Monooxygenases incorporate one atom of oxygen of O2 into the substrate with the second atom being reduced to H2O. Dioxygenases on the other hand, incorporate both atoms into the substrate. Different microbes, however, employ different genetic and hence different enzymatic systems to oxidize alkanes.
**Monoterminal oxidation**

Alkanes are usually activated by terminal oxidation to the corresponding alcohol. The conversion of alkanes to alkanol is generally catalyzed by a group of protein collectively referred to as the "alkane hydroxylase system." It has three main components: alkane 1-monoxygenase, and the two soluble proteins rubredoxin, and rubredoxin reductase (Staijen et al., 2000). Rubredoxin reductase transfers electrons from NADH to rubredoxin, an iron sulfur electron transfer protein, which provides AlkB, a non haem alkane-monoxygenase localized in the cytoplasmic membrane, with two electrons to reduce one atom of molecular oxygen to water. The other oxygen atom being incorporated into the terminal methyl group of the substrate (Staijen et al., 1997) (figure 2.1).

![Figure 2.1: Oxidation of n-alkane by oxygenase enzyme](image)

**Alkane hydroxylase (Rubredoxin dependent)**

In 1992, van Beilen et al, studied the topology of alkane hydroxylase in *E. coli* using protein fusions linking different amino terminal fragments of the alkane hydroxylase (AlkB) to alkaline phosphatase and β-galactosidase. They, thus, predicted a model for alkane hydroxylase containing six transmembrane segments. The membrane-bound alkane hydroxylases possess 6 transmembrane helices and 4 conserved histidine rich motifs with two iron atoms in the active site (van Beilen et al, 2003). Enzymes containing diiron clusters are capable of oxidizing inactivated C-H bonds similar to alkane hydroxylase (Alk B). Shanklin et al (1997) expressed this alkane hydroxylase gene in *E. coli* and purified it to near homogeneity. Mössbauer spectroscopy revealed a dinuclear iron cluster similar to that found in diiron enzymes like, the aerobic component of ribonucleotide reductase, methane monooxygenase and a desaturase (Shanklin et al, 1997). In 2003, Shanklin and Whittle further, experimentally proved
that while there wasn’t overall sequence similarity between AlkB and desaturase like enzymes; the histidine motifs in AlkB are equivalent to desaturase-like enzymes and the these conserved histidine residues are important for coordinating the Fe ions at the active site.

Several bacterial strains are known to harbour alkane hydroxylases homologous to the AlkB of *Pseudomonas* sp. The marine bacterium *Alcanivorax borkumensis* that almost exclusively grows on hydrocarbons and utilizes aliphatic hydrocarbons in the carbon range of C_{12} to C_{20}. It contained the alkane hydroxylases AlkB1 and AlkB2, of which AlkB1 was closely related to *P. putida* while AlkB2 was more closely related to the alkane hydroxylase of *P. aeruginosa* (van Beilen et al., 2004). In another study van Beilen et al. (2002) isolated Gram positive alkane degraders from near a car repair shop and used degenerate alkane hydroxylase primers to show that most *Rhodococcus* isolated contained three to five homologues of the GPo1 alkB gene. The aliphatic hydrocarbon degrading *Mycop bacterium* sp. strain CH1 showed DNA hybridization with alkB gene of *P. oleovorans* under low stringency conditions but not under high stringency conditions hinting at a probable distant relationship between the genes involved in alkane oxidation (Churchill et al., 1999). In the *Nocardioides* sp. strain CF8 that could utilize alkanes from C_{2} to C_{16}, two distinct monooxygenases were present for alkane oxidation (Hamamura et al., 2001).

## Rubredoxin

Rubredoxins (Rd), the electron transfer components of membrane-bound alkane hydroxylases, are iron sulfure redox active proteins usually containing a single Fe(S-Cys)_{4} site. Rd shuttles electrons from Rd reductase to alkane hydroxylase (van Beilen et al. 2003). The OCT plasmid of *Pseudomonas* GPo1 codes for two rubredoxins AlkF and AlkG of 14 and 18 KDa molecular mass (Kok et al., 1989). AlkG, which contains two rubredoxin domains, AlkG1 and AlkG2 (connected by a 70 amino acid linker), is more than 3 times the size of other bacterial rubredoxins. Since some structural features necessary for electron transfer are conserved, rubredoxins involved in alkane degradation can be exchanged between alkane hydroxylase systems of Gram negative and Gram positive bacteria (van Beilen et al., 2002). Also, sequence analysis had revealed that AlkG1 was conserved across phylogenetic boundaries.

The ADP1 strain of *Acinetobacter* sp. which can grow on alkanes of 12 or more carbon atoms contain genes *rubA* and *rubB* that code for rubredoxin and a NAD(P)H dependent rubredoxin reductase. Even single base pair substitutions in *rubA* or *rubB*
lead to defects in alkane degradation, indicating that both are essential for alkane utilization (Geißdörfer et al., 1999).

This primary alcohol is further oxidized by alcohol and aldehyde dehydrogenases (Figure 2.2).

**Cytochrome P450 dependent alkane hydroxylase**

Cytochrome P450s derive their name from their typical spectral properties. The reduced P450 on binding with CO produces an absorption maximum at 450 nm which is used for estimating the P450 content. Cytochrome P450 monooxygenases are abundantly available in nature and are involved in the oxidation of several xenobiotic and endogenous hydrophobic substances. This family of enzymes is also involved in the biotransformation of drugs, metabolism of carcinogens as also the biosynthesis of fatty acids, steroids, fat-soluble vitamins in addition to xenobiotic transformation. The Cyt P450 enzymes have also been implicated in the conversion of alkanes and aromatic compounds. The role of Cyt P450 as biocatalysts has been reviewed by Bernhardt in 2006. Their molecular weight generally ranges from 50000 to 60000 daltons and are generally the terminal oxygenases in electron transport chains where they oxidize inter hydrophobic substances in the presence of oxygen and the reducing equivalent NAD(P)H (Sato and Omura (1978) as cited in Narhi and Fulco (1987)). The general catalysis reaction of cytochrome P450 systems is

$$\text{RH} + \text{O}_2 + \text{NAD(P)H} + \text{H}^+ \rightarrow \text{ROH} + \text{H}_2\text{O} + \text{NAD(P)^+}$$

The catalytic reaction takes place in multiple steps starting with the binding of the substrate and then followed by the introduction of the first electron from NAD(P)H via an electron transfer chain. This is followed by oxygen binding which by accepting the second electron produces a ferric peroxy anion which is protonated to from the ferric hydroperoxy complex. This complex undergoes cleavage to form the putative ferryl species that attacks the substrate yielding the hydroxylated product (Bernhardt, 2006).

Farinas et al (2001) reported the oxidation of octane to octanol and octanone by a Cyt P450 fatty acid monoxygenase BM-3 (from *Bacillus megaterium*). After this study, in 2002, Glieder et al converted the cytochrome P450 BM-3 from a medium chain fatty acid monoxygenase into a highly efficient biocatalyst for the conversion of small to medium chain length alkanes into alcohols which would not require additional proteins for catalysis. Some Cyt P450s are also notoriously famous for the activation of
environmental Polycyclic aromatic hydrocarbons into more reactive and carcinogenic forms (Shimada and Kuriyama, 2004).

Cytochrome P450s of yeast have been reviewed by Kappeli in 1986. Among eukaryotic microbes the Cyt P450s were initially found in *Saccharomyces cerevisiae*

**Alcohol dehydrogenase**

Alkanol dehydrogenases oxidize alkanol to alkanal with concomitant reduction of NAD(P)^+ to NAD(P)H. In alkane utilizing bacteria, alcohol dehydrogenases are generally constitutive, soluble and require NAD^+ and NADP^+ as co-factors. Some of these enzymes have been purified and are relatively non-specific for primary alcohols within a certain chain length. The *Pseudomonas aeruginosa* alcohol dehydrogenase catalyzes the oxidation of primary and secondary alcohols to aldehydes and ketones using its coenzyme NAD. Levin *et al* (2004) crystallized the ternary complex of this enzyme along with its coenzyme and substrate and determined its structure to be a tetramer with four identical chains of 342 amino acids. It was similar to the alcohol dehydrogenase monomers of vertebrates, archea and bacteria.

The membrane-bound alcohol dehydrogenase purified from *P. aeruginosa* was induced by growth on hydrocarbons (especially long chain hydrocarbons like hexadecane) and had an affinity for long chain primary alcohols. Interestingly, this enzyme did not use NAD or NADP as coenzymes (Tassin *et al*, 1973). More recently, Kato *et al* (2001) cloned a gene encoding an alcohol dehydrogenase from a thermophilic *Bacillus thermoleovorans* B23. This strain conferred 1-tetradecanol dehydrogenase activity on *E. coli* cells.
Figure 2.2: Alkane degradation by terminal, sub-terminal and diterminal oxidation. Terminal oxidation leads to the formation of fatty acids that enter the $\beta$-oxidation pathway. $\omega$-hydroxylation by a fatty acid monooxygenase leads to dicarboxylic acids. Subterminal oxidation gives rise to secondary alcohols oxidizing further to ketones (van Beilen et al, 2003).

Aldehyde dehydrogenase

Like alcohol dehydrogenase, bacteria also possess multiple aldehyde dehydrogenase. In a recent study (Ishige et al, 2000), reported a long-chain aldehyde dehydrogenase, Ald1, in the soluble fraction of Acinetobacter sp. strain M1. The deduced amino acid sequence showed high similarity to those of various aldehyde dehydrogenase. The Ald1 gene was cloned from the chromosomal DNA and stably expressed in E. coli and the gene product was purified to apparent homogeneity. The recombinant gene product showed enzyme activity towards $n$-alkanals with the highest activity for tetradecanal.
The resultant fatty acids enter the β-oxidation (Figure 2.3).

**Figure 2.3:** The β-oxidation pathway includes repeated cycles of four reactions with each fatty acid molecule. At the end of each cycle the fatty acid is progressively shortened by two carbon atoms until the even chain oxidation results in the formation of acetyl-coA and the odd chain converted to propionyl coA (Croston G, 2001 www.biocarta.com).

The paraffin degrading strain *Acinetobacter sp. ODDK71* yielded the monomeric oxidation products 1-hexadecanol and 1-hexadecanoic acid when grown with hexadecane (Koma *et al.,* 2001). The thermophilic long chain alkane degrading strain *B. thermoleovorans* that was isolated from subterranean oil reservoirs, also degraded alkanes via the terminal oxidation pathway followed by the β-oxidation pathway (Kato *et al.,* 2001). Kato *et al.* (2001) had also cloned the gene encoding an alcohol dehydrogenase from this strain. The alkane degradation system of *Pseudomonas oleovorans* (now *putida*) strain GP01 that can degrade medium chain alkanes from pentane to dodecane (van Beilen *et al.,* 1994) is among the most thoroughly characterized systems.

**Diterminal oxidation**

In this type of oxidation, both the terminal methyl groups are sequentially hydroxylated resulting in the formation of a diol which on further oxidation, yields a dicarboxylic acid (Kester and Foster, 1963; Jurtshuk and Cardini, 1971).

**Subterminal oxidation**

In this mode of oxidation, secondary alcohol is the first product of alkane oxidation, which is further oxidized to a ketone. Forney and Markovetz (1970) studied the degradation of tridecane by *Pseudomonas aeruginosa* strain Sol20 and detected the presence of tridecan-2-ol and undecan-1-ol in the cell free fluid by gas-liquid chromatography indicating a subterminal catabolic pathway. Some fungi have also long known to oxidize alkanes by subterminal oxidation. The oxidation of tetradecane by *Cunninghamella blakesleeana* and *Penicillium* revealed a monoterinal oxidation by *Cunninghamella* but oxidation products of *Penicillium* included tetradecan-2-ol, dodecan-1-ol, tetradecan-2-one and tetradecan-4-one (Allen and Markovetz, 1970). The psychrotroph *Rhodococcus* sp. strain Q15, on the other hand, oxidizes alkane via the terminal as well as the subterminal oxidation pathway (Whyte *et al*, 1998).

### 2.1.2. Genetics of alkane degradation

**Genetics of alkane oxidation in Pseudomonas**

In 1975, the ability of *Pseudomonas putida* PpG6 to utilize alkanes and grow on their primary terminal oxidation products was known (Nieder and Shapiro, 1975). They had concluded that the growth specificity of the strain PpG6 was due to specificity of the whole cell alkane hydroxylation and the physiological role of the alkane hydroxylase complex was to initiate monoterinal oxidation. Grund *et al* (1975) subsequently indicated that the OCT plasmid codes for the inducible alkane hydroxylase and primary alcohol dehydrogenating activity while the oxidizing activities for primary alcohols, aliphatic aldehydes and fatty acids is constitutively coded for by chromosomes. In 1977, Benson *et al* had identified two plasmid alkane hydroxylase loci: alkA, determining a soluble component, and alkB, determining a particulate component. The alkane hydroxylase system of *Pseudomonas putida* GPo1, consists of three components: alkane hydroxylase (Alk B), rubredoxin (Alk G) and rubredoxin reductase (Alk T) (Smits *et al*, 2002). The Alk B, F and G (the alkane hydroxylase and the 2 rubredoxins) alongwih the alcohol and aldehyde dehydrogenases (Alk J and AlkH) are coded by the *alkBFGHJKL* operon. The third part of the electron transfer component the rubredoxin reductase (Alk
T) and the positive regulator AlkS are coded for by the alkST locus. Staijen et al (2000) tested the synthesis and function of Alk B, G and T of GPo1 in E. coli recombinants and found that these components were synthesized at different rates in E. coli and after induction produced these alk components many folds. However, they found that the enzymes were less stable in the new host and the specific activity of Alk B was lower in E. coli than in P. oleovorans hinting at the requirement of a molecular environment and certain factors that were available in P. oleovorans but not in the E. coli host. The marine hydrocarbonoclastic bacterium Alcanivorax borkumensis which shares 65% identity with P. aeruginosa AlkB and has substrate ranges similar to P. aeruginosa and P. putida, is regulated differently from P. putida GPo1. Unlike GPo1 alkS gene expression was not induced by alkanes (van Beilen et al, 2004).

A comparison of the membrane-bound alkane hydroxylase of P. putida GPo1, an AlkB homolog of Mycobacterium tuberculosis H37Rv and that of Acinetobacter ADP1, showed that four histidine motifs were conserved and highly degenerate primers, that amplified internal gene fragments, based on two of these motifs were developed (van Beilen et al, 2003). Several strains of the Acinetobacter sp., Pseudomonas sp., Mycobacterium and Rhodococcus sp. contained homologs of the P. putida GPo1 alkB gene.

**Genetics of alkane oxidation in Acinetobacter**

Alkane degradation is the rule rather than exception among Acinetobacter sp. While the genetics of the first step of alkane oxidation are very well known in Pseudomonas sp., in Acinetobacters there are more than one means for the primary oxidation step.

*Acinetobacter calcoaceticus S19* follows a monoterminal oxidation pathway for alkane degradation as evident from the oxidation substrates (Bajpai et al, 1998). Asperger et al (1981), on the other hand, had studied the occurrence of cytochrome P450 on Acinetobacters grown with hexadecane. It has also been proposed that there is a rubredoxin- and rubredoxin reductase-dependent terminal alkane hydroxylase involved in alkane oxidation in *Acinetobacter calcoaceticus 69-V* (Asperger et al 1991, as cited in Ratajczak, 1998) and *Acinetobacter* sp. strain ADP1 (Geissdörfer et al, 1995). The *Acinetobacter* strain ADP1 which uses long carbon chain length alkanes (atleast 12 carbon atoms) as the sole carbon source requires at least five genes for alkane degradation. The alkane hydroxylase is encoded by alkM, Rubredoxin and NAD(P)H dependent Rubredoxin reductase are coded by rubA and rubB, the transcriptional regulator alkR and xcpR which is a part of the general secretory pathway (Ratajczak et al, 1998). alkR is the transcriptional activator of alkM and both genes are essential for
alkane degradation by ADP1 (Ratajczak et al., 1998). A single base pair substitution in rubA and rubB can lead to defects in alkane degradation showing that both are essential for alkane degradation in ADP1 (Geißdörfer et al., 1999). The critical first step here is the formation of the primary alcohol, similar to that in P. oleovorans.

However, Finnerty had postulated a new pathway for alkane oxidation by Acinetobacter sp. strain HO1-N. According to this pathway, the initial reaction was catalyzed by a dioxygenase, so the pathway would be $\text{RCH}_3 \rightarrow \text{RCH}_2\text{OOH} \rightarrow \text{RCO(O)OH} \rightarrow \text{RCHO} \rightarrow \text{RCOOH}$. While there were experimental bases for this hypothesis, there was no direct evidence for the initial reaction catalyzed by the dioxygenase (as cited in Maeng et al., 1996). Maeng et al. (1996) isolated an Acinetobacter sp. strain M-1, that used long chain $n$-alkanes from C$_{13}$ to C$_{44}$ as the sole carbon source and their investigation of its metabolic pathway, found a new alkane oxidizing enzyme that required molecular oxygen but not NAD(P)H. The purification of this enzyme and a study of its properties revealed it to be a dioxygenase thus providing evidence and supporting the Finnerty pathway for alkane oxidation. Sakai et al. (1996) provided further biochemical evidence to the Finnerty pathway by purifying the alkane oxidizing enzyme to apparent homogeneity by SDS-PAGE. They inferred that this enzyme was a flavoprotein and required molecular oxygen and Cu but not NAD(P)H for its activity. Geißdörfer et al. (1999) have noted the peculiarity of the genetic organization of Acinetobacter where related genes may be apart of functionally unrelated genes linked together. Unlike Pseudomonas oleovorans, only the alkM mono oxygenase gene is regulated while the rub AB genes are constitutive. Ratajczak et al. (1998) had found that AlkM (408 aa) exhibited homology to AlkB (401 aa) from P. oleovorans.

**Gene probing for screening of alkane-degrading microbial population**

Catabolic gene probes have been designed for screening bacterial populations from various habitats for hydrocarbon oxidizing bacteria. Smits et al. (1999) chose two conserved regions of the non-haem iron alkane hydroxylase sequences of P. oleovorans GP01 (AlkB) and Acinetobacter sp. ADP1 (AlkM) to design degenerate oligonucleotide primers. These primers were then used to screen several Gram positive and Gram negative bacteria. They noted that the strains that did not degrade alkanes did not yield PCR products with homology to alkane hydroxylases. Whyte et al. (2002) aligned the predicted amino acid sequences of alkane monooxygenase genes of P. putida (alkB), Acinetobacter (alkM), and Rhodococcus (alkB1 and alkB2) and then used the corresponding DNA sequences for primer design. These primers were used to study the prevalence of alkane mono oxygenase genotypes in hydrocarbon contaminated and...
pristine polar soils. Vomberg and Klinner (2000) derived a gene probe from the *alkB* gene of *P. oleovorans* ATCC 29347 and screened fifty four bacterial strains that could utilize gaseous and liquid alkanes. They found the strongest hybridization signals of *alkB* with short chain alkane degrading Pseudomonads and lesser sequence identity with *Rhodococcus, Nocardiodides, Gordona* and *Sphingomonas sp.* Kohno et al (2002), on the other hand, designed three sets of primers for the detection of alkane degrading bacteria. The PCR primers and probes were again based on the homologous regions of many alkane hydroxylase genes. The primers and probes were tested on known bacterial strains as also unknown environmental samples. The first group included genes encoding *alkB* that catalyzed medium chain length alkanes (C₆ –C₁₂) via the monoterminal oxidation pathway using monooxygenases. The second group included genes coding for *alkM* which catalyzed long chain alkanes (C₁₂−) via the monoterminal pathway using monooxygenases or dioxygenases. However, the third group included *alkB* or *alkB₁* are of unknown substrate specificity and unknown oxidation systems. The ALK primer sets showed specificity for the respective alkane hydroxylase groups. So the first group strains would be detected by the primers and probes of the first group, but would not detect the strains of the second and third groups.
2.2 Generation of acidic oily sludge while processing of paraffins

Paraffin wax obtained from crude oil has several uses including candle making, coating of surfaces, food additives etc. Refining of petroleum wax, in earlier times, employed the use of acids such as sulfuric acid for removal of impurities and improvement of colour, odour etc. However, the residual sulfuric acid left after refining found its way into the oily sludge making it extremely toxic and difficult to degrade. Oily sludge itself is carcinogenic and immunotoxic in nature (Mishra et al., 2001). India, while processing approximately 128 million metric tonnes of crude oil per annum (TEDDY, 2006) through its 18 refineries, generates 20000 tonnes of this oily sludge every year (0.002-0.1% of the weight of crude processed). This oily sludge contains alkanes, aromatic hydrocarbons, NSO (nitrogen, sulphur, oxygen containing) compounds and asphaltenes (Bhattacharya et al., 2003). Safe disposal of this oily sludge is a major problem for refineries since it threatens the environment through soil and ground water pollution. Oily sludge disposal strategies include land filling, chemical fixation, sludge farming, incineration etc. However, while all these treatments show limited success microbial bioremediation offers an environmentally benign and economically feasible alternative. Bioremediation, the use of microbes to degrade crude oil/oily sludge, is now an established technology for the detoxification of oily sludge and reclamation of land for reuse. But, when the sludge is acidic in nature, these hydrocarbon-degrading bacteria are unable to survive. The Digboi refinery in Assam, North Eastern India, is faced with such a problem. The sulfuric acid contaminated oily sludge dumped inside the refinery premises is an inventory of 20,000 to 50,000 tonnes at a pH ranging from 1.5 to 3. Since all other approaches to remediate this sludge were met with limited success, bioremediation was explored as an environmentally benign and economically feasible alternative. The two approaches to bioremediation could be biostimulation of indigenous microbes through the addition of fertilizers, aeration etc or bioaugmentation by the addition of adapted microbial hydrocarbon degraders (Balba et al., 1998) or a combination of the two approaches. As bioremediation candidates, both bacteria and fungi are relatively plentiful in soil and both the groups contribute substantially to the biodegradation of hydrocarbons (Bartha and Bossert, 1984).

Several organisms with varying capabilities of crude oil and oily sludge degradation are known. Lazar et al (1999) had screened several bacterial consortia in the laboratory using waste hydrocarbons of the oily sludge of Romanian oil fields.

2.2.1 Factors affecting biodegradation

TERI University-PhD. Thesis, 2008
Biodegradation is affected and limited by many factors, including the type of hydrocarbons, bioavailability of the hydrocarbon to the microbe, salinity, pH, temperature and nutrients in the environment and the absence or presence of oxygen. In the words of Hamer and Al-Awadhi (2000), “Typical soil microenvironments that require remediation comprise three principal inorganic particulates, sand, silt and clay, a chemically and physically diverse organic fraction comprising both natural components and pollutants, an aqueous phase that is frequently discontinuous and contains unevenly distributed dissolved and dispersed organic and inorganic matter, a gaseous phase of different composition from the overlying atmosphere, and, of course, a diversity of both indigenous and non-indigenous microorganisms.”

**Physical and chemical factors**

**Composition of the oil**

Crude oil is produced by high pressure and temperature action on biological material over huge geological time scales. The variability of these factors possibly translates into the variability of the composition and properties of crudes. Crude oils are comprised of four broad fractions: the alkane, the aromatic, the nitrogen-sulphur-oxygen (NSO) containing fraction and the asphaltene fraction. The alkane fraction generally forms the principal constituent of the crude oil. This fraction mainly consists of the saturated hydrocarbons with varying carbon chain length and whose relative proportions vary in different crudes. Alkanes could be straight chain or branched chain compounds. The aromatic fraction contains hydrocarbons with ring structures ranging from benzene to multi ring polycyclic aromatic hydrocarbons (PAHs). The NSO fraction contains compounds, which are mainly composed of nitrogen, oxygen and sulphur derivatives of the hydrocarbons generated via geochemical reactions with inorganic sulphur compounds etc. these compounds are extremely toxic and more recalcitrant than the aromatic and alkane fractions. The most recalcitrant fraction in the crude oil is the asphaltene fraction, which contains higher hydrocarbons formed primarily by cross linking of the NSO units (Westlake et al., 1974; Atlas, 1981; Morgan and Watkinson, 1994). Experiments done by Huesemann in 1995 (cited in Salanitro et al.,1997) to test the extent and limit of bioremediation of oily soils showed that overall bioremediation effectiveness was dependent on the types of hydrocarbons present, more than the soil types, nutrients etc.
Temperature

R. M. Atlas had studied seven different crude oils, way back in 1975 and found biodegradability to be highly dependent on the incubation temperature and the composition of these crude oils. Temperature influences petroleum biodegradation by its effect on the physical nature and chemical composition of the oil, rate of hydrocarbon metabolism by microorganisms and composition of the microbial community. At low temperatures, the viscosity of the oil increases, the volatilization of toxic short chain alkanes is reduced and their water solubility is increased, delaying the onset of biodegradation while at high temperatures there is an increased rate of metabolism but at temperature ranges of more than 40°C the membrane toxicity of hydrocarbons increases (as cited in Rahman et al, 2002). Rates of degradation are generally observed to decrease with decreasing temperature believed to be because of decreased rates of enzyme activity (Leahy and Colwell, 1990).

Oxygen

The initial steps in the catabolism of aliphatic (Singer and Finnerty, 1984a), cyclic (Perry, 1984) and aromatic hydrocarbons (Cerniglia, 1984) by bacteria and fungi involve the oxidation of the substrate by oxygenases, for which molecular oxygen is required. Aerobic conditions are therefore for this route of microbial oxidation of hydrocarbons in the environment. Anaerobic degradation of petroleum hydrocarbons by microorganisms has been shown in some studies to occur only at negligible rates and its ecological significance has been generally considered to be minor (Leahy and Colwell, 1990).

Nutrients

The effect of nutrients on degradation is varied and can be attributed to the variable and complex composition of soils. However, nitrogen and phosphorus are considered to be the most important as they are required for incorporation of carbon into the biomass (Fedorak and Westlake, 1981). Stimulation of crude oil degradation has been seen to be stimulated by the addition of nitrogen in the form of oleophillic fertilizers, including paraffinized urea, octylphosphate, ferric octoate, paraffin supported MgNH₄PO₄ and 2-ethylhexyldipolyethylene oxide phosphate (Atlas and Bartha, 1972; Bergstein and Vestal, 1978; Horowitz and Atlas, 1977). The form time and quantity of nitrogen addition is also important for effective bioremediation. Chaillan et al (2006) found detrimental effects of nitrogen urea concentration on the hydrocarbon degrading fungal population.
pH

A neutral pH is optimal for biodegradation while extremes in pH are known to negatively influence biodegradation (as cited in Rahman et al, 2002). Soil pH can be highly variable, ranging from 2.5 in mine spills to 11.0 in alkaline deserts (Bossert and Bartha, 1984) unlike aquatic ecosystems. Most heterotrophic bacteria and fungi favor a pH near neutrality, with fungi being more tolerant to acidic conditions (Atlas, 1988). Extremes in pH, as can be observed in some soils, therefore, have a negative effect on the degradation capabilities of microorganisms. Acidophilic and acid tolerant microbes are not unknown. *Thiobacillus oxidans* and *T. ferroxidans* of the *Thiobacillus* group are known sulphur oxidizers and are thought to grow at low pH by maintaining their cytoplasmic environments near neutrality. They are thought to do this by pumping protons out of the cell or by maintaining a cell surface barrier which is impermeable to protons. Hydrocarbon contaminated environment that have a low pH, however, pose a rather extreme environment where the microbe has to withstand the acidic conditions and at the same time degrade hydrocarbons (Sharp and Munster, 1986).

**Pressure and Water Activity**

The importance of pressure as a variable in the biodegradation of hydrocarbons is mostly confined to deep sea environments. Because of high pressures in deep ocean environments oil will be degraded very slowly by the microbial populations because of which certain recalcitrant fractions of the oil could persist for years or decades (Colwell and Walker, 1977).

The water activity or water potential of soils can range from 0.0 to 0.99, in contrast to aquatic environments, in which water activity is stable at a value near 0.98 (Bossert and Bartha, 1984). Hydrocarbon biodegradation in terrestrial ecosystems may therefore be limited by the available water for microbial growth and metabolism.

**Biological Factors**

**Adaptation by prior exposure**

Prior exposure of a microbial community to hydrocarbons, either from anthropogenic sources such as accidental oil spills, petroleum exploration and transportation activities, and waste oil disposal, or from natural sources such as seeps and plant derived hydrocarbons (NAS,1985; Bartha and Bossert,1984), is important in determining how
rapidly subsequent hydrocarbon inputs can be biodegraded. This phenomenon, which results from increases in the hydrocarbon oxidizing potential of the community, is known as ‘adaptation’ (Spain et al, 1980). The three interrelated mechanisms by which adaptation can occur are (i) induction and/or depression of specific enzymes (ii) genetic changes which result in new metabolic capabilities and (iii) selective enrichment of organisms able to transform the compounds of interest (Spain and Veld, 1983). Olivera et al (1997) studied the biodegradation of a mixture on n-alkanes by microbial communities from pristine and hydrocarbon contaminated sediments. They found that the control community grew poorly and degraded only 5-10% of the hydrocarbons while the microbes from the polluted sediments decreased the hydrocarbons to less than 5%.

**Adaptation by alteration in genetic composition**

The primary genetic mechanism for the adaptation of the microbial community is the amplification, by means of selective enrichment and gene transfer and mutation, of genes which are involved in the metabolism of the chemical contaminant (Barkay and Pritchard, 1988; Spain and Veld, 1983). Direct monitoring of this process with respect to adaptation to hydrocarbons has been made possible by the development of DNA probes specific for the genes encoding hydrocarbon catabolic pathways (Trevors, 1985).

**Role of plasmids in adaptation**

Plasmid DNA may play a particularly important role in genetic adaptation in that it represents a highly mobile form of DNA which can be transferred via conjugation or transformation and can impart novel phenotypes, including hydrocarbon oxidizing ability to recipient organisms. The pathways for the metabolism of for instance, naphthalene, salicylate, camphor, octane, xylene and toluene have been shown to be encoded on plasmids in *Pseudomonas* spp. (Chakrabarty, 1976). Exposure of natural microbial populations to oil or other hydrocarbons may impose a selective advantage to strains possessing plasmids encoding enzymes for hydrocarbon catabolism, resulting in an overall increase in the plasmid frequency in the community.

**Seeding**

Seeding involves the introduction of allochthonous microorganisms into the natural environment for the purpose of increasing the rate or extent, or both, of biodegradation of pollutants. The rationale for this approach is that the autochthonous microbial
populations may not be capable of degrading the wide range of potential substrates present in complex mixtures such as petroleum. The criteria to be met by effective seed organisms include the ability to degrade most petroleum components, genetic stability, and viability during storage, rapid growth following storage, a high degree of enzymatic activity and growth in the environment, the ability to compete with indigenous microorganisms, non pathogenicity and the inability to produce toxic metabolites (Atlas, 1977). While some microbes may be effective bioremediation agents as single cultures, in other cases using a consortium would enhance degradation. Rahman et al (2002), in a laboratory study for the degradation of Bombay High crude oil, concluded that a bacterial consortium consisting of Micrococcus, Corynebacterium, Flavobacterium, Bacillus and Pseudomonas strains showed more growth and degradation of the crude oil than the individual isolates.

It has been known for almost a century now certain microorganisms are able to degrade petroleum hydrocarbons and use them as a sole source of carbon and energy for growth. There are two essential characteristics that define hydrocarbon-oxidizing microbes:

1. membrane bound group specific oxygenases and
2. mechanisms for optimizing contact between the microbes and the water insoluble hydrocarbon.

As mentioned earlier, for microbes to be able to degrade hydrophobic hydrocarbon substrates, the substrate has to be bioavailable to the degrading microbe. The hydrocarbon uptake can be by the adherence of the cell to the hydrocarbon and direct uptake or through the emulsification of the hydrocarbon through surface active compounds like biosurfactants. Husain et al (1997) had noted, that the adherence and emulsification were the two main modes of hydrocarbon uptake in P. nautical strain 617, evident from the morphological changes that accompanied the transfer of this strain from the hydrophilic acetate to hydrophobic eicosane substrate.

2.2.2. Hydrocarbon biodegradation

The ability to degrade hydrocarbon substrates is present in a wide variety of bacteria and fungi. It has been observed that there is an increase in the number of hydrocarbon oxidizing bacteria in areas that suffer from oil pollution. Hydrocarbon degradation is predominantly an aerobic process. The specificity of the degradation process however, can be related to the genetic potential of the particular microorganism to introduce molecular oxygen into the hydrocarbon and with relatively few reactions to generate the...
intermediates that subsequently enter the general energy yielding catabolic pathways of the cell. The specific genetic capacity is expressed in the hydrocarbon substrate specificity of the oxygenase and in the ability of the carbon source to induce the various enzyme activities necessary for its biodegradation.

There are some general observations, however, on the microbial degradation of hydrocarbons:

1. The saturates are efficiently degraded by most microbes, with the length of the carbon chain being the deciding factor for degradation.
2. Saturated alkane hydrocarbons are more amenable to degradation than their branched chain and unsaturated counterparts.
3. Aromatic hydrocarbon degradation is more difficult than that of aliphatic hydrocarbons. With many microbes either partially oxidizing them or co metabolically degrading aromatic hydrocarbons.
4. The resins and asphaltenes are the most recalcitrant group of hydrocarbons.

So hydrocarbons can be ranked in the following order of susceptibility to microbial degradation: $n$-alkanes > branched alkanes > low molecular weight alkanes > high molecular weight aromatics. There may however, be certain exceptions to this trend.

**Biodegradation of Aliphatic hydrocarbons**

Of the different constituents of crude oil, the alkane fraction forms the maximum percentage in crude oil.

Degradation of aliphatic hydrocarbons and the mechanisms of alkane degradation have been known for a long time now. Nieder and Shapiro had studied the monterminal oxidation of alkanes (six to ten carbon atoms) for growth by *Pseudomonas putida* PpG6 (*oleovorans*) in 1975. Oxidation of $n$-paraffins proceeds, in general, via terminal oxidation to the corresponding alcohol, aldehyde and fatty acid (McKenna and Kallio, 1965). The acetic acid bacterium *Acetobacter rancens* degraded tetradecane and hexadecane by monterminal oxidation as evidenced by the formation of $n$-alkanoic acids and alcohols and the activity was constitutive (Hommel and Kleber, 1984). The filamentous fungus *Cladosporium resinae* also follows the monterminal oxidation pathway similar to bacteria and yeasts, as evidenced by its growth on dodecane and hexadecane and the oxidation of the monterminal intermediates by its cell free extracts (Walker and Cooney, 1973). Hydro peroxides may serve as unstable intermediates in the
formation of the alcohol (Watkinson and Morgan, 1990 as quoted by Rosenberg and Ron, 1996). Fatty acids derived from alkanes are then further oxidized to acetate and propionate (odd chain alkanes) by inducible β oxidation systems.

The group specificity of the alkane oxygenase system is different in various bacterial species.

Subterminal oxidation occurs in some bacterial species (Forney and Markovetz, 1971). This type of oxidation is responsible for the formation of secondary alcohols and ketones. Several yeast strains are also known to degrade alkanes. Lodderomyces elongisporus could degrade the n-alkanes tetradecane, hexadecane and heptadecane when precultured on glycerol.

The polyethylene degrading fungus Penicillium simplicissimum YK could degrade the long chain alkanes triacontane (C\textsubscript{30}) and tetracosane (C\textsubscript{40}) in addition to branched chain alkanes in two weeks (Yamada-Onodera et al, 2002).

**Biodegradation of Aromatic compounds**

Polycyclic aromatic hydrocarbons (PAH) found in the aromatic fraction of the oily sludge are a group of compounds with two or more condensed benzene rings. They exist in several isomeric forms. Only the four and six ring PAH can have more than 70 isomers. Their strong hydrophobic nature causes the PAH to show a strong tendency to absorb on surfaces and thus show little or slow decomposition. Because of their toxic effects and frequent occurrence in the environment the EPA numbers them among “priority pollutants”. The toxicological significance of PAH and their residues is because of its carcinogenic, mutagenic and teratogenic potential. The EPA has selected 16 non-substituted PAH as model standards for analysis. These include naphthalene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benz(a)pyrene, acenaphthene (Kästner, 2000). PAH are metabolized not only by cytochrome P450 monoxygenases in mammalian cells, but also by a large number of enzymes in bacteria, fungi and algae. While, both prokaryotic and eukaryotic microorganisms have the enzymatic potential to oxidize aromatic hydrocarbons that range in size from a single ring (e.g. benzene, toluene and xylene) to naphthalene to polycyclic aromatics such as anthracene, phenanthrene etc, the molecular mechanisms by which bacteria and higher microorganisms degrade aromatic compounds are fundamentally different.

Bacteria initially oxidize aromatic compounds by incorporating both atoms of molecular oxygen into the aromatic substrate to from a cis-dihydrodiol. The reaction is catalyzed by a multicomponent dioxygenase, leading to enzymatic fission of the aromatic ring. This
pathway that can lead to complete metabolization proceeds via the formation of cis-dihydrodiols, dehydrogenation to form dihydroxy PAH, extradiol ring cleavage, elimination of the first ring, decarboxylation of the hydroxy naphthoic acid and extradiol ring cleavage and degradation of the second ring.

In eukaryotes and also in some prokaryotes, PAH are oxidized by the activity of cytochrome P450 monooxygenases to form arene oxides. These are subsequently hydrolysed to trans-dihydrodiols or non-enzymatically to phenols which can form conjugates with cell components (Rosenberg and Ron, 1996; Kästner, 2000).

The third possibility of PAH degradation comprises non-specific radical oxidation by lignolytic enzymes of white rot fungi. This degradation leads to the formation of reactive compounds and quinines, which in some cases may be further degraded or mineralized to CO₂. Some saprophytic fungi are also able to produce quinines.
Biodegradation of pyrene

The four-ringed PAH, pyrene is not a carcinogen but byproduct of the manufactured gas process and other incomplete combustion processes and is in one of the Top 20 Hazardous Substances on the CERCLA priority list (http://umbbd.msi.umn.edu). Pyrene can be biodegraded by bacteria and fungi through initial dioxygenase (bacteria) and monooxygenase (bacteria and fungi) reactions.

The initial attack by a dioxygenase occurs at different points of the pyrene molecule leading to 1,2- or 4,5- pyrene diol. The trans-4,5 pyrene dihydro diol is not converted further. Similarly, the 4-hydroxyperinaphenon formed by the oxidation of 1,2-diol is also not metabolized further. However, the 4, 5- pyrene diol undergoes cleavage which after decarboxylation to 4-phenantheric acid after one ring elimination enters the phenanthrene oxidation pathway. Heitkamp et al (1988) were among the first to report the enzymatic mechanisms and ring fission products for the microbial metabolism of pyrene. They found the formation of both cis- and trans- 4,5 dihydrodiols of pyrene by their Mycobacterium sp. Indicating the possible presence of multiple oxidation pathways for pyrene degradation. The Mycobacterium sp. strain CH1 could mineralize pyrene while using it as the sole source of carbon and energy but had a three day lag phase during pyrene mineralization (Churchill et al, 1999). The genetics of PAH metabolism in bacteria has been reviewed by Van Hamme et al (2003).

Fungi are known to oxidize PAH but are unable to utilize them as the sole carbon source and energy (Field et al, 1992). While most attention has been focused on lignolytic fungi that can cause oxidation of PAH by their non-specific extracellular enzymes, PAH metabolic pathways known in other fungi include the hydroxylation by cytochrome P450 monooxygenases. This pathway is known in many non-lignolytic fungi. Capotorti et al (2004) isolated a fungal strain Aspergillus terreus from a PAH polluted soil and found that this isolate mainly metabolized pyrene to pyrene sulfate. The filamentous strain Penicillium janthinellum SFU403, on the other hand, oxidized pyrene to pyrene 1,6 and 1,8 quinones (Launen et al, 1995). Launen et al (2000) also demonstrated that the intracellular reductants NADPH and glutathione can reduce both these quinones to their corresponding semiquinone anion radicals. Cyclothyrium sp. produced four metabolites from pyrene after 96 h incubation which were trans-4,5-dihydroxy-4,5-dihydropyrene (pyrene trans-4,5-dihydrodiol), pyrene-1,6-quinone, pyrene-1,8-quinone and 1-hydroxypyrene (da Silva et al, 2003, 2004). Pyrene metabolism by Aspergillus niger yielded two major and one minor metabolite. The spectrum of the metabolite matched that of 1,6 dihydroxy pyrene (Sacka et al 1997). Lange et al (1994) isolated and identified two novel metabolites during the cometabolism of pyrene, by the plant-inhabiting
basidiomycete *Crinipellis stiparia* JK364, in medium containing malt extract, glucose and yeast extract.

**Figure 2.4** The oxidation of pyrene by monooxygenases. This pathway was contributed by Nathaniel Keith, University of Minnesota.
Biodegradation of phenanthrene

The PAH phenanthrene is not mutagenic or carcinogenic it is toxic to marine diatoms, crustaceans and fish. It is a tricyclic aromatic hydrocarbon and the smallest aromatic hydrocarbon to have a "bay-region" and a "K-region" making it a model substrate for metabolic studies of carcinogenic PAHs (http://umbbd.msi.umn.edu).

Phenanthrene can be metabolized through various routes. It is initially oxidized by bacteria in 1,2- or 3,4- position to form the corresponding cis-dihydriodols (Figure 2.5). Some *Pseudomonas* and *Nocardia* sp. oxidize this to 3,4 dihydroxyphenanthrene which is further converted to 1-hydroxy-2-naphthoic acid. After decarboxylation to 1,2-dihydroxy naphthalene it can enter the naphthalene metabolic pathway (Evans, 1965 as cited in Kästner, 2000). Certain other bacteria like *Aeromonas*, *Vibrio*, *alcaligenes* and *Micrococcus* sp. form dicarboxylic acid intermediates on further oxidation from 1-hydroxy-2-naphthoic acid (Kiyohara *et al*, 1976 in Kästner, 2000). The *Mycobacterium* sp. strain CH1 was capable of mineralizing phenanthrene while using it as the sole carbon and energy source with a lag phase of just one day as compared to three days in pyrene mineralization (Churchill *et al*, 1999).
Figure 2.5 The oxidation of phenanthrene. This pathway was contributed by Jun Ouyang, University of Minnesota.
**Biodegradation of NSO containing hydrocarbons**

Heterocyclic aromatic hydrocarbons (PAH) containing N, S and O atoms can also be considered PAH in a broad sense because of some comparable characteristics (Sims and Overcash, 1983: orns).

Microbial metabolism of homocyclic nitroaromatics occurs by:

1. an initial oxygenation reaction yielding a nitrite
2. a reduction yielding aromatic amines that could be further metabolized
3. complete reduction of the nitro group yielding nitrite
4. partial reduction of the nitro group to hydroxylamine which after a replacement can be metabolized further

as explained by Marvin-Sikkema and De Bont (1994) and cited in Blotevogel and Gorontzy (2000). The aromatics resulting from this reaction are further degraded by known routes of hydrocarbon degradation. Nitroaromatic compounds can serve as nitrogen as well as carbon sources. While some bacteria oxidatively remove the nitro group leaving the benzene ring intact, other bacteria are able to degrade nitrophenols and nitrobenzoates. These include *Pseudomonas* and *Nocardia* strains. As reviewed by Blotevogel and Gorontzy (2000), there are four different ways for the transformation of the nitro group by aerobic microorganisms. First is by a monooxygenase catalyzed reaction converting 4-nitrophenol to catechol. The second is by the insertion of two hydroxyl groups by a dioxygenase with the subsequent removal of the nitro group as nitrite. Third is the reduction of nitro group to hydroxylamine as observed in *Comamonas acidovorans*. And the fourth is the partial reduction of the benzene ring, nucleophilic addition of the hydride ion and elimination of the nitro group as nitrite subsequently.

Biodesulfurization of DBT, on the other hand, occurred via the 4S pathway (sulfoxide-sulfone-sulfonate-sulfate) (Isbister 1986; Isbister and Kobylnski, 1985), which lead to the formation of biphenyl or hydroxyphenyl derivatives (o’-o’- biphenyl and monohydroxybiphenyl) with the release of sulfur as sulfate (Kilbane, 1990). This pathway retains the full fuel value of DBT while removing sulphur selectively. This mechanism is based on a progressive oxidation of the thiophene heteroatom, followed by the cleavage of the C-S bonds. The 4S pathway reactions are shown in figure 2.6.
Figure 2.6 The DBT 4S oxidation pathway. This pathway was contributed by Dr. Margie Romine, Pacific Northwest National Lab, and updated by Dr. Kevin Gray, Energy BioSystems Corp. (University of Minnesota: umbbd.ahc.umn.edu)
Biodegradation of resins and asphaltenes

Asphaltenes are a general class of aromatic types substances that form a non-volatile high molecular weight fraction of crude oil. They are generally referred to as the heptane (or pentane) insoluble fraction of crude oil. Asphaltenes are a stack of non homogenous sheets of condensed aromatic rings that may also contain Nickel and Vanadium. The complexity of their structures causes them to have no fixed melting point and hence generally remain solid in the oil. The asphaltenes that are dispersed in the crude oil are held together by resins. Resins and asphaltenes are believed to be the most recalcitrant fraction of crude oil and oily sludge. Capelli et al (2001) studied the degradation of crude oil contaminated mud formed as a result of secondary extraction collected from oil wells of south-west Argentina. While they noted extensive degradation of saturates and aromatic hydrocarbons, they also noted a degradation of approximately 20% of the resin fraction. The asphaltene fraction however, could not be degraded by the indigenous microbial flora. Hao et al (2004) also found a degradation of the resin and asphaltene fraction of crude oil alongwith the aliphatic and aromatic fraction using their thermophilic strain TH2. Pineda-Floris et al (2004) enriched a microbial consortium using asphaltenes as the sole source of carbon and energy. Identification of the bacterial strains after a steady state was reached in 10 weeks, revealed the presence of bacteria of the genera *Bacillus, Brevibacillus, Corynebacterium* and *Staphylococcus*. This bacterial consortium was shown to mineralize asphaltenes at room temperature and a pH of 7.4.