Biochemical and Genetic Studies on PAH degradation

Structure of catechol 2,3 dioxygenase
5.0 Discussion

Hydrocarbons are ubiquitous contaminants present in the environment. They are to be removed from the environment because of their toxic and mutagenic properties. Biotechnological intervention in managing hazardous waste is being recognized as ecologically sound and cost effective technology (Forsyth et al., 1995).

Polycyclic aromatic hydrocarbons are one of the main constituents present in crude oil. Their affinity to bind to biological molecules such as proteins and DNA render them carcinogenic and mutagenic (IARC, 1983; Klaassen et al., 1986). The lower molecular weight PAHs (containing 2 benzene rings fused together, e.g. naphthalene), have been studied most extensively amongst all PAHs. The studies on the degradation of higher molecular weight PAHs are limited because of extremely poor solubility and therefore low availability to the cells. PAHs in addition to being health risk also alter the microbial community in soil owing to the toxicity of not just the parent compounds but also the degradation intermediates formed either due to abiotic or biotic factors if not completely mineralized.

In any environment crude oil is subjected to both abiotic and biotic factors. Physicochemical methods of removing PAHs from the environment have been attempted successfully (Bossert and Compeau, 1995), however, the general practice for removing PAHs from the environment remains to be biological. A full-scale implementation of bioremediation for the treatment of hydrocarbon contaminated site is a multidisciplinary task involving components of microbiology, chemistry, engineering etc. Biological removal of PAH is either done by increasing the native microbial population through bioventing, nutrient addition etc., or by adding very high
concentration of laboratory grown native microflora (Shuttleworth and Cerniglia, 1995).

Microbial degradation of the environmental pollutant is an established fact. Invariably the strains that are found to be degrading the recalcitrant compound are obtained from an environment that has a history of contamination for which the remediation is sought (Schnieder et al., 1996).

In order to isolate a bacterial strain capable of degrading various aromatic hydrocarbons particularly high molecular weight PAHs, three consortia were developed from crude oil contaminated soils. The ability of crude oil degradation by these consortia were tested using Bombay High and Gujarat crude oil. All three consortia that were tested for their ability to degrade crude oil showed higher degradation of Bombay High crude oil. This was due to the fact that the sources of the crude oil at these two places vary in their composition. The Bombay High crude oil was found to be richer in lower alkanes C\textsubscript{12} onwards, while Gujarat crude oil had predominantly alkanes from C\textsubscript{18} onwards. This is consistent with the observation made by Cook et al., (1974) who demonstrated that biodegradation rates for crude oil vary with the source, due to the difference in their composition particularly the alkane fraction. Among the three consortia tested Consortium 3 showed the maximum ability to degrade aromatic fraction of the crude oil (16% of the 48% total crude oil degradation). Our observation also indicates that the 3 consortia had bacterial isolates that could degrade a variety of PAH compounds either individually or synergistically. Bouchez et al. (1995), demonstrated that individual bacterial strains that were capable of degrading some PAH as sole source of carbon, were inhibited by naphthalene. This inhibition effect was overcome when the strains were mixed together, indicating synergism. Similarly Brodkrob and Legge, (1992) showed that addition of \textit{Phanerochaete chrysosporium}
enhanced the mineralization of pyrene by 18%, over and above the degradation by native microflora.

In the present study we found that the major PAHs that were found to be degraded by Consortium 3 belonged to three major categories.

Homonuclear: naphthalene, phenanthrene, pyrene and chrysene.

Heteronuclear: fluoranthene, fluorene, cyclopentaphenanthrene and acenaphthene.

Heterocyclic: Dibenzothiophene.

Degradation of PAHs belonging to such diverse category by bacterial consortium could be due to the synergistic effect of various bacterial isolates or the consortia might contain predominantly certain class of bacteria that have wide range of substrate specificity (Stringfellow and Aitken, 1995).

Consortium 3 degraded both three and four-benzene ringed PAHs (high molecular weight PAHs) significantly compared to the other two consortia. Degradation of high molecular weight PAH by Consortium 3 was between 50-60% while those by Consortium 1 & 2 were less than 10%. The, Consortium 3 was further screened for isolating an efficient PAH degrading bacterial strain.

In general, degradation of hydrocarbon is more extensive by mixed culture than pure isolates, however, in few cases the degradation of hydrocarbons has been seen to be more extensive by pure isolates than mixed population (Walker and Colwell, 1976). We obtained 205 bacterial isolates from three bacterial consortia. These were screened for their crude oil degradation efficiency. The final selection was narrowed down to three bacterial isolates. The three bacterial strains designated as S19, S30 and P20 that were obtained had higher efficiency of crude oil degradation. Degradation of lower
molecular weight PAHs (mainly naphthalene and acenaphthene) was accomplished by all the three strains. However, P20 could degrade higher PAHs such as DBT, phenanthrene, anthracene, carbanthracene, cyclopentaphenanthrene, pyrene and chrysene, very efficiently, while S19 and S30 were unable to degrade these PAHs. Interestingly, when the alkane degradation efficiency of the three strains were compared, it was observed that the strains S19 and S30 could degrade both low and high molecular weight n-alkanes efficiently. Whereas P20 was inefficient in degrading the low molecular weight n-alkane but an efficient degrader of high molecular weight n-alkanes. This is in contrast to the general view that bacterial strains that utilize complex substrates can also degrade simpler substrate more readily. Therefore preferential utilization of aromatic and high molecular alkane reflects the diversity at the genetic level (Leahy and Colwell, 1990).

Such a property could be of significant implication in bioremediation of crude oil contaminated sites using a defined mixture of bacteria where the higher alkane fraction may be degraded by P20 while the degradation of lower fraction may be achieved by other bacterial strain either synergistically or concomitantly. However, it is important to point out that the maximum carbon chain length whose degradation could be quantified, was limited by

a. Extraction procedure: alkanes that are classified as waxes (>C₆₀) do not get dissolved at an intermediate temperature of 40⁰-50⁰C and therefore are eliminated from the analysis.

b. Chromatography: The Gas Chromatography uses columns whose matrices have certain range of resolution (C₃₈ in our case), beyond that the compounds are not detected even if they are present in the organic extracts.
The analysis of alkane fraction or their efficiency of degradation does not reflect the entire range of n-alkanes (or PAHs by similar argument). Therefore the comparison is relative rather than absolute.

The bacterial strain P20 was identified as *Alcaligenes odorans*, which is a Gram-negative bacterium. Alcaligenes sp. are known to metabolize recalcitrant compounds and heavy metals in the environment. For instance, *Alcaligenes eutropus* is known to metabolize heavy metals (Springael et al., 1993).

*A. odorans* in our observation was found to be resistant to seven different antibiotics. Bacterial multi-drug resistance is common for the strains that are isolated from the environment (Mathew et al., 1999; Nakaune et al., 1998). In view of the capacity of *A. odorans* to degrade high molecular weight PAHs biochemical investigations with pyrene as a model compound was chosen for the present study owing to its higher concentration than chrysene.

In order to determine the success of bioremediation a reliable estimate of *A. odorans* population surviving in the environment was required. Many strategies are available to enumerate microbial population in the environment such as most probable number (MPN), antibiotic resistance pattern, ELISA based detection systems, plasmid profiling, DNA:DNA colony hybridization using specific gene probes, PCR amplification of specific genes involved in degradation process etc. (Power et al., 1998; Stapleton et al., 1998). All these techniques are replete with their own limitations it is therefore essential to obtain the survival data from a combination of these techniques to arrive at any definite conclusion. RAPD primers that are not directed to any specific sequence have been extensively used in fingerprinting prokaryotes as well as eukaryotes. Often, the fingerprints produced by these RAPD primers are not
reproducible, and therefore are of little importance in monitoring/enumerating specific bacterial isolate in soil (Gao et al., 1996). Several investigators demonstrated that ERIC-PCR fingerprinting was highly reproducible and strain specific (Di Giovanni et al., 1999; Gao et al., 1996).

DNA fingerprinting based on families of short repetitive sequences found in enteric bacteria namely, repetitive extragenic palindrome (REP) and enterobacterial repetitive intragenic consensus (ERIC) have become popular in monitoring microorganisms in the environment because of high sensitivity of detection within a short time (de Bruijn, 1992; Versalovic et al., 1991). rep-PCR based fingerprinting approach have been successfully used for diagnosis of largely uncharacterized organisms as it requires no previous knowledge of DNA sequence. This method has an advantage that it does not rely on the presence of well-characterized genes such as those involved in degradation of hydrocarbons etc. Use of multiple screening procedure that are in agreement with the observations made by other techniques minimize the chance of error in making reliable/ exact estimates of population counts.

*Alcaligenes odorans* with its capacity to degrade a variety of high molecular weight PAHs and alkanes seemed to be a good candidate for bioremediation applications at sites contaminated with hydrocarbons predominated with alkane compounds. Bioremediation experiments were conducted in crude oil contaminated microcosm which was know to contain approximately 33% (w/w) of aromatic hydrocarbons. A combination of plate counting method and PCR-fingerprinting based on microbial repeat sequences was used to arrive at a realistic population estimate. We standardized the PCR conditions for generating species specific DNA fingerprint for monitoring the fate of *Alcaligenes odorans* in crude-oil contaminated microcosm.
In our study, we found that the microbial population in the microcosm declined significantly over a period of 30 days. This was not very unusual since microorganisms that are released into the environment often have difficulty in establishing themselves in the new ecological niche. There may be several reasons for the microbial population to decline constantly over a period of time in the field studies. These include nutrition status of the soil, moisture levels, microbial antagonism etc., apart from competing with the native microflora for nutrition, they must find effective means to survive the nematode and protozoan predators that thrive in such environments (Morgan et al., 1997).

We observed that the conventional plate counting method resulted in higher rates of survival of *A. odorans* (47.23%) as compared to the survival obtained from PCR fingerprinting (27.22%). This is expected since in natural environment several microorganisms have multi-drug resistance and therefore are selected on antibiotic plates. The sudden loss of *A. odorans* (like) population from the control plot can not be explained, as this loss is not reflected in the percentage (log %) survival of the population on kanamycin plate.

Survival of different toluene degrading *Pseudomonas* strains in soil was also monitored by Heurtas et al. (1998). They observed that when soil containing 10% of toluene was augmented with $10^8$ cfu/g cells of kanamycin resistant *P. putida* (capable of tolerating upto 10% of toluene in liquid medium) there was a decrease in population of kanamycin resistant bacterial count by 5-6 log units within 24 hours. Subsequently, they observed that the bacterial population increased to $10^6$ cfu/g. In absence of proper control mere increase in kanamycin counts can not be related to the survival of specific (introduced) bacterial isolate in a complex environment like soil. Heurtas et al., (1998) also demonstrated that recovery of bacterial strains from soil depended on the
temperature of incubation and humidity in soil and increased in the recovery was linked to the increase in moisture content. Another plausible explanation for obtaining low population count on the plate could be that many bacterial species have been shown to adapt a survival strategy known as 'non-culturable but viable' (NCBV), in response to environmental stress or when their population decrease below certain level (Vahjen et al., 1997; Tso and Tghon, 1999). Bacterial cells can not be cultivated using conventional techniques during NCBV phase. This may be another reason why bacterial population after some time in the environment reaches a minimal level.

Despite very low survival rates of A. odorans it was observed that degradation of PAHs was higher in the treated microcosm (23.49%) as compared to the control microcosm (16.97%). Further it was observed that the quantitative difference in PAH degradation was more striking when the aromatic fractions were analyzed on GC. In the treated microcosm, degradation of naphthalene, pyrene and DBT was very high compared to the control microcosm. The alkane degradation in the control microcosm was higher (37.73%) as compared to the treated microcosm (36.54%). This could be because A. odorans was grown on pyrene and therefore the genes and enzymes responsible for the metabolism of pyrene and other PAHs were induced. Therefore our strain could degrade pyrene and other PAHs more efficiently. Grosser et al., (1991) demonstrated that degradation of pyrene in soil was enhanced by 48% by introducing a native pyrene degrading bacteria at a concentration of $2 \times 10^9$ cfu/g. However, inoculation of the soil with $10^8$ cfu/g cells did not increase the degradation of pyrene above that of control because of high microbial activity at the site. Degradation of PAHs are subject to catabolite repression, e.g., succinate inhibited expression of toluene metabolism pathways (Duetz et al., 1994). Mlynarz and Wardop, (1995) showed that in
presence of an easily metabolisable carbon source such as glucose, the degradation of PAH was suppressed.

We conclude from our observation on quantification of biodegradation that, gravimetric analysis to quantify the degradation of complex contaminants like crude oil does not reveal the true picture. The difference in pyrene degradation of control and treated microcosm is approximately 58% whereas gravimetrically the degradation is not so obvious. Therefore, it is important to realize the limitations of each of the quantification methods before deriving any inference from such studies.

In order to utilize the capacities of microorganisms to their optimal level of efficiency it is important to understand the biochemical pathways of metabolism of recalcitrant compounds.

Alcaligenes odorans has the ability to degrade many high molecular weight PAHs. Two such compounds were pyrene and chrysene. The concentration of pyrene and chrysene (four benzene ringed PAHs) in crude oil was found to be 3.37% and 2.41% respectively in the contaminated microcosm soil used in this study. The concentrations of these compounds in Bombay High crude oil were different with pyrene having significantly higher levels than chrysene (Fig. 9). In view of the toxic effect of the oxygenated intermediates of pyrene, it was of interest to study the degradation pathway for pyrene degradation to ascertain whether the oxygenated pyrene intermediates were being accumulated inside the cell.

Growth of A. odorans on pyrene proceeded without any apparent lag phase. This observation is consistent with the fact that pyrene degradation is limited by its solubility. Tiehm, A. (1994) also observed linearity in bacterial growth when phenanthrene was supplied in crystalline form as carbon source. The near linear
growth follows with a concomitant linear disappearance of pyrene from the medium. We observed that the addition of non-ionic detergents to increase the availability of pyrene resulted in enhanced growth. After 48 hours of growth the lysis of cells prevented further metabolism of pyrene (data not shown). Tiehm (1994) argued that for surfactants/detergent to act effectively on increasing the bioavailability, the critical micellar concentration (CMC) should be determined for individual surfactants. CMC values can also be influenced by microbial growth and therefore the CMC value should be determined in presence of microbial growth.

Several reports of PAH degradation by cometabolism are known (Mueller et al., 1990; Walter et al., 1991), however degradation as sole carbon source are limited. Alcaligenes odorans grown on pyrene as sole carbon source had a specific growth rate of 0.087 h\(^{-1}\) which is faster than that of Mycobacterium (Boldrin et al., 1993), which had a specific growth rate of 0.056 h\(^{-1}\). These growth rates are far too slow compared to other bacteria that grow on easily metabolizable substrates such as glucose. Since PAHs have limited water solubility and therefore the availability of these compounds for degradation are limited by solubility (Shuttleworth and Cerniglia, 1995). A. odorans grown on pyrene produced several degradation intermediates (Fig. 18 & 19). The identity of only two of these could be ascertained using automated search of the fragmentation pattern database on GC-MS, they were 1,2,3,4-tetrahydro-1,2-dihydroxy naphthalene and 4-phenanthene carboxylic acid. Similarly, Dean-Ross and Cerniglia (1996) obtained several peaks on the total ion chromatogram, yet identity of only three of them could be established. This could be due to the limitation of the database, which may not contain all possible degradation intermediates in its library. Growth of A. odorans on the degradation intermediate could provide an insight into the pathway.
Degradation pathway for pyrene in *Mycobacterium flavence* involves the formation of a dihydroxy-pyrene (DHP) by dioxygenase activity. DHP is metabolized further to phenanthrene dicarboxylic acid (three ring intermediate) followed by its decarboxylation to form 4-phenanthrene carboxylic acid. Dean-Ross and Cerniglia (1996) detected 4-phenanthroic acid during bacterial metabolism of pyrene. We could also detect this intermediate in *A. odorans*, and propose a mechanism for pyrene degradation in *A. odorans* during initial stages, analogous to that operative in *Mycobacterium flavence*. Subsequent metabolism of pyrene to 1,2,3,4-tetrahydro-1,2-dihydroxy naphthalene (two ringed intermediate) occurs by some unknown mechanism, which has not been reported so far in any study on pyrene degradation. The detection of three and two benzene ring compounds from pyrene gives an indication that sequential ring cleavage reactions might take place during the metabolism of pyrene. However, we do not have a time course experiment's data showing appearance of the 3 ringed intermediate before the 2 ringed intermediate. The two ringed intermediate is a novel intermediate in pyrene metabolism and could be metabolized further to CO₂. We could detect upto 3% of mineralization of pyrene, over and above the cell minus control. The low levels of mineralization could be either due to asymmetric labeling of carbon in pyrene or due to toxicity of the intermediates. It would be of interest to study the toxicity of the intermediates formed during PAH metabolism. Mineralization by Gram-positive organisms *Mycobacterium* and *Rhodococcus* sp. have been reported to very high [58-62%] (Heitkamp and Cerniglia, 1988; Walter *et al*., 1991). The available information on mineralization of pyrene by Gram-negative organisms and fungi range from 0.4-4% (Bezalel *et al*., 1996; Sack *et al*., 1997a).
Inhibitor studies on uptake of pyrene suggest that the uptake of pyrene is blocked completely by certain Ionophores, ATPase inhibitors and electron transport chain inhibitors. Contrary to the notion that hydrocarbons enter cells by passive diffusion our observation seems to suggest that uptake of pyrene depends on divalent metal ion and active energy generation system. Lal and Khanna (1996) also showed that the uptake of a lipohilic n-alkane, C18 (hydrocarbon) was energy dependent.

*Alcaligenes odorans* was screened extensively for the presence of a degradative plasmid using specific isolation methods and PFGE. The presence of plasmid in this strain was not detected, indicating that the genes responsible for pyrene degradation were present on the chromosomal DNA. Goyal and Zylstra (1996) have also observed presence of PAH degrading genes on chromosome. Screening of genomic libraries constructed in from *A. odorans* revealed no positive clone on mineral agar plates containing pyrene. Non availability of degradation intermediates prevented us from growing the clones on the intermediates to test if the clones had genes that are expressed lower down in the metabolic sequence of pyrene degradation. Almost all of the reports of gene cloning for PAH degradation that have come up so far have been through naphthalene or phenanthrene degradation. The clones from naphthalene degrading bacterial isolates were tested for the substrate utilization pattern by Zylstra *et al.*, (1994). It was seen that besides naphthalene the clone could utilize several PAHs biphenyl, anthracene, fluorene, fluoranthene, pyrene, acenaphthylene and chrysene. However little or no homology of the genes cloned from *Pseudomonas* was seen across species or among strains capable of utilizing higher molecular weight PAHs. This indicates that there exists a diversity of genetically distinct pathways for PAH degradation.
Dagher et al., (1997) cloned genes for upper pathway of naphthalene degradation using a consensus primer made from *pah*, *nah* and *dox* operons. We attempted a similar approach to clone the gene(s) involved in PAH degradation. We observed that there was no homology between the genes that code for benzoate degradation and genes encoded for pyrene degradation in *A. odorans*. Degenerate primer (Daly et al., 1997) for catechol-2,3- dioxygenase was used to amplify the gene from *A. odorans* using a PCR. The positive control (plasmid pY3; kindly gifted by Yujing Yang) showed a single band at 763 bp (expected size) while *A. odorans* did not produce any band (data not shown).

In order to isolate genes that are expressed in response to stress (PAH being a carbon stress), subtractive hybridization is extensively used in eukaryotes. Presence of polyadenylated mRNA contributes to the success of this technique in eukaryotes. However, Gething et al. (1980) used *in-vitro* polyadenylation and subtractive hybridization successfully to isolate a gene from influenza virus. It is intriguing that such a strategy has not been very popular in prokaryotes. The use of a yeast poly(A)poymerase has been demonstrated in specific poly adenylation of mRNA (Amara and Vijaya, 1997). We used *E. coli* poly(A)polymerase to non specifically polyadenylate the total RNA and construct an immobilized cDNA library. Three rounds of subtraction leads to the generation of a normalized cDNA library. Because of the kinetics of hybridization the most abundant RNA is removed faster than low abundance RNA. Subtractive hybridization using immobilized cDNA from glucose grown *A. odorans* and RNA from pyrene grown cells was performed without much success, suggesting that the genes for pyrene degradation in *A. odorans* could be expressed constitutively. The strategy outlined in this work can be used for isolating genes from prokaryotes that are expressed differentially.