Biochemical and Genetic Studies on PAH degradation

Results

Structure of catechol 2,3 dioxygenase
4.0 Results

4.1 Composition of crude oil

In the present study Bombay High crude oil was used extensively, whose composition was determined by fractionating the crude oil on silicagel column. Upon gravimetric analysis of individual fraction obtained from silicagel column chromatography, Bombay High crude oil was found to be containing approximately 60% alkanes, 25% aromatics and 8% NSO compounds (Fig. 1). Since asphaltenes are a class of compounds that are insoluble in most organic solvents, the weights of the three fractions were added and the value was subtracted from the total weight of crude oil taken for fractionation to determine the amount of asphaltenes present in the crude oil. Thus, Bombay High crude oil was found to contain 7% of asphaltenes.

4.2 Degradation of crude oil by bacterial consortia

In order to isolate a bacterial strain capable of degrading polycyclic aromatic hydrocarbons (PAHs), three bacterial consortia were developed from crude oil contaminated soil. These consortia were tested for their ability to utilize crude oil as sole source of carbon and energy. The bacterial consortia were grown on either Bombay High crude oil or Gujarat crude oil (1%; w/v) as described in methods (section 3.2.1.1). After 15 days of growth the residual crude oil was extracted and quantified gravimetrically to determine the degradation efficiency of each consortium. Consortium 1 could degrade up to 28% of Gujarat crude oil and 38% of Bombay High crude oil.

Consortium 2 could degrade up to 40% of Gujarat crude oil while it degraded up to 61% of Bombay High crude oil. Similarly, Consortium 3 could degrade up to 35% of
Figure 1: Composition of Bombay High crude oil

Bombay High crude oil was fractionated on silicagel column (section 3.2.1.2). The percent composition was determined gravimetrically with respect to the total crude oil loaded on silicagel column.
Gujarat crude oil and 49% of Bombay High crude oil (Fig. 2). The degradation percentages were calculated by taking the natural weathering of respective crude oils in mineral salt medium at 37°C as control.

4.3 Degradation of individual fractions of crude oil by bacterial consortia

After 15 days of incubation and subsequent extraction of the residual crude oil and determination of degradation efficiency, the residual Bombay High crude oil showing higher degradation than Gujarat crude oil, was fractionated on silicagel column. It was observed that Consortium 1 could degrade up to 27% of alkanes (total crude oil degradation 38%), 9% of aromatics and 2% of NSO. Consortium 2 could degrade up to 48% of alkanes (total crude oil degradation 60%), 9% of aromatics and 3% of NSO. Similarly Consortium 3 could degrade up to 28% of alkanes (total crude oil degradation 48%), 16% of aromatics and 4% of NSO (Fig. 3). In all cases the degradation of NSO fraction was obtained from the difference in the weight of the total crude oil degradation and the sum of the degradations of alkanes and aromatics fractions.

4.4 Degradation of various PAH compounds by bacterial consortia

The aromatic fraction obtained after 15 days of growth of bacterial consortia on Bombay High crude oil and fractionation of the residual crude oil was further analyzed by Gas Chromatography. The reduction in peak areas of individual PAHs compared to the uninoculated control was determined after integrating the chromatogram peak areas. Degradation percentages of individual PAH compounds were calculated as described in methods (section 3.2.1.3). The major PAHs that were degraded substantially were naphthalene, acenaphthene, fluoranthene, dibenzothiophene, phenanthrene, anthracene, cyclopentaphenanthrene, fluorene, pyrene and chrysene. As shown in figure 4, Consortium 1 could degrade the PAHs in the following manner,
Figure 2: Degradation of Gujarat and Bombay High crude oil by bacterial consortia.

Bacterial consortia were grown on mineral salt medium with 1% (w/v) Gujarat or Bombay High crude oil as sole source of carbon and energy. Residual crude oil was extracted after 15 days of growth and degradation was quantified gravimetrically (section 3.2.1). Degradation percentage represents the loss compared to the initial oil concentration at zero time. The values above the bar represent degradation percent.
Figure 3: Degradation of different fractions of Bombay High crude oil by bacterial consortia.

Bacterial consortia were grown on mineral salt medium with Bombay High crude oil (1%; w/v) as sole source of carbon and energy. After 15 days of growth the residual crude oil was extracted, fractionated on silicagel column and quantified gravimetrically (section 3.2.1). Degradation percentage represents the loss compared to the initial oil concentration at zero time. Degradation of NSO fraction was quantified by the difference in the weights of total crude oil degradation with the sum of the degradation of alkane and aromatic fractions. The values above the bars represent degradation percent.
Figure 4: Degradation of aromatic fraction of Bombay High crude oil by bacterial consortia.

Bacterial consortia were grown on mineral salt medium with Bombay High crude oil as sole source of carbon and energy. Residual crude oil extracted after 15 days of growth was fractionated on silicagel column. Aromatic fractions were analyzed using Gas Chromatography and degradation percentages were calculated using the regression factor obtained for each of the standard PAH compound (section 3.2.1.3).
naphthalene 60%; acenaphthene 54%; fluorene 8.2%; dibenzothiophene 9.74%;
phenanthrene 33.3%; anthracene 6.15%; cyclopentaphenanthrene 2%; fluoranthene
2%, pyrene 4.6% and chrysene 2%. Consortium 2 could degrade the PAHs in the
following manner, naphthalene 72%; acenaphthene 58.5%; fluorene 33.33%;
dibenzothiophene 42.6%; phenanthrene 52.3%; anthracene 15%;
cyclopentaphenanthrene 2%; fluoranthene 6.15%; pyrene 10.25% and chrysene 6.15%.
Similarly, Consortium 3 could degrade the PAHs in the following manner, naphthalene
100%; acenaphthene 100%; fluorene 97.4%; dibenzothiophene 90.96%; phenanthrene
80%; anthracene 80%; cyclopentaphenanthrene 47.7%; fluoranthene 52%; pyrene 45%
and chrysene 51%.

The degradation percentages represent the reduction in peak area of the respective
PAHs compared to the uninoculated control kept for the same period.

4.5 Degradation of various n-alkanes by bacterial strains

As shown in figures 3 & 4, Consortium 3 had the most efficient aromatic hydrocarbon
degrading bacterial population. Therefore, this consortium was further screened to
isolate the most efficient PAH degrading bacterial strain. Three bacterial strains S19,
S30 and P20 were found to be the most efficient crude oil degrading bacterial strains.
These bacterial strains were grown on crude oil and individual fractions (alkane and
aromatic) of the crude oil were analyzed. Degradation percent of the individual alkane
was determined as described previously. The major straight chain alkanes that were
found to be degraded significantly were C_{12}, C_{14}, C_{16}, C_{18}, C_{20}, C_{22}, C_{24}, C_{26}, C_{28}, C_{30} and
C_{32}. As shown in figure 5, bacterial strain S30 degraded various alkanes in the following
manner, C_{12} 89.4%; C_{14} 89.4% C_{16} 89.4%; C_{18} 97.5%; C_{20} 92.4%; C_{22} 94.6%; C_{24} 93.5%;
C_{26} 91.7%; C_{28} 91.2%; C_{30} 91.7% and C_{32} 92.4%, while bacterial strain S19 degraded the
Figure 5: Degradation of various n-alkanes by bacterial strains.

Bacterial strains were grown on mineral salt medium with Bombay High crude oil as sole source of carbon and energy. Residual oil extracted after 15 days of growth was fractionated on silicagel column. Alkane fraction was analyzed using Gas Chromatography and degradation percentages were quantified using the regression factor obtained for each of the standard alkanes (section 3.2.1.3).
alkanes in following manner, C_{12} 89.1%; C_{14} 91% C_{16} 91.7%; C_{18} 97.5%; C_{20} 87.6%; C_{22} 83.5%; C_{24} 91%; C_{26} 90.5%; C_{28} 90.5%; C_{30} 91.7% and C_{32} 91.7%. Similarly the bacterial strain P20 could degrade various alkanes in the following manner, C_{12} 73.5%; C_{14} 40% C_{16} 37%; C_{18} below detection; C_{20} 49%; C_{22} 74%; C_{24} 78.4%; C_{26} 77.7%; C_{28} 68.2%; C_{30} 83.6% and C_{32} 87.6%.

The degradation percent represent the reduction in peak area compared to the uninoculated control kept for the same period of time.

4.6 Degradation of various PAH compounds by bacterial strains

The aromatic fraction obtained after 15 days of growth of bacterial stains on Bombay High crude oil was further analyzed by Gas Chromatography. The reduction in peak areas of individual PAHs compared to the uninoculated control was determined after integrating the chromatogram peak areas. Degradation percentages of individual PAH compounds were calculated as described in methods (section 3.2.1.3). The major PAHs that were degraded substantially were naphthalene, acenaphthene, fluoranthene, dibenzothiophene, phenanthrene, anthracene, cyclopentaphenanthrene, fluorene, pyrene and chrysene. As shown in figure 6, bacterial strain S30 could degrade the PAHs in the following manner, naphthalene 67.74%; acenaphthene 69.68%; fluorene 23.7%; dibenzothiophene 0.48%; phenanthrene 0.48%; anthracene 0.48%; carbanthracene 0.48%; cyclopentaphenanthrene 0.48%; pyrene 0.48% and chrysene 0.48%; while the bacterial strain S19 could degrade the PAHs in the following manner, naphthalene 80.32%; acenaphthene 49.84%; fluorene 0.48%; dibenzothiophene 0.48%; phenanthrene 0.48%; anthracene 12.58%; carbanthracene 0.48%; cyclopentaphenanthrene 0.48%; pyrene 0.48% and chrysene 0.48%. Similarly, bacterial strain P20 could degrade the PAHs in the following manner, naphthalene...
Figure 6: Degradation of various PAHs by bacterial strains

Bacterial strains were grown on mineral salt medium with Bombay High crude oil as sole source of carbon and energy. Residual crude oil extracted after 15 days of growth was fractionated on silicagel column. Aromatic fraction was quantified using Gas Chromatography (section 3.2.1.3). The degradation percentage was quantified using the regression factor obtained for each of the PAH.
83.71%; acenaphthene 83.76%; fluorene 70.65%; dibenzothiophene 45%;
phenanthrene 60%; anthracene 48.87%; carbanthracene 47.9%;
cyclopentaphenanthrene 27.10%; pyrene 61.45% and chrysene 64.84%.

The degradation percentages represent the reduction in peak area of the respective
PAHs compared to the uninoculated control kept for the same period.

4.7 Identification of the bacterial strain P20

Amongst the three bacterial strains P20 was found to be the most efficient PAH
degrading bacteria (Figs. 5&6). Morphological characterization of the bacterial strain
revealed that the bacteria was Gram-negative cocci and formed colonies of 2-3 mm
diameter. On the basis of morphological, physiological and biochemical tests the
bacterial strain was characterized by Institute of Microbial Technology (IMTECH;
Chandigarh) as Alcaligenes odorans. We shall refer to the strain P20 as Alcaligenes
odorans henceforth throughout the text.

4.8 Antibiotic resistance profile of Alcaligenes odorans

In order to have a selectable antibiotic marker in Alcaligenes odorans, 14 different
antibiotics were screened. Alcaligenes odorans was plated on LA plate and filter discs
containing antibiotics (50 µg/disc) were placed on them. A zone of clearance was
produced around the filter discs indicating sensitivity of the strain, while mat growth
was observed when the strain was resistant to antibiotics. Based on this screening it
was observed that Alcaligenes odorans was resistant to kanamycin, ampicillin,
penicillin, vancomycin, carbenicillin, spectinomycin and amoxycillin at a final
concentration of 50 µg/ml.
4.9 Degradation of crude oil by \textit{Alcaligenes odorans}

\textit{Alcaligenes odorans} was grown on mineral salt agar Petri plate containing 1\% (w/v) Bombay High crude oil as sole source of carbon and energy. The Petri plate containing crude oil but without any bacterial inoculum was used as negative control. The Petri plate containing \textit{Alcaligenes odorans} produced distinct zones of clearance (Fig. 7) with colonies growing at the centre of each circular zone of clearance produced. The control Petri plate did not produce any zone of clearance after having incubated under identical conditions.

\textit{Alcaligenes odorans} was grown in mineral salt medium containing crude oil (1\%; w/v) from either Bombay High or Gujarat refinery, as described previously. After 15 days of growth, the residual crude oil was extracted and quantified gravimetrically. \textit{Alcaligenes odorans} degraded 45\% of Bombay High crude oil and 37\% of Gujarat crude oil. The residual crude oil was fractionated to determine the degradation of individual fraction. It was observed that \textit{Alcaligenes odorans} degraded up to 27\% of alkanes, 15\% of aromatics and 3\% of NSO fractions of Bombay High crude oil, while it could degrade up to 17.5\% of alkanes, 18.4\% of aromatics and 1.1\% of NSO fractions of Gujarat crude oil (Fig. 8). Determination of the degradation of NSO fraction was done as described previously.

4.10 Quantification of PAH degradation in crude oil by \textit{Alcaligenes odorans}

The residual aromatic fraction from the degradation of Bombay High crude oil by \textit{Alcaligenes odorans} was analyzed by Gas Chromatography. Degradation of the major PAH compounds present in the crude oil was quantified as described previously. Gas Chromatographic profile of the aromatic fraction shows significant reduction in peak areas of fluorene, dibenzothiophene (DBT), cyclopentaphenanthenrene, fluoranthene,
Figure 7: Degradation of crude oil on plate by Alcaligenes odorans.

Alcaligenes odorans was plated on mineral salt medium with Bombay-High crude oil as sole source of carbon and energy. The plates were incubated at 37°C for 15 days and the zone of clearance was observed.
Figure 8: Degradation of crude oil and its various fractions by *Alcaligenes odorans*

*Alcaligenes odorans* was grown on mineral salt medium containing crude oil for 15 days. Degradation was quantified gravimetrically by solvent extraction and fractionation on silicagel column (section 3.2.1.2). Percentage degradation of alkane, aromatic and NSO fractions represent the loss compared to the initial oil concentration at zero time. Degradation of NSO was quantified by difference of total crude oil degradation from the sum of alkane and aromatic fractions. The values above the bars represent degradation percent.
pyrene, chrysene and perylene (Fig.9). Degradation of individual PAH was quantified from the reduction in peak area of the individual PAH compound. It was observed that *Alcaligenes odorans* degraded up to 51% of fluorene, 50.6% of DBT, 37.2% of cyclopentaphenanthrene, 22% of fluoranthene, 52.8% of pyrene, 81.4% of chrysene and 94.8% of perylene (Fig. 10).

Owing to its very high concentration in crude oil and complexity in structure (four-ringed PAH; Table 2) we chose pyrene as a model compound in the present study.

**4.11 Generation of molecular marker**

Success of any bio-augmentation study depends on the enumeration of the added microbial population and disappearance of the target compounds. In order to track the added bacteria, it is essential to have enumeration methods that are quick and accurate. DNA fingerprinting of *Alcaligenes odorans* using microbial repeat sequences (ERIC-PCR) was standardized to develop molecular marker that could become a useful tool in enumerating *A. odorans* in the environment.

**4.11.1 Strain specificity of ERIC-PCR**

DNA fingerprinting of various *E. coli* strains was performed to test if the banding pattern generated by ERIC-PCR was specific enough (Fig. 11). Fingerprints generated for three *E. coli* strains, JM109, K12 and HB101 were different from each other. The strain JM109 had distinct bands at approximately 1.5Kb, 900bp and 100bp (Lane III; Fig. 11). *E.coli* K12 had a single band at 100bp (Lane IV), while *E. coli* HB101 had major bands at 1Kb and 500bp (Lane V).

DNA fingerprinting of *Alcaligenes odorans* was standardized to give identical fingerprints over a wide range of DNA concentrations. It is clear from figure 11 (Lanes
Figure 9: Gas Chromatographic profile of the degradation of aromatic fraction of crude oil by *Alcaligenes odorans*.

Aromatic fraction was obtained from Bombay High crude oil after silica gel chromatography as described in figure 8. The aromatic fraction was dissolved in acetone and analysed by Gas Chromatograph (section 3.2.1.3).
Figure 10: Degradation of pure PAH compounds by Alcaligenes odorans.

Alcaligenes odorans was grown on aromatic fraction and the PAHs were analyzed by Gas Chromatography. The degradation percentage was quantified using the regression factor obtained for each of the PAH (section 32.1.3). The values above the bar represent degradation percent.
Figure 11: Sensitivity of ERIC-PCR in differentiating between bacterial strains and effect of bacterial DNA concentration.

DNA of various bacterial strains were fingerprinted using ERIC-PCR (3.2.5.6).

M= 100 bp marker DNA; Lane II= Negative Control; Lane III= E. coli JM109 DNA; Lane IV= E.coli K12 DNA; Lane V= E.coli HB101; Lanes VI-IX= 1, 10 and 100 fold diluted Alcaligenes odorans DNA respectively.
VI-IX) that ERIC fingerprint of *Alcaligenes odorans* remains identical at 1, 10 and 100 fold dilution of DNA, while at 1000 fold dilution some of the major bands disappear from the fingerprint.

### 4.11.2 Species and genus specificity of ERIC-PCR

ERIC-PCR was standardized and checked for its sensitivity. ERIC fingerprint was obtained for various *Alcaligenes* typed strains obtained from Microbial Type Culture Collection, IMTECH, Chandigarh, India. We found that the fingerprints generated were highly species and genus specific (Fig. 12). *Alcaligenes odorans* had four major bands at 2Kb, 900bp, 500bp and 450bp under the conditions standardized for its fingerprinting (Lanes V & VI; Fig. 12). ERIC fingerprint was genus specific as seen in Lanes I & II, *Pseudomonas putida* had two major bands at approximately 800bp and 600bp. Similarly, other *Alcaligenes* species produced characteristic fingerprint but none that match those produced by *Alcaligenes odorans*. Their banding pattern showed variance depending on DNA concentration because the PCR parameters were standardized for *Alcaligenes odorans* DNA.

### 4.12 Degradation of crude oil in microcosm constructed from refinery soil

Total extractable petroleum hydrocarbon from soil at zero time was 1.680 g/10 g (16.8% w/w). Gravimetric quantification of crude oil degradation was done at intervals of 10 days (Fig. 13). The total extractable crude oil degraded in microcosms treated with *A. odorans* after 30 days was approximately 60.03% (w/w; of 1.680 g extractable at zero time), while degradation of crude oil in control microcosm was 54.7%. The total extractable hydrocarbon obtained from the treated microcosms after 30 days was fractionated into alkane and aromatic fraction on silicagel column. The fractions were analyzed on GC.
Figure 12: Species specific fingerprinting by ERIC-PCR of various *Alcaligenes* species.

Typed strains obtained from IMTECH were grown on IA, their genomic DNA was extracted and fingerprinted using ERIC PCR. The amplicons were resolved on 2% (w/v) agarose gel. Major bands of interest in *Alcaligenes odorans* are marked 1, 2, 3 and 4.

M = 1 Kb ladder; Lane II = *Pseudomonas putida* DNA; 1μl; Lane III = *Pseudomonas putida* DNA 2μl; Lane IV = *Alcaligenes paradoxus* DNA 1μl; Lane V = *Alcaligenes paradoxus* DNA 2μl; Lane VI = *Alcaligenes odorans* DNA 1μl; Lane VII = *Alcaligenes odorans* DNA 2μl; Lane VIII = *Alcaligenes eutropus* DNA 1μl; Lane IX = *Alcaligenes eutropus* DNA 2μl.
Figure 13: Degradation of various fractions of total petroleum hydrocarbon by *Alcaligenes odorans* in microcosm.

Residual crude oil from microcosm was extracted and quantified gravimetrically after 30 days. The crude oil was fractionated into alkanes and aromatic fractions on silicagel column and the fractions were quantified gravimetrically (section 3.2.1). *Alcaligenes odorans* was added to the treated microcosm with mineral salt medium as source of nutrient. Control plate contained TPH contaminated soil and mineral salt medium without *Alcaligenes odorans*, but contained indigenous microflora. The values above the bars represent degradation percent.
It was seen that the control microcosm had microbial population that contained efficient alkane degrader. Total alkanes degraded in control microcosm was 37.73% while in the Alcaligenes odorans treated microcosm the degradation of alkane was 36.54% (Fig. 13). Total aromatic hydrocarbon degradation in the microcosm augmented with Alcaligenes odorans was higher, 23.49%, compared to its degradation in control microcosm of 16.97%. Degradation of pyrene was quantified in the control and Alcaligenes odorans treated microcosm. In the control microcosm the degradation of pyrene was only 4% while, Alcaligenes odorans treated microcosm degraded 66.55% of pyrene (Fig. 14a). GC analysis of the aromatic fraction showed that apart from pyrene several other PAHs were also degraded significantly in the microcosm augmented with A. odorans. These were: 65.96% naphthalene, 68.43% fluorene, 58.05% dibenzothiophene, 74.35% cyclopentaphenanthrene, 65.26% chrysene and 45.92% perylene. Similarly, in the control microcosm the degradation of these PAHs were as following: 1.86% naphthalene, 36.86% fluorene, 4.35% dibenzothiophene, 50.16% cyclopentaphenanthrene, 27.35% chrysene and 19.44% perylene (Fig. 14a & 14b).

4.13 Microbial population count in microcosm constructed from refinery soil

The total bacterial count obtained on LA palates without any antibiotic (total count) revealed that the soil had a bacterial load of $10^7$ cfu/g soil that could be grown on LA plate, while the antibiotic selective plating on kanamycin gave $5.5 \times 10^3$ resistant bacterial strain. The microcosm was augmented with $3 \times 10^9$ cfu/g of A. odorans and the population was found to decrease continuously with time to $5 \times 10^3$ cfu/g in 30 days from a kanamycin resistant bacterial population at zero time of $4.7 \times 10^7$ cfu/ml (immediately after augmentation) (Fig.15). Total microbial count in the control
Figure 14a: Degradation of various PAHs present in crude oil by *Alcaligenes odorans* in microcosm.

Residual crude oil from microcosm was extracted and quantified gravimetrically after 30 days. The crude oil was fractionated and the degradation of PAHs was quantified by Gas Chromatography using the regression factor obtained using standard PAHs (section 3.2.1.3). *Alcaligenes odorans* was added to the treated plate with mineral salt medium as source of nutrient. Control plate contained TPH contaminated soil and mineral salt medium without *Alcaligenes odorans*, but contained indigenous microflora.
Aromatic fraction was obtained from the residual crude oil from both treated as well as control microcosm. The aromatic fraction was dissolved in acetone and analyzed by Gas Chromatography (section 3.2.1.3).
Figure 15: Survival of *Alcaligenes odorans* and degradation of total petroleum hydrocarbon in microcosm constructed from contaminated refinery soil.

Crude oil contaminated microcosm was constructed using crude oil contaminated soil in petriplates (150 mm diameter) with 16.5% total petroleum hydrocarbon (TPH) 150gm/plate. *Alcaligenes odorans* was added to the treated microcosm with mineral salt medium as a source of nutrient. Control microcosm contained TPH contaminated soil and mineral salt medium without any bacterial inoculum. CFU and TPH remaining in the soil were determined periodically (sections 3.2.4).
microcosm decreased marginally to $3.7 \times 10^3$ cells on kanamycin plates from the zero time kanamycin resistant bacterial count of $5.5 \times 10^3$ cfu/ml.

4.14 Fingerprinting of *Alcaligenes odorans* isolated from crude oil contaminated microcosm (constructed from refinery soil)

In order to assess the utility of ERIC-PCR in enumerating specific bacterial isolate in the natural environment we constructed a microcosm using soil that was contaminated with crude oil for a long time. Estimate for viable bacterial counts were performed on LA kanamycin plates, colonies showing different morphology was randomly picked and fingerprinted. A total of 85 (after 30 days) kanamycin resistant colonies were fingerprinted using ERIC-PCR. These different bacterial isolates belonged to various ERIC-PCR profiles on gel electrophoresis (Fig. 16). Of the 85 isolates 49 isolates (like those in Lanes 3,4,8, 9 and 10) had similar fingerprint as that of *Alcaligenes odorans*.

4.15 Survival of *Alcaligenes odorans* in microcosm constructed from refinery soil

Enumeration of *Alcaligenes odorans* was done by plate selective counting as well as ERIC-PCR based finger printing. It was observed that the microbial population in control microcosm remained constant. The population of *Alcaligenes odorans*, determined by selective plate counting, declined continuously. Despite the low survival pyrene degradation in *Alcaligenes odorans* treated microcosm was significantly high compared to the control. The survival data obtained from plate counting were higher as compared to the data obtained from DNA fingerprinting (Table 3). With PCR based
Figure 16: ERIC-PCR of selected bacterial isolates obtained from crude oil contaminated soil of microcosm

Kanamycin resistant bacterial isolates were fingerprinted using ERIC primers (Section 3.2.5.6). The amplicons were resolved on 2% (w/v) agarose gel and visualized by UV M=λ HinDIII marker; A.od.= Standard *Alcaligenes odorans*; Lanes 3-26 = fingerprints of bacterial isolates obtained from the microcosm.
Table 3: Survival and PCR identity of *Alcaligenes odorans* in microcosm

Soil suspension diluted in physiological saline was plated either on LA plate or LA-kanamycin plate. The values (count) represent an average of three replicates. ERIC-PCR was performed with the cells obtained on LA plates (section 3.2.5.6). Values given in parenthesis represent the number of isolates that gave a complete match with standard *A. odorans* versus total number of isolates fingerprinted.

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enumeration 27.22% of survival was obtained while with simple plate counting method the survival was 47.23%.

4.16 Growth of *Alcaligenes odorans* and uptake of pyrene during growth

*Alcaligenes odorans* was grown in mineral salt medium with pyrene (100 ppm) as sole source of carbon and energy. Growth of the bacterial strain *Alcaligenes odorans* was monitored by measuring the increase in the total cell protein and utilization of pyrene was quantified by Gas Chromatography. Growth of *Alcaligenes odorans* proceeded without any apparent lag and started to plateau after 68 hours of growth. The doubling time for *Alcaligenes odorans* with pyrene as sole source of carbon and energy, was found to be 8 hours (specific growth rate, $\mu = 0.087 \text{ h}^{-1}$). During this period of growth, the uptake of pyrene was also found to be linear in the beginning and started to plateau after 60 hours of growth. During the entire period of 72 hours of growth (when the culture entered stationary phase), approximately 80% of the added pyrene disappeared from the medium (Fig. 17). We found that using 100 ppm of pyrene resulted in loss of growth during batch to batch transfer, probably due to toxicity of pyrene at 100 ppm. We therefore reduced the concentration of pyrene used for the growth of *Alcaligenes odorans* from 100 ppm to 50 ppm. However, this reduction did not alter the growth rate and uptake of pyrene (data not shown). We therefore continued all our work with 50 ppm pyrene.

4.17 Degradation of pyrene by *Alcaligenes odorans*

*Alcaligenes odorans* was grown on mineral salt medium with pyrene (50 ppm) as sole source of carbon and energy. The degradation intermediates were extracted using ethyl acetate after 72 hours of growth and analyzed by Gas Chromatograph. Figure 18 shows several degradation intermediates from the ethyl acetate extract of
Figure 17: Growth and uptake of pyrene by *Alcaligenes odorans*.

*Alcaligenes odorans* was grown on mineral salt medium with pyrene (50 ppm) as sole source of carbon and energy. Growth of *Alcaligenes odorans* on pyrene was monitored by measuring total cell protein (section 3.2.7.2), while the uptake of pyrene was measured by extracting the residual pyrene and analyzing by Gas Chromatography (section 3.2.1.3).
Control

Alcaligenes odorans

Figure 18: Degradation of pyrene in mineral salt medium by Alcaligenes odorans

*Alcaligenes odorans* was grown with pyrene (50 ppm) as sole source of carbon and energy for 72 hours. The degradation intermediates were extracted from the culture and analysed by Gas Chromatography (3.2.2.5). The control flask contained pyrene (50 ppm) in mineral salt medium without any bacterial inoculum.

105
pyrene grown culture. Some of the intermediates that were in significant concentrations had Rt of 45.6 min., 48.4 min., 49.73 min., 53.45 min. and 54.57 min. These peaks were not present in the ethyl acetate extract obtained from control flask without any bacterial inoculum. Peaks having Rt of 9.6 min. and 10.26 min. increased significantly compared to the control.

4.18 Identification of degradation intermediates by GC-MS

Degradation intermediates of pyrene degradation from *Alcaligenes odorans* grown on mineral salt media containing pyrene as sole source of carbon and energy, was extracted using ethyl acetate for the analysis on Gas Chromatograph coupled with Mass Spectrograph (GC-MS). The ethyl acetate extract on GC-MS produced several peaks as seen on the total ion chromatogram (TIC; Fig. 19) The peaks were analyzed using MS-Chemstation computer software having Weily’s mass spectrograph database as described in methods (section 3.2.2.8) Two peaks at Rt 63.8 min. (peak I) and at 79.3 min. (peak II), showed more than 95% match with the corresponding standard intermediate whose fragmentation pattern was stored in the Weily’s database. Therefore these two peaks were considered as authentic intermediates. The remaining peaks did not produce significant match with the standard intermediates stored in the Weily’s database and therefore could not be analyzed further.

4.19.1 Peak I (63.8 min.)

Analysis of the fragmentation pattern of the peak at 63.8 min showed that the compound has a molecular ion of 164 atomic mass unit (amu) and other significant fragments had 51, 61, 91, 108, 120 and 146 amu. Based on the probabilistic search performed by the computer on Weily’s MD-database, the compound was identified as
Alcaligenes odorans was grown on pyrene (50 ppm) and the degradation intermediates were extracted (section 3.2.2.7). The extract was analysed by Gas Chromatograph coupled with Mass Spectrograph (GC-MS). The total ion chromatogram (TIC) peaks were scanned and probabilistic search of the fragmentation pattern database was performed to identify the degradation products. Time shown on X-axis is in minutes.
1,2-dihydroxy 1,2,3,4-tetrahydro naphthalene. The fragmentation pattern had 96% match with the fragmentation pattern of the standard intermediate (Fig. 20).

4.19.2 Peak II (79.3 min.)

Fragmentation pattern of the peak at 79.3 min. had a molecular ion at 222 amu and major fragmentation ions having the mass of 33, 55, 91, 129, 141, 183, 199 and 212 amu. The probabilistic search of Weily's database identified this peak as 4-phenanthrene carboxylic acid (Fig. 21). The peak had 95% similarity with the fragmentation pattern of the standard intermediate.

4.20 Uptake and mineralization of radiolabelled pyrene by whole cells of *Alcaligenes odorans*

*Alcaligenes odorans* grown on mineral salt medium with pyrene (50 ppm) was harvested in the log phase of growth and the cells were resuspended in phosphate buffer. The cell suspension having total protein 1mg/ml, in phosphate buffer (pH 7.0; 50 mM) was incubated at 37°C. Incorporation of radiolabelled pyrene (specific activity 58.7 mCi/mmol) into the cell was monitored by extracting the residual radiolabelled pyrene and quantifying it by liquid scintillation counting. It was observed that 1.35 nmol of radiolabelled pyrene were incorporated into the cells in 120 minutes when the total concentration of pyrene was 1.70 nmol (Fig. 22). No incorporation of the radiolabelled pyrene was observed beyond 120 minutes. In order to determine the mineralization of radiolabelled pyrene, $^{14}\text{CO}_2$ liberated during growth on radiolabelled pyrene was trapped into KOH and precipitated as BaCO$_3$. The precipitate was analyzed on liquid scintillation counter to quantify the amount of $^{14}\text{CO}_2$ liberated. It was observed that 0.39 nmol of radiolabelled pyrene were liberated as $^{14}\text{CO}_2$ when the total radiolabelled pyrene concentration was 17.04 nmol.
Intermediates of pyrene degradation were isolated and analyzed on GC-MS (section 3.2.2.7). TIC peak at 63.8 min. was scanned using probabilistic search. The fragmentation pattern of the standard intermediate is shown in the lower panel while the fragmentation pattern of the TIC peak at 63.8 min. is shown in the upper panel.

Figure 20: Mass spectrum of the TIC peak at 63.8 min.
Intermediates of pyrene degradation were isolated and analyzed on GC-MS (section 3.2.2.7). TIC peak at 79.3 min. was scanned using probabilistic search. The fragmentation pattern of the standard intermediate is shown in the lower panel while the fragmentation pattern of the TIC peak at 79.3 min. is shown in the upper panel.
Figure 22: Uptake and mineralization of [$^{14}C$]-pyrene by *Alcaligenes odorans*

Whole cells of *Alcaligenes odorans* (protein 1mg/ml) were incubated with $^{14}C$-pyrene. Uptake of the radiolabelled substrate was followed by ethyl acetate extraction of the residual pyrene and scintillation counting. Mineralization of pyrene was followed by trapping the $^{14}CO_2$ evolved in KOH and precipitation to yield BaCO$_3$. The BaCO$_3$ precipitate was filtered, dried and quantified on scintillation counter (section 3.2.2.6).
The mass balance of $^{14}$C-pyrene in *Alcaligenes odorans* indicating that, of the total radiolabelled pyrene added to the whole cell suspension 79% is taken up by the cells in 120 minutes while 21% remains outside the cells. Out of the 79% of pyrene that is taken up by *Alcaligenes odorans* 2.9% is mineralized to $^{14}$CO$_2$ and 75.1% of radiolabelled pyrene remains inside the cells (Fig. 23).

### 4.21 Pathway of pyrene degradation in *Alcaligenes odorans*

Based on the intermediates obtained from *Alcaligenes odorans* grown on pyrene, the pathway for pyrene degradation in *Alcaligenes odorans* can be constructed in the following manner (Fig. 24):

Pyrene was found to be converted to 4-phenanthrene carboxylic acid (peak II; Fig. 15) and then by some unknown route to 1,2-dihydroxy 1,2,3,4-tetrahydro naphthalene (peak I; Fig 14), which is then converted to CO$_2$ (as detected in mineralization studies; Fig. 23)

### 4.22 Effect of various inhibitors on pyrene uptake by whole cells of *Alcaligenes odorans*

Inhibitors belonging to four different categories were selected in order to study the uptake of pyrene by *Alcaligenes odorans*. These categories include sulfhydrol group inhibitors, electron transport inhibitors, ATPase inhibitors and ionophores. *Alcaligenes odorans* cells grown on pyrene were harvested at logarithmic phase of growth and pre-incubated with the respective inhibitors before measuring their effect on pyrene uptake. Valinomycin, an ionophore, caused complete inhibition of pyrene uptake at 50µM (Table 4a). The second ionophore, nigracin, also inhibited pyrene uptake by 46.2% at a final concentration of 1mM. Sulfhydrol group inhibitors used in the present
Figure 23: Mass balance of $^{14}$C-Pyrene in *Alcaligenes odorans*.

Whole cell experiment was conducted to determine the mass balance of $^{14}$C-pyrene in *Alcaligenes odorans* (section 3.2.2.6). The uptake and mineralization of the radiolabelled pyrene was followed to determine the amount of carbon remaining inside the cell.
Figure 24: Proposed pathway of Pyrene degradation in *Alcaligenes odorans*

The initial sequence of the pathway (shown in bracket) is documented in literature (Dean-Ross and Cerniglia, 1996). The compounds typed in bold face were identified by GC-MS during this study.
Table 4a: Effect of various inhibitors on uptake of pyrene by whole cell of *Alcaligenes odorans*.

*Alcaligenes odorans* was harvested at logarithmic phase and pre-incubated with appropriate inhibitors for 15 minutes. Uptake of pyrene was quantified by measuring the residual pyrene using Gas Chromatography (section 3.3.1).

| Inhibitors | Concentrations | Inhibition (%)
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Valinomycin</td>
<td>10 µM</td>
<td>39.3</td>
</tr>
<tr>
<td></td>
<td>50 µM</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>100 µM</td>
<td>100</td>
</tr>
<tr>
<td>Nigrecin</td>
<td>0.25 mM</td>
<td>8.6</td>
</tr>
<tr>
<td></td>
<td>0.50 mM</td>
<td>21.2</td>
</tr>
<tr>
<td></td>
<td>1.0 mM</td>
<td>46.2</td>
</tr>
<tr>
<td>IAA</td>
<td>0.5 mM</td>
<td>24.0</td>
</tr>
<tr>
<td></td>
<td>1.0 mM</td>
<td>39.0</td>
</tr>
<tr>
<td>NEM</td>
<td>0.25 mM</td>
<td>13.0</td>
</tr>
<tr>
<td></td>
<td>0.5 mM</td>
<td>29.8</td>
</tr>
<tr>
<td></td>
<td>1.0 mM</td>
<td>40.1</td>
</tr>
<tr>
<td>HgCl₂</td>
<td>0.25 mM</td>
<td>15.8</td>
</tr>
<tr>
<td></td>
<td>0.50 mM</td>
<td>29.5</td>
</tr>
<tr>
<td></td>
<td>1.0 mM</td>
<td>40.3</td>
</tr>
</tbody>
</table>

Control whole cell experiment, without inhibitor had an uptake of 79% of pyrene (50 ppm) in 20 minutes with 1mg/ml total cell protein.
study were iodo-acetic acid, n-ethyl maelimide (NEM) and mercuric chloride who inhibited pyrene uptake maximally at 1mM final concentration by 39%, 40.1% and 40.3%, respectively (Table 4a).

The electron transport chain inhibitors tested include, 2-n-heptyl-4-hydroxy quinoline N oxide (Hydroxy Quinoline) and roteonone caused complete inhibition of pyrene uptake at 100µM final concentration. Oligomycin also inhibited the uptake nearly completely (93.7%) at 375µM concentration. Another electron transport chain inhibitor 1,10 phenanthroline caused 68% inhibition of pyrene uptake at a concentration of 100µM (Table 4b). Similarly, n-chlorophenyl hydrazone (CCCP), an uncoupler, caused complete inhibition of pyrene uptake at 100µM. Two other inhibitor that belong to uncoupler category, dinitrophenol (DCP) and dichlorophenol (DCP) inhibited pyrene uptake by 59.7% and 79% respectively at 100µM final concentration (Table 4b).

4.23 Molecular analysis of Alcaligenes odorans

In order to ascertain whether the degradative capacity of Alcaligenes odorans was plasmid borne or not, extensive plasmid preparation using various techniques were carried out as described in methods (section 3.2.8). It became clear that the strain Alcaligenes odorans did not harbor any plasmid. This was further confirmed by pulse field gel electrophoresis (PFGE; section 3.2.12). In order to locate the genes responsible for pyrene degradation, genomic library was constructed using pBR322 as a vector after appropriate size fractionation as mentioned in methods (section 3.2.9.5). The library was screened using mineral salt medium plate having pyrene as sole carbon source, by patch-plating the clones. No clone containing the genomic DNA insert from Alcaligenes odorans showed any growth on mineral salt agar medium containing pyrene.
Table 4b: Effect of various inhibitors on uptake of pyrene by whole cell of *Alcaligenes odorans*.

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Concentrations</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hydoxy Quinoline</strong></td>
<td>10 µM</td>
<td>17.2</td>
</tr>
<tr>
<td></td>
<td>50 µM</td>
<td>23.0</td>
</tr>
<tr>
<td></td>
<td>100 µM</td>
<td>100</td>
</tr>
<tr>
<td><strong>1,10-Phenanthrolene</strong></td>
<td>10 µM</td>
<td>6.0</td>
</tr>
<tr>
<td></td>
<td>50 µM</td>
<td>12.0</td>
</tr>
<tr>
<td></td>
<td>100 µM</td>
<td>68.0</td>
</tr>
<tr>
<td><strong>Roteonone</strong></td>
<td>10 µM</td>
<td>12.2</td>
</tr>
<tr>
<td></td>
<td>50 µM</td>
<td>30.7</td>
</tr>
<tr>
<td></td>
<td>100 µM</td>
<td>100</td>
</tr>
<tr>
<td><strong>Oligomycin</strong></td>
<td>0.125 mM</td>
<td>48.0</td>
</tr>
<tr>
<td></td>
<td>0.250 mM</td>
<td>49.4</td>
</tr>
<tr>
<td></td>
<td>0.375 mM</td>
<td>93.7</td>
</tr>
<tr>
<td><strong>DNP</strong></td>
<td>50 µM</td>
<td>21.01</td>
</tr>
<tr>
<td></td>
<td>100 µM</td>
<td>59.7</td>
</tr>
<tr>
<td><strong>DCP</strong></td>
<td>0.25 mM</td>
<td>23.0</td>
</tr>
<tr>
<td></td>
<td>0.5 mM</td>
<td>44.1</td>
</tr>
<tr>
<td></td>
<td>1.0 mM</td>
<td>79.0</td>
</tr>
<tr>
<td><strong>CCCP</strong></td>
<td>10 µM</td>
<td>60.2</td>
</tr>
<tr>
<td></td>
<td>50 µM</td>
<td>82.0</td>
</tr>
<tr>
<td></td>
<td>100 µM</td>
<td>100.0</td>
</tr>
</tbody>
</table>

Control whole cell experiment, without inhibitor had an uptake of 79% of pyrene (50 ppm) in 20 minutes with 1mg/ml total cell protein.
Similarly, genomic library constructed using Lambda Fix-II cloning system did not yield any gene. Subtractive hybridization was used as an alternative strategy to clone the genes that may differentially expressed in presence of pyrene. Since subtractive hybridization requires highly pure cDNA its use in prokaryotic gene cloning has not been with great success. We standardized a novel method of generating a highly pure reusable cDNA library from bacterial RNA.

4.24 In-vitro polyadenylation of bacterial RNA

Marker RNA containing 16S and 23S RNA was polyadenylated using $\alpha^{32}$P-ATP and E. coli polyadenylate polymerase. The ethidium staining of poly(A) tailed 16S+23S marker RNA revealed that after polyadenylation the markers ran slower, when compared to the standard marker (Panel A, Lanes 2&4; Fig. 25). Panel B (Fig. 25) shows the incorporation of $\alpha^{32}$P-ATP into RNA (Panel B, Lanes 2&4) while the non-polyadenylated RNA did not show any incorporation.

4.25 Fingerprinting of cDNA of Alcaligenes odorans

To prove that using the method established by us, it is possible to generate highly pure cDNA we synthesized polyadenylated RNA from A. odorans. These were converted to single stranded cDNA and fingerprinted using M13 core sequence as primer. As shown in figure 26 the fingerprint containing aliquots of immobilized cDNA were similar to each other with a maximum size of amplification product of approximately 2 Kb (lanes 3 & 4), and these fingerprints did not match with those obtained from RNA prep that had not been treated with DNase. Higher amounts (5ul) of immobilized cDNA did not produce any fingerprint with M13 primers (Lane 5) probably due to high concentration of template. RNA prep not treated with DNase, also showed fingerprinting with M13 indicating some DNA contamination (Lanes 6 & 7). This observation is further
Figure 25: In-vitro polyadenylation of standard RNA.

Standard 16S and 23S marker RNA were used to check for the *in-vitro* polyadenylation. $\alpha^{32}$P-ATP was used as substrate and auto-radiography was used to confirm the incorporation of adenylate residues (section 3.2.5.1).

Lanes 1 & 5: Marker RNA without polyadenylation
Lanes 2 & 4: Polyadenylated Marker RNA
Panel A: Agarose gel; Panel B: Autoradiograph
Figure 26: Fingerprinting of cDNA captured on oligo(dT)$_{30}$ magnetic bead.

After in-vitro polyadenylation of total RNA, immobilized cDNA was synthesized and fingerprinting of the cDNA using M13 core sequence primers was performed (section 3.2.5.3).

Lanes 1, 8 & 11: λ HindIII marker DNA
Lane 2: Negative control (without cDNA)
Lanes 3-5: Solid phase cDNA from polyadenylated total RNA
Lanes 6 & 7: Total RNA without DNase treatment
Lanes 9 & 10: Total RNA with DNase treatment
corroborated by the presence of a very high molecular weight band near 23Kb of \( \lambda \text{HindIII} \) marker, which may be of an intact genomic DNA. DNase treated RNA that was used for polyadenylation reaction, did not show any finger printing with M13 primer (Lanes 9 & 10). This observation suggests that after \textit{in-vitro} polyadenylation of the total RNA, it is possible to generate immobilized cDNA library.

Subtractive hybridization using cDNA from glucose grown \textit{Alcaligenes odorans} with RNA from pyrene grown \textit{Alcaligenes odorans} was performed as described in methods (section 3.2.5.4). After three rounds of subtraction to generate normalized population of cDNA the subtracted RNA were converted to cDNA as described previously and the cDNA were resolved on agarose gel. The gel analysis did not reveal any band from the subtracted RNA probably because the genes responsible for pyrene degradation are expressed at a low level in all populations of \textit{Alcaligenes odorans} irrespective of the carbon source.