CHAPTER V

EFFICIENCY OF ALCALIGENES SP. STRAIN GU110 IN THE MINERALIZATION OF CRUDE OIL AND CRUDE OIL COMPONENTS.
were used from a 20% autoclaved stock solution. The nutrient broth yeast extract peptone medium (NBYP) was formulated by incorporating yeast extract (0.2%) and peptone (0.3%) in nutrient broth (Hi Media). Alkanes; hexadecane and heneicosane and phenanthrene were of Sigma grade, DBT and fluorene from E Merck. Bombay High crude oil (BHCO) was obtained from ONGC, Bombay; parrafin oil (PCL laboratories, India); engine oil (Servo Indian Oil). All organic solvents (HPLC grade) were from E Merck. Silica gel (60-120 mesh) for column chromatography from S.D.Fine-Chem Ltd. and alumina from SRL Ltd.

A model hydrocarbon mixture was formulated as follows;

<table>
<thead>
<tr>
<th>Nature of hydrocarbon</th>
<th>Name of component</th>
<th>Concentration used in ASW</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkane</td>
<td>Hexadecane</td>
<td>0.1% (v/v)</td>
</tr>
<tr>
<td>Di-aromatic</td>
<td>Naphthalene</td>
<td>10mg%</td>
</tr>
<tr>
<td>Tri-aromatic</td>
<td>Phenanthrene</td>
<td>10mg%</td>
</tr>
<tr>
<td></td>
<td>Fluorene</td>
<td>10mg%</td>
</tr>
<tr>
<td>Sulfur heterocyclic</td>
<td>DBT</td>
<td>10mg%</td>
</tr>
</tbody>
</table>

All solid hydrocarbon components were separately weighed, dissolved in hexadecane and then used as growth substrate.

Methods used

Alcaligenes sp. strain GU110 was grown on a host of compounds, as sole source of carbon and energy in an ASW containing media which included easily utilisable, non-aromatic carbon sources like lactate, mannitol, yeast extract, tryptone or on rich media like nutrient broth yeast extract peptone (NBYP). Besides, alkanes like hexadecane and heneicosane, simple aromatics like benzoate, PAH’s; DBT and phenanthrene were used individually as growth substrates. Model hydrocrabon mixture, Bombay High crude oil (BHCO), paraffin oil, and engine oil also served as growth substrates. Incubations were
done at room temperature on a shaker (100 rpm) in conical flasks. Utilization of the various carbon sources, PAH's, alkanes and oils was determined as increase in biomass as measured by optical density at 560nm on a Milton Roy (Spectronic1201) spectrophotometer.

Volumes of samples (1ml) from these flasks were taken at different time intervals and centrifuged at 12,500 rpm for 10 minutes in a microfuge (Beckman E™), or centrifuged in a RC5C (Sorvall) at 8000rpm for 20 minutes at 4°C. The supernatants were subjected to the D₆₁₀ assay (Reddy et al., 1983, Roy et al., 1979) and the cell pellets were washed and subjected to the BATH (Bacterial Adherence to Hydrocarbon) assay (Deziel, 1996; Rosenberg et al., 1980). These assays were described as follows;

Assessment of Hydrocarbon Emulsifying ability by D₆₁₀ assay:-

1ml volumes of the cell free culture supernatant was vigorously mixed with 10μl of hexadecane, in a quartz cuvette and the stability of the emulsion formed over a period of time was measured as turbidity at 610nm on a Milton Roy (Spectronic1201) spectrophotometer. Plain un-inoculated media's treated in a similar manner served as controls. The emulsifying activity of these cell free supernatants is a function of the degree of stability of the emulsion and is expressed as D₆₁₀.

Determination of cell hydrophobicity by BATH assay:-

The cells of strain GU110 grown on the appropriate substrate were washed and resuspended in phosphate buffer (0.05M) or plain distilled water to give an OD at 560nm of 1.0. The cell suspension (2ml) in testubes, was vortexed with 500μl of hexadecane. After allowing the hexadecane and aqueous phases to separate (for 1 hour) the turbidity of the aqueous phase was measured at 560nm and hydrophobicity was
expressed as the percentage of adherence to hexadecane and calculated as follows;

$$100 \times \left(1 - \frac{OD \text{ at 560nm of the aqueous phase}}{OD \text{ at 560nm of the initial cell suspension}}\right).$$

**Assessment of crude oil degradation by Alcaligenes sp. strain GU110**

*Alcaligenes* sp. strain GU110 (2% v/v) from maintenance flasks was inoculated in 50mls of ASW with Bombay High crude oil (0.1% v/v) as sole carbon source and also with Bombay High crude oil supplemented with DBT (0.01% w/v) in 500ml conical flasks and incubated at room temperature on a shaker (100 rpm). Appropriate uninoculated controls were also kept. Growth was monitored at 560nm and formation of polar hydroxylated aromatic metabolites was recorded in terms of RE (μg/ml) as discussed before.

**Fractionation of crude oil by Column Chromatography**

After allowing for growth of strain GU110 on crude oil, the entire flask contents were extracted with dichloromethane, dried over anhydrous sodium sulfate and the solvent removed by evaporation at 60°C. After drying, the residual oil samples were fractionated into the aliphatic, aromatic and NSO or polar components by an adaptation of the column chromatographic method described by ONGC (Dehradun).

A 45 cms x 2.0 cms glass column was packed with activated silica gel (60-120 mesh) and alumina in an equal ratio to a height of 15 cms. The residual oil from the control and test flasks were adsorbed on 0.5gms of activated silica gel and overlayered on the column. The saturate, aromatic and polar fraction were eluted sequentially with 80ml each of petroleum ether, benzene and methanol respectively. Solvents were evaporated at 60°C and the residues were transferred to pre weighed vials and weighed on a Mettler
Gas Chromatographic analysis of crude oil fractions

The crude oil saturate and aromatic fractions were monitored before and after degradation by injection (0.5μl) into a Shimadzu Gas chromatograph (GC-14B) fitted with flame ionization detector (FID) and capillary column (DB-1). Carrier gas was nitrogen. The injector and detector were maintained at 320°C and the oven temperature was programmed to rise from 60°C to 300°C at the rate of 6°C per minute increment and then held at 300°C for 5 minutes.

Design of marine microcosms for oil spill simulation studies

Filtered natural seawater was filled in 4 glass tanks of 300 liter capacity equipped with mechanical stirrers and independent aeration line, and maintained at constant temperature (25°C) (Fig. 5.1). Seawater in these tanks was supplemented with nitrogen [8mM(NH₄)₂SO₄] and phosphorous [5mM each K₂HPO₄ and KH₂PO₄] when required.

Formulation of mixed marine bacterial consortium

A mixture of hydrocarbon degraders comprising of the *Alcaligenes* sp. strain GU110 and other marine bacterial strains that had been isolated and maintained in the laboratory was designed and referred to as consortium 1 (Table 5.1) and consortium 2 (Table 5.2) respectively. 500μl of each of the pure cultures in consortium 1 and 2, pre-grown on its principal substrate (in ASW) was inoculated into 100ml of ASW containing a mixture of m-toluic acid (0.07%), naphthalene (0.04%), xylene (0.04%), quinoline (0.02%) pyridine (0.04%), phenanthrene (0.08%) and DBT (0.05%) for the bacterial cultures in consortium 1 and on, m-toluic acid (0.07%), quinoline (0.02%), phenanthrene (0.08%) and DBT (0.05%) for the bacterial cultures composing
Fig. 5.1 Line diagram of artificial microcosms used in this study (Coelho et al., 1995); (a) 324 litre of glass tank, (b) filtered sea water, (c) tarballs/crude oil, (d) stirrer, (e) aerators, (f) sampling site
### Table No. 5.1

Consortium I: List of bacterial cultures used in tarball simulation studies.

<table>
<thead>
<tr>
<th>Place isolated</th>
<th>Strain No.</th>
<th>Principal Substrate</th>
<th>Strain identified as</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goa</td>
<td>GU 101</td>
<td>m-Toluic acid</td>
<td><em>P. stutzeri</em></td>
<td>Satyakam.C.(1993)</td>
</tr>
<tr>
<td>Vengurla</td>
<td>GU 102</td>
<td>Naphthalene</td>
<td>Unidentified</td>
<td>J. Coelho <em>et al.</em>, (1995)</td>
</tr>
<tr>
<td>Vengurla</td>
<td>GU 103</td>
<td>Xylene</td>
<td>Unidentified</td>
<td>J. Coelho <em>et al.</em>, (1995)</td>
</tr>
<tr>
<td>Vengurla</td>
<td>GU 105</td>
<td>Pyridine</td>
<td>Unidentified</td>
<td>J. Coelho <em>et al.</em>, (1995)</td>
</tr>
<tr>
<td>Goa</td>
<td>GU 110</td>
<td>DBT</td>
<td><em>Alcaligenes sp.</em></td>
<td>J. Rodrigues (this study)</td>
</tr>
</tbody>
</table>

### Table No. 5.2

Consortium II: List of bacterial cultures used in oil spill simulation studies.

<table>
<thead>
<tr>
<th>Place isolated</th>
<th>Strain No.</th>
<th>Principal Substrate</th>
<th>Strain identified as</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goa</td>
<td>GU 101</td>
<td>m-Toluic acid</td>
<td><em>P. stutzeri</em></td>
<td>Satyakam.C.(1993)</td>
</tr>
<tr>
<td>Goa</td>
<td>GU 110</td>
<td>DBT</td>
<td><em>Alcaligenes sp.</em></td>
<td>J. Rodrigues (this study)</td>
</tr>
</tbody>
</table>
consortium 2. Flasks were incubated at room temperature on a rotary shaker (200 rpm). These mixed cultures were then cryo-preserved at -70°C by a method developed in our laboratory (N. Fernandes, personal communication). This mixture was then amplified on the above substrates, and used as inoculum (0.5% v/v) in artificially created microcosms.

**Determination of growth of Alcaligenes sp. GU110 as part of the consortium**

50ml aliquots of seawater samples from each of the 2 inoculated tanks were filtered using a Millipore filter which was then dispensed in 5ml of ASW. Viable counts of all of the components of the mixed culture in consortium 1 and 2, including GU110 was established by the MPN (Most Probable Number) method by carrying out a series of 10 fold dilutions from these filtered seawater aliquots, in tubes containing 0.08% sodium benzoate as sole carbon source. To specifically determine the viability of *Alcaligenes* sp. strain GU110, similar dilution's were performed using 0.05% (w/v) DBT as the sole carbon source. The highest dilution showing growth was taken as a measure of the DBT utilizing bacterial population. This was compared to the viable counts using sodium benzoate (0.08%) as sole substrate, since it supported the growth of all the cultures in the consortium.

**RESULTS**

The marine strain *Alcaligenes* sp. strain GU110 could effectively grow on a range of hydrocarbon and non-hydrocarbon compounds as sole carbon source.
Cell Surface Hydrophobicity (BATH) and Emulsifying ability ($D_{610}$) of Alcaligenes sp. strain GU110 grown on various aromatic and alkane moieties as carbon sources

The BATH for adherence to hydrocarbons and the $D_{610}$ assay was performed during the growth of GU110 on different substrates, which represent the different components of crude oil. These included, the simple aromatic compound, benzoate; the PAH’s, DBT and phenanthrene and components of the saturate alkane fraction, hexadecane and heneicosane. GU110 cells grown on these substrates displayed cell surface hydrophobicities ranging from 20% for phenanthrene grown cells to 80% for DBT grown cells but the emulsifying activities exhibited by strain GU110 grown on these different substrates were almost similar (Table 5.3).

A) Polycyclic aromatic hydrocarbons (PAH’s): The PAH’s, DBT and phenanthrene are prominent components of crude oil and serve as sole sources of carbon and energy when provided to Alcaligenes sp. strain GU110 in an ASW containing medium.

In the Chapter 3 we have proposed a model for utilization of DBT by Alcaligenes sp. strain GU110 based primarily on its ability to adhere to crystals to DBT as observed under the microscope. Like all other PAH’s the DBT moiety, is hydrophobic and insoluble in nature. Growth of GU110 on 1% DBT, yielded cells with an increasing degree of hydrophobicity (Fig. 5.2A & Fig. 5.3). Infact these cells revealed cell surface hydrophobicities as high as 80%. Cell free culture supernatants obtained during different stages of growth on 1% DBT revealed low levels of extracellular emulsifying activity. Growth of GU110 at increasing concentrations of DBT revealed that the degree of emulsification activity and the hydrophobic nature of GU110 cells was not dependent on the increasing DBT concentrations (Fig. 5.4).
Table No. 5.3: *Emulsifying ability and cell surface hydrophobicities of strain GU110 cells grown on aromatic and alkane carbon sources*

<table>
<thead>
<tr>
<th>Carbon Source</th>
<th>Cell hydrophobicities (% adherence)</th>
<th>Emulsifying activity ((D_{610}))</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Simple aromatic compound</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sodium benzoate</td>
<td>57.83</td>
<td>0.109</td>
</tr>
<tr>
<td><strong>PAH's</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenanthrene</td>
<td>23.77</td>
<td>0.156</td>
</tr>
<tr>
<td>Dibenzothiophene</td>
<td>86.4</td>
<td>0.182</td>
</tr>
<tr>
<td><strong>n-Alkanes</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hexadecane (C-16)</td>
<td>79.85</td>
<td>0.153</td>
</tr>
<tr>
<td>Heneicosane (C-21)</td>
<td>72.85</td>
<td>0.199</td>
</tr>
</tbody>
</table>

Table No. 5.4: *Emulsifying ability and cell surface hydrophobicities of strain GU110 cells grown on easily utilisable carbon sources*

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>Cell hydrophobicities (% adherence)</th>
<th>Emulsifying activity ((D_{610}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium lactate</td>
<td>78.80</td>
<td>0.084</td>
</tr>
<tr>
<td>Mannitol</td>
<td>13.00</td>
<td>0.150</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>24.00</td>
<td>0.339</td>
</tr>
<tr>
<td>Tryptone</td>
<td>15.5</td>
<td>0.475</td>
</tr>
<tr>
<td>NBYP medium</td>
<td>22.40</td>
<td>0.423</td>
</tr>
<tr>
<td>NBYP medium + n-hexadecane</td>
<td>27.76</td>
<td>0.737</td>
</tr>
</tbody>
</table>
Fig 5.2A Bacterial adherence to hexadecane displayed by strain GU110 cells grown on DBT. (A) Control: DBT grown GU110 washed cell suspension (B) Test: DBT grown GU110 cell suspension vortexed with hexadecane. Note, the upper hexadecane layer exhibits a distinct red color due to adherence of cells from the lower aqueous layer.

Fig 5.2B Bacterial adherence to hexadecane displayed by GU110 cells grown on hexadecane. (A) Control: Hexadecane grown GU110 washed cell suspension. Test (B & C): Hexadecane grown washed cell suspension vortexed with hexadecane. The decrease in the turbidity of the lower aqueous cell suspension after vortexing with hexadecane, was due to adherence of cells to hexadecane.
Fig. 5.3 Cell surface hydrophobicities (% Adherence) and emulsifying activity ($D_{610\text{nm}}$) displayed by strain GU110 cells during growth on DBT (1%).
Fig. 5.4 Cell surface hydrophobocities (% Adherence) and emulsifying activity (A610nm) displayed by strain GU110 cells grown on different concentrations of DBT.
Growth of GU110 on 1% phenanthrene produced cells with a lower degree of adherence compared to cells grown on 1% DBT (Fig 5.5). Emulsifying ability ($D_{610}$) increased during initial log period and then remained steady.

B) Aliphatic hydrocarbons: Both hexadecane and heneicosane are representative alkane moieties present in the saturate fraction of crude oil and these differ only on the basis of the chain length.

Strain GU110 grows very rapidly on hexadecane (C-16) and greater biomass was obtained at higher concentrations. After 27 hours of growth on 0.1% hexadecane the optical density at 560nm decreases with the concomitant increase in the formation of orange colored waxy clumps of cells in the ASW media. These clumps tend to persist in the medium (even in the late stationary phase) and the turbidity increases in the lower aqueous phase abruptly, after 71 hours. When these orange clumps were observed under the phase contrast microscope (40X) they revealed aggregates of cells. Growth of Alcaligenes sp. strain GU110 on 1% hexadecane resulted in a rapid increase in the optical density at 560nm with the formation of a pinkish orange cellular aggregates or scum at the surface of the medium. When the culture broth was centrifuged at 8000 RPM in a Sorvall RC5C cooling centrifuge at 4°C at different time intervals, the pink scum along with the remaining hexadecane solidifies at the surface of the supernatant at this low temperature. The scum was collected and observed under the microscope and revealed high densities of cells in the hexadecane layer itself. The cell pellets obtained from the lower aqueous layer were washed and their cell hydrophobicities were determined. Although these cells, were not associated with the hexadecane, they had a high degree of adherence to hexadecane (Fig. 5.2B).
Fig. 5.5 Cell surface hydrophobicities (% Adherence) and emulsifying activity (D_{610nm}) displayed by strain GU110 cells during growth on phenanthrene (1%).
Fig. 5.6 revealed that GU110 cells grown on 1% hexadecane exhibited hydrophobicities as high as 90% which decreased on prolonged incubation of cells with hexadecane. The emulsifying ability or $D_{610}$ was not very constant and varied with the growth of the culture on hexadecane (Fig. 5.6) but cell free supernatants of late stationary phase GU110 cultures grown at different hexadecane concentrations revealed the ability to form stable emulsions with hexadecane over a period of 30 minutes (Fig. 5.7).

Unlike hexadecane, utilization of heneicosane (C-21) carbon for growth of GU110 in ASW media resulted in very low levels of biomass being obtained over an extended period of time. These cells displayed hydrophobicities of 72% (Table 5.3).

Cell surface hydrophobicities (BATH) and emulsifying ability ($D_{610}$) during growth of GU110 on easily utilisable carbon sources

To determine whether Alcaligenes sp. strain GUI10 displayed hydrophobicities and emulsifying activities in the absence of crude oil components, this strain was grown on simple carbon substrates such as lactate and mannitol, yeast extract and tryptone as well as a rich medium like NBYP. Growth on sodium lactate yielded GU110 cells which exhibited the highest degree of hydrophobicity compared to cells grown on all the other simple carbon sources tested but emulsifying activities in these lactate grown cell free supernatants were considerably lower than those obtained by growth of strain GU110 on other substrates (Table 5.4). On yeast extract, tryptone and NBYP media, cell hydrophobicities and $D_{610}$ values were similar. When the NYBP medium was supplemented with hexadecane, emulsifying activity was higher than on NYBP without hexadecane although the cell hydrophobicities remained same.
Fig. 5.6 Cell surface hydrophobicities (%Adherence) and emulsifying activity (D_{610nm}) of strain GU110 cells during growth on 1% hexadecane.
Fig. 5.7 Emulsifying ability \( (D_{610nm}) \) of GU110 cell free supernatants after growth on ASW with different concentrations of hexadecane.
Mode of Utilization of crude oil and other oils by GU110

*Alcaligenes* sp. strain GU110 could utilize Bombay High crude oil, paraffin oil and engine oil as growth substrates (Fig. 5.8) and the cell surface hydrophobicities and emulsifying abilities of this marine strain after growth on these different oils was determined (Table 5.5). Uniform turbid GU110 cell suspensions were not observed on all these hydrophobic substrates and cells formed aggregates or clumps. Growth of GU110 on crude oil and tarball resulted in a breaking up or emulsification of oil into small brown clumps (Fig. 5.9). Microscopic examination revealed the presence of cellular aggregates within these clumps. Higher cell densities were achieved by strain GU110 on increasing the concentration of crude oil in the medium. Cell hydrophobicities varied from between 10 to 20% and increased with growth of the culture on 1% crude oil (Fig. 5.10). Low degrees of emulsifying activity were also observed in these cell free supernatants.

Degradation of DBT in presence of crude oil

We determined the utilization of sulfur heterocycle DBT, in presence of crude oil by growing the culture on crude oil supplemented with DBT. Growth was recorded as an increase in biomass at 560nm with the concomitant production of a visible vermilion color in the ASW medium which is characteristic of DBT oxidation products and is measured as Resorcinol equivalents or RE (µg/ml). Fig. 5.11 shows the comparison of the phenolic metabolites formed during growth of GU110 on crude oil in the presence and absence of DBT. Results clearly indicate that DBT is attacked by *Alcaligenes* sp. strain GU110 even in presence of crude oil.
Fig. 5.8 Comparison of growth of GU110 on different oils. Paraffin oil, Crude oil, and Engine oil were used as sole source of carbon (0.1%).

Table 5.5: Cell hydrophobicities and emulsifying ability of GU 110 cells grown on different oils

<table>
<thead>
<tr>
<th>Oil Used</th>
<th>Concentration used at</th>
<th>Emulsifying activity(D610)</th>
<th>Hydrophobicity (% Adherence)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude Oil</td>
<td>0.1%</td>
<td>0.293</td>
<td>38.61</td>
</tr>
<tr>
<td></td>
<td>1%</td>
<td>0.208</td>
<td>31</td>
</tr>
<tr>
<td>Engine Oil</td>
<td>0.1%</td>
<td>0.136</td>
<td>23.12</td>
</tr>
<tr>
<td></td>
<td>1%</td>
<td>0.140</td>
<td>29.60</td>
</tr>
<tr>
<td>Paraffin Oil</td>
<td>0.1%</td>
<td>0.083</td>
<td>16.01</td>
</tr>
<tr>
<td></td>
<td>1%</td>
<td>0.076</td>
<td>5.3</td>
</tr>
</tbody>
</table>
Fig. 5.9a Growth of strain GU110 on crude oil. (A) Control: Un-inoculated ASW with crude oil. Test with 0.1% (B) and 1% crude oil (C) inoculated with strain GU110.

Fig. 5.9b Growth of strain GU110 on tarball.
(A) Control: Un-inoculated ASW with tarball.
(B) Test: Growth of strain GU110 on tarball resulted in the emulsification and dispersion of the tarball and in the formation of colored polar metabolites which is typical of this strain.
Fig. 5.10 Cell surface hydrophobicities (% Adherence) and emulsifying activity (D_{610nm}) displayed by GU110 cells during growth on 1% crude oil.
Fig. 5.11 Growth ($A_{590nm}$) and formation of polar metabolites (Total phenolics as RE) by strain GU110 cells grown on crude oil and crude oil supplemented with DBT.
Efficacy of strain GU110 in the degradation of crude oil fractions

To determine the extent of degradation of crude oil by *Alcaligenes* sp. strain GU110, the extracted residual crude oil from GU110 grown flasks, were analyzed separately for the three different fractions, as described in Methods.

Table 5.6 shows the gravimetric analysis of the crude oil components after growth of strain GU110 on crude oil and on crude oil supplemented with DBT. Both, the alkane and the aromatic fractions were reduced considerably but the polar or NSO fraction was enriched when compared to sterile un-innoculated controls.

To confirm the contribution of GU110 towards the mineralization of crude oil we analyzed the different fractions on the gas chromatograph (GC). Gas chromatographic analysis of the alkane fraction revealed depletion of all the major peaks in comparison to sterile controls, and few of these peaks could be observed after increasing the sensitivity by a factor of 10 (Fig. 5.12). In comparison only a few of the peaks were removed in the aromatic fraction of degraded crude oil (Fig. 5.13). GC analysis of the fractions obtained after growth of GU110 on crude oil supplemented with DBT revealed that DBT eluted out in the alkane saturate fraction of crude oil. Fig 5.14 reveals the simultaneous removal of DBT along with the other peaks from the saturate fraction.

Utilization of Model Hydrocarbon mixture

Utilization of the artificially formulated mixture of hydrocarbon components consisting of DBT and different aromatic substrates, including the alkane, hexadecane (described in Materials) by *Alcaligenes* sp. strain GU110 was assessed by an increase in the optical density at 560nm as well as by determining the release of water-soluble phenolic polar
### Table No. 5.6: Degradation of Bombay High Crude Oil by *Alcaligenes sp.* strain GU110

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Amount remaining in</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control sample</td>
<td>Degraded sample</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>mg</td>
<td>%</td>
<td>mg</td>
<td>%</td>
</tr>
<tr>
<td>Culture grown on crude oil</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alkanes</td>
<td>16.9</td>
<td>100</td>
<td>6.3</td>
<td>37.27</td>
</tr>
<tr>
<td>Aromatics</td>
<td>8.6</td>
<td>100</td>
<td>7.3</td>
<td>84.88</td>
</tr>
<tr>
<td>NSO</td>
<td>12.7</td>
<td>100</td>
<td>15.5</td>
<td>122</td>
</tr>
<tr>
<td>Culture grown on crude oil and DBT</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alkanes</td>
<td>15.7</td>
<td>100</td>
<td>7.6</td>
<td>48</td>
</tr>
<tr>
<td>Aromatics</td>
<td>9.6</td>
<td>100</td>
<td>6.2</td>
<td>64.58</td>
</tr>
<tr>
<td>NSO</td>
<td>7</td>
<td>100</td>
<td>11.2</td>
<td>166</td>
</tr>
</tbody>
</table>
Fig. 5.12 GC profile of n-alkane fraction of Bombay high crude oil (BHCO)
(A) Un-inoculated sterile control.
(B) Test: After growth of strain GU110 (14 days). Recorder sensitivity was increased 10 fold to depict residual peaks. Complete removal of certain peaks may be noted.
Fig. 5.13 GC profiles of aromatic fraction of BHCO
(A) Un-inoculated sterile control.
(B) Test : After growth of strain GU110 (14 days).
Fig. 5.14 GC profiles of n-alkane fraction after growth of strain GU110 on BHCO supplemented with DBT:
(A) Un-inoculated sterile control
(B) Test: After growth of strain GU110 (14 days).
DBT eluted out in the alkane fraction by the crude oil fractionation method employed. Test samples revealed absence of the DBT peak after degradation.
metabolites into the medium which were estimated as Resorcinol Equivalents (RE in μg/ml). Growth and utilization of this model hydrocarbon mixture by *Alcaligenes* sp. strain GU110 resulted in an increase in biomass and in the formation of water-soluble polar metabolites (Fig. 5.15). The whole cell culture broth was then extracted with ethyl acetate, the organic extract dried over anhydrous sodium sulfate and injected into a GC (NETEL Omega QC+) fitted with a stainless steel (SE 30) packed column and FID. Carrier gas was nitrogen. GC analysis of these organic extracts revealed removal of these hydrocarbon substrate peaks when compared to sterile control, as shown in Fig. 5.16. It was interesting to note the removal of fluorene by strain GU110 cells when it was provided as part of the hydrocarbon mixture since fluorene did not serve as growth substrate for GU110 when supplied as the sole carbon source.

**Oil Spill Simulation studies**

Two sets of experiments were separately designed using tarballs and crude oil as growth substrates. **Set 1:** Tarball's from Bombay High was used as substrate and introduced into the 'test' and 'control' tanks at a concentration of 0.15% wt/v. The test tanks (with and without N & P) were inoculated with a mixture of seven different bacterial cultures, which we termed as consortium 1.

**Set 2:** Bombay High crude oil (0.1% v/v) was used as substrate in the tanks in a similar manner as described above, and the test tanks were inoculated with a mixture of four different bacterial cultures which we termed as consortium 2.

The 4 different glass tanks used for both of these experiments were designated as follows;

1) Tank A [Control]: Seawater + crude oil / tarball + nitrates + phosphates.
Fig. 5.15 Growth of strain GU110 on a Model Hydrocarbon mixture as determined by an increase in turbidity and in the formation of polar phenolic metabolites (Resorcinol Equivalents, RE)
Fig. 5.16  GC profile of Model Hydrocarbon Mixture before (A) and after (B) growth of strain GU110. DBT and all other components within this mixture were utilized. Note: Although fluorene is not used individually as a sole source of carbon by strain GU110 it is attacked when present within a mixture of other HC compounds.
2) Tank A [Test]: Seawater + crude oil/tarball + nitrates + phosphates + microbial inocula.

3) Tank B [Control]: Seawater + crude oil/tarball.

4) Tank B [Test]: Seawater + crude oil/tarball + microbial inocula.

Fig. 5.17A shows the growth and survival of *Alcaligenes* sp. strain GU110 on tarballs as part of a seven-membered consortium (consortium 1) in an artificially created microcosm with and without nitrogen and phosphorous when compared to the growth of all of the bacterial components of consortium 1 as described in Fig. 5.17B. Similarly, the increase in the GU110 bacterial population on crude oil, when used as a member of consortium 2 was seen in Fig. 5.18A. Survival of all of the bacterial components from consortium 2 was determined on sodium benzoate as shown in Fig. 5.18B. *Alcaligenes* sp. strain GU110 cells remained viable for more than 20 days on both crude oil and tarballs.

**DISCUSSION**

The biodegradation of hydrocarbons has a high ecological significance as it constitutes the major process for remediation of sites, which are contaminated with hydrocarbons. A better understanding of the fate of polluting hydrocarbons in the natural environments would help in the biotechnological improvement of the biodegradation processes thereby enabling the use of engineered bioremediative processes in such ecosystems. A key point which is only partially understood is the mechanism of uptake by microorganisms of, of these strongly hydrophobic compounds (Bouchez, 1999). Two uptake modes are generally considered for the biodegradation of PAH's (Deziel *et al.*, 1986...
Fig. 5.17  (A) Growth and proliferation of strain GU110 on tarball in artificially created microcosms as part of consortium 1. Bacterial counts were performed using the MPN method with DBT as the growth substrate.

(B) Growth of 7 membered mixture of bacterial HC degraders (consortium 1) on tarball in artificial microcosms containing seawater (Tank B) and seawater supplemented with nitrates and phosphates (Tank A). Growth was estimated using benzoate as substrate by the MPN method.
Fig. 5.18  (A) Growth of strain GU110 on crude oil, in artificially constructed microcosms as part of consortium 2. Bacterial counts were performed using the MPN method with DBT as the growth substrate. (B) Growth of the 4 membered mixture of bacterial HC degraders on crude oil in artificially constructed microcosms containing seawater (Tank B) and seawater supplemented with nitrates and phosphates (Tank A) estimated by the MPN method using benzoate as the carbon source.
1996; Bouchez et al., 1997) and long chained alkanes (Boulton and Ratledge, 1984; Singer and Finnerty 1984; Haferburg et al., 1986; Hommel, 1994). These include;

1) **Interfacial accession** or direct contact of cells with hydrocarbon, a function of the cell surface hydrophobicity expressed in terms of % adherence. The main characteristics of interfacial uptake were a high cellular hydrophobicity to allow cell adherence (to the hexadecane phase) and a high interfacial tension of culture supernatant fluid proving that there was no biosurfactant production. (Bouchez et al., 1999).

2) **Biosurfactant-mediated HC uptake** or cell contact with pseudosolubilized or solubilized hydrocarbons; hydrocarbon uptake is most often associated with the production of surface active compounds called biosurfactants, that enable (pseudo) solubilization and or emulsification of hydrocarbons (Foght et al., 1989; Goma et al., 1973; Goswami and Singh 1991; Reddy et al., 1983; Rosenberg 1986; Zhang and Miller 1992; Deziel et al., 1996). Several researchers have reported the production of biosurfactant by marine bacteria (Reisfeld et al., 1972; Floodgate 1978; Zajic et al., 1974; Rosenberg 1986). These surface active molecules are either released extracellularly or are bound at the bacterial cell surface (Rosenberg, 1986). This extracellular emulsifying agent was assayed for by measuring the ability of the cell free supernatants to form stable emulsions with hexadecane over a period of time ($D_{610}$ assay). *Alcaligenes* sp. strain GU110 was isolated from the marine environment, primarily for its ability to attack a sulfur heterocycle, DBT, but it also possessed the ability to grow on other water-immiscible hydrocarbon substrates.

By growing this marine bacterial isolate on a variety of hydrocarbon substrates, we tried...
to discern the mode of hydrocarbon uptake in this strain.

Our studies revealed that;

(i) *Alcaligenes* sp. strain GU110 possessed high degrees of cell surface hydrophobicities but these levels depended on the nature and type of substrate on which strain GU110 was grown.

(ii) External emulsifying activity was low but not absent and could only lead to enhanced hydrocarbon uptake.

This confirmed the fact that adherence or direct contact of cell with hydrocarbon is the primary mechanism of uptake of these strongly hydrophobic compounds by GU110.

GU110 cells grown on DBT and hexadecane revealed high degrees of hydrophobicities compared to cells grown on other aromatic or alkane compounds. Deziel *et al.*, (1996) have reported a *Pseudomonas aeruginosa* strain 19SJ which exhibited surface hydrophobicities of the order of 50% when grown on 2% (w/v) naphthalene. The *Alcaligenes* sp. strain GU110 revealed surface hydrophobicities as high as 80% when grown at different concentrations of DBT ranging from 0.01% to 1.0%. The cell surface hydrophobicities displayed by this strain are therefore independent of the concentration of the hydrocarbon on which it is grown.

Growth on n-hexadecane was very rapid, as seen by the high turnover of bacterial cells within 24 hours when grown in an ASW medium containing hexadecane as sole source of carbon and energy. n-Hexadecane has been shown to enhance oxygen supply to organisms, resulting in rapid growth. (Rols and Goma, 1991). Growth of GU110 on longer chained alkanes (C-21) was considerably slower.

The growth of GU110 on hexadecane was accompanied by breakage of the upper
hydrocarbon layer into droplets and the formation of a pink colored surface cream. Microscopic examination of this upper phase indicated that these droplets were covered with patches of adhering cells. These hexadecane droplets were not observed in the aqueous phase. As growth increases, the turbidity of the lower aqueous phase also increases and this was due to the unbound cells. The values presented for growth of GU110 on hexadecane (Fig. 5.6) should be considered minimal, since surface adhered bacteria did not contribute to turbidity at 560nm. Although these cells were unbound, they still possessed the ability to adhere to hydrocarbon and exhibited % hydrophobicities as high as 80%. A similar phenomenon was exhibited by Acinetobacter calcoaceticus RAG-1 cells grown on hexadecane (Rosenberg and Rosenberg, 1981) but this organism was known to produce a potent extracellular emulsifying agent, referred to as emulsan. Hexadecane grown Alcaligenes sp. strain GU110 cells also produced emulsions which remained stable for more than 30 minutes at 610nm when vortexed with hexadecane and we could therefore infer the production of external emulsifying activity in these cell free supernatants. Bouchez et al., (1999) have described an Alcaligenes faecalis, isolated from soils polluted by hydrocarbons, which exhibited a 49% hydrophobicity when grown on hexadecane as sole carbon source. The high adherence (80%) to hexadecane, by GU110 cells could be attributed to the fact that these cells had already been exposed to hexadecane containing medium, when it was used as sole carbon source by the cells.

Microorganisms are known to produce surface active compounds not only during growth on hydrophobic substrates but also on non-hydrocarbon substrates (Cooper and Goldenberg 1987; Guerra-Santos et al., 1994; Mulligan et al., 1989; Reiling et al.,
These surface active compounds may therefore be divided according to the type of carbon source used to produce them, such as hydrocarbons, water-soluble molecules, or both (Haferburg et al., 1986). When the Alcaligenes sp. strain GU110 was grown on a variety of water-soluble carbon sources, excreted biosurfactant as assayed by the D610 assay, was low except when cells were grown on tryptone. Surface hydrophobicities of these cells, which were grown on hydrophilic moieties, varied depending upon the type of the substrate and its concentration in the media. Lactate grown GU110 cells were highly hydrophobic at low concentrations of lactate (0.2%) and exhibited hydrophobicities as high as 70% but when these cells were grown on other easily utilisable carbon sources the hydrophobicities ranged from 10 to 20%. According to Rosenberg & Rosenberg (1985) adherence prevails in natural environments (open systems) and emulsification under artificial closed systems. But the hydrophobic nature of GU110 cells prevailed even under in vitro flask conditions.

Growth of Alcaligenes sp. strain GU110 on different oils was observed as turbidity, but greater biomass turnover was produced on crude oils, and engine oil than on paraffin oil. Visual examination of flask contents during growth of GU110 on these different oils, as well as on n-alkane hexadecane, as described above, revealed a common phenomenon; the oil-water interface is a hydrophobic interface and after inoculation of bacterial culture GU110, into such a closed system containing oil and ASW, the physical nature of these oils change, where in they tend to get dispersed within the media into fine fiber like threads, immediately followed by an increase in turbidity of the aqueous media. Obviously, cell proliferation is preceded by an emulsification of the
hydrophobic moiety, whereby the increased surface area of the oil droplets leads to
greater surface area being made available for better cell adherence. As the cells gain
access to these molecules substrate utilization is enhanced and therefore biomass
increases. Microbial surface active compounds are known to be ideally suited to
mediate the interaction (adhesion and de-adhesion) between micro-organisms and such
hydrophobic interfaces (Neu, 1996). Alcaligenes sp. strain GU110 grown on the
different oils revealed low emulsifying activities but these cells did display surface
hydrophobicities ranging from 10 to 30%.

Prominent amongst the oil degrading capacities of strain GU110 is its ability to grow on
and utilize crude oil as sole source of carbon and energy. Rambeloarisoa et al., (1984)
have shown that crude oil degradation is inversely proportional to the concentration of
oil in the medium. The marine species we isolated could produce higher biomass at
higher concentrations of crude oil, thus proving that increasing concentrations of crude
oil do not adversely affect degradation by strain GU110.

Crude oil is a major sea pollutant and its susceptibility to attack by indigenous marine
microbial populations will depend on its chemical composition. Studies concerning the
relationship of chemical composition and biodegradability of crude oil have shown that
crude oil containing a higher concentrations of alkanes was found to be more
susceptible to microbial attack (Walker et al., 1975, 1976). However PAH components
of crude oil are toxic and carcinogenic, and their biodegradability decreases with
increase in the number of benzene rings (Herbs & Schwall, 1978) viz. ; mono-
aromatics > di-aromatics > tri-aromatics > tetra-aromatics > penta-aromatics.
Aromatic nuclei containing sulfur were twice as refractory as non-sulfur analogs.
Crude oil and hydrocarbon degradation by heterotrophic consortia has been extensively reviewed by Leahy and Colwell (1990). It has been observed that individual organisms could metabolize only a limited range of hydrocarbon substrates (Britton 1984). Few environmental microbial isolates have been isolated that could degrade both alkanes and PAH compounds. Atlas & Bartha (1972a) have isolated a *Brevibacterium* and *Flavobacterium*, which were capable of degrading only the aliphatic fractions of crude oil. Foght et al., (1990) screened 138 isolates for degradation of hexadecane and phenanthrene and observed that none of the isolates mineralized both these compounds. Several environmental strains such as *Acinetobacter calcoaceticus* and *Alcaligenes odorans* (Lal & Khanna, 1996) *Arthrobacter* sp. (Efroymson and Alexander, 1991) and *Rhodococcus* strains (Malachowsky et al., 1994) and a *Pseudomonas* sp. (Whyte et al., 1997) were found to degrade both.

The results obtained in the present study are in contrast to the findings of Atlas & Bartha (1972a) and Foght & Westlake (1990). *Alcaligenes* sp. strain GU110 was capable of degrading both the alkane and aromatic fractions of BHCO with preferential and considerable degradation of alkane fraction as compared to the aromatic fraction. Similar findings have been reported by Lal & Khanna (1996) where-in bacterial soil isolates *Acinetobacter calcoaceticus* S30 and *Alcaligenes odorans* P20 degraded BHCO by 50% and 45% respectively, with both strains showing preferential degradation of n-alkane fraction.

Only 15% (w/w) of the aromatic fraction of BHCO was degraded by strain GU110 when crude oil was used as sole carbon source. When strain GU110 was supplied with
crude oil in combination with S-heterocycle DBT, we observed a greater degradation (35.42%) of aromatic fractions compared to when crude oil was provided alone. The depletion of dibenzothophene from such a crude oil supplemented medium was concomitant with the decrease in the aromatic fraction of BHCO and the increase of polar water-soluble metabolites in the NSO fraction.

In the previous chapter we have seen that *Alcaligenes* sp. strain GU110 possesses a constitutive broad specificity dioxygenase enzyme which encompasses a broad range of aromatic moieties which include mono-aromatics, di and tri-aromatics. When confronted with both DBT and alkane rich crude oil in the medium, the culture attacks the alkanes as well as constitutively attacks the DBT molecule. The same set of enzymes involved in DBT degradation is also responsible for the removal of other aromatic moieties present in crude oil, thereby accounting for the increased degradation of the aromatic crude oil fraction in the presence of DBT. The fact that strain GU110 could remove all the components, including DBT, when grown on the model hydrocarbon mixture, which we formulated in the laboratory, is further evidence that this marine strain could attack alkanes as well as di, tri and substituted aromatics with comparative ease.

After the strain GU110 degraded crude oil, the weight of the NSO or polar fraction increased. Similar findings were reported by Zajic *et al.*, (1974) and Jobson *et al.*, (1972). Increases in this asphaltene fraction after microbial degradation of crude oil probably arise from the production of extracellular compounds during growth which include phenolic acids, carboxylic acids, esters and ketones (Walker *et al.*, 1975). After degradation of crude oil supplemented with DBT, by strain GU110, the weight of
the polar fraction increases considerably, than when it was grown on crude oil alone. We could attribute this to the formation of polar metabolites or water-soluble compounds produced by strain GU110 on utilization of DBT. Oxidation of other aromatic compounds present in the crude oil by GU110 in the presence of DBT, would also contribute to the enhanced levels of phenolic polar intermediates, when compared to those obtained by growing the culture on crude oil alone. These water-soluble metabolites did not elute out from the silica gel column with the aromatic fraction and thus became part of the polar fraction in crude oil. Similar findings have also been described by Sagardia et al., (1975), Malik (1978) and Fedorak & Westlake (1983) when studying the fate of sulfur heterocycles in the environment.

The complete removal of DBT, which is a representative of the organic sulfur containing component of crude oil, by Alcaligenes sp. strain GU110, helps to predict the fate of sulfur heterocycles within crude oil, when it is attacked by such a DBT degrading marine microbe. Biodesulfurizations, where-in bacterial cultures specifically cleave only the sulfur atom from the thiophenic moiety without attacking the carbon ring, is the commercially useful and preferred alternative for removal of sulfur compounds from crude oil. But the role of microbial systems like the Alcaligenes sp. strain GU110 to desulfurize crude oil cannot be underestimated as such microorganisms formed water-soluble compounds by the oxidation of sulfur heterocycles like DBT, which could be readily separated from crude oil, thereby enhancing the sulfur removal process.

In situ experiments wherein oil spills are simulated help to predict the fate of oil when it is spilt in the open marine environment, as well as help to determine the remediation
capabilities of microbial seed cultures. In our laboratory, we have isolated, maintained and preserved a host of marine bacterial isolates capable of attacking different toxic hydrocarbon moieties present in crude oils. It has previously been reported (Lal & Khanna, 1996) that a faster rate of degradation of crude oil is achieved by the action of a combination of microorganisms with different hydrocarbon degrading capabilities rather than by a single versatile organism with the capability to degrade both alkanes and aromatic compounds. When these cultures were used as members of a mixed microbial consortium (consortium 1 & 2) to seed oil spills in closed systems, we could track down the culture of our choice and assess its growth by its unique degradative ability. The fate of GU110 in such a closed system, in presence of other aromatic hydrocarbon degraders is quite prominent as seen by the high bacterial counts obtained over a period of more than 30 days. The bacterial numbers obtained were also a measure of the growth of culture on crude oil / tarball and therefore of their degradation. GU110 was therefore not only capable of surviving as a part of the mixed microbial consortium but it was also capable of proliferating on both crude oil and tarball as carbon source.

**IN CONCLUSION**

*Alcaligenes* sp. strain GU110 was capable of growing on and emulsifying a variety of hydrophobic moieties ranging from straight chain alkanes to polyaromatics and sulfur containing compounds, and different types of oils. The increasingly hydrophobic property of these cells when grown on such water insoluble molecules confirms that a specific adhesion mechanism is involved in HC uptake wherein the cell comes in direct
contact with the hydrocarbon.

This marine bacterium is capable of growing effectively on crude oil but biodegradation of the crude is by preferential attack of the alkane fraction, although S - heterocycle, DBT when present, is simultaneously removed.

Bioremediative and biodegradative potential of GU110 was evident even when it was present as a member of a mixed marine bacterial population for seeding crude oil contaminated closed aquatic systems, therefore confirming its ability to be used commercially to enhance the oil spill clean up processes.