Chapter 2

Isolation, Characterization and Construction of Consortia of Crude Oil Degradating and Biosurfactant or Bioemulsifier Producing Bacteria from Various Oil Fields of Gujarat
2.1. Introduction

As industrialization expands, petroleum hydrocarbons become a greater potential source of contaminants in water and soil environments (Margesin and Schinner 2001). Chang and Lin (2006) reviewed 242 accidents related to storage tanks in industrial facilities over the last 40 years. According to their reports, 74% of the accidents occurred in petroleum refineries, oil terminals, or storage facilities.

To remediate petroleum contaminants in these environments, microbial biodegradation appears to be a promising tool to control petroleum pollution. It is not surprising that much research is in progress to study the biochemistry and the genetics involved in this activity. One of the mechanisms by which microorganisms assimilate hydrocarbons is through production of metabolites, or specific agents that cause dispersion of liquid hydrocarbons as hydrocarbons in water emulsions, micro droplets or micelles which are subsequently transported into the cell. These metabolites are called biosurfactants.

Biosurfactants are surface-active substances synthesized by living cells. They have the properties of reducing surface tension, stabilizing emulsions, promoting foaming and are generally non-toxic and biodegradable. Interest in microbial surfactants has been steadily increasing in recent years due to their diversity, environmental friendly nature, possibility of large-scale production, selectivity, performance under extreme conditions and potential applications in environmental protection (Banat et al., 2000; Rehman et al., 2002). Rosenberg and Ron (1999) have extensively studied the nature of microbial biosurfactants. The use of chemicals for the treatment of a hydrocarbon polluted site may contaminate the environment with their by-products, whereas biological treatment efficiently destroys pollutants, while its agents are biodegradable themselves. Biosurfactants enhance the emulsification of hydrocarbons, and have the potential to solubilize hydrocarbon contaminants and increase their availability for microbial degradation. Hence, biosurfactants producing microorganisms may play an important role in the accelerated bioremediation of hydrocarbon contaminated sites (Rosenberg and Ron 1999; Rehman et al., 2002). These compounds can also be used for enhanced oil recovery and may be considered for other potential applications in environmental protection (Rehman et al., 2002; Shulga et al., 1999). Other applications include integration in herbicide and pesticide formulations, detergents, health care and cosmetics products, pulp and paper, coal, textiles, ceramic processing,
Several microorganisms are known to synthesize surface-active agents and most of them are bacteria and yeasts (Banat 1995; Kim et al., 2000), when grown on hydrocarbon substrate as the carbon source. These microorganisms synthesize a wide range of chemicals with surface activity, such as glycolipids, phospholipids and others (Muriel et al., 1996; Desai et al., 1997). These chemicals are apparently synthesized to emulsify the hydrocarbon substrate and facilitate its transport into the cells. Recent studies have demonstrated the successful use of biosurfactants for facilitating the degradation of organic pollutants in soil and for the dispersion of oil in oil spills (Rosenberg and Ron 2001; Oberbremer et al., 1990; Banat et al., 2000). Very recently, Kumar et al. (2006) investigated the biodegradation of oil by hydrocarbon degrading *Pseudomonas putida* in the presence of a biosurfactant producing bacterium. The authors reported that the co-culture exhibited improved degradation capabilities in a reproducible fashion, in aqueous and soil matrices in comparison with individual cultures.

In this chapter, the isolation and identification of several bacterial cultures originating in oil contaminated sites, and capable of growing on hydrocarbon containing media is described. The relationship between biosurfactant production and emulsification activity is also investigated. The construction of microbial consortia was also carried out from biosurfactant producing bacteria to check whether they are applicable for bioaugmentation strategies.

**2.2. Materials and methods**

**2.2.1. Screening of samples**

The soil samples (100 g each) were collected from, three crude oil spilled and one diesel spilled stations for the isolation of crude oil degrading microorganisms. The samples were collected in pre-sterilized glass bottles and transported to the laboratory for analysis.

The crude oil spill sample collection sites were as listed below:

1. Navagam ONGC (Navagam, Ahmedabad, India)
2. Unava GSPC (Unava, Mehasana, India)
3. Sanand ONGC (Sanand, Ahmedabad)
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For diesel spill sample collection was done at:
4 Sanand ONGC (Sanand, Ahmedabad)

2.2.2. Enumeration and isolation of microorganisms
Enumeration and isolation of heterotrophic bacteria, fungi and actinomycetes were carried out through serial dilution agar plating technique using nutrient agar, sabouraud dextrose agar (SDA) and actinomycetes isolation agar medium (HIMEDIA) respectively. Bushnell-Hass agar was employed for determining the density of oil degrading microorganisms.

2.2.3. Growth of bacteria on crude oil
The bacterial cultures isolated from oil spilled environments were inoculated in Bushnell-Hass medium with 1% paraffinic crude oil as carbon source and kept in the shaker at 160rpm at 35°C for a period of seven days. The growth was recorded and categorized spectrophotometrically at 600nm as low growth with optical density (O.D.) in the range (O.D. 0.25–0.5), moderate growth (O.D. 0.51–0.75), high growth (O.D. 0.76–1.0) and excellent growth (O.D.>1). Those bacteria which showed excellent growth were further characterized by their ability to emulsify crude oil and the BATH test.

2.2.4. Characterization of bacteria
The isolates were grouped to various genera as per Bergey's Manual of Determinative Bacteriology (Holt et al., 1994). These cultures were characterized depending on their morphology, gram staining, spore staining, motility, oxidase, catalase, oxidation, fermentation, gas production, ammonia formation, nitrate and nitrite reduction, indole production test, methyl-red and Voges-Proskauer test, citrate and mannitol utilization test, hydrolysis of casein, gelatin, starch, urea and lipid (Aaranson et al., 1970).

2.2.5. Emulsification index (E$_{24}$%)
The emulsifying capacity was evaluated by an emulsification index ($E_{24}$%). The $E_{24}$% of culture samples was determined by adding 3 ml of kerosene and 3 ml of the cell-free broth in a test tube, which was vortex at high speed for 2 min and allowed to stand for 24h. The $E_{24}$ index is given as the percentage of the height of emulsified layer (cm) divided by the total height of the liquid column (cm). The percentage of emulsification index was calculated by using the following equation as described by Cooper and Goldenberg (1987).

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2.2.6. Analysis of cell surface hydrophobicity

The bacterial adhesion to hydrocarbons (BATH) assay was used in order to test the hydrophobicity of the bacterial strains (Rosenberg et al., 1985). Bacteria were harvested from growth cultures by centrifugation at 8000g for 10 min at 4°C, washed twice, and suspended in PUM buffer to an initial absorbance at 400nm of 1.2±1.4. Acid-washed test tubes containing 1ml hexadecane and 2ml of the adjusted cell suspension were vortex at high speed for 2 min and incubated at 30°C for 15 min. The bottom aqueous phase was carefully removed with a Pasteur pipette and transferred to disposable cuvettes. The absorbance at 400nm was measured on a spectrophotometer (Shimadzu, UV 1800). The adherence was expressed as the percentage decrease in optical absorbance of the lower aqueous phase following the mixing procedure, compared with that of the cell suspension prior to mixing. Those bacteria which showed high emulsification value over 60% and high BATH values were further identified by 16S ribotyping method.

2.2.7. Identification of bacteria by 16S ribotyping method

Universal bacterial 16S rDNA gene primers corresponding to Escherichia col: position 8f and 1527r were used for polymerase chain reaction (PCR) of the 16S rDNA gene (Pidiyar et al., 2002). Purified PCR products were sequenced using internal overlapping primers (Pidiyar et al., 2002). Sequences were initially analyzed at RDPII and NCBI server using BLAST (blastn) tool and corresponding sequences were downloaded. MEGA4 package were used to construct the phylogenetic tree.

2.2.8. Preparation of consortia

The bacterial strains that exhibited excellent growth on crude oil and also showed high emulsification activity were chosen to prepare consortia. The consortia included several bacterial species. Achromobacter sp. XRF-1, Ochrobactrum anthropi XRF-2, Pseudomonas aeruginosa XRF-4, Pseudomonas putida XRF-5, Bacillus cereus XRF-7 and Rhodococcus ruber XRF-8 were selected. A loop-full of each culture were inoculated into 100 ml sterile individual nutrient broth containing flasks. The flasks were incubated and agitated at 160 rpm, for 12 h at 30°C. One ml volumes of the
culture broth from each of the above five strains were mixed to prepare a bacterial consortium, and from that one ml consortium was used for degradation of crude oil.

2.2.9. Degradation of crude oil

Individual bacterial cultures and a bacterial consortium (1.0%) were transferred to 250 ml conical flasks, each containing 100 ml of sterile Bushnell-Hass broth with 1% paraffinic crude oil. An un-inoculated control was also studied concurrently. The flasks were incubated at 30°C and shaken at 160 rpm for 7 days. At 24h intervals, a set of flasks were used for the enumeration of microbial population by means of optical density measurements and the estimation of crude oil content gravimetrically. Degradation was estimated as difference between the initial and final concentration of the oil content in the medium by using the following formula and also by means of GC analysis.

\[
\text{Crude oil degradation (\%)} = \frac{\text{Control-Experimental}}{\text{Control}} \times 100
\]

2.2.10. Gas chromatographic analysis

A small portion of prepared extract (2µl) was injected into a gas chromatograph (Shimadzu GC 2014) equipped with a flame ionization detector and RTX-1 column. The carrier gas used was nitrogen, at a flow rate of 45 ml/min. The injector port and detector were maintained at 300°C. The oven temperature was programmed as follows: 5 min at 100°C followed by 10°C /min to 300°C for 30 min as described by Xiao et al. (2006).

2.3. Result and discussion

2.3.1. Isolation and characterization of crude oil degrading microbes

In present investigation from the Navagam soil samples, the highest hydrocarbon content (3.10 mg/g) and the lowest (0.87 mg/g) were observed in the samples labeled S1 and S4 respectively. The highest bacterial population was observed in sample S3 containing 1.52mg/g hydrocarbon content. The total population of bacteria was \(2.4 \times 10^7\). The highest fungi and actinomycetes populations were observed in sample S2. They were \(4.2 \times 10^4\) and \(2.8 \times 10^4\) respectively and the hydrocarbon content was 1.52mg/g (Table 2.1).
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Among the Unava station soil samples, the highest hydrocarbon content of 3.23 mg/g in sample S1 and lowest of 1.22 mg/g in sample S3 was observed respectively. The highest bacteria and fungi populations were observed in sample S2, which contains 2.58 mg/g hydrocarbon. The total populations of bacteria and fungi were $7.8 \times 10^7$ and $5.6 \times 10^3$ respectively. The highest actinomycetes population was observed in sample S3 which was $2.3 \times 10^4$ and the hydrocarbon content was 2.7 mg/g (Table 2.1).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Bacteria</th>
<th>Fungi</th>
<th>Actinomycetes</th>
<th>Hydrocarbon Content (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Navagam crude oil station soil</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S1</td>
<td>$5.2 \times 10^6$</td>
<td>$3.2 \times 10^3$</td>
<td>$1.8 \times 10^4$</td>
<td>3.10</td>
</tr>
<tr>
<td>S2</td>
<td>$3.8 \times 10^6$</td>
<td>$4.2 \times 10^4$</td>
<td>$2.8 \times 10^4$</td>
<td>2.34</td>
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<tr>
<td>S3</td>
<td>$2.4 \times 10^7$</td>
<td>$3.4 \times 10^2$</td>
<td>$1.1 \times 10^4$</td>
<td>1.52</td>
</tr>
<tr>
<td>S4</td>
<td>$8.4 \times 10^6$</td>
<td>$1.4 \times 10^3$</td>
<td>$2.3 \times 10^4$</td>
<td>0.87</td>
</tr>
<tr>
<td>Unava crude oil station soil</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S1</td>
<td>$5.3 \times 10^6$</td>
<td>$3.7 \times 10^3$</td>
<td>$2.3 \times 10^3$</td>
<td>3.23</td>
</tr>
<tr>
<td>S2</td>
<td>$7.8 \times 10^7$</td>
<td>$5.6 \times 10^3$</td>
<td>$4.2 \times 10^3$</td>
<td>2.58</td>
</tr>
<tr>
<td>S3</td>
<td>$6.2 \times 10^6$</td>
<td>$7.8 \times 10^2$</td>
<td>$2.3 \times 10^4$</td>
<td>2.7</td>
</tr>
<tr>
<td>S4</td>
<td>$6.4 \times 10^7$</td>
<td>$4.7 \times 10^3$</td>
<td>$2.6 \times 10^4$</td>
<td>1.22</td>
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<tr>
<td>Sanand crude oil station Soil</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S1</td>
<td>$7.2 \times 10^6$</td>
<td>$3.3 \times 10^2$</td>
<td>$2.7 \times 10^3$</td>
<td>3.47</td>
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<td>$5.4 \times 10^3$</td>
<td>$3.6 \times 10^3$</td>
<td>2.85</td>
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<tr>
<td>S3</td>
<td>$4.8 \times 10^5$</td>
<td>$5.6 \times 10^2$</td>
<td>$4.3 \times 10^4$</td>
<td>2.1</td>
</tr>
<tr>
<td>S4</td>
<td>$8.2 \times 10^7$</td>
<td>$5.2 \times 10^4$</td>
<td>$4.7 \times 10^3$</td>
<td>2.53</td>
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<tr>
<td>Sanand diesel station Soil</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S1</td>
<td>$5.3 \times 10^6$</td>
<td>$3.7 \times 10^2$</td>
<td>$2.7 \times 10^4$</td>
<td>3.03</td>
</tr>
<tr>
<td>S2</td>
<td>$6.2 \times 10^8$</td>
<td>$5.6 \times 10^2$</td>
<td>$4.1 \times 10^3$</td>
<td>2.77</td>
</tr>
<tr>
<td>S3</td>
<td>$6.3 \times 10^7$</td>
<td>$8.2 \times 10^2$</td>
<td>$3.8 \times 10^3$</td>
<td>0.98</td>
</tr>
<tr>
<td>S4</td>
<td>$5.4 \times 10^7$</td>
<td>$4.8 \times 10^3$</td>
<td>$2.3 \times 10^3$</td>
<td>3.2</td>
</tr>
</tbody>
</table>

In the Sanand crude oil station soil samples, the highest hydrocarbon content 3.47 mg/g and lowest 2.1 mg/g were observed in samples S2 and S3 respectively. The highest bacteria and fungi population was observed in sample S4, in which the hydrocarbon content was 2.53 mg/g. The total populations of bacteria and fungi were $8.2 \times 10^7$ and $5.2 \times 10^3$ respectively. The highest actinomycetes population was observed in sample S3 which was $4.3 \times 10^4$ where hydrocarbon content was 2.1 mg/g (Table 2.1).

Amongst the Sanand diesel soil samples, the highest hydrocarbon content 3.2 mg/g and lowest 0.98 mg/g were observed in samples S4 and S3 respectively. The highest bacterial population was observed in sample S2 where the hydrocarbon content was 2.77 mg/g. The total population of bacteria was $6.2 \times 10^8$. The highest fungi population

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was observed in sample S4 and was $4.8 \times 10^3$ and the hydrocarbon content was 3.2 mg/g. The highest actinomycetes population was observed in sample S1 and was $2.7 \times 10^4$ whereas the hydrocarbon content was 3.03 mg/g (Table 2.1).

Table 2.2 Distribution of bacterial genera showing different levels of growth on crude oil

<table>
<thead>
<tr>
<th>Genera</th>
<th>No. of isolates</th>
<th>Optical density at 620 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.25–0.5</td>
</tr>
<tr>
<td>Pseduomonas</td>
<td>100</td>
<td>52</td>
</tr>
<tr>
<td>Bacillus</td>
<td>62</td>
<td>35</td>
</tr>
<tr>
<td>Aeromonas</td>
<td>18</td>
<td>4</td>
</tr>
<tr>
<td>Alcaligenes</td>
<td>25</td>
<td>13</td>
</tr>
<tr>
<td>Enterobacter</td>
<td>28</td>
<td>14</td>
</tr>
<tr>
<td>Corynebacterium</td>
<td>73</td>
<td>36</td>
</tr>
<tr>
<td>Flavobacterium</td>
<td>36</td>
<td>17</td>
</tr>
<tr>
<td>Acinetobacter</td>
<td>13</td>
<td>5</td>
</tr>
<tr>
<td>Vibrio</td>
<td>14</td>
<td>9</td>
</tr>
<tr>
<td>Micrococcus</td>
<td>18</td>
<td>13</td>
</tr>
<tr>
<td>Moraxella</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td>Brucella</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>400</td>
<td>202</td>
</tr>
<tr>
<td>Percentage</td>
<td></td>
<td>50.50%</td>
</tr>
</tbody>
</table>

0.25–0.5=low growth, 0.51–0.75=moderate growth, 0.76–1.0=high growth and >1.0=excellent growth.

A total of 400 isolates were screened from all four sites (Table 2.2). They were further characterized by their ability to grow on crude oil and then further classified. The isolates which showed an optical density between 0.25–0.5 were classified as low growth isolates. Those isolates showed an O.D. between 0.51–0.75 were classified as moderate growth, and with O.D. between 0.76–1.0 were classified as high growth, while those which showed O.D. >1 were classified as excellent growth isolates. Of the 400 isolates 50.50% showed low growth, 28.25% showed moderate growth, 14.25% isolates showed high growth and 7% showed excellent growth. These 7% isolates were selected for further study (Table 2.2).

2.3.2. Genus wise distribution of bacteria from all four oil spilled sites

Colony morphology, gram staining and biochemical tests were used to establish genus wise distribution of bacteria from all four sites. The percentage distribution of different genera of bacteria from both crude oil station spill soils and diesel oil station spill soil is presented in Figure 2.1.
In the Navagam crude oil station soil samples, the members of genus Pseudomonas (27%) were at a higher percentage followed by Bacillus (17%), Corynebacterium (10%), Alcaligenes (9%), Flavobacterium (8%), Aeromonas and Enterobacter (7%), Acinetobacter (5%), Vibrio and Moraxella (3%), and Micrococcus and Brucella (2%) (Figure 2.1).

In the Unava crude oil station soil samples, the members of genus Bacillus (23%), were at a higher percentage followed by Pseudomonas (19%), Corynebacterium (17%), Flavobacterium (12%), Alcaligenes and Enterobacter (6%), Acinetobacter and Vibrio (4%), Aeromonas and Micrococcus (3%), Brucella (2%) and Moraxella (1%) (Figure 2.1).

In the Sanand crude oil station soil samples, the members of genus Pseudomonas (31%) were at a higher percentage followed by Corynebacterium (15%), Bacillus and Enterobacter (10%), Alcaligenes (8%), Acinetobacter (7%), Aeromonas, Flavobacterium and Micrococcus (5%), Moraxella (2%) and Vibrio and Brucella (1%) (Figure 2.1).

In the Sanand diesel spill soil samples, the members of genus Corynebacterium (31%) were at a higher percentage followed by Pseduomonas (23%), Bacillus (23%), Flavobacterium (11%), Vibrio (6%), Enterobacter (5%), Aeromonas and Micrococcus (3%), Alcaligenes and Acinetobacter (2%), and Moraxella and Brucella (1%) (Figure 2.1).

![Figure 2.1 Genus wise distributions of hydrocarbon degrading bacteria isolated from different sites.](image-url)
2.3.3. *Emulsification index (E$_{24}$%)*  
All of the 28 isolates (7% of total isolates), which showed excellent growth on crude oil (O.D. > 1.0) were further characterized by their ability to emulsify hydrocarbon. Of these 10 isolates showed $\geq$60% emulsification index (Figure 2.2). Hence, these 10 isolates were chosen for further study. These isolates were further identified using the 16S ribotyping method. These isolates were thereafter designated XRF-1 to XRF-10.

![Emulsification Index of Bacterial Isolates](image)

*Figure 2.2 Emulsification index of bacterial isolates which excellent growth on crude oil >1.0 O.D.*

2.3.4. *BATH test*  
Ten best isolates which showed $>60\%$ emulsification indexes were further characterized with respect to cell surface hydrophobicity by the BATH test. The highest BATH value, 72% was observed in *Rhodococcus ruber* XRF-8 followed to it *Pseudomonas aeruginosa* XRF-4, which showed the second highest BATH value of 66.66%. The isolates XRF-1, XRF-2, XRF-3, XRF-5, XRF-6, XRF-7, XRF-9 and XRF-10 showed BATH values of 48.66%, 66%, 46.66%, 62.66%, 64.33%, 59.66%, 46% and 48% respectively (Figure 2.3). Almost all isolates showed good cell surface hydrophobicity.
2.3.5. Identification of crude oil degrading bacteria by 16S ribotyping method.

The partial sequences of the all 10 strains were determined. Initial analysis of the sequences was done at RDPII and NCBI, where relevant sequences from these databases were downloaded for further analysis as described by Cole et al. (2005).

The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei 1987). The optimal tree with the sum of branch length=0.58388163 is shown in Figure 2.4. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches as in Felsenstein (1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and were in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 296 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 as per Tamura et al (2004).

The first sequence showed 99.9% sequence similarity with *Achromobacter sp.* In the phylogenetic analysis, it was shown to cluster with *Achromobacter sp.* R-4 (FR682929), and *Achromobacter sp MT-E3* (EU727196). The identity of XRF-1 thus, is confirmed as *Achromobacter sp.* and so we have labeled it as *Achromobacter sp.*
XRF-1. The sequence of the 16S rDNA gene of the strain XRF-1 is deposited and available under the GenBank accession number GQ922335 (Table 2.3).

The second sequence showed 99.9% sequence similarity with *Ochrobactrum anthropi*. In the phylogenetic analysis, it was shown to cluster with *Ochrobactrum anthropi* strain W-7 (EU187487), and *Ochrobactrum anthropi* strain P23 (FJ374126). The identity of XRF-2 thus, is confirmed as *Ochrobactrum anthropi*, and so we have labeled it as *Ochrobactrum anthropi* XRF-2. The sequence of the 16S rDNA gene of the strain XRF-2 is deposited and available under the GenBank accession number GQ922336 (Table 2.3).

The third, fourth and fifth sequences showed more than 99% sequence similarity with *Pseudomonas aeruginosa*. In the phylogenetic analysis, they are shown to clusters with *Pseudomonas aeruginosa* (FJ665510), *Pseudomonas aeruginosa* strain MML2212 (EU344794) and *Pseudomonas aeruginosa* strain JC2 (AM900348) and each other as well. The identities of XRF-3, XRF-4 and XRF-6, were thus confirmed as *Pseudomonas aeruginosa* and so we have labeled them as *Pseudomonas aeruginosa* XRF-3, *Pseudomonas aeruginosa* XRF-4 and *Pseudomonas aeruginosa* XRF-6 respectively. The sequences of the 16S rDNA gene of the strains, XRF-4, XRF-5, XRF-6 are deposited and available under the GenBank accession number GU212670, GU212672 and GU212673 respectively (Table 2.3).

The sixth sequence showed more than 99% sequence similarity with *Pseudomonas aeruginosa*. In the phylogenetic analysis, it was shown clustered with *Pseudomonas putida* strain BASUP87 (GU396283) and *Pseudomonas putida* strain YJF3-34 (FJ405889). Thus, the identity of strain XRF-5 was confirmed as *Pseudomonas putida*, so we have labeled it as *Pseudomonas putida* XRF-5. The sequence of the 16S rDNA gene of the strain XRF-5 is deposited and available under the GenBank accession number GU212672 (Table 2.3).

The seventh sequence showed more than 99% sequence similarity with *Bacillus cereus*. In the phylogenetic analysis, XRF-7 was shown to cluster with *Bacillus cereus* strain TCCC11017 (EU231628) and *Bacillus cereus* (FN674520). Thus, the identity of XRF-7 was confirmed as *Bacillus cereus* and hence forward we have labeled it as *Bacillus cereus* XRF-7. The sequence of the 16S rDNA gene of the strain XRF-7 is deposited and available under the GenBank accession number GU212674 (Table 2.3).

The eighth sequence showed more than 99% sequence similarity with *Rhodococcus ruber*. In the phylogenetic analysis, the XRF-8 was shown to cluster with:
Rhodococcus ruber strain SP2B (FJ705352), Rhodococcus ruber strain PB1 (DQ665849) and Rhodococcus ruber strain ATT505 (AY758191). Thus, XRF-8 was confirmed as Rhodococcus ruber and we have labeled it as Rhodococcus ruber XRF-8. The sequence of the 16S rDNA gene of the strain XRF-7 is deposited and available under the GenBank accession number GU212675 (Table 2.3).

The ninth and tenth sequences also showed more than 99% sequence similarity with Brevendomonas dimuneta. In the phylogenetic analysis, they were shown to cluster with Brevendomonas dimuneta strain B34 (GU397389) and Brevendomonas dimuneta strain KACC (DQ979376). Thus, XRF-9 and XRF-10 were confirmed as Brevendomonas dimuneta. Hence we have labeled them Brevendomonas dimuneta XRF-9 and Brevendomonas dimuneta XRF-10. The sequences of the 16S rDNA gene of the strains XRF-9 and XRF-10 are deposited and available under the GenBank accession numbers GU212676 and GU212677 (Table 2.3).

Table 2.3 list of strain used in particular study along with strain designation and Gene bank accession no.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Strain designation</th>
<th>Gene bank no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Achromobacter sp.</td>
<td>XRF-1</td>
<td>GQ922335</td>
</tr>
<tr>
<td>Ochrabactrum anthopi</td>
<td>XRF-2</td>
<td>GQ922336</td>
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<td>Pseudomonas aeruginosa</td>
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<td>GU212676</td>
</tr>
<tr>
<td>Brevendomonas dimuneta</td>
<td>XRF-10</td>
<td>GU212677</td>
</tr>
</tbody>
</table>
Figure 2.4 The phylogenetic tree, based on 16S rDNA sequences, was generated by using the Neighbour-Joining method (mega4) Jukes and Cantor distance: 1000 bootstrap replicates. The bootstrap values (%) are shown besides the clades; accession numbers are written besides the names of type strains, and scale bars represent distance values.
2.3.6. Growth of isolates

Growth of all the 10 isolates in crude oil was accompanied by the formation of an emulsion from a slick. When crude oil was used as sole carbon source, a similarity was observed in the growth pattern of most isolates. Significant growth of most isolates occurred after 24 hours and most isolates entered the stationary phase after 48 hours as shown in Figure 2.5. Almost all the isolates showed excellent growth on crude oil, but high O.D. near about 3 was obtained in case of *Rhodococcus ruber* XRF-1.

![Graph showing growth of isolates](image)

**Figure 2.5 Growth of selected isolates on BH agar containing crude oil.**

2.3.7. Crude oil degradation

In initial screening of bacteria, those bacteria which showed high surface activity were selected for crude oil degradation. High percentage of degradation was achieved with all the ten isolates. The highest percentage of crude oil degradation was achieved with *Rhodococcus ruber* strain XRF-9. It was about 84% gravimetrically. Gas chromatographic profile also supported this observation. GC profile showed virtually all the peaks in profile A (control) were degraded by *R. ruber* as seen in profile B (exp.) (Figure 2.7W). The least degradation was achieved in case of *Pseudomonas aeruginosa* XRF-3 at 52%. In case of other isolates XRF-9, XRF-10, XRF-7, XRF-2, XRF-7, XRF-5, XRF-6 and XRF-4, the total degradations that were achieved gravimetrically were 60%, 63%, 65%, 71%, 73%, 75%, 78%, and 80% respectively. Crude oil was readily degraded by almost all isolates which were determined by the
GC profiles (Figure 2.7P to 2.7Z). Gas chromatographic profiles of all tested isolates showed that almost all the peaks in the GC profile were eliminated (B) in comparison with control (A).

The ability of mixed consortia of these strains to degrade crude oil was also tested. Consortia were prepared using the following strains: *Achromobacter sp.* XRF-1, *Ochrobactrum anthropi* XRF-2, *Pseudomonas aeruginosa* XRF-4, *Pseudomonas putida* XRF-5, *Bacillus cereus* XRF-7, *Rhodococcus ruber* XRF-8. These showed high percentage of crude oil degradation as compared with their individual capacities. These consortia exhibit total 94% degradation. The gas chromatographic profile also supports this (Figure 2.7Z). During crude oil degradation it was noticed that there was excessive foam production by consortia mix.

![Figure 2.6](image)

*Figure 2.6 Excessive foam produced by mix consortia (strains XRF-1+XRF-2+XRF-4+XRF-5+XRF-7+XRF-8) during crude oil degradation.*
Figure 2.7(P). Gas chromatographic analysis of Crude oil (A) (control) and (B) degraded after 7 days by Achromobacter sp. XRF-1.

Figure 2.7(Q). Gas chromatographic analysis of Crude oil (A) (control) and (B) degraded after 7 days by Ochrobactrum anthropi sp. XRF-2.

Figure 2.7(R). Gas chromatographic analysis of Crude oil (A) (control) and (B) degraded after 7 days by Pseudomonas aeruginosa XRF-3.
Figure 2.7(S) Gas chromatographic analysis of Crude oil (A) (control) and (B) degradation products after 7 days by Pseudomonas aeruginosa XRF-4.

Figure 2.7(T) Gas chromatographic analysis of Crude oil (A) (control) and (B) degraded after 7 days by Pseudomonas putida XRF-5.

Figure 2.7(U) Gas chromatographic analysis of Crude oil (A) (control) and (B) degraded after 7 days by Pseudomonas aeruginosa XRF-6.
Figure 2.7(V) Gas chromatographic analysis of Crude oil (A) (control) and (B) degraded after 7 days by Bacillus cereus XRF-7.

Figure 2.7(W) Gas chromatographic analysis of Crude oil (A) (control) and (B) degraded after 7 days by Rhodococcus ruber XRF-8.

Figure 2.7(X) Gas chromatographic analysis of Crude oil (A) (control) and (B) degraded after 7 days by Brevendomonas dimuneta XRF-9.
Bacteria, actinomycetes and fungal populations were high in most of all samples from all four sites, where higher quantities of oil contents were noticed. Similarly large population of bacteria, actinomycetes and fungi were recorded from oil-spilled environment (Venkateswaran et al. 1993; Wrenn and Venosa 1996; Cabello 1997). Population levels of hydrocarbon utilizers and their population within the microbial community appear to be a sensitive index of environmental exposure to hydrocarbons. In unpolluted ecosystem, hydrocarbon utilizers generally constitute less than 0.1% of the microbial community and in oil polluted ecosystems they can constitute up to 100% of the viable microorganisms. The microbial populations quantitatively reflect
the degree or extent of exposure of that ecosystem to hydrocarbon contamination (Atlas., 1981; Al-gounaim et al., 1995). In diesel station soil samples contained Corynebacterium species at higher proportion. The dominance of the genus Corynebacterium revealed that it might have developed the ability to acclimatize to the oil environment. We observed bacteria belonged to diversified groups, which may be due to the oil enrichment. The changes in the dynamic equilibrium state of soil leads to a corresponding change in microbial population and composition. The added oil enriched for the species that have inherent petroleum hydrocarbon assimilating potential, whereas the less adapted species among the total heterotrophic population are gradually eliminated, resulting in qualitative shifts in species composition (Amadi 1990). The biochemical characteristics of the bacteria varied. Austin et al. (1977) reported that the biochemical reactions of the petroleum degraders, as a whole, were not uniform and no pattern of relatedness were discernible based on the reactions. Microorganisms are known to attack specific compounds present in crude oil that is a complex mixture of saturates, aromatics and polar compounds (Bharathi and Vasudevan 2001). An effective degradation of crude oil would require simultaneous action of several metabolically versatile microorganisms with favourable environmental conditions such as pH, temperature and availability of nutrients (Venkateswaran and Harayama 1995). An oil spill in the environment leads to an adaptive process and if metabolically active hydrocarbon utilizing microorganisms could be added quickly, the long period before the indigenous population could respond would be reduced considerably. The necessity for seeding with complementary hydrocarbon degrading bacteria arises from the rationale that indigenous microbial populations may not be capable of degrading a wide range of potential substrates in a complex mixture such as crude oil (Chhatre et al., 1996).

In Navagam station and Sanand station soil sample the predominant genera was Pseudomonans similar result observed in studies by Patel and Ghosh (2003) on petroleum biodegrading organisms present in the soil and oil of Mehsana and Ahmedabad oil field areas of Gujarat have shown that all the 47 strains isolated belonged to genus Pseudomonas.

The biosurfactant producing bacteria showed oil degradation in range between 52 to 85%. Microbial consortia which produce biosurfactants showed excellent results a total of 94% oil degradation in comparison with individual bacterium. Similar study carried out by Kumar et al. (2006) investigated co-culture exhibited improved
degradation capabilities in a reproducible fashion, in aqueous and soil matrices in comparison with individual cultures, in comparison to that our consortia showed higher percentage of crude oil degradation.

2.4. Conclusion

A large number of crude oil degrading microbes were isolated from all four sites but the predominant microbiota was bacteria. The Type of genus present in each sample varied from sample to sample.

The predominant genus present in Navagam, and Sandand crude oil spill soil samples was *Psudomonas* species whereas, Unava crude oil and Sanand diesel soil samples had *Bacillus* and *Corynebacterium* respectively. Amongst all the tested isolates a small proportion of bacteria showed high emulsification capacity along with high BATH value.

Microbial consortia which produce biosurfactants showed excellent results of over 90% oil degradation in comparison with individual bacterium. Hence such microbial consortia can be used as bioaugmentation strategy to cure oil spills.

References


