Part A: Investigation of Physico-chemical Properties and Uncultured Diversity of Formation Water Samples Collected from Oil Reservoirs in Western India

Introduction

Water occurring naturally within the pores of rock is called as formation water. During extraction of crude oil, this water comes to the surface along with oil. Formation water plays a significant role in many geological processes like mineralization of metals, migration and accumulation of hydrocarbons (Cathels et al., 1983; Land et al., 1988; Bethke et al., 1990). By analyzing geochemical changes in formation water, important rock-fluid interactions can be identified. Formation water is associated with crude oil and also present in the form of oil-water emulsion. As living organisms need water for their biological activities; the probability of occurrence of thermophilic bacteria is more in formation water than in oil.

3.1 Sample Collection and Analysis

Formation water samples were collected from a total of 10 oil wells from Mehsana and Ahmedabad region of Gujarat, India (figure 3.1). Out of 10 samples, seven were collected from Bechraji field of Mehsana region. Two samples were collected from Limbodra and a single sample from Kalol region. Formation water from Limbodra well numbers 73, 28 and Kalol 463 was separated only when fraction of demulsifier was added in the oil-water emulsion. Temperature of all the samples was checked using digital thermometer.
Table 3.1: Sampling locations, sample designations, oil well depth and temperature of samples

<table>
<thead>
<tr>
<th>Sr No.</th>
<th>Region</th>
<th>Field</th>
<th>Area</th>
<th>Depth(m) Approx.</th>
<th>Well no.</th>
<th>Well head temperature °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Mehsana</td>
<td>Bechraji</td>
<td>GGS 1</td>
<td>950-1000</td>
<td>71</td>
<td>65-70</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td>950-1000</td>
<td>74</td>
<td>65-70</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td>950-1000</td>
<td>103</td>
<td>65-70</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
<td>GGS 2</td>
<td>950-1000</td>
<td>144</td>
<td>65-70</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td>950-1000</td>
<td>100</td>
<td>65-70</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td></td>
<td>GGS 1</td>
<td>-</td>
<td>Group sample GGS 1</td>
<td>65-70</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td></td>
<td>GGS 2</td>
<td>-</td>
<td>Group sample GGS 2</td>
<td>65-70</td>
</tr>
<tr>
<td>8</td>
<td>Limbodra</td>
<td>Limbodra</td>
<td>GGS 1</td>
<td>850-900</td>
<td>73</td>
<td>75-80</td>
</tr>
<tr>
<td>9</td>
<td></td>
<td></td>
<td></td>
<td>1400-1500</td>
<td>28</td>
<td>75-80</td>
</tr>
<tr>
<td>10</td>
<td>Kalol</td>
<td>Kalol</td>
<td>GGS IV</td>
<td>1400-1500</td>
<td>463</td>
<td>80-85</td>
</tr>
</tbody>
</table>

The depth of each oil well was on average 950-1000 m in Mehsana region. In case of well no. 73 from Limbodra region, the depth was 850-900 m whereas well no. 28 and 463 has depth in the range of 1400-1500 m (table 3.1).
It was found that samples from Mehsana region have temperature in the range of 65-70°C. Samples from Limbodra were having temperature in the range of 75-80°C whereas Kalol 463 was having temperature in the range of 80-85°C. To minimize the probable volatization or biodegradation between sampling and analysis; samples were kept at 4°C.

3.1.1 Microscopic Observation

Aqueous fraction separated from oil was observed under the phase contrast microscope to detect the presence of bacteria or other microorganisms. Similarly, the same samples were observed under UV (Ultra Violet) microscope to detect the presence of methanogens. It may be noted that methanogens alone and not other bacteria or archaea exhibit auto-florescence when illuminated with UV light because of the presence of co-factor F$_{420}$. Microscopic detection of bacteria and methanogens is described in table 3.2. It was found that all formation water samples showed presence of bacteria. Methanogens, however, were detected only in GGS 1, GGS 2 and Bechraji 144 samples.
Table 3.2: Microscopic Observation of the samples

<table>
<thead>
<tr>
<th>Sr. No</th>
<th>Well No.</th>
<th>Microscopic observation for the presence of</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Bacteria</td>
</tr>
<tr>
<td>1</td>
<td>GGS 1</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>GGS 2</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>Meh 74</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>Meh-71</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>Bech 100</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>Bech 144</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>Bech 103</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>Kalol 463</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>Limb 73</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>Limb 28</td>
<td>+</td>
</tr>
</tbody>
</table>

3.1.2 Physico-chemical analysis of formation water samples

The physical and chemical properties of formation water are mainly depending on the geographic location of the oil field, the geological formation with which the formation water has been in contact for many years and the nature of hydrocarbon being produced. Properties of formation water and quantity can differ during the life of a reservoir. Physico-chemical analysis of formation water samples was done in order to check whether formation water alone can support the growth of the native bacteria. It included pH, salinity, toxic heavy metal ions, total nitrogen, elemental analysis (CHNS), total solids and volatile solids.
3.1.2.1 pH and Salinity

The pH and the salinity (g % of NaCl) of the formation water samples were measured immediately after bringing the samples to the laboratory. The pH and salinity of the formation water was dependent on the source of the injection water as well as presence of types of rocks in the reservoir. It was observed that all samples were slightly alkaline. The salinity was less than one percent (table 3.3).

Table 3.3: pH and Salinity of the formation water samples

<table>
<thead>
<tr>
<th>Well no.</th>
<th>pH</th>
<th>Salinity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Meh 71</td>
<td>8.02</td>
<td>0.86</td>
</tr>
<tr>
<td>Meh74</td>
<td>8.00</td>
<td>0.86</td>
</tr>
<tr>
<td>Meh 103</td>
<td>8.0</td>
<td>0.86</td>
</tr>
<tr>
<td>Bech100</td>
<td>8.20</td>
<td>0.87</td>
</tr>
<tr>
<td>Bech 144</td>
<td>8.01</td>
<td>0.88</td>
</tr>
<tr>
<td>Limb 73</td>
<td>8.00</td>
<td>0.87</td>
</tr>
<tr>
<td>GGS 1</td>
<td>8.01</td>
<td>0.88</td>
</tr>
<tr>
<td>GGS 2</td>
<td>7.82</td>
<td>0.89</td>
</tr>
<tr>
<td>Limb 28</td>
<td>8.01</td>
<td>0.85</td>
</tr>
<tr>
<td>Kalol 463</td>
<td>8.01</td>
<td>0.86</td>
</tr>
</tbody>
</table>

Therefore, pH and salinity values were not too high, to affect the growth of native microbial flora negatively. The pH is indirect measurement of acidity and alkalinity. The presence of strong mineral acids, weak acids such as carbonic acid, acetic acid and other hydrolyzing salts such as iron or aluminum sulfate may have contributed to the acidity of formation water. Alkalinity of formation water is because of carbonate, bicarbonate and
hydroxide content. In addition to these components other basic compounds like borates, phosphates, silicates etc. may contribute to the alkalinity.

There are many factors which are responsible for the origin of salinity of the formation water. The mechanisms like such as reverse chemical osmosis (Graf, 1982), dissolution of potash and halite (Land and Prezbinowski, 1981), evaporated seawater (Carpenter, 1978), and water rock interactions in sediments (Hardie, 1990) are found to be responsible for the salinity of the formation water. Honor (1987) reported that salinity of formation water in the Shiwu depression of China ranges from 0.024 to 1.0 g%. Carpenter (1978) analyzed 295 formation water samples and he observed 18 samples were having salinity less than one percent.

3.1.2. 2 Total solids and Volatile solids

Total solids in the formation water samples were comprised of volatile solids and fixed solids. Volatile solids are organic in nature and are available as a substrate for organisms. In all samples, total solids contributed to less than one gram percent (0.85-0.89 g %). In case of volatile solids, the gram percentage is in the range of 0.01-0.14 which was very low. These results indicate that there was insufficient organic matter as a substrate in the samples to support the growth of the bacteria (table 3.4). Honor (1987) reported that total dissolved solids are significantly less than 3.5g% of formation water in the Shiwu depression of China. Similar results were observed by Carpenter (1978). He also observed that the TDS values of formation water in the hydro-pressured zone usually are less than 4000 mg/L.
Table 3.4: Total and volatile solids content of formation water

<table>
<thead>
<tr>
<th>Well no.</th>
<th>Total solids (g %)</th>
<th>Volatile solids (g %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Meh 71</td>
<td>0.86</td>
<td>0.02</td>
</tr>
<tr>
<td>Meh 74</td>
<td>0.865</td>
<td>0.14</td>
</tr>
<tr>
<td>Meh 103</td>
<td>0.86</td>
<td>0.012</td>
</tr>
<tr>
<td>Bech 100</td>
<td>0.87</td>
<td>0.010</td>
</tr>
<tr>
<td>Bech 144</td>
<td>0.88</td>
<td>0.028</td>
</tr>
<tr>
<td>Bech 150</td>
<td>0.87</td>
<td>0.013</td>
</tr>
<tr>
<td>GGS 1</td>
<td>0.88</td>
<td>0.013</td>
</tr>
<tr>
<td>GGS 2</td>
<td>0.89</td>
<td>0.012</td>
</tr>
<tr>
<td>Limb 28</td>
<td>0.85</td>
<td>0.024</td>
</tr>
<tr>
<td>463</td>
<td>0.86</td>
<td>0.021</td>
</tr>
</tbody>
</table>

3.1.2.3 Nitrogen Content

Total nitrogen content (Kjeldahl Nitrogen) of the formation water samples represents both, inorganic nitrogen as well as organic nitrogen. The organic nitrogen is also called as ammonical nitrogen. It is the representative of proteinaceous nitrogen or biomass present in the formation water. Ammonical nitrogen can be used by the bacteria as a nitrogen source. The total and ammonical nitrogen content of individual formation water samples is given in the table 3.5. It was observed that all formation water samples showed low level of ammonical nitrogen to support the growth of the bacteria. The percentage of the organic nitrogen is in the range of 0.00019-0.00048 g%. The percentage of total nitrogen is in the range of 0.00026-0.00050 g%.
Table 3.5: Nitrogen content of the formation water samples

<table>
<thead>
<tr>
<th>Well no.</th>
<th>Nitrogen Content (ppm)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Ammonical</td>
<td>Total Kjeldahl</td>
</tr>
<tr>
<td>Meh 71</td>
<td>44.06</td>
<td>55.0</td>
<td></td>
</tr>
<tr>
<td>Meh74</td>
<td>34.59</td>
<td>42.0</td>
<td></td>
</tr>
<tr>
<td>Meh 103</td>
<td>32.54</td>
<td>40.0</td>
<td></td>
</tr>
<tr>
<td>Bech100</td>
<td>38.38</td>
<td>50.0</td>
<td></td>
</tr>
<tr>
<td>Bech 144</td>
<td>34.92</td>
<td>41.0</td>
<td></td>
</tr>
<tr>
<td>Limb 73</td>
<td>37.30</td>
<td>45.0</td>
<td></td>
</tr>
<tr>
<td>GGS 1</td>
<td>19.68</td>
<td>26.0</td>
<td></td>
</tr>
<tr>
<td>GGS 2</td>
<td>38.59</td>
<td>48.0</td>
<td></td>
</tr>
<tr>
<td>Limb 28</td>
<td>48.11</td>
<td>26.0</td>
<td></td>
</tr>
<tr>
<td>Kalol463</td>
<td>35.68</td>
<td>44.0</td>
<td></td>
</tr>
</tbody>
</table>

3.1.2.4 Elemental analysis

CHNS elemental analysis is used for the rapid determination of C, H, N and S. It was observed here that all the samples were having low percentage of carbon and nitrogen, indicating oligotrophic environment for the native bacteria. None of the formation water sample contained sulfur. Nazina et al (2006) reported 0.5-3.3% nitrogen, 0.0027% sulfur and 0.06-0.77% carbon dioxide in formation water samples of Dagang oil field, China.
Table 3.6: C, H, N and S analysis of formation water samples.

<table>
<thead>
<tr>
<th>Well no.</th>
<th>C %</th>
<th>H %</th>
<th>N %</th>
<th>S %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Meh 71</td>
<td>0.53</td>
<td>12.59</td>
<td>0.130</td>
<td>ND</td>
</tr>
<tr>
<td>Meh 74</td>
<td>0.143</td>
<td>12.54</td>
<td>0.480</td>
<td>ND</td>
</tr>
<tr>
<td>Meh 103</td>
<td>0.125</td>
<td>12.23</td>
<td>0.325</td>
<td>ND</td>
</tr>
<tr>
<td>Bech 100</td>
<td>0.109</td>
<td>12.54</td>
<td>0.944</td>
<td>ND</td>
</tr>
<tr>
<td>Bech 144</td>
<td>0.104</td>
<td>12.72</td>
<td>0.357</td>
<td>ND</td>
</tr>
<tr>
<td>Limb 73</td>
<td>0.098</td>
<td>12.68</td>
<td>0.097</td>
<td>ND</td>
</tr>
<tr>
<td>GGS 1</td>
<td>0.252</td>
<td>12.31</td>
<td>0.574</td>
<td>ND</td>
</tr>
<tr>
<td>GGS 2</td>
<td>0.128</td>
<td>12.31</td>
<td>0.947</td>
<td>ND</td>
</tr>
<tr>
<td>Limb 28</td>
<td>0.140</td>
<td>12.24</td>
<td>0.692</td>
<td>ND</td>
</tr>
<tr>
<td>Kalol 463</td>
<td>0.136</td>
<td>12.30</td>
<td>0.458</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND: Not detected

3.1.2.5 Toxic heavy ion content

The presence of toxic heavy metal ions like Cu, Cd, Pb, As and Hg was checked in the formation water samples. It was observed that the concentration of Cu and Cd were much below the concentrations that are inhibitory to the bacteria. Pb, As and Hg were not detected in any of the formation water sample tested. The presence of Cu and Cd ions in such low concentration was not expected to affect the growth of the native bacteria.

The concentration of metals in formation water depends on the field properties and geology of the formation. According to Utvik (2003), there is no correlation between concentration of metal in the crude oil and in formation water associated with it.
Stephenson (1992) observed that concentration of metals in formation water is generally higher than those in sea water.

Table 3.7: Toxic heavy metal ion content of formation water samples

<table>
<thead>
<tr>
<th>Well No</th>
<th>Metal ion concentration (ppm)</th>
<th>Cu</th>
<th>Cd</th>
<th>Pb</th>
<th>As</th>
<th>Hg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Meh-71</td>
<td></td>
<td>0.154</td>
<td>0.095</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Meh 103</td>
<td></td>
<td>0.296</td>
<td>0.081</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Meh 74</td>
<td></td>
<td>0.104</td>
<td>0.08</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Bech 100</td>
<td></td>
<td>0.081</td>
<td>0.095</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Bech 144</td>
<td></td>
<td>0.06</td>
<td>0.221</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>GGS-2</td>
<td></td>
<td>0.079</td>
<td>0.137</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>GGS-1</td>
<td></td>
<td>0.080</td>
<td>0.099</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Kalol 463</td>
<td></td>
<td>0.137</td>
<td>0.081</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Limb 73</td>
<td></td>
<td>0.181</td>
<td>0.085</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Limb 28</td>
<td></td>
<td>0.073</td>
<td>0.089</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND: Not detected

3.2 Culture independent bacterial diversity of Indian oil reservoirs

Petroleum reservoir is a complex ecosystem with a wide range of eco-physiological parameters. The temperature of typical Indian oil reservoir may vary from 50°C to 136°C. The environment is almost always oxygen limiting, however, presence of dissolved oxygen or in the form of oxyanions may not be ruled out. A wide range of petroleum hydrocarbons as well as dissolved organics from the sediments are available for the microorganisms to be used as source of carbon and energy. Also, presence of sulfates, phosphates, nitrates, etc. may serve as terminal electron acceptor. In the view of above, it may be appreciated that microbial flora of the petroleum reservoir may not be entirely accounted by the culture
dependent methods. Hence, in the present investigation PCR based DNA fingerprinting techniques were used for the investigation of the microbial diversity of the petroleum reservoirs. Culture independent approach to investigate microbial diversity has been widely used in the published literature over past few decades. Initially culture-independent analysis of microbial community was done based on assessment of metabolically active microorganisms with the help of different stains like (INT)-formazan, 5-cyano-2, 3-ditolyltetrazolium chloride (CTC), etc. Other molecular techniques like in situ hybridization, analysis of total protein banding pattern and phospholipid fatty acid analysis were also used for the analysis of microbial communities. Rapid revolutions in the area of molecular techniques, PCR-based approaches have become most popular technique to study the community profile. These techniques involve DGGE, SSCP, intergenic spacer analysis, RFLP, DNA microarrays etc. A number of studies have documented microbial communities in hot oil reservoirs. Indigenous microbial communities have also been detected in core samples and water saturated regions of reservoirs. Members of indigenous reservoir communities may include strictly anaerobic, sulfate-reducing prokaryotes and methanogens, as well as other microbes. It is essential to establish databases of the microbial diversity in petroleum reservoirs so that genetic tools for exploration and production can then be developed.

There has been paucity of information about the microbial communities associated with Indian oil reservoirs. In this study the phylogenetic diversity of microorganisms based on 16S rRNA gene analysis of Indian oil reservoirs using nested PCR-DGGE was done. The subsequent sequences and phylogenetic analyses were expected to elucidate the bacterial taxonomic picture in poorly studied Indian oil reservoirs.
3.2.1 Extraction of nucleic acids from formation water

Total DNA of formation water samples was extracted by CTAB method as described by Zhou et al (1996). Agarose gel electrophoresis of the total DNA did not reveal visible bands when 3µl sample was loaded. Low concentration of the extracted DNA could be attributed to the low microbial counts (approximately $10^3$/ml) in the formation water samples. Absorbance observed at 260 nm, however, indicated the presence of DNA and hence, was used for the further downstream procedures. Low yield as well as relatively poor quality of DNA has been previously reported, especially in case of environmental samples. Such obstacles can be overcome in diversity studies by adopting the PCR based DNA fingerprinting approach.

Figure 3.2: PCR amplification of 600bp fragment of 16S rDNA. Lane 1- 100 bp ladder, Lane 2-GG2, Lane 3-Bech 144, Lane 4- Meh 74, Lane 5-Meh 103, Lane 6-GGS1, Lane 7-Kalol 463, Lane 8- Meh 71, Lane 9-Bech 100, Lane 10-Limb 28, Lane 11-Limb 73
In the present investigation, 16S rRNA gene fragments were amplified from the total environmental DNA using nested PCR approach. The outer PCR was performed using FDD2-RPP2 primer pair whereas SRV3-1 and Com2 primers (targeting V3-V5 hyper variable regions) were used for the nested PCR. A 40 bp GC clamp was attached to the 5’ end of the SRV3-1 primer to avoid complete melting of PCR amplicons during Denaturing Gradient Gel Electrophoresis (Wang et al., 2008). Specific amplification of desired 16S rRNA gene fragments (~600 bp) was verified by agarose gel electrophoresis (figure 3.2).

3.2.2 Culture independent profiling of microbial community associated with oil reservoirs by PCR-DGGE

A series of parameters were optimized to achieve optimum resolution of 16S rRNA gene fragments on DGGE. These parameters included polyacrylamide gel strength, use of PCR primers with GC clamp attached to the 5’ end, electrophoresis voltage, run time, denaturing gradient range etc. It was found that the resolution of the V3-V5 fragments was better when polyacrylamide gel strength was 6% than with 8% gel strength. This observation was contradictory to the earlier report mentioning reported better resolution on 8% gel strength. However, the 16S rRNA gene fragments resolve in that study were smaller in size (<400bp). The better resolution observed on 6% gel strength could be attributed to the longer fragments of 16S rRNA used in the present study. It is a well-known fact that better separation of larger fragments is achieved on gels with relatively lower strength. Early DGGE profile of V3-V5 fragments in the present investigation revealed that separated bands were smudgy or less sharp. However, the resolution as well
as sharpness of bands was significantly improved when PCR fragments were obtained with 40 bp GC clamp attached to the 5’ end of forward primer (GCclamp-SRV3-1).

**Figure 3.3:** DGGE patterns of the PCR products amplified from primers GC Clamp and Com 2.

- (a) Lane 1- GGS2, Lane 2- GGS1, Lane 3- Limb 73, Lane 4- Bech 100, Lane 5- Meh 74

- (b) Lane 6- Limb 28, Lane 7- Kalol 463, Lane 8- Meh 71, Lane 9 -Meh 103, Lane 10- Bech 144
This observation was consistent with the earlier reports where in, the GC clamp was explained to be responsible for preventing complete melting of the PCR fragments even after excessive exposure to the increasing concentration of chemical denaturants in DGGE. A range of various denaturing gradients, voltages and run times was tried out in the present investigation to achieve maximum resolution of the V3-V5 fragments of 16S rRNA genes of bacterial community associated with oil reservoirs of Western India. Amount of DNA loaded in each well was adjusted to 200 ng. DGGE profile of bacterial community DNA associated with individual oil wells is represented in figure 3.3

Total 36 well separated bands were eluted and subsequently re-amplified by PCR using SRV3-1 and Com 2 primers and sequenced using automated sequence (ABI prism 3100 Genetic Analyzer Applied Biosystems).

3.2.3 Diversity indices

Microbial community composition of different oil wells was statistically analyzed and compared using various diversity indices such as Shannon Weiver Index, Simpson’s Index etc. The diversity and relationship among the community constituents was interpreted from the DGGE profiles obtained in this study.

Simpson’s index, Simpson’s index of diversity; Simpson’s reciprocal index (1/D); Shannon-Weaver index; Equitability index \( J = \frac{H}{H_{max}} \), were calculated for the population examined, assuming each species has only one number. Simpson's index \( 1 - D \) and the Shannon-Weaver index \( H \) revealed more diversity in Meh 71 and Limb 28. The inverse of Simpson’s index \( 1/D \) was used to quantitatively measure microbial diversity associated with ten different samples, as reciprocal of Simpson’s Index is sensitive to the
dominance of species in community (Zhou et al., 2002). Reciprocal of Simpson's index ensures that an increase in the reciprocal index reflects an increase in diversity (Bento et al., 2005).

Table 3.8: Diversity indices to analyze bacterial community data in oil reservoirs.

<table>
<thead>
<tr>
<th>Sample</th>
<th>D</th>
<th>1-D</th>
<th>1/D</th>
<th>H</th>
<th>J</th>
</tr>
</thead>
<tbody>
<tr>
<td>GGS 2</td>
<td>0.14</td>
<td>0.86</td>
<td>7.14</td>
<td>2.28</td>
<td>0.841</td>
</tr>
<tr>
<td>GGS1</td>
<td>0.43</td>
<td>0.62</td>
<td>2.32</td>
<td>2.34</td>
<td>0.844</td>
</tr>
<tr>
<td>Limb73</td>
<td>0.24</td>
<td>0.76</td>
<td>4.16</td>
<td>1.80</td>
<td>0.753</td>
</tr>
<tr>
<td>Meh 74</td>
<td>0.20</td>
<td>0.80</td>
<td>5.00</td>
<td>1.75</td>
<td>0.90</td>
</tr>
<tr>
<td>Bech 100</td>
<td>0.17</td>
<td>0.83</td>
<td>5.88</td>
<td>2.35</td>
<td>0.74</td>
</tr>
<tr>
<td>Bech 144</td>
<td>0.45</td>
<td>0.55</td>
<td>2.22</td>
<td>2.22</td>
<td>0.841</td>
</tr>
<tr>
<td>Meh 71</td>
<td>0.092</td>
<td>0.090</td>
<td>10.86</td>
<td>2.59</td>
<td>0.879</td>
</tr>
<tr>
<td>Meh 103</td>
<td>0.12</td>
<td>0.88</td>
<td>8.33</td>
<td>2.27</td>
<td>0.862</td>
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<tr>
<td>Limb 28</td>
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<td>0.90</td>
<td>10.10</td>
<td>2.53</td>
<td>0.875</td>
</tr>
<tr>
<td>Kalol 463</td>
<td>0.124</td>
<td>0.88</td>
<td>8.06</td>
<td>2.39</td>
<td>0.827</td>
</tr>
</tbody>
</table>

Bech 144 and GGS 1 showed relatively low values of inverse of Simpson’s index (1/D), indicating less diversity in the formation water samples. This apparently low diversity may be a characteristic of the extreme conditions of the subsurface petroleum reservoir environment. Absence of dominant population in any of the oil reservoir tested was evident from the equitability (J) data (table 3.8).
3.2.4 Phylogenetic analysis of bacterial community in oil reservoirs by DGGE

Prominent 16S rRNA V3-V5 gene fragments separated by DGGE and representing microbial community of oil reservoirs were excised from acryl amide gel. A total of 36 bands were separated and after elution of each band they were reamplified by PCR using SRV3-1 and Com 2. The gel bands were purified and submitted to the automated sequencing in ABI prism 3100 Genetic Analyzer (Applied Biosystems). The formation of chimeric DNA fragments was not observed in those sequences. The nucleotide sequences obtained in this study have been deposited at the GenBank database under the accession numbers JX036286 to JX036321 (table 3.9). The phylogenetic relationships of the 36 sequenced bands are shown in figure 3.4. Significantly, a number of bands related to hydrocarbon-degrading strains previously found in oil wells and oil-contaminated soil samples. Cyclohexane degrading Brachymonas petroleovorans was isolated from oil refinery wastewater sludge (Rouviere and Chen, 2003). Chryseobacterium sp. was reported as PAH degrading bacteria (Kumar et al., 2011, Guo et al., 2008). Pelobacter carbinolicus was detected in the clone library obtained from the produced water from a high-temperature North Sea oil-field (Dahle et al., 2008).

Species of the Bacillus were reported as candidates for hydrocarbon degradation. Al-Sharidah et al (2000) used crude oil degrading Bacillus subtilis for hydrocarbon degradation studies. Cunha et al (2006) isolated sporulating gram-positive bacteria from an oil reservoir in Brazilian deep-water production basin. Fusibacter paucivorans was obtained from an African saline oil-producing well (Ravot et al., 1999). A few Operational Taxonomic Units (OTUs) in bacterial community analysis of the Huabei oilfield, China were related to Hydrogenophaga, Sphingomonas and Brachymonas species (Tang et al.,
2012). Microbial community analysis of oil reservoirs situated in South Brazilian offshore basin revealed 16S rRNA gene clones that were related to the Propionibacterium, Bacillus, (Sette et al., 2007).

Table 3.9: Identification of 16S rRNA gene sequences from bacterial community of formation water from western India oil reservoirs.

<table>
<thead>
<tr>
<th>Sample</th>
<th>DGGE band ID (accession no.)</th>
<th>Closest cultivable species (% identity) [accession no]</th>
<th>Maximum identity to GenBank (% identity) [accession no.]</th>
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<td>GGS 2</td>
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<td><em>Pelobacter carbinolicus</em> (97) [NC007498.2]</td>
<td><em>Pelobacter carbinolicus</em> (97) [NC007498.2]</td>
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<td></td>
<td>H2 (JX036287)</td>
<td><em>Fusibacter paucivorans</em> (96) [NR024886.1]</td>
<td><em>Fusibacter paucivorans</em> (96) [NR024886.1]</td>
</tr>
<tr>
<td>GGS 1</td>
<td>F1 (JX036288)</td>
<td><em>Brachymonas petroleovorans</em> (97) [AY275432.1]</td>
<td>Uncultured bacterium (97) [FN429400.1]</td>
</tr>
<tr>
<td></td>
<td>F2 (JX036289)</td>
<td><em>Hydrogenophaga sp.</em> (98) [AB681449.1]</td>
<td>Uncultured bacterium (99) [EF459884.1]</td>
</tr>
<tr>
<td>Limb73</td>
<td>1a (JX036290)</td>
<td><em>Hydrogenophaga sp.</em> (97) [EF179863.1]</td>
<td>Uncultured bacterium (99) [EF459884.1]</td>
</tr>
<tr>
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<td>1b (JX036291)</td>
<td><em>Brachymonas petroleovorans</em> (94) [AY275432.1]</td>
<td>Uncultured bacterium (95) [FN429400.1]</td>
</tr>
<tr>
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<td>1c (JX036292)</td>
<td><em>Nitrospira sp.</em> (97) [AJ224041.1]</td>
<td>Uncultured bacterium (98) [J0003189.1]</td>
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<tr>
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<td>M1 (JX036293)</td>
<td><em>Bacillus thioparans</em> (97) [JN999834.1]</td>
<td><em>Bacillus thioparans</em> (97) [JN999834.1]</td>
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<tr>
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<td>M2 (JX036294)</td>
<td><em>Thalassospira sp.</em> (99) [EU440812.1]</td>
<td><em>Thalassospira sp.</em> (99) [EU440812.1]</td>
</tr>
<tr>
<td></td>
<td>M3 (JX036295)</td>
<td><em>Thalassospira sp.</em> (92) [EU440812.1]</td>
<td><em>Thalassospira sp.</em> (92) [EU440812.1]</td>
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<tr>
<td>Bech 100</td>
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<td><em>Sphingomonas panni</em> (97) [HQ739092.1]</td>
<td>Uncultured bacterium (97) [GQ158668.1]</td>
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<td>C2 (JX036298)</td>
<td><em>Paracoccus sp.</em> (99) [681877.1]</td>
<td><em>Paracoccus sp.</em> (99) [681877.1]</td>
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<td>C3 (JX036299)</td>
<td><em>Paracoccus pantotrophus</em> (96) [JQ246875.1]</td>
<td>Uncultured bacterium (96) [JF189197.1]</td>
</tr>
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<td>C4 (JX036300)</td>
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<td><em>Paracoccus sp.</em> (99) [JN681877.1]</td>
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<tr>
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<td>Uncultured bacterium (98) [EF459884.1]</td>
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<td>A2 (JX036302)</td>
<td><em>Sulfurspirillum sp.</em> (94) [AF357199.1]</td>
<td>Uncultured bacterium (98) [FJ469320.1]</td>
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<tr>
<td></td>
<td>A3 (JX036303)</td>
<td><em>Pelomonas parvaue</em> (94) [JQ600112.1]</td>
<td>Uncultured bacterium (95) [AB240287.1]</td>
</tr>
<tr>
<td></td>
<td>A4 (JX036304)</td>
<td><em>Thauera sp.</em> (97) [AY570693.1]</td>
<td>Uncultured <em>Thauera</em> sp. (97) [JN648270.1]</td>
</tr>
<tr>
<td>Meh 71</td>
<td>a1 (JX036305)</td>
<td><em>Pelomonas saccharophila</em> (90) [AB681917.1]</td>
<td>Uncultured bacterium (91) [AB240287.1]</td>
</tr>
</tbody>
</table>
### Chapter III: Results and Discussion (A)

<table>
<thead>
<tr>
<th></th>
<th>Accession No.</th>
<th>Organism</th>
<th>Sequence Identity</th>
</tr>
</thead>
<tbody>
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<td><em>Pelomonas puraquae</em> (93)</td>
<td>Uncultured bacterium (91)</td>
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<td>[AB240287.1]</td>
</tr>
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<td>a3</td>
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</tr>
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<td>Uncultured organism (99)</td>
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<td></td>
<td></td>
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<td>b3</td>
<td>JX036311</td>
<td><em>Staphyloccocus</em> sp. (97) [JQ361085.1]</td>
<td>Uncultured bacterium (97)</td>
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</tr>
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<td></td>
<td>[JQ612532.1]</td>
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<td>Uncultured organism (99)</td>
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<td></td>
<td></td>
<td>[HQ749197.1]</td>
<td></td>
</tr>
<tr>
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<td>[JF208716.1]</td>
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</tr>
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<td>d3</td>
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<tr>
<td></td>
<td></td>
<td>[DQ532277.1]</td>
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<td></td>
<td></td>
<td>[HQ749197.1]</td>
<td></td>
</tr>
</tbody>
</table>

Bacterial phylotypes detected by PCR-DGGE of high temperature oil reservoir contained the sequence related to *Thermoanaerobacter brockii* (Lan et al., 2011). *Thauera* sp. was reported to be toluene degrader under denitrifying conditions (Van Hamme et al., 2003). *Staphyloccocus aureus, Pseudomonas aeruginosa and Bacillus* sp. were isolated from hydrocarbon-polluted site in Ilaje, Ondo state, Nigeria (Boboye et al., 2010). Sulfate reducing bacteria of the genus *Nitrospira* was detected in 16S rRNA clone library of Hubei oil field, China (Li et al., 2007). Recently two novel species of genus *Thalosospira* were isolated from surface water of waste oil pool (Liu, et al., 2007). Hubert et al., (2007) isolated *Sulfurospirillum* sp. from Up-flow, packed-bed bioreactors inoculated with water.
produced from an oil field. Paracoccus sp. was isolated from Microcoleus consortium and was reported to be growing at extremely high/low pH, high/low temperature and high salinity.

The same strain has ability to grow on petroleum hydrocarbons and tolerate heavy metal ions such as Cu\(^{2+}\) and Pb\(^{2+}\) (Diestra et al., 2007). Pelomonas sp. was found to degrade benzene anaerobically (Weelink, 2008). Carazole degrading Lysobacter sp. was isolated from onshore and freshwater sites by Maeda et al (2010). Majority of bands could be grouped in to Proteobacteria and Firmicutes. The total 36 sequences assigned to the 24 phylogenetic clades clustered among Firmicutes (10 sequences), Proteobacteria (22 sequences), Actinobacteria (3 sequences) and Bacteriodetes (1 sequence).

Formation water appears to harbor novel microbial species as many of the sequences shared less than 95% homology with the closest phylogenetic affiliates in the GeneBank database. Most bacteria detected in the PCR-DGGE library cluster among fermentative bacteria. The ten sequences that clustered within the firmicutes lineage are indicative of the large fraction of highly diverse Gram positive organisms (table 3.9; figure 3.4). In the present study, molecular analysis was used to characterize the diversity of bacterial community in the formation water from petroleum reservoirs of western India. Analysis showed four major bacterial classes in the sample: Firmicutes, gamma-proteobacteria, beta-proteobacteria and Actinobacteria. This observation revealed the presence of significantly diverse microbial population in the Indian oil reservoirs. Although the level of efforts expended in this study did not profoundly explain bacterial diversity, however, we may conclude that it is sufficient to understand the types of bacterial communities present in western India oil field which are otherwise not reported anywhere.
Figure 3.4: Phylogenetic tree demonstrating the relationship of the thirty six predominant band sequences based on 16S rRNA gene V3-V5 region from the petroleum reservoir with other sequences obtained from Blast searches. The scale bar represents 0.05 inferred substitutions per nucleotide position.
Part B: Enrichment and Isolation and Characterization of Petroleum Hydrocarbon Degrading Bacteria

Introduction

The physical characteristics and chemical composition of the ecosystem play a major role in survival of living organism especially in environment like oil field. Among physical characteristics, temperature is the major restrictive aspect for microbial growth in oil wells. In oil reservoirs, temperature increases along with depth at a rate of ca. 3°C per 100 m. Therefore deep oil reservoirs having an in situ temperature more than 130–150°C cannot sustain bacterial growth (Magot et al., 2000). It is known that oil-bearing strata are predominantly anoxic environments, due to which they are dominated by fermentative, sulfate-reducing and methanogenic bacteria. Bacteria in the oil reservoir mostly thrive at an oil/water interface. Crude oil is the most prominent organic matter in the oil reservoir; hence, the probability of isolating hydrocarbon degrading microorganisms from the oil well formation water is high. Along with hydrocarbons sulphur-, oxygen- and nitrogen-containing compounds are also susceptible to biodegradation (Fedorak et al., 1984, Huang et al., 2003). During degradation, intermediates such as acyclic and cyclic, saturated and aromatic carboxylic acids and phenols are produced from various hydrocarbons (Meredith et al., 2000, Taylor et al., 2001). The heterocyclic acids with multiple, different hetero atoms are the main cause of corrosion problems during processing of heavy, degraded oils (Tomczyk et al., 2001). Light hydrocarbons and gases like ethane, propane and butane can also be biodegraded (Boreham et al., 2001). As formation water is not a rich source of nutrients, the cell count
of microorganisms is low. This may necessitate the enrichment of hydrocarbon degrading bacteria before their isolation. By selective enrichment techniques, petroleum hydrocarbon degrading bacteria have been isolated successfully (Bastiaens et al.; 2000, Boldrin et al. 1993; Dagher 1996). Most of the times, these studies are based on selective enrichment in minimal media supplemented with various hydrocarbons.

3.3 Enrichment of hydrocarbon degrading bacteria

In the present investigation, the formation water samples were pooled to make a composite sample. This composite sample was used as an inoculum for the enrichment of petroleum hydrocarbon degrading bacteria. An enrichment medium contains only crude oil as a carbon source that permits the growth of specific types or species of bacteria which alone can utilize the components from crude oil. Therefore, an enrichment medium may have selective features. For this enrichment, the minimal medium was used which contained NH4Cl and KNO3 as nitrogen sources, phosphates, sodium chloride, trace element solution and 0.025% yeast extract. One percent (v/v) composite sample as the inoculum and crude oil as sole carbon and energy source were added to the medium. The enrichment of hydrocarbon degrading bacteria was performed at 37, 60, 70 and 80°C. Enrichment cultures were agitated at 130 rpm for 20 days. Positive growth was determined by an increase in the turbidity of the flasks containing crude oil as a sole carbon and energy source compared to the negative control flasks. No growth was observed in the enrichments at 70°C and 80°C whereas growth was observed at 37°C and at 60°C. In enrichments at 60°C, cell density reached up to 5 x 10^7 cells/ml after 20 days. Crude oil used as a sole source of carbon and energy, dispersed uniformly during the
enrichments probably due to the production of the biosurfactants by growing consortium of microorganisms (figure 3.5). It was reported that hydrocarbon-degrading microbes produce a variety of biosurfactant molecules. These molecules either remain attached to the cell surface or are released as extracellular molecules (van Hamme et al., 2003). Biosurfactants can effectively reduce the interfacial tensions of oil and water \textit{in situ} as well as the viscosity of the oil (Al-Sabagh, 2000; Liu et al., 2004).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3.5.png}
\caption{Dispersion of oil by biosurfactant produced via bacterial consortium}
\end{figure}

\begin{enumerate}
\item \textbf{Enrichment at 37°C}
\item \textbf{Enrichment at 60°C}
\end{enumerate}
The transfer was given after 20 days for both the mesophilic as well as thermophilic consortia in the same medium. After three successive transfers the bacterial consortium was used for the further experiments. This same enrichment method was used by Horowitz et al., (1975), Adebusoye et al (2007) and Etoumi et al (2008) for the isolation of oil degrading bacteria.

3.3.1 Growth on crude oil using semi solid media (Chemotaxis)

Chemotaxis is the phenomenon whereby, bacteria, direct their movements according to certain chemicals in their environment. Migrating towards the highest concentration of carbon and energy source is important for bacterial growth. In the present investigation chemotaxis assay was performed and the movement of bacteria towards carbon and energy source (crude oil) was considered as an indication of their possible ability to utilize petroleum hydrocarbons as a carbon and energy source.

**Figure 3.6: Chemotaxis shown by the consortium**

![Chemotaxis shown by the consortium](image)
Chemotaxis assay revealed the presence of biofilm below the oil layer. It was also observed that CO₂ (as seen as gas bubbles) and water (at the bottom) were generated due to bacterial respiratory metabolism in the test tube (figure 3.6). This experiment, thus, helped in the separation of crude oil utilizing bacteria from other bacteria from the consortium. The bacterial biofilm formed below the oil layer was re-inoculated in a minimal medium containing 1% dextrose. The incubation was carried out separately at 37°C as well as 60°C. After two days of incubation, growth was seen (10⁸ cells/ml). This consortium was then used for the isolation of bacteria on solid media.

3.3.2 *Utilization of vapors of n-alkanes on solid media*

The most important advantage of vapor-phase growth methods is that they can be performed faster than more complex chemical analytical tests. In the present study when thermophilic consortium was spread on a minimal medium containing n-alkanes in a vapor phase the colonies were observed. In this experiment the mixture of n-alkanes (C12-C18) was added on a Whatman filter paper and it was placed in the headspace of slant made up of minimal medium in a bottle (figure 3.23)

![Figure 3.7: Experimental set up for the utilization of n-alkanes on solid media](image)
These methods allow the investigation of large numbers of bacteria for their ability of utilizing multiple hydrocarbon substrates in a short period (Ridgway et al., 1990). It was observed that bacteria from consortium having ability to utilize alkanes at 50°C formed colonies in the test bottles in 3-4 days. Results were reproducible when transfer was given in same medium. For the isolation of hydrocarbon degrading bacteria use of oil agar has been reported previously (Walker and Colwell et al, 1976, Diaz et al, 2000). Ridgway et al (1990) isolated gasoline degrading bacteria by providing hydrocarbon vapors for the growth bacteria in minimal medium plates using small plastic containers.

### 3.4 Isolation of the hydrocarbon degrading bacteria

Bacterial cultures used in all experiments were isolated by selective enrichment technique. The thermophilic consortium of putative oil degrading bacteria obtained from the chemotaxis experiment was serially diluted and plated out to obtain isolated colonies on solidified nutrient media.

<table>
<thead>
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<th>Accession number</th>
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<td>X2</td>
<td>30 to 42</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>M3</td>
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</tr>
<tr>
<td>5</td>
<td>CB</td>
<td>30 to 42</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>L</td>
<td>30 to 42</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>S</td>
<td>30 to 42</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>GP</td>
<td>45 to 65</td>
<td>MCM B-882</td>
</tr>
<tr>
<td>9</td>
<td>HT</td>
<td>45 to 65</td>
<td>MCM B-883</td>
</tr>
<tr>
<td>10</td>
<td>Col</td>
<td>45 to 65</td>
<td>MCM B-885</td>
</tr>
<tr>
<td>11</td>
<td>ND</td>
<td>45 to 65</td>
<td>MCM B-886</td>
</tr>
</tbody>
</table>
Eleven morphotypes were scored after 48 hours of incubation (seven were at 37°C and four were at 60°C (table 3.10). It was observed that the isolates X2, M3, M1, CB, L and S were able to grow in the range of 30-42°C. Isolates GP, HT, Col and ND were able to grow in the range of 45-65°C. Culture F was able to grow in the range of 30-55°C. On the basis of growth temperature range, five cultures which could grow at 55°C or above were selected for further studies. These selected cultures F, GP, HT, Col and ND were further identified on the basis of 16S rRNA gene sequencing.

3.4.1 16S rRNA sequencing

Each of the five thermophilic isolates selected as described above, were identified on the basis of 16S rRNA gene sequence homology with the reference sequences available in the GenBank database. Table 3.11 illustrates the identification of the thermophilic oil degrading isolates.

Table 3.11: Identification based on sequencing of 16S rRNA gene

<table>
<thead>
<tr>
<th>Code</th>
<th>MCM B Code</th>
<th>Closest Phylogenetic Affiliation (GenBank Database)</th>
<th>Query coverage %</th>
<th>Homology %</th>
</tr>
</thead>
<tbody>
<tr>
<td>GP</td>
<td>882</td>
<td>Aeribacillus pallidus</td>
<td>98</td>
<td>99</td>
</tr>
<tr>
<td>HT</td>
<td>883</td>
<td>Hydrogenophilus hirschii</td>
<td>97</td>
<td>99</td>
</tr>
<tr>
<td>F</td>
<td>884</td>
<td>Bacillus pumilus</td>
<td>98</td>
<td>99</td>
</tr>
<tr>
<td>ND</td>
<td>885</td>
<td>Aeribacillus pallidus</td>
<td>98</td>
<td>99</td>
</tr>
<tr>
<td>Col</td>
<td>886</td>
<td>Aeribacillus pallidus</td>
<td>98</td>
<td>99</td>
</tr>
</tbody>
</table>

The nucleotide sequences have been deposited in the NCBI nucleotide sequence database under accession numbers JN701184.1 to JN701184.1. A comparative analysis of the
nucleotide sequence of the 16S rRNA gene revealed (table 3.11) that isolate GP, ND and Col were closely related to *Aeribacillus pallidus* (99% homology). Isolate HT was most closely affiliated to *Hydrogenophilus hirschi* (99% homology) and isolate F was closely associated with *Bacillus pumilus* (99% homology). The main aim of this investigation was to study the taxonomic identity and biochemical properties of the isolated oil degrading bacteria. This will be helpful in allowing them to adapt to the habitat conditions. In order to complete this aim, temperature range of growth as well as pH and salinity range for growth were determined.

### 3.5 Phenotypic and biochemical characterization of thermophilic isolates

The oil reservoir is characterized by extreme environmental conditions like high temperature and hydrostatic pressure, elevated concentrations of heavy metals, sharp gradients of all physicochemical parameters and low water content required for metabolism.

Like temperature, salinity and pH of formation waters can also limit bacterial growth. The salinity of the formation water may be low to very high. The pH of the formation water is in the range of 5 to 8.

In the present study, a total of 10 bacterial strains were isolated from the enrichment technique. Based on the ability to grow above 45°C, five strains were selected for their further characterization like their phenotypic, genotypic and physiological properties.
3.5.1 MCM B-882

*Morphological characterization*

The thermophilic strain, MCM B-882 was isolated from formation water at 60°C. Colony characteristics of this strain were studied on Luria medium solidified using gelrite as a solidifying agent. The colonies were rounded with wavy edges (1-3 mm in diameter) smooth, convex, transparent, colorless, structurally homogeneous and viscous. Cells of MCM B-882 were Gram-positive, rod-shaped occurring either solitarily or arranged in pairs and were 0.38 (0.33-0.47) x 2.75 (1.52-2.88) μm in size. Cells of a larger size were presumably due to impaired cell division. The vegetative cells of strain were straight and motile. MCM B-882 cells displayed pleomorphism, a characteristic feature of thermophiles (figure 3.8). Sporulating cells appeared at the end of the growth retardation phase.

*Nutritional requirements and physiological properties*

MCM B-882 was an obligate thermophile, which exhibited oxidase positive and catalase negative characters. It could grow under micro-aerobic conditions (in a rubber stoppered bottles half-filled with liquid medium and N₂ gas in the head space). The strain showed good growth on LB medium as well as in minimal medium with glucose. It was capable of utilizing a wide range of carbohydrates, alcohols, polycarboxylic acids, and hydrocarbons. MCM B-882 was able to utilize glucose, maltose, lactose, mannose, sucrose, glycerol, salicin, glucosamine, sorbitol, mannitol, esculin and D-arabinose as source of carbon and energy (table 3.12). MCM B-882 could grow in the pH range of 6.0-10.0 and optimally at 7.0; temperature range of 45 – 65°C and optimally at 60°C; as
well as in salt (NaCl) range of 1-4% and optimally at 2%. Specific growth rate of MCM B-882 determined in Luria broth under optimum growth conditions was found to be 0.65 (figure 3.9).

**FAME analysis**

The fatty acid spectrum of MCM B-882 lacked hydroxyl acids (table 3.13), which is typical of Gram-positive bacteria. Branched fatty acids prevailed in this strain. Isopentadecanoic, palmitic, and isoheptadecanoic acids were the predominant species. The prevalence of isopentadecanoic and isoheptadecanoic acids was earlier established in other representatives of the genus *Geobacillus*. The major fatty acid composition of MCM B-882 revealed isopentadecanoic acid (i15:0) as major component followed by isopalmitic acid. (i16:0). However, a difference could be seen in the content of iso-17:0 (isoheptadecanoic acid). It was present in low amount in MCM B-882. Myristic acids, palmitic acids and lauric acids also occurred in small amounts in MCM B-882 cells.

**Genotype Characterization**

The G+C content in the DNA of strain MCM B-882 was 42.24 %, a value close to those reported for the species of the genus *Geobacillus*. The Tm value was found to be 84.

**Phylogenetic Analysis**

The nucleotide sequence of a large portion of the 16S rRNA gene (1369 nucleotides) was determined. A comparative analysis of the nucleotide sequence of the 16S rRNA gene revealed (figure 3.10) that strain belonged to the cluster of species of the genus *Geobacillus*. Among the *Geobacillus* species, strain was most closely affiliated to
G. pallidus (99%) (table 3.14) which was later reclassified in a new genus *Aeribacillus*. Hence, MCM B-882 was identified as *Aeribacillus pallidus*.

**Comparison with the closest phylogenetic affiliates:**

The phylogenetic analysis revealed that strain MCM B-882 was affiliated with genus *Aeribacillus* as *Aeribacillus pallidus*. *A. pallidus* DSM 3670T is the only type strain and species of genus *Aeribacillus*. *Bacillus pallidus* first described by Scholz and colleagues in 1987 was renamed in 2004 by Banat and colleagues as “*Geobacillus pallidus*” (Yasawong et al., 2011). The same strain was subsequently transferred to the new genus *Aeribacillus* as *A. pallidus* by Minana-Galbis et al (2010) on the basis of 16S rDNA sequence divergence and as well as unique phenotypic characteristics (Minana-Galbis et al., 2010). Another strain *A. pallidus* TD1 was recently reported by Yasawong et al (2011). *A. pallidus* TD1 as well as MCM B-882, in the present study, contained linear and branched fatty acids, and some unsaturated fatty acids; branched saturated fatty acids were dominant. By contrast, no unsaturated fatty acids were detected in strain *A. pallidus* DSM 3670 and linear fatty acids were dominant. Palmitic acid was the main constituent (25% and 50% respectively) in TD1 and DSM 3670. However, palmitic acid constituted only 4.23% of the cellular fatty acids in MCM B-882. Further 16:1 w+c alcohol constituted 9.26% of the cellular fatty acids in MCM B-882 but the same fatty acid was absent in both TD1 as well as DSM 3670. Same observation was true even in case of 16:1 w7c alcohol. Uniqueness of MCM B-882 was further confirmed in DNA base composition studies which revealed 42.24 mol% G+C of MCM B-882 as significantly different from 38.9% and 39-41% reported for TD1 and DSM 3670 respectively. Carbohydrate utilization pattern observed for MCM B-882 was significantly
different from that for TD1 as well as DSM 3670. Only strain TD1 was able to produce acid from cellobiose, ribose, and xylose where as both TD1 and MCM B-882 produced acid from arabinose and mannose. DSM 3670 did not produce acid from any of these five carbohydrates. Thus it was concluded on the basis of genetic, biochemical characterization and FAME analysis that MCM B-882 was a novel species belonging to the genus *Aeribacillus* and hence designated as *Aeribacillus sp. nov.* MCM B-882.

**Figure: 3.8 Colony morphology and Microscopic observation of MCM B-882**

(A) Colony morphology of MCM B-882

(B) Gram staining of cells of MCM B-882

(C) SEM Photograph of cells of MCM B-882 (20000X)

(D) SEM Photograph of a single cell of MCM B-882 (20000X)
Table 3.12: Carbohydrate utilization profile of MCM B-882

<table>
<thead>
<tr>
<th>Sugars</th>
<th>Results (Acid production)</th>
<th>Sugars</th>
<th>Results (Acid production)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactose</td>
<td>-</td>
<td>Sorbitol</td>
<td>+</td>
</tr>
<tr>
<td>Xylose</td>
<td>-</td>
<td>Mannitol</td>
<td>+</td>
</tr>
<tr>
<td>Maltose</td>
<td>+</td>
<td>Adonitol</td>
<td>-</td>
</tr>
<tr>
<td>Fructose</td>
<td>+</td>
<td>α-Methyl D-Glucoside</td>
<td>+</td>
</tr>
<tr>
<td>Dextrose</td>
<td>+</td>
<td>Ribose</td>
<td></td>
</tr>
<tr>
<td>Galactose</td>
<td></td>
<td>Rhamnose</td>
<td></td>
</tr>
<tr>
<td>Raffinose</td>
<td>-</td>
<td>Cellobiose</td>
<td>-</td>
</tr>
<tr>
<td>Trehalose</td>
<td>+</td>
<td>Melezitose</td>
<td>-</td>
</tr>
<tr>
<td>Melibiose</td>
<td></td>
<td>α-Methyl D-Mannoside</td>
<td>-</td>
</tr>
<tr>
<td>Sucrose</td>
<td>+</td>
<td>Xylitol</td>
<td></td>
</tr>
<tr>
<td>L-arabinose</td>
<td>-</td>
<td>ONPG</td>
<td>-</td>
</tr>
<tr>
<td>Manose</td>
<td>+</td>
<td>Esculin</td>
<td>+</td>
</tr>
<tr>
<td>Inulin</td>
<td></td>
<td>D-Arabinose</td>
<td>+</td>
</tr>
<tr>
<td>Sodium gluconate</td>
<td>-</td>
<td>Citrate</td>
<td></td>
</tr>
<tr>
<td>Glycerol</td>
<td>+</td>
<td>Malonate</td>
<td></td>
</tr>
<tr>
<td>Salicin</td>
<td>+</td>
<td>Sorbose</td>
<td>-</td>
</tr>
<tr>
<td>Glucosamine</td>
<td>+</td>
<td>Inositol</td>
<td></td>
</tr>
<tr>
<td>Dulcitol</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 3.9: Growth of MCM B-882 at different (A) pH (B) Temperature (C) Salt concentrations. Growth of MCM B-882 in LB was shown in (D)
### Table 3.13: Cellular fatty acid composition (% w/w) of the reported *Geobacillus* species

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>MCM B-882</th>
</tr>
</thead>
<tbody>
<tr>
<td>i 14:0 isomyristic acid</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1.4</td>
<td>&lt;10</td>
<td>2.57</td>
<td></td>
</tr>
<tr>
<td>14:0 myristic acid</td>
<td>3.0</td>
<td>-</td>
<td>0.6</td>
<td>1.0</td>
<td>7.22</td>
<td>1.0</td>
<td>&lt;10</td>
<td>1.59</td>
<td></td>
</tr>
<tr>
<td>i 15:0 isopentadecanoic</td>
<td>24.3</td>
<td>34.03</td>
<td>40.0</td>
<td>34.80</td>
<td>22.80</td>
<td>32.42</td>
<td>22.60</td>
<td>&lt;10</td>
<td>27.86</td>
</tr>
<tr>
<td>a15:0 anteisopentadecanoic</td>
<td>0.4</td>
<td>1.30</td>
<td>4.40</td>
<td>1.90</td>
<td>1.01</td>
<td>1.30</td>
<td></td>
<td>7.28</td>
<td></td>
</tr>
<tr>
<td>16:1 w+c alcohol</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>9.26</td>
<td></td>
</tr>
<tr>
<td>i 16:0 isopalmitic acid</td>
<td>3.5</td>
<td>17.46</td>
<td>7.2</td>
<td>5.90</td>
<td>15.10</td>
<td>6.14</td>
<td>21.0</td>
<td>&lt;10</td>
<td>17.16</td>
</tr>
<tr>
<td>16:1 w 7 c alcohol</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>8.86</td>
<td></td>
</tr>
<tr>
<td>16:1 w 11 c alcohol</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>5.40</td>
<td></td>
</tr>
<tr>
<td>16:0 palmitic acid</td>
<td>23.3</td>
<td>5.80</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>4.23</td>
<td></td>
</tr>
<tr>
<td>17:1 iso w 10 c</td>
<td>-</td>
<td>1.90</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>5.10</td>
<td></td>
</tr>
<tr>
<td>i 17:0 isoheptadecanoicacid</td>
<td>16.6</td>
<td>34.86</td>
<td>33.80</td>
<td>23.50</td>
<td>26.30</td>
<td>39.77</td>
<td>18.50</td>
<td>&lt;10</td>
<td>7.32</td>
</tr>
<tr>
<td>a 17:0 anteisoheptadecanoic</td>
<td>5.8</td>
<td>6.10</td>
<td>15.30</td>
<td>10.10</td>
<td>4.97</td>
<td>4.6</td>
<td>&lt;10</td>
<td>10.01</td>
<td></td>
</tr>
<tr>
<td>12.0 lauric</td>
<td>1.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15:0 pentadecanoic</td>
<td>1.1</td>
<td>1.40</td>
<td>-</td>
<td>2.80</td>
<td>0.82</td>
<td>2.10</td>
<td>&lt;10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16:0 palmitic acid</td>
<td>23.3</td>
<td>-</td>
<td>14.50</td>
<td>17.10</td>
<td>4.98</td>
<td>11.20</td>
<td>&gt;10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17:0 heptadecanoic acid</td>
<td>3.2</td>
<td>1.20</td>
<td>-</td>
<td>1.70</td>
<td>0.53</td>
<td>1.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:1° Oleic</td>
<td>6.1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:0 Stearic</td>
<td>8.3</td>
<td>-</td>
<td>-</td>
<td>0.9</td>
<td>-</td>
<td>&lt;10</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a 14:0 anteisomyrisyic acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>&lt;10</td>
<td></td>
</tr>
</tbody>
</table>
Table 3.14: 16S rRNA gene sequencing based identification of MCM B-882.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Closest phylogenetic affiliation</th>
<th>Score</th>
<th>% homology</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCM B-882</td>
<td><em>Aeribacillus pallidus</em></td>
<td>2512</td>
<td>99%</td>
</tr>
</tbody>
</table>

> MCM B-882 (GenBank: JN701184.1)

TCCGGATTTCGGTGACGAGTTGTGATNTGGCTCAGGACGAACGCTGGCGGCGTGCCCTAA
TACATGCAAGTGCCGGACCGGAAGGAGCTGTGCTCCTTTAGTTAGTGCGGCACGGG
GAGTAAACACGTGGGCAACCTGGCAGACTGGGATAACTTNNGAAGACGAGCTAA
TACCCGATAACACCGAAAAACCGCATGTTTCTGGTGAAGGCGGCTTTTAGCTGTCAC
TGAGGATGGGCCGCCGCGCATTAGCTAGTTGAGTTGAAACGCTGCTCGAAGGAGAAGCGAC
GATGCGTAGCGACACCTGAGAGGTGACCGCGCCACACTGNACTGAGACACGGCCAGAC
ACCCTGAAACAGGGCGTACCCTTGAGCAGTACCCTGAGCAGAGAAGCGAAGCCCATCAGG
TGCCAGAAGCCGCTATTACGTAGTTGGCAAGCGTGTGCTCCGGGAATTATTTGGCGGTAA
GCCGCGGCGAGGGGCTTTCTAAGTCTGTATGGAAATTCTCGCCGCCAACCGGCAGGGG
CATTTGAAAACCTGGGAACCTTTGAGTACAGGAGGAGGGAGGAGGAATTCACGCTGTAGCGT
GAAATGCGCTAGAGATGGAGAGAACACAGTGGCGGAGGGCGCTCTCCTGGCCTGTAAC
GACGCTGAAGGGCCGAAAGCGTGGGGGACGCGAACGAGTTAGATACCCCTGTTAGTCCACGC
CGTAAACGATGAGTGTCAATGCTGTAGGATACCCCTTGTGCTGCTAGCAGCAACGC
ATTAGACACTCCCGCTTGGGAGTACCGGNTGAAGGTTAATTAAAGGAAATTTGAGGG
GGAACACCGACAACGTGGGAACAGCTGTTGTTTTAATTNGAAGCGCAACGCAAGCCTTAC
AGGCTCTGGACTNCCGTCGACAACCCTAGAGATAGGCCCTCCCCTCTCGGAGAGCGAG
TGACAGGTGGTCATGTTGTGCTAGCTGCTGTGCTGTGACGTGTTGTTGTTAAGCTCCTCC
AACAGCGGAACCTGGACTTTAGTTGGCGACATTAGTGGGCCACTCTAAGGGTGCAGT
CCGGCTAAAGTGGAGGAAGTGGGAGATGACGCTAAATCATCATCGCCCTTTATGACTC
GGCTACACACGTGTACAATGGGTTGTAAGGAGCAGGGCAACCGGAGATGGTGAGGCG
AATCCCCAAAAACACACTCTGAGTTGGAGATGAGCGTGACACTCGCTGCTGAGAAGCCG
GAATCGCTAGTA
Figure 3.10: Phylogenetic tree showing the position of strain MCM-B 882 within the radiation of the genus *Geobacillus* and related taxa. Bootstrap values (expressed as percentages of 1000 replications) greater than 50% are shown at branching points.
Table 3.15: Comparison of characteristics of all recognized Geobacillus species including strain MCM B-882

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>13</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell width (um)</td>
<td>0.5-1</td>
<td>0.8- 0.9</td>
<td>0.7-1</td>
<td>1.5</td>
<td>0.7-1</td>
<td>0.5- 1.2</td>
<td>0.6-1</td>
<td>0.7- 1</td>
<td>0.5-1</td>
<td>0.9-1</td>
<td>1-</td>
<td>0.5-</td>
<td>1.2</td>
</tr>
<tr>
<td>Cell length (um)</td>
<td>1.0- 14.2</td>
<td>2-5</td>
<td>3.7</td>
<td>3.5</td>
<td>3- &gt;100</td>
<td>3.7</td>
<td>2-3.5</td>
<td>1.5- 3.5</td>
<td>1.5-2.5</td>
<td>4.7-8</td>
<td>4.7-7</td>
<td>3-7</td>
<td>3.75</td>
</tr>
<tr>
<td>Motility</td>
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<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>ND</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Temp range for</td>
<td>50-70</td>
<td>30- 70</td>
<td>44- 80</td>
<td>43-80</td>
<td>42- 69</td>
<td>37- 60</td>
<td>40- 70</td>
<td>45-65</td>
<td>45-65</td>
<td>45-65</td>
<td>42-</td>
<td>45-65</td>
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<td>growth (0C)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>G+C (mol%)</td>
<td>50</td>
<td>39- 41</td>
<td>44</td>
<td>54</td>
<td>4</td>
<td>45- 46</td>
<td>52</td>
<td>52- 58</td>
<td>48- 58</td>
<td>50-</td>
<td>50-</td>
<td>50-</td>
<td>55</td>
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</table>

Acid produced from

<p>| | | | | | | | | | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
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<td>-</td>
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<td>Arabinose</td>
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<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>d</td>
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<td>-</td>
<td>-</td>
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</table>

Hydrolysis of

<p>| | | | | | | | | | | | | | |</p>
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<td>ND</td>
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</tbody>
</table>

3.5.2 MCM B-883

Morphological features

The thermophilic MCM B-883 was isolated from oil well formation water by an enrichment technique at 60°C in a Luria medium containing gelrite as a solidifying agent. The colonies were circular with even edges (1-2 mm in diameter) smooth, convex, transparent, colorless, and homogeneous and with butyrous consistency. Cells of MCM B-883 were Gram-negative, rod-shaped occurring either solitarily or arranged in pairs and were 0.35 (0.33-0.44) x 2.2 (1.55-2.7) μm in size (figure 3.11). The vegetative cells of strain were straight and motile. Sporulating cells did not appear at the end of the growth retardation phase.

Nutritional requirements and physiological properties

MCM B-883 was an obligate thermophile and exhibited oxidase positive and catalase negative characters. It could grow under micro-aerobic conditions (in a rubber stoppered bottles half-filled with liquid medium and N₂ gas in a head space). The strain showed good growth on LB medium as well as in minimal medium with glucose. It was capable of utilizing a wide range of carbohydrates, alcohols, polycarboxylic acids, and hydrocarbons. MCM B-883 was able to utilize dextrose, maltose, fructose, mannose sucrose, ribose and esculin as source of carbon and energy (table 3.16). MCM B-883 could grow in the pH range of 6.0-9.0 and optimally at 7.0. It could grow in the temperature range of 45-65°C and optimally at 60°C. It could grow in the salt concentration (NaCl) range of 1- 4% and optimally at 2% The MCM B-883 has specific growth rate 0.68 in Luria broth when grown under optimum growth conditions (figure 3.12).
**FAME analysis**

Fatty acids were analyzed as methyl ester derivatives prepared from dry cell material. It was observed that i15:0, i16:0 and a17:0 were the major fatty acids in MCM B-883. When compared with its closest affiliate match *H. hirschii*, it was observed that 16:0 is the major fatty acid present in the *H. hirschii*. It was also observed that cyclo C17:0, 18:1 w7c and 19:0 cyclo were present in *H. hirschii* whereas they were absent in MCM B-883 (table 3.17)

**Genotype Characterization**

The G+C content in the DNA of strain MCM B-883 was 58.54 %, a value close to those reported for the species of the genus *Hydrogenophilus*. The Tm value was found to be 92.26.

**Phylogenetic Analysis**

The nucleotide sequence of a large portion of the 16S rRNA gene (1335 nucleotides) was determined. A comparative analysis of the nucleotide sequence of the 16S rRNA gene revealed (figure.3.13) that strain belongs to the cluster of species of the genus *Hydrogenophilus*. It showed closest affiliation (99%) with *Hydrogenophilus hirschii* (table 3.18).

**Comparison of MCM B-883 with the closest phylogenetic affiliates:**

The phylogenetic analysis revealed that strain MCM B-883 was affiliated with genus *Hydrogenophilus* as *Hydrogenophilus hirschii*. *H. hirschii* DSM 11420 and *H. hirschii* JCM 10831 are the type strains of genus *Hydrogenophilus*. *H. hirschii* was first described by Stohr and colleagues in 2001. It was isolated from the Angel Terrace
Spring, Yellowstone National Park. *H. hirschii* DSM 11420 as well as MCM B-883 in the present study contained linear and branched fatty acids. No unsaturated fatty acids were detected in *H. hirschii* DSM 11420 as well as MCM B-883. Palmitic acid was the main constituent (55.34% respectively) in DSM 11420, whereas isopalmitic acid was the main constituent (14% respectively) in MCM B-883. However, palmitic acid constituted only 4.55% of the cellular fatty acids in MCM B-883. Further 16:1 w7alcohol alcohol constituted 6.58 % of the cellular fatty acids in MCM B-883 whereas it was only 3.12% in DSM 11420. a17:0 (anteisoheptadecanoic acid) constituted only 4.55% of the cellular fatty acids in MCM B-883 but the same fatty acid was absent in DSM 11420. Same observation was true even in case of a15:0 anteisopentadecanoic acid 16:1 w7c alcohol.

Uniqueness of MCM B-883 was further confirmed in DNA base composition studies which revealed 58.54 mol% G+C of MCM B-883 as significantly different from 61.0 % reported for DSM 11420. Carbohydrate utilization pattern observed for MCM B-883 was significantly different from that for DSM 11420. Only MCM B-883 was able to produce acid from fructose, sucrose and ribose as both DSM 11420 and MCM B-883 produced acid from glucose.

Thus it was concluded on the basis of genetic, biochemical characterization and FAME analysis that MCM B-883 was a novel species belonging to the genus *Hydrogenophilus* and hence designated as *Hydrogenophilus sp. nov.* MCM B-883 (table 3.19).
Figure 3.11: Colony morphology and microscopic observation

Table 3.20: Carbohydrate utilization profile of MCM B-883

<table>
<thead>
<tr>
<th>Sugars</th>
<th>MCM B-883</th>
<th>Sugars</th>
<th>MCM B-883</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactose</td>
<td>-</td>
<td>Sorbitol</td>
<td>-</td>
</tr>
<tr>
<td>Xylose</td>
<td>-</td>
<td>Mannitol</td>
<td>-</td>
</tr>
<tr>
<td>Maltose</td>
<td>+</td>
<td>Adonitol</td>
<td>-</td>
</tr>
<tr>
<td>Fructose</td>
<td>+</td>
<td>α-Methyl D-Glucoside</td>
<td>-</td>
</tr>
<tr>
<td>Dextrose</td>
<td>+</td>
<td>Ribose</td>
<td>+</td>
</tr>
<tr>
<td>Galactose</td>
<td>-</td>
<td>Rhamnose</td>
<td>-</td>
</tr>
<tr>
<td>Raffinose</td>
<td>-</td>
<td>Cellobiose</td>
<td>-</td>
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<td>Trehalose</td>
<td>+</td>
<td>Melezitose</td>
<td>-</td>
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<tr>
<td>Melibiose</td>
<td>-</td>
<td>α-Methyl D-Mannoside</td>
<td>-</td>
</tr>
<tr>
<td>Sucrose</td>
<td>+</td>
<td>Xylitol</td>
<td>-</td>
</tr>
<tr>
<td>L-arabinose</td>
<td>-</td>
<td>ONPG</td>
<td>-</td>
</tr>
<tr>
<td>Mannose</td>
<td>+</td>
<td>Esclusin</td>
<td>+</td>
</tr>
<tr>
<td>Inulin</td>
<td>-</td>
<td>D-Arabinose</td>
<td>-</td>
</tr>
<tr>
<td>Sodium gluconate</td>
<td>-</td>
<td>Citrate</td>
<td>-</td>
</tr>
<tr>
<td>Glycerol</td>
<td>+</td>
<td>Malonate</td>
<td>-</td>
</tr>
<tr>
<td>Salicin</td>
<td>+</td>
<td>Sorbose</td>
<td>-</td>
</tr>
<tr>
<td>Glucosamine</td>
<td>+</td>
<td>Inositol</td>
<td>-</td>
</tr>
<tr>
<td>Dulcitol</td>
<td>-</td>
<td></td>
<td>-</td>
</tr>
</tbody>
</table>
Figure 3.12: Growth of MCM B-883 at different (A) pH (B) Temperature (C) Salt concentrations. Growth of MCM B-883 in LB was shown in figure D.
Table 3.17: Cellular fatty acid composition (% w/w) of the reported *Hydrogenophilus* species

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>MCM B-883</th>
<th><em>H. themoluteolus</em></th>
<th><em>H. hirschi</em></th>
<th><em>H. islandicus</em></th>
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<tbody>
<tr>
<td>14:0 myristic acid</td>
<td>1.38</td>
<td>ND</td>
<td>2.48</td>
<td>ND</td>
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<tr>
<td>i 14:0 isomyristic acid</td>
<td>1.70</td>
<td>ND</td>
<td>NA</td>
<td>ND</td>
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<tr>
<td>i15:0 isopentadecanoic acid</td>
<td>24.12</td>
<td>ND</td>
<td>NA</td>
<td>ND</td>
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<tr>
<td>a15:0 anteisopentadecanoic acid</td>
<td>5.87</td>
<td>ND</td>
<td>NA</td>
<td>ND</td>
</tr>
<tr>
<td>16:1 w7 alcohol</td>
<td>6.58</td>
<td>ND</td>
<td>3.12</td>
<td>ND</td>
</tr>
<tr>
<td>i16:0 isopalmitic acid</td>
<td>13.99</td>
<td>ND</td>
<td>NA</td>
<td>ND</td>
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<tr>
<td>16:1 w c</td>
<td>3.96</td>
<td>ND</td>
<td>NA</td>
<td>ND</td>
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<tr>
<td>16:0 palmitic acid</td>
<td>4.55</td>
<td>Major</td>
<td>55.34</td>
<td>44.98</td>
</tr>
<tr>
<td>17: 1 isow10c</td>
<td>4.35</td>
<td>ND</td>
<td>NA</td>
<td>ND</td>
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<tr>
<td>i17:0 isoheptadecanoic acid</td>
<td>7.41</td>
<td>ND</td>
<td>NA</td>
<td>ND</td>
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<tr>
<td>a17:0 antisoheptadecanoic acid</td>
<td>12.53</td>
<td>ND</td>
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<td>ND</td>
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<tr>
<td>18:1 w9 c</td>
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<td>ND</td>
<td>not detected</td>
<td>ND</td>
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<tr>
<td>18:0 stearic acid</td>
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<td>Major</td>
<td>1.16</td>
<td>ND</td>
</tr>
<tr>
<td>19:0 cyclo w8c</td>
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<td>ND</td>
<td>NA</td>
<td>4.67</td>
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<tr>
<td>3-OH C10:0</td>
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<td>major 3-hydroxy cellular fatty acid.</td>
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<td>ND</td>
<td>17.92</td>
<td>13.90</td>
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<tr>
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<td>-</td>
<td>ND</td>
<td>2.26</td>
<td>17.93</td>
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<tr>
<td>19:0 cyclo</td>
<td>-</td>
<td>ND</td>
<td>12.98</td>
<td>ND</td>
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Table 3.18: 16S rRNA gene sequencing based identification of MCM B-883.

<table>
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<th>Strain</th>
<th>Closest phylogenetic affiliation</th>
<th>Score</th>
<th>% homology</th>
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<tr>
<td>MCM B-883</td>
<td><em>Hydrogenophilus hirschi</em></td>
<td>2523</td>
<td>99%</td>
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</tbody>
</table>
Chapter III: Results and Discussion (B)

>MCM B-883 (GenBank: JN701185.1)

ATTTGAAACGCTGGCGGCATGCNTTACACATGCAAGTCGAACGGCATCGCGGGAAAGCTT
GCTTTCCTGGCAGCAGTCGTAACGGGTGAGTAACACATCGGAACGTACCGGCTAGTG
GGGGATAACCCGTCGAAAGACGGGCTATACCGCATACGTTTTCGGAGNAAGCAGGGACC
TTGATCGCTCCAGCAATGCGCCGTGGTGAAGAAGGCTTTCCGGTGTAAAGCCCTTTTCGGCA
GGGAAGAAATCGCCGCGGGCAGAATACCTCTGCTGAGATACGACTGACAGAAGAAGGAC
CGCTAACTACGTGCAACAGCGCCCGTGTAATACGTAGGGTGGCAGCGTTAATCGGATTT
ACTGGCGTAAAGCGTGGCAGCGCGCCCGCCGTTAAGACAGGTGTAAGAATCCCTGCTGGCTCA
ACCTAGGAATTTGCGCTTTGTAGCTGCAGGCAGGCTGTAACGTAGGAAGGCTGGAATTC
TGTTGTAGCACTGAAATGCGTAGAGATCAGGAGAAGGAGCTGCAAACCTCCTGCTGAGATGG
CGGGATTGTGGCAGGAAGGCTGAAACGTAGGAAGGCTGGAATTC

**Figure 3.13:** Unrooted phylogenetic tree based on 16S rRNA gene sequence showing the position of MCM B-883 within the radiation of the genus *Hydrogenophilus*
Table 3.19: Comparison of characteristics of all recognized *Hydrogenophilus* species including strain MCM B-883

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<td>Cell length (um)</td>
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<td>Motility</td>
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<td>+</td>
<td>+</td>
<td></td>
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<td>Temp range for growth (0C)</td>
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<td>G+C (mol%)</td>
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<td>63-65</td>
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<td>NA</td>
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<td>-</td>
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<td>D-Glucose</td>
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<td>Alkane utilization</td>
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<td>NA</td>
<td>NA</td>
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</tr>
</tbody>
</table>

3.5.3 MCM B-884

**Morphological characterization**

The thermophilic strain, MCM B-884 was isolated from formation water at 55°C. Colony characteristics of this strain were studied on Luria medium solidified using gelrite as a solidifying agent. The colonies were circular (1-2 mm in diameter) smooth, convex, even-edged, transparent, colorless, and structurally homogeneous and butyrous. Cells of MCM B-884 were Gram-positive, rod-shaped occurring either solitarily or arranged in pairs and were 0.35 (0.33-0.42) x 1.75 (1.5-2.0) μm in size. Cells of a larger size were presumably due to impaired cell division. The vegetative cells of strain were straight and motile (figure 3.14). Sporulating cells appeared at the end of the growth retardation phase.

**Nutritional requirements and physiological properties**

MCM B-884 was thermotolerant, exhibiting oxidase positive and catalase negative characters. It could grow under micro-aerobic conditions (in a rubber stoppered bottles half-filled with liquid medium and N₂ gas in a head space). The strain showed good growth on LB medium as well as in minimal medium with glucose. It was capable of utilizing a wide range of carbohydrates, alcohols, polycarboxylic acids and hydrocarbons. MCM B-884 was able to utilize glucose, maltose lactose, mannose, sucrose, glycerol, salicin, glucosamine, sorbitol, mannotol, esculin and D-arabinose as source of carbon and energy (table 3.20). MCM B-884 could grow in the pH range of 6.0-10.0 and optimally at 7.0; temperature range of 30-55°C and optimally at 37°C; as well as salt concentration (NaCl) range of 1- 4% and optimally at 2%. Specific growth
rate of MCM B-882 determined in Luria broth under optimum growth conditions was 1.08 (figure 3.15).

**FAME analysis**

The fatty acid spectrum of MCM B-884 lacked hydroxyl acids (table 2), which is typical of Gram-positive bacteria. Branched fatty acids prevailed in this strain. In FAME analysis it was observed that 17:0 (cycloheptadecanoic acid) was the major fatty acid followed by 14:0 (myristic acid). Lauric acids and heptadecanoic acid also occurred in small amounts in MCM B-884 cells (table 3.21).

**Genotype Characterization**

The G+C content in the DNA of strain MCM B-884 was 56.82 %, a value close to those reported for the species of the genus *Bacillus*. The Tm value was found to be 92.05.

**Phylogenetic Analysis**

The nucleotide sequence of a large portion of the 16S rRNA gene (1395 nucleotides) was determined. A comparative analysis of the nucleotide sequence of the 16S rRNA gene revealed (figure 3.16) that the strain belonged to the cluster of species of the genus *Bacillus*. Among the *Bacillus* species, strain is most closely affiliated to *B. pumilus* (99%) (table 3.22). Species of the *Bacillus* has been reported as candidates for hydrocarbon degradation and has been reported to be present in the oil reservoirs.
Figure 3.14: Colony morphology and microscopic observation of MCM B-884

(C) SEM photograph of cells of MCM B-884

(D) SEM photograph of cells of MCM B-884

Table 3.20: Carbohydrate utilization profile of MCM B-884

<table>
<thead>
<tr>
<th>Sugars</th>
<th>MCM B-884</th>
<th>Sugars</th>
<th>MCM B-884</th>
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<tbody>
<tr>
<td>Lactose</td>
<td>+</td>
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<td>+</td>
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<td>Xylose</td>
<td>+</td>
<td>Sorbitol</td>
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<td>+</td>
<td>Mannitol</td>
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<td>Fructose</td>
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<td>Adonitol</td>
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<td>Dextrose</td>
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<td>α-Methyl D-Glucoside</td>
<td>+</td>
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<tr>
<td>Galactose</td>
<td>+</td>
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</tr>
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<td>Raffinose</td>
<td>+</td>
<td>Rhamnose</td>
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<tr>
<td>Trehalose</td>
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<td>Cellobiose</td>
<td>-</td>
</tr>
<tr>
<td>Melibiose</td>
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<td>Melezitose</td>
<td>-</td>
</tr>
<tr>
<td>Sucrose</td>
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<td>α-Methyl D-Mannoside</td>
<td>-</td>
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<tr>
<td>L-arabinose</td>
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<td>Manose</td>
<td>+</td>
<td>ONPG</td>
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<tr>
<td>Inulin</td>
<td>+</td>
<td>Esculin</td>
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<td>Sodium gluconate</td>
<td>-</td>
<td>D-Arabinose</td>
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</tr>
<tr>
<td>Glycerol</td>
<td>-</td>
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<td>Salcin</td>
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Figure 3.15: Growth of MCM B-884 at different (A) pH (B) Temperature (C) Salt concentrations. Growth of MCM B-884 in LB was shown in (D).

Table 3.21: Cellular fatty acid composition (% w/w) of MCM B-884

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>% total fatty acids</th>
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<td>12:0 Lauric acid</td>
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<td>14:0 myristic acid</td>
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<tr>
<td>17:0 cycloheptadecanoic acid</td>
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Table 3.22: 16S rRNA gene sequencing based identification of MCM B-884.

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<th>Closest phylogenetic affiliation</th>
<th>Score</th>
<th>% homology</th>
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<td>MCM B-884</td>
<td>Bacillus pumilus</td>
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<td>99%</td>
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Chapter III: Results and Discussion (B)

> MCM B-884 (GenBank: JN701186.1)

```
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AAGGACTCAGCGCCGGTTTCTATAAGTCTGATGTGAAGCCCGTCACACCAGGCGC
TAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTA
AAGGACTCAGCGCCGGTTTCTATAAGTCTGATGTGAAGCCCGTCACACCAGGCGC
TAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTA
```

Figure 3.16: Unrooted phylogenetic tree based on 16S rRNA gene sequence showing the position of MCM B-884 within the radiation of the genus *Bacillus*. 
3.5.4 MCM B-885

*Morphological characterization*

The thermophilic strain, MCM B-885 was isolated from formation water at 60°C. Colony characteristics of this strain were studied on Luria medium solidified using gelrite as a solidifying agent. The colonies were rounded with wavy edges (1-3 mm in diameter) smooth, convex, transparent, colorless, structurally homogeneous and viscous. Cells of MCM B-885 were gram-positive, rod-shaped occurring either solitarily or arranged in pairs and were 1.55 (0.2-0.24) x 3.00 (2.16-3.0) μm in size. The vegetative cells of strain were straight and motile. MCM B-885 cells displayed pleomorphism, a characteristic feature of thermophiles (figure 3.17). Sporulating cells appeared at the end of the growth retardation phase.

*Nutritional requirements and physiological properties*

MCM B-885 was an obligate thermophile, which exhibited oxidase positive and catalase negative characters. It could grow under micro-aerobic conditions (in a rubber stoppered bottles half-filled with liquid medium and N2 gas in a head space). The strain showed good growth on LB medium as well as in minimal medium with glucose. It was capable of utilizing a wide range of carbohydrates, alcohols, polycarboxylic acids, and hydrocarbons. MCM B-885 was able to utilize glucose, maltose lactose, mannose, sucrose, glycerol, salicin, glucosamine, sorbitol, mannitol, esculin and D-arabinose as source of carbon and energy (table 3.23). MCM B-885 could grow in the pH range of 6.0-10.0 and optimally at 7.0; temperature range of 45-65°C and optimally at 60°C; as well as salt concentration (NaCl) range of 1- 4% and optimally at 1%. Specific growth
rate of MCM B-885 determined in Luria broth under optimum growth conditions was 0.53 (figure 3.18), which is lower than that of MCM B-882 (i.e. 0.65).

**FAME analysis**

Branched fatty acids prevailed in the MCM B-885. Iso-pentadecanoic, anteisopentadecanoic acid and anteisoheptadecanoic acid were the predominant acids. The prevalence of isopentadecanoic and isoheptadecanoic acids had been earlier established in other representatives of the genus *Geobacillus*. Myristic acids and palmitic acids also occurred in small amounts. However, a difference could be seen in the content of iso-17:0: it was present in low amount in MCM B-885. Myristic acids, palmitic acids and lauric acids also occurred in small amounts in MCM B-885 cells (table 3.24)

**Genotype Characterization**

The G+C content in the DNA of strain MCM B-885 was 40.19 %, a value close to those reported for the species of the genus *Geobacillus*. The Tm value was found to be 86.

**Phylogenetic Analysis**

The nucleotide sequence of a large portion of the 16S rRNA gene (1398 nucleotides) was determined. A comparative analysis of the nucleotide sequence of the 16S rRNA gene revealed (figure 3.19) that strain belongs to the cluster of species of the genus *Geobacillus*. Among the *Geobacillus* species, strain is most close to *G. pallidus* (99%) (table 3.25). *G. pallidus* is now reclassified in a new genus *Aeribacillus*. So MCM B-885 was identified as *Aeribacillus pallidus*. 
Comparison with the closest phylogenetic affiliates:

The phylogenetic analysis revealed that strain MCM B-885 was affiliated with genus *Aeribacillus as Aeribacillus pallidus*. *A. pallidus* DSM 3670T is the only type strain and species of genus *Aeribacillus*. Another strain *A. pallidus* TD1 was recently reported by Yasawong *et al.* (2011). *A. pallidus* TD1 as well as MCM B-885, in the present study, contained linear and branched fatty acids, and some unsaturated fatty acids; branched saturated fatty acids were dominant. By contrast, no unsaturated fatty acids were detected in strain *A. pallidus* DSM 3670 and linear fatty acids were dominant. Palmitic acid was the main constituent (25% and 50% respectively) in TD1 and DSM 3670. In case of MCM B-885 palmitic acid constituted only 2.37% of the cellular fatty acids.

Uniqueness of MCM B-885 was further confirmed in DNA base composition studies which revealed 40.19 mol% G+C of MCM B-885 as significantly different from 38.9% and 39-41% reported for TD1 and DSM 3670 respectively. Carbohydrate utilization pattern observed for MCM B-885 was significantly different from that for TD1 as well as DSM 3670. Only strain TD1 was able to produce acid from cellobiose, ribose and xylose where as both TD1 and MCM B-885 produced acid from arabinose and mannose. DSM 3670 did not produce acid from any of these five carbohydrates. Thus it was concluded on the basis of genetic, biochemical characterization and FAME analysis that MCM B-885 was a novel species belonging to the genus *Aeribacillus* and hence designated as *Aeribacillus sp. nov.* MCM B-885 (table 3.26).
Figure 3.17 Colony morphology and Microscopic observation of MCM B-885

(A) Colony morphology of MCM B-885

(B) Gram Staining of MCM B-885

(C) SEM photograph of cells of MCM B-885

Table 3.23: Carbohydrate utilization profile of MCM B-885

<table>
<thead>
<tr>
<th>Sugars</th>
<th>MCM B-885</th>
<th>Sugars</th>
<th>MCM B-885</th>
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<td>Lactose</td>
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</tr>
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<td>Fructose</td>
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<td>Adonitol</td>
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<td>Dextrose</td>
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<td>Galactose</td>
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</tr>
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<td>Trehalose</td>
<td>+</td>
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<td>Melibiose</td>
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<td>Melezitose</td>
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</tr>
<tr>
<td>Sucrose</td>
<td>+</td>
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<td>-</td>
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<td>Xylitol</td>
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<td>Esculin</td>
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</tr>
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<td>Glucosamine</td>
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<td>Dulciol</td>
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</tbody>
</table>
**Figure 3.18:** Growth of MCM B-885 at different (A) pH (B) Temperature (C) Salt concentrations. Growth of MCM B-885 in LB was shown in (D).

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>MCM B-885</th>
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</thead>
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<td>i 14:0 isomyristic acid</td>
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<td>i 17:0 isoheptadecanoic acid</td>
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<td>a 17:0 antisoheptadecanoic acid</td>
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Table 3.25: 16S rRNA gene sequencing based identification of MCM B-885.

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<th>Strain</th>
<th>Closest phylogenetic affiliation</th>
<th>Score</th>
<th>% homology</th>
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<td>MCM B-885</td>
<td>Aeribacillus pallidus</td>
<td>2523</td>
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</table>

> MCM B-885 (GenBank: JN701187.1)

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Figure 3.19: Phylogenetic tree showing the position of strain MCM-B 885 within the radiation of the genus *Geobacillus* and related taxa. Bootstrap values (expressed as percentages of 1000 replications) greater than 50% are shown at branching points.
Table 3.26: Comparison of characteristics of all recognized *Geobacillus* species including strain MCM B-885

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</table>

3.5.5 MCM B-886

*Morphological characterization*

The thermophilic strain, MCM B-886 was isolated from formation water at 60°C. Colony characteristics of this strain were studied on Luria medium solidified using gelrite as a solidifying agent. The colonies were circular with wavy edges (2-3 mm in diameter) smooth, convex, transparent, colorless, structurally homogeneous and viscous. Cells of MCM B-886 were Gram-positive, rod-shaped occurring either solitarily or arranged in pairs and were 0.38 (0.35-0.5) x 3.75 (2.28-4.3) μm in size. The vegetative cells of strain were straight and motile. Like MCM B-882 and MCM B-885, cells of the MCM B-886 displayed pleomorphism (figure 3.20). Sporulating cells appeared at the end of the growth retardation phase.

*Nutritional requirements and physiological properties*

MCM B-886 was an obligate thermophile, which exhibited oxidase positive and catalase negative characters. It could grow under micro-aerobic conditions (in a rubber stoppered bottles half-filled with liquid medium and N2 gas in a head space). The strain showed good growth on LB medium as well as in minimal medium with glucose. It was capable of utilizing a wide range of carbohydrates, alcohols, polycarboxylic acids, and hydrocarbons. MCM B-886 was able to utilize glucose, maltose lactose, mannose, sucrose, glycerol, salicin, glucosamine, sorbitol, mannitol, esculin and D-arabinose as source of carbon and energy (table 3.27). MCM B-886 could grow in the pH range of 6.0-10.0 and optimally at 7.0; temperature range of 45-65°C and optimally at 60°C; as well as salt concentration (NaCl) range of 1- 4% and optimally at 1%. Specific growth
rate of MCM B-886 determined in Luria broth under optimum growth conditions was 0.41 (figure 3.21).

**FAME analysis**

Branched fatty acids prevailed in the MCM B-886. Iso-pentadecanoic, isoplamitric acid and anteisoheptadecanoic acid were the predominant species. The prevalence of isopentadecanoic and isoheptadecanoic acids had been earlier established in other representatives of the genus *Geobacillus*. However, a difference could be seen in the content of iso-17:0: it was present in low amount in MCM B-886. Myristic acids and plamitic acids also occurred in small amounts in MCM B-886 cells (table 3.28)

**Genotype Characterization**

The G+C content in the DNA of strain MCM B-886 was 39.41 %, a value close to those reported for the species of the genus *Geobacillus*. The Tm value was found to be 85.

**Phylogenetic Analysis**

The nucleotide sequence of a large portion of the 16S rRNA gene (1400 nucleotides) was determined. A comparative analysis of the nucleotide sequence of the 16S rRNA gene revealed (figure 3.21) that strain belongs to the cluster of species of the genus *Geobacillus*. Among the *Geobacillus* species, strain is most close to *G. pallidus* (99%) (table 3.29). *G. pallidus* is now reclassified in a new genus *Aeribacillus*. So MCM B-886 was identified as *Aeribacillus pallidus*. 
Comparison with the closest phylogenetic affiliates:

The phylogenetic analysis revealed that strain MCM B-886 was affiliated with genus *Aeribacillus* as *Aeribacillus pallidus*. *A. pallidus* DSM 3670T is the only type strain and species of genus *Aeribacillus*. Recently Yasawong et al (2011) reported another strain *A. pallidus* TD1. *A. pallidus* TD1 as well as MCM B-886, in the present study, contained linear and branched fatty acids, and some unsaturated fatty acids; branched saturated fatty acids were dominant. By contrast, no unsaturated fatty acids were detected in strain *A. pallidus* DSM 3670 and linear fatty acids were dominant. Palmitic acid was the main constituent (25% and 50% respectively) in TD1 and DSM 3670. However, Palmitic acid constituted only 4.23% of the cellular fatty acids in MCM B-886.

Uniqueness of MCM B-886 was further confirmed in DNA base composition studies which revealed 39.41 mol% G+C of MCM B-886 as different from 38.9% reported for TD1. Carbohydrate utilization pattern observed for MCM B-886 was significantly different from that for TD1 as well as DSM 3670. Only strain TD1 was able to produce acid from cellobiose, ribose and xylose where as both TD1 and MCM B-886 produced acid from arabinose and mannose. DSM 3670 did not produce acid from any of these five carbohydrates. Thus it was concluded on the basis of genetic, biochemical characterization and FAME analysis that MCM B-886 was a novel species belonging to the genus *Aeribacillus* and hence designated as *Aeribacillus sp. nov*. MCM B-886 (table 3.30).
Figure 3.20: Colony morphology and Microscopic observation of MCM B-886

(A) Colony morphology of MCM B-886
(B) Gram Staining of MCM B-886
(C) SEM photograph of cells of MCM B-886

Table 3.27: Carbohydrate utilization profile of MCM B-886

<table>
<thead>
<tr>
<th>Sugars</th>
<th>MCM B-886</th>
<th>Sugars</th>
<th>MCM B-886</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactose</td>
<td>-</td>
<td>Inositol</td>
<td>-</td>
</tr>
<tr>
<td>Xylose</td>
<td>-</td>
<td>Sorbitol</td>
<td>+</td>
</tr>
<tr>
<td>Maltose</td>
<td>+</td>
<td>Mannitol</td>
<td>+</td>
</tr>
<tr>
<td>Fructose</td>
<td>+</td>
<td>Adonitol</td>
<td>+</td>
</tr>
<tr>
<td>Dextrose</td>
<td>+</td>
<td>α-Methyl D-Glucoside</td>
<td>+</td>
</tr>
<tr>
<td>Galactose</td>
<td>-</td>
<td>Ribose</td>
<td>-</td>
</tr>
<tr>
<td>Raffinose</td>
<td>-</td>
<td>Rhamnose</td>
<td>-</td>
</tr>
<tr>
<td>Trehalose</td>
<td>+</td>
<td>Cellobiose</td>
<td>-</td>
</tr>
<tr>
<td>Melibiose</td>
<td>Melezitose</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Sucrose</td>
<td>+</td>
<td>α-Methyl D-Mannoside</td>
<td>-</td>
</tr>
<tr>
<td>L-arabinose</td>
<td>-</td>
<td>Xylitol</td>
<td>-</td>
</tr>
<tr>
<td>Manose</td>
<td>+</td>
<td>ONPG</td>
<td>+</td>
</tr>
<tr>
<td>Inulin</td>
<td>-</td>
<td>Esculin</td>
<td>+</td>
</tr>
<tr>
<td>Sodium gluconate</td>
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<td>D-Arabinose</td>
<td>+</td>
</tr>
<tr>
<td>Glycerol</td>
<td>+</td>
<td>Citrate</td>
<td>+</td>
</tr>
<tr>
<td>Salicin</td>
<td>+</td>
<td>Malonate</td>
<td>-</td>
</tr>
<tr>
<td>Glucosamine</td>
<td>+</td>
<td>Sorbose</td>
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</tr>
<tr>
<td>Dulciol</td>
<td>-</td>
<td></td>
<td>-</td>
</tr>
</tbody>
</table>
Figure 3.21: Growth of MCM B-886 at different (A) pH (B) Temperature (C) Salt concentrations. Growth of MCM B-886 in LB was shown in (D).

Table 3.28: Fatty acid profile of MCM B-886

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>MCM B-886</th>
<th>Fatty acid</th>
<th>MCM B-886</th>
</tr>
</thead>
<tbody>
<tr>
<td>i 14:0 isomyristic acid</td>
<td>2.69</td>
<td>16:1 w 7 c alcohol</td>
<td>8.86</td>
</tr>
<tr>
<td>14:0 myristic acid</td>
<td>1.81</td>
<td>16:1 w 11 c alcohol</td>
<td>5.40</td>
</tr>
<tr>
<td>i 15:0 isopentadecanoic acid</td>
<td>26.34</td>
<td>16:0 palmitic acid</td>
<td>4.23</td>
</tr>
<tr>
<td>a15:0 anteisopentadecanoic acid</td>
<td>6.57</td>
<td>17:1 iso w 10 c</td>
<td>5.10</td>
</tr>
<tr>
<td>i 16:0 isopalmitic acid</td>
<td>17.16</td>
<td>i 17:0 isopentadecanoic acid</td>
<td>7.32</td>
</tr>
<tr>
<td>a 17:0 anteisopentadecanoic acid</td>
<td>10.11</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 3.29: 16S rRNA gene sequencing based identification of MCM B-886.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Closest phylogenetic affiliation</th>
<th>Score</th>
<th>% homology</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCM B-886</td>
<td><em>Aeribacillus pallidus</em></td>
<td>2412</td>
<td>99%</td>
</tr>
</tbody>
</table>

> MCM B-886 (GenBank: JN701188.1)

CTCCGGATTCGTTNNGACAGAGTTTAGTTGATCTGCTCGCTACGGACGAACGGCTGGGCCGCTGCCTA
ATACTAGCTAAGTCGAGGCGAAGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT
TGGATAACGTCGAGGCGAAGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT
CTCCGGATTCGTTNNGACAGAGTTTAGTTGATCTGCTCGCTACGGACGAACGGCTGGGCCGCTGCCTA
ATACTAGCTAAGTCGAGGCGAAGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT
Figure 3.22: Phylogenetic tree showing the position of strain MCM-B 886 within the radiation of the genus *Geobacillus* and related taxa. Bootstrap values (expressed as percentages of 1000 replications) greater than 50% are shown at branching points.
Table 3.30: Comparison of Characteristics of all recognized *Geobacillus* species including strain MCM B-886

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>13</th>
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<td>Cell width (um)</td>
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<td>0.7-</td>
<td>1.5</td>
<td>0.7-</td>
<td>0.5-</td>
<td>0.6-</td>
<td>0.7-</td>
<td>0.5-</td>
<td>0.9-</td>
<td>1-</td>
<td>0.5-</td>
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<td>3.5</td>
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<td>4.7-</td>
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<td>45-65</td>
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<td>39-41</td>
<td>44</td>
<td>54</td>
<td>4</td>
<td>45-46</td>
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<td>52-58</td>
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<td>Trehalose</td>
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<td>-</td>
<td>ND</td>
<td>d</td>
<td>+</td>
<td>+</td>
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<td>+</td>
<td>+</td>
<td>ND</td>
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</table>

Part C: Optimization of Process Parameters for Microbial Degradation of Petroleum Hydrocarbons

Introduction

In the next 25 years requirement for fossil energy in the world is likely to grow by more than 50%. There are many advances in alternative technologies but the demand for fossil fuel is continuously increasing. Conventional oil recovery methods are able to recover approximately one-third of the oil in place. Hence, to supply adequate quantity of oil as per the ever increasing demand there is a need of either new oil reservoirs or advanced techniques for the recovery of oil from existing reservoirs. Biotransformation of straight chain alkanes to methane under anaerobic condition now has been reported. It is accepted that in situ methanogenesis in matured oil reservoirs will be valuable for getting energy in the form of natural gas. Under anaerobic conditions, using hydrocarbon degrading methanogenic consortium residual oil from the matured oil reservoirs can be transformed to methane. The recovery of crude oil in the form of methane (major component of natural gas) can become important technique to obtain natural gas as an alternate form of energy from the matured oil reservoirs.

Upon microbial degradation of hydrocarbons many polar compounds like fatty acids, organic acids, and alcohols are produced. These low molecular weight compounds further act as substrates for the fermentative, acetogenic and sulfate-reducing bacteria. Further metabolic transformations of these compounds produce H₂ and acetate that can then be used by methanogenic bacteria to produce methane. In past it was thought that
microbial oil degradation was an aerobic process. Therefore *in situ* methanogenesis in the oil reservoir was not accepted because methanogenesis has always been recognized as an obligate anaerobic process. Recent geochemical considerations and microbiological data strongly indicate that oil biodegradation in the deep terrestrial subsurface proceeds mainly through anaerobic metabolism (Roling et al., 2003; Head et al., 2003; Larter et al., 2003). Recent studies have now shown that petroleum hydrocarbon biodegradation can be directly coupled to methane production. For example, the production of methane from the decay of toluene, o-xylene, benzene, alkanes and some alicyclic compounds has been documented (Townsend et al., 2004). The conversion of hexadecane to CH$_4$ might require three groups of microorganisms: acetogenic (or syntrophic) bacteria converting hexadecane to acetate and H$_2$, and acetoclastic and hydrogenotrophic archaea producing CH$_4$ from acetate or H$_2$ and CO$_2$, respectively. The following part of the thesis describes the optimization of process parameters for microbial degradation of petroleum hydrocarbons/ crude oil.

### 3.6 Standardization of analytical techniques

Crude oil is a mixture of different hydrocarbons which can be separated into aliphatic and aromatic hydrocarbons, asphaltenes and resins using chromatographic techniques (Rosini et al., 1960). As alkanes are the major fraction of the crude oil, the methods for extraction and GC analysis of alkanes were standardized. For the analysis of residual crude oil, a spectrophotometric technique was standardized. Components like H$_2$, CO$_2$ and VFA were analyzed on GC.
3.6.1 Standardization of GC for aliphatic hydrocarbon degradation

The detailed analysis of crude oil for its characterization can be done by gas chromatography (GC) and gas chromatography/mass spectrometry (GC/MS). The analysis of crude oil on GC or GC/MS is a common technique. The fractions of crude oil i.e. aliphatic and/or aromatic hydrocarbons can also be analyzed using GC or GC/MS (Hostettler and Kvenvolden, 1994; Wang et al., 1995; Barakat et al., 1999). Aliphatic hydrocarbons (n-alkanes and branched alkanes) are the major part of most of the crude oil in the world.

During present investigation GC/MS facility was not available. Therefore, n-alkanes were analyzed on GC. The average response factors of C12 to C18 alkanes and total peak area of the sample chromatogram are used to calculate the concentration of total alkanes.

Table 3.31: Optimum conditions for GC for n-alkane analysis

| Column               | Petrocol DH 50.2, 50m x 0.20mm ID,  
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Oven</td>
<td>80°C (30 min) to 260°C at 2 C/min (15min)</td>
</tr>
<tr>
<td>Carrier</td>
<td>Helium, 19-21 cm/sec (0.72 ml/min) at 35°C</td>
</tr>
<tr>
<td>Detector</td>
<td>FID (280°C)</td>
</tr>
<tr>
<td>Injector</td>
<td>0.2 µl, Split was put off, 280°C.</td>
</tr>
<tr>
<td>Fuel gas</td>
<td>Hydrogen (30 ml/min)</td>
</tr>
<tr>
<td>Oxidizing gas</td>
<td>Air (250 ml/min)</td>
</tr>
</tbody>
</table>
An n-alkane mixture along with aromatic compounds was run for performance and calibration verification and to ensure GC conditions were adequate for residual alkane analysis. The analysis also ensured that the initial calibration met the quality control criteria. In the present study gas chromatograph (Perkin Elmer Autosystem XL fitted with Petrocol DH 50.2, 50m x 0.20mm ID capillary column) coupled with a flame ionization detector (FID) was used for the analysis of residual alkanes.

![Gas chromatograph of standard hydrocarbons](image)

**Figure 3.23: Gas chromatograph of standard**

Helium was used as a carrier gas. Parameters like flow rate of carrier gas, detector and oven temperatures, injection volume were standardized to provide separation of the target analytes. The optimum parameters for n-alkane analysis are mentioned in table 3.31.
Optimum resolution of the peaks was obtained and peaks of the hydrocarbon components were resolved according to their molecular weight (figure 3.23). Aromatic components appeared before aliphatic components i.e. n-alkanes. The retention time of individual component in the chromatogram was given in the table 3.32.

Table 3.32: Retention time of the individual hydrocarbons

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Retention time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexane</td>
<td>12.0</td>
</tr>
<tr>
<td>Benzene</td>
<td>17.00</td>
</tr>
<tr>
<td>Toluene</td>
<td>36.19</td>
</tr>
<tr>
<td>Xylene</td>
<td>52.50</td>
</tr>
<tr>
<td>C12</td>
<td>88.0</td>
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<tr>
<td>C13</td>
<td>96.55</td>
</tr>
<tr>
<td>C14</td>
<td>103.40</td>
</tr>
<tr>
<td>C15</td>
<td>110.81</td>
</tr>
<tr>
<td>C16</td>
<td>117.84</td>
</tr>
<tr>
<td>C17</td>
<td>126.18</td>
</tr>
<tr>
<td>C18</td>
<td>137.49</td>
</tr>
</tbody>
</table>

3.6.2 Extraction of n-alkanes

The hydrocarbons are chemically non polar and therefore non-miscible in the nutrient media. Residual alkanes have to be extracted with suitable solvent before analysis on GC, to avoid traces of water in the samples. In the present investigation petroleum
hydrocarbons were extracted in solvents such as n-pentane, n-hexane or n-heptane. Typically hexane was found to be the most suitable for the extraction of the alkanes (C12-C18) from aqueous phase. 100 µl Mixture of n-alkanes (C12-C18) was added to the 10ml of both minimal medium and n-hexane. 5 ml hexane was added to the minimal medium for the extraction of alkanes using separating funnel. The extracted alkanes were analyzed on GC.

![Figure 3.24: GC profile of extracted alkanes from the medium](image)

Alkanes extracted in hexane were analyzed on the GC to determine the efficiency of extraction/recovery (figure 3.23, 3.24). The percent recovery of n-alkanes was compared
with concentrations of n-alkanes which were added directly to the n-hexane. GC analysis of hexane extracts revealed the efficiency of alkane extraction to be ca. 91%.

Hexane extraction followed by GC analysis was subsequently used throughout the investigation to monitor the microbial degradation of petroleum hydrocarbon, especially alkanes.

3.6.3 Spectrophotometric estimation of toluene extract of crude oil

GC/MS is extensively used for the analysis of crude oil. GC/MS facility was not available during the present investigation; hence, spectrophotometric analysis was used to measure petroleum hydrocarbon concentration because of the simplicity and accuracy of
the process. The results were consistent and sensitive enough to cater to the need of the experimentation. The residual oil in the treatments was determined spectrophotometrically (Odu et al., 1985; Sathishkumar et al., 2009). Residual oil from the broth was extracted using toluene and its quantity was detected using spectrophotometer at 420 nm. A standard curve was prepared using known concentrations of crude oil and it was used to calculate approximately the quantity of toluene extractable hydrocarbons in the residual oil (figure 3.26). A linear dose response relationship was observed for 0.1 to 1 mg of crude oil per ml of toluene as solvent. Degradation of crude oil was calculated as the difference between the initial and final concentrations of total hydrocarbons.

![Figure 3.26: Standard graph showing relation between concentration of toluene extract and its OD at 420 nm.](image_url)

**spectrophotometric estimation of toluene extract of oil.**

\[ y = 0.715x + 0.1398 \]

\[ R^2 = 0.9865 \]
3.7 Production of surface active agents

Hydrocarbons are chemically non-polar and very sparingly soluble in water. These chemical properties of the hydrocarbons make them less susceptible to microbial attack. Therefore, to access the hydrocarbons, bacteria commonly secrete bio-surfactants which cause dispersion of crude oil facilitating microbial attack on petroleum hydrocarbons. Biosurfactants can enhance the microbial growth by increasing the bioavailability of water insoluble hydrocarbons in aqueous medium (Ron and Rosenberg, 2002). Solubility in water is the rate limiting factor for the microbial degradation of hydrocarbons (Bognolo, 1998). Surface active compounds help to reduce the surface tension between two immiscible solvents (Banat et al., 1995; Banat et al., 2000; Satpute et al., 2010). Bio-emulsifier activity of the microorganisms can be determined in terms of emulsion index (E$_{24}$) which is the ratio of the percent height of the emulsion layer relative to the total height of cell free supernatant mixed with kerosene (or any hydrocarbon) in a test tube and kept stationary for 24 hours (Cooper and Goldenberg, 1987). Biosurfactants have many applications in petroleum industry, like in microbial-enhanced oil recovery (MEOR), emulsion-based fuels, emulsion-facilitated petroleum transport, oil tank clean-up and environmental protection and remediation.

3.7.1 Detection of biosurfactant activity

Several types of biosurfactant have been described in the published scientific literature. It has also been frequently reported that no single assay can detect/ quantify the biosurfactant produced/ secreted by the microbial cultures. Hence, in the present study multiple assays were performed to detect and quantify the ability of individual isolate to
produce/secrete bio-surfactants in the culture broths. Most of these techniques used in this study were based on the ability of culture supernatant to reduce the surface tension. Some techniques screened bacteria for cell-surface hydrophobicity. These properties suggest of biosurfactant production. The biosurfactant activity of culture supernatant can be checked by drop collapse assay (Jain et al, 1998). This assay depends on the destabilization of liquid droplets by surfactants. The stability of the liquid drops is inversely proportional to the surfactant concentration.

This assay is simple and easy to perform. It requires a small volume of sample and can be carried out in microtitre plates. This assay has been routinely used in preliminary screening studies. Another assay commonly called as oil spread assay is also based on the phenomenon of surface tension. Cell free culture broth will displace oil forming clear zone only if biosurfactant is secreted by the culture in supernatant.

Table 3.33: Qualitative analysis of biosurfactant production

<table>
<thead>
<tr>
<th>Isolate Designation</th>
<th>Analytical techniques</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Drop Collapse Method</td>
</tr>
<tr>
<td>MCM B-882</td>
<td>+</td>
</tr>
<tr>
<td>MCM B-883</td>
<td>+</td>
</tr>
<tr>
<td>MCM B-884</td>
<td>+</td>
</tr>
<tr>
<td>MCM B-885</td>
<td>+</td>
</tr>
<tr>
<td>MCM B-886</td>
<td>+</td>
</tr>
</tbody>
</table>
The diameter of this clearing zone is directly proportional to the surfactant activity. The qualitative analysis of biosurfactant production by individual isolate was checked by drop collapse method and oil spread method. It was observed that all the isolates were able to produce biosurfactant (table 3.33). Bio-emulsifier activity of individual isolates was determined in terms of emulsion index ($E_{24}$) as well as oil dispersion assay.

![Figure 3.27: Determination of emulsion index using kerosene](image)

**Calculation of Emulsion Index**

(after 24 hr)

$$E_{24} (%) = \frac{H_{EL}}{H_S} \times 100$$

Where, $H_{EL} = \text{Height of the emulsion layer}$, $H_S = \text{Height of the solution}$

<table>
<thead>
<tr>
<th>Strains</th>
<th>$E_{24}$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCM B-882</td>
<td>69</td>
</tr>
<tr>
<td>MCM B-883</td>
<td>63</td>
</tr>
<tr>
<td>MCM B-884</td>
<td>71</td>
</tr>
<tr>
<td>MCM B-885</td>
<td>65</td>
</tr>
<tr>
<td>MCM B-886</td>
<td>64</td>
</tr>
</tbody>
</table>
Emulsion indices of individual cultures are illustrated in figure 3.25 and table 3.34. It was observed that each isolated culture displayed significant emulsification activity. The emulsions formed by these isolates were stable up to 72hrs. Kumar et al (2007) reported emulsion index, $E_{24} = 50$ for *Planococcus matriensis* when kerosene was used. Plaza et al (2005) reported that stable emulsion was obtained by *Ralstonia picketti* (BP20) and *Alcaligenes piechaudii* (CZOR L-1B), isolated from petroleum hydrocarbon-contaminated soil. Comparatively, significantly higher emulsion indices were displayed by the isolated strains obtained in the present investigation.

Biosurfactant production in many cases has been reported to be an inducible property. Hence, in this study biosurfactant production by individual isolates was evaluated in a minimal medium supplemented with crude oil as sole source of carbon and energy. Figure 3.26 illustrates the dispersal of oil as a consequence of microbial metabolism of crude oil. Dispersion of crude oil was observed only in media inoculated with bacterial isolates but not in uninoculated controls. Five of the bacterial isolates with ability to disperse oil were selected for further study.

![Dispersion of Crude oil](image)

**Figure 3.28:** Production of surface active agents causes dispersion of crude oil
3.7.2 Extraction of biosurfactant

Extraction of the biosurfactant was carried out by the method described by Darvishi et al (2011). Biosurfactant was extracted from nutrient broth inoculated with 2% mixture of alkanes. Biosurfactant was precipitated with 5N HCl and then extracted with Chloroform- Ethanol. Biosurfactant was observed as a light honey colored compound after extraction (figure 3.29).

![Figure 3.29: Extraction of biosurfactant using chloroform-ethanol](image)

All five isolates (MCM B-882, MCM B-883, MCM B-884, MCM B-885 and MCM B-886) selected for further studies, showed production of biosurfactant. Detailed characterization of biosurfactants was out of scope of this study, and hence was not pursued. Inducible biosurfactant production was indicative of the ability of microbial isolates to utilize crude oil. However, the ability of isolates to utilize petroleum hydrocarbons as a source of carbon and energy was confirmed by INT assay. Under oxygen limiting conditions in the oil reservoir, oxyanions such as nitrate, phosphate, sulphate etc. act as terminal electron acceptors. Chromogenic INT can be used as a
substitute for above terminal electron acceptor. INT gets reduced to form a colored compound if electrons are generated as a part of microbial metabolism of crude oil.

3.8 INT Assay

Salts of tetrazolium are often used as indicators of microbial respiratory activity and viability of microorganisms in physiological and ecological studies (Smith and McFeters, 1996). Microorganisms generally have enzymes like dehydrogenases and reductases which reduce the salts of tetrazolium to form formazan dye (Packard, 1971; Rodriguez et al., 1992) (figure 3.30). These dyes are artificial chromogenic substrates and of various colors. One such salt, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT) has a variety of applications in study of microbial physiology. Methods have been developed using INT to indicate the presence of respiring or viable bacteria in populations. In the present investigation minimal medium containing crude oil as a sole carbon source was used to evaluate the ability of selected bacterial isolates to utilize petroleum hydrocarbons as carbon and energy source.

![Figure 3.30: Pictorial representation of reduction of tetrazolium to formazan](image)

INT acts as electron acceptor and gets reduced to brown colored formazan as a consequence of microbial metabolism of crude petroleum oil.
Table 3.35: Details of experimental set up in microtitre plate for INT assay

<table>
<thead>
<tr>
<th>Well number</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1, 2</td>
<td>Min. Med + oil + culture + INT (Test)</td>
</tr>
<tr>
<td>4, 5</td>
<td>Min. Med + culture + INT (Control 1)</td>
</tr>
<tr>
<td>7, 8</td>
<td>Min. Med + oil + INT (Control 2)</td>
</tr>
</tbody>
</table>

The experiments were performed using individual cultures isolated by enrichment technique. The details of the experimental set up are described in table 3.35.

Petroleum hydrocarbon degrading ability of all five bacterial isolates (namely, MCM B-882, MCM B-883, MCM B-884, MCM B-885 and MCM B-886) was evident from the brown colored formazan precipitate formed (figure 3.31). Similar method was used by Haines et al (1996) for the detection of positive dilutions in a hydrocarbon degrader MPN procedure.
3.9 Degradation of alkanes by individual cultures and consortium

Screening of hydrocarbon degrading microorganisms isolated from formation water by selective enrichment technique, resulted in the isolation of oil degrading bacteria belonging to three different genera namely *Aeribacillus*, *Bacillus* and *Hydrogenophilus*. All strains were cultivated in liquid media with either crude oil or n-alkanes as a sole source of carbon and energy. Evolution of Carbon dioxide was used as the major indicator of microbial degradation of petroleum hydrocarbons in the stoppered bottles. Further, increase in the cell density was also measured to enumerate microbial growth as a consequence of biodegradation of petroleum oil or hydrocarbon. Residual alkanes were quantified by gas-chromatographic techniques and residual crude oil was quantified by spectrophotometric analysis. Although enrichment technique selected only those indigenous microorganisms that have been especially acclimated to degrade hydrocarbons, it was necessary to characterize the biodegradation potential for individual isolates.

For the degradation of alkanes by individual isolates as well as mixture of isolates (consortium), minimal medium with 1% mixture of C12 to C18 alkanes and KNO₃ (1% w/v) and NH₄Cl (0.1% w/v) used as a nitrogen source. Sample aliquots were removed after 14 days and analyzed for cell density, CO₂ and residual alkanes. The percent degradation of n-alkanes by individual cultures is given in the figure 3.32. The increased cell density and CO₂ produced was given in the table 3.36. Significant efficiency of hydrocarbon degradation was observed for each of five isolates. Three strains, MCM B-882, MCM B-885 and MCM B-886 exhibited relatively higher efficiency of hydrocarbon degradation and were all identified as members of *Aeribacillus pallidus* (previously known as *Geobacillus pallidus*).
Figure 3.32: Degradation of alkanes by thermophilic isolates
It was observed that total utilization of alkanes by MCM B-882, MCM B-885 and MCM B-886 was found to be 30, 33 and 34 % respectively (figure 3.32). These results were in line with earlier reports describing degradation of n-hexadecane using Geobacilli. Marchant et al (2006) reported more than 60% degradation of hydrocarbons in 40 days at 60°C using microbial consortium. Many Geobacillus strains have been obtained from high-temperature environments. According to Rahman et al (2004), most of the members of the genus Geobacillus were capable of hydrocarbon degradation.

Table 3.36: Degradation of alkanes monitored in terms of increase in the cell density and CO₂ production

<table>
<thead>
<tr>
<th>Isolate/Consortium</th>
<th>Cell density (cells/ml)</th>
<th>CO₂ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>After ‘0’ days</td>
<td>After ‘14’ days</td>
</tr>
<tr>
<td>MCM B-882</td>
<td>1.2 x 10⁶</td>
<td>4.8 x 10⁷</td>
</tr>
<tr>
<td>MCM B-883</td>
<td>1.0 x 10⁶</td>
<td>3.8 x 10⁷</td>
</tr>
<tr>
<td>MCM B-884</td>
<td>1.2 x 10⁶</td>
<td>5.4 x 10⁷</td>
</tr>
<tr>
<td>MCM B-885</td>
<td>1.0 x 10⁶</td>
<td>3.6 x 10⁷</td>
</tr>
<tr>
<td>MCM B-886</td>
<td>1.0 x 10⁶</td>
<td>4.0 x 10⁷</td>
</tr>
<tr>
<td>Consortium</td>
<td>1.0 x 10⁶</td>
<td>6.6 x 10⁷</td>
</tr>
</tbody>
</table>

In the present investigation MCM B-884, isolated from oil well formation water was closely affiliated to Bacillus pumilus. It could degrade hydrocarbons at 55°C. It was observed that total utilization of alkanes by MCM B-884 was 39%. Majority of the species of Bacillus have been reported in the literature as strong candidates for hydrocarbon degradation. Toledo et al (2006) isolated fifteen bacterial strains capable of hydrocarbon degradation from solid waste oil samples. Most of these strains were identified as, Bacillus
*pumilus* (eight strains) and *Bacillus subtilis* (two strains). This could be related with the property of those microorganisms to colonize environments contaminated with hydrocarbons. *Bacillus* species were the predominant microorganisms in highly polluted soil samples. Pyrene, naphthalene and phenanthrene were utilized by *B. pumilus* strains as sole carbon source isolated by Toledo et al (2006). Thermophilic petroleum hydrocarbon degradation was reported for *Bacillus subtilis* DM-04 and *Pseudomonas aeruginosa*. Both strains were obtained from the soil that was contaminated with crude oil in North-East India. These thermophilic bacteria can be used for the bioremediation of petroleum contaminated soils in tropical countries. (Das et al, 2007). Bioaugmentation of TPH-contaminated soils with consortium of *P. aeruginosa* and *B. subtilis* decreased the TPH levels in soil from 84 to 21 g/kg of soil, respectively. Sequence of *B. pumilus* was also detected in the DGGE analysis of petroleum samples from Brazilian reservoirs (Oliveira et al, 2008). It is reported that mixed cultures carry out more extensive biodegradation of petroleum than pure cultures (Ghazali et al., 2004; Sun et al., 2005; Gerdes et al., 2005).

In the present study, individual isolates were mixed together to form a consortium and was tested for its ability to degrade hydrocarbons. The total utilization of alkanes by consortium at 60°C was found to be 51%. It was greater than the degradation capacity of individual isolates. It was also confirmed by the production of CO₂ (56 µM) and increase in the cell density. The optimization of the alkane degradation process was done by evaluating the effect of pH, temperature and nitrogen source on the biodegradation process.
3.10 Optimization of process parameters for biodegradation of alkanes

Oil degrading bacteria isolated and used in the present investigation exhibited significant alkane degrading efficiency. Various process parameters were optimized to maximize the alkane degradation efficiency of the cultures as well as of consortium of these isolates. The parameters optimized included pH, temperature and nitrogen supplements. This information was to be subsequently used for the sand pack trials, a step necessary before in situ pilot trials.

3.10.1 Effect of initial pH on n-alkane biodegradation

Effect of pH on hydrocarbon degradation efficiency of the isolates was evaluated in minimal medium supplemented with mixture of alkanes as a source of carbon and energy. Early investigation revealed the ability of each isolate to thrive over a pH range of 6 to 10. Hence, the same pH range was included in the process parameter optimization studies. Progress of biodegradation of alkanes was monitored in terms of change in initial pH, CO₂ evolution, and increase in biomass yield/ cell density. It was observed that each isolate could degrade n-alkanes over the entire range of pH used (table 3.38). However, maximum biodegradation of alkanes was observed at pH 8 for almost all isolates. Alkaline pH favored alkane biodegradation as compared to neutral or acidic pH. Among all cultures MCM B-885 showed highest alkane degradation (54.2%) at pH 8.0. Similarly, maximum growth as well as CO₂ production was also observed at pH 8.0. However, almost comparable growth was observed for each isolate over the entire pH range tested. CO₂ production was, however, significantly higher at pH 8.0 as compared to acidic or extreme alkaline pH.
Table 3.37: Alkane degradation at various pH

<table>
<thead>
<tr>
<th>Cultures</th>
<th>Parameters</th>
<th>pH</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>6.0</td>
<td>7.0</td>
<td>8.0</td>
<td>9.0</td>
</tr>
<tr>
<td>MCM B-882</td>
<td>Degradation %</td>
<td>35.57</td>
<td>45.93</td>
<td>52.50</td>
<td>45.21</td>
</tr>
<tr>
<td></td>
<td>CO₂ (µM)</td>
<td>36.40</td>
<td>40.38</td>
<td>46</td>
<td>44.64</td>
</tr>
<tr>
<td></td>
<td>Cells/ml</td>
<td>2.6 x10⁷</td>
<td>3.0 x10⁷</td>
<td>3.3 x10⁷</td>
<td>3.3 x10⁷</td>
</tr>
<tr>
<td>MCM B-883</td>
<td>Degradation %</td>
<td>41.72</td>
<td>45.36</td>
<td>46.66</td>
<td>42.28</td>
</tr>
<tr>
<td></td>
<td>CO₂ (µM)</td>
<td>40.20</td>
<td>42.0</td>
<td>41</td>
<td>40.50</td>
</tr>
<tr>
<td></td>
<td>Cells/ml</td>
<td>2.8 x10⁷</td>
<td>3.4 x10⁷</td>
<td>3.2 x10⁷</td>
<td>2.8 x10⁷</td>
</tr>
<tr>
<td>MCM B-884</td>
<td>Degradation %</td>
<td>31.40</td>
<td>42.83</td>
<td>38.64</td>
<td>33.31</td>
</tr>
<tr>
<td></td>
<td>CO₂ (µM)</td>
<td>32.00</td>
<td>45.20</td>
<td>37.60</td>
<td>36.8</td>
</tr>
<tr>
<td></td>
<td>Cells/ml</td>
<td>1.1 x10⁷</td>
<td>2.8 x10⁷</td>
<td>2.3 x10⁷</td>
<td>1.6 x10⁷</td>
</tr>
<tr>
<td>MCM B-885</td>
<td>Degradation %</td>
<td>37.34</td>
<td>49.87</td>
<td>54.21</td>
<td>45.74</td>
</tr>
<tr>
<td></td>
<td>CO₂ (µM)</td>
<td>35.20</td>
<td>44.50</td>
<td>49</td>
<td>42.70</td>
</tr>
<tr>
<td></td>
<td>Cells/ml</td>
<td>3.0 x10⁷</td>
<td>3.8 x10⁷</td>
<td>4 x10⁷</td>
<td>3.3 x10⁷</td>
</tr>
<tr>
<td>MCM B-886</td>
<td>Degradation %</td>
<td>36.19</td>
<td>51.82</td>
<td>49.94</td>
<td>42.13</td>
</tr>
<tr>
<td></td>
<td>CO₂ (µM)</td>
<td>32.14</td>
<td>43.52</td>
<td>42.56</td>
<td>38.69</td>
</tr>
<tr>
<td></td>
<td>Cells/ml</td>
<td>2.4 x10⁷</td>
<td>3.2 x10⁷</td>
<td>3.6 x10⁷</td>
<td>3.5 x10⁷</td>
</tr>
<tr>
<td>Consortium</td>
<td>Degradation %</td>
<td>42.5</td>
<td>55.81</td>
<td>57.70</td>
<td>49.77</td>
</tr>
<tr>
<td></td>
<td>CO₂ (µM)</td>
<td>41.23</td>
<td>47.26</td>
<td>53.0</td>
<td>41.23</td>
</tr>
<tr>
<td></td>
<td>Cells/ml</td>
<td>2.5 x10⁷</td>
<td>4.2 x10⁷</td>
<td>4.5 x10⁷</td>
<td>3.4 x10⁷</td>
</tr>
</tbody>
</table>

The highest cell density as well as CO₂ production was exhibited by MCM B-885 at pH 8 among all the isolates tested. The alkane degradation efficiency of consortium was found to be higher when compared with efficiency of individual cultures. It was observed that the higher alkane degradation efficiency was almost also accompanied by the higher cell density and CO₂ released in the head space in contained environment.
3.10.2 Effect of temperature on alkane degradation

Effect of temperature on n-alkanes degradation was evaluated using minimal medium containing mixture of n-alkanes (C12-C18) as carbon and energy source. It was observed in the initial studies that each isolate was capable of growing over a temperature range of 45 to 65°C (except MCM B-884 which could grow up to 55°C).

Table 3.38: Alkane degradation at various temperatures

<table>
<thead>
<tr>
<th>Cultures</th>
<th>Parameters</th>
<th>Temperature °C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>45</td>
</tr>
<tr>
<td>MCMB-882</td>
<td>Degradation %</td>
<td>42.41</td>
</tr>
<tr>
<td></td>
<td>CO₂ (µM)</td>
<td>35.26</td>
</tr>
<tr>
<td></td>
<td>Cells/ml</td>
<td>2.6 x 10⁷</td>
</tr>
<tr>
<td>MCMB-883</td>
<td>Degradation %</td>
<td>54.86</td>
</tr>
<tr>
<td></td>
<td>CO₂ (µM)</td>
<td>46.26</td>
</tr>
<tr>
<td></td>
<td>Cells/ml</td>
<td>2.8 x 10⁷</td>
</tr>
<tr>
<td>MCM B-884</td>
<td>Degradation %</td>
<td>41.50</td>
</tr>
<tr>
<td></td>
<td>CO₂ (µM)</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>Cells/ml</td>
<td>3.1 x 10⁷</td>
</tr>
<tr>
<td>MCMB-885</td>
<td>Degradation %</td>
<td>49.50</td>
</tr>
<tr>
<td></td>
<td>CO₂ (µM)</td>
<td>45.52</td>
</tr>
<tr>
<td></td>
<td>Cells/ml</td>
<td>2.3 x 10⁷</td>
</tr>
<tr>
<td>MCMB-886</td>
<td>Degradation %</td>
<td>39.91</td>
</tr>
<tr>
<td></td>
<td>CO₂ (µM)</td>
<td>41.68</td>
</tr>
<tr>
<td></td>
<td>Cells/ml</td>
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</tr>
<tr>
<td>Consortium</td>
<td>Degradation %</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td>CO₂ (µM)</td>
<td>57</td>
</tr>
<tr>
<td></td>
<td>Cells/ml</td>
<td>4.3 x 10⁷</td>
</tr>
</tbody>
</table>
Therefore, in the process parameter optimization studies selection of temperature range was done according to results of initial observations. Biodegradation of alkanes was monitored in terms of change in CO₂ evolution, increase in biomass yield/cell density and analysis of residual alkanes on GC. The results are shown in the table 3.39.

It was found that each isolate (except MCM B-884) could degrade n-alkanes over the 45-60°C range of temperature. In case of cultures MCM B-882 and MCM B-885, maximum biodegradation of alkanes was observed at 50°C. However, MCM B-883 and MCM B-884 showed maximum alkane degradation at 45°C. MCM B-886 showed highest alkane degradation at (48%) at 60°C. Consortium could degrade alkanes optimally at 50°C. Among all cultures MCM B-883 showed highest alkane degradation (54.86%) at 45°C. At 50°C, consortium showed maximum alkane degradation (65%) at 50°C. Higher alkane degradation efficiency was always accompanied by the increase in the cell density as well as CO₂ production. Even though, significant hydrocarbon degradation was observed over a temperature range of 40 to 60°C, 50°C was found to be the optimum temperature for hydrocarbon degradation in case of each isolate tested except MCM B-884.

3.10.3 Effect of different nitrogen sources on alkane degradation

Nitrogen, being an essential element for the growth as well as metabolic activity, effect of nitrogen source on the hydrocarbon degradation was investigated for each isolate as well as for the bacterial consortium. For the selection of ideal nitrogen source in alkane degradation process, minimal medium was supplemented with either of four different nitrogen sources (200mM each) in separate experiments. First one was ammonium
chloride which is common and readily used by most of the microorganisms. The second one was potassium nitrate for those microorganisms having ability to use it during denitrification, third one was the urea which is a commercially cheap nitrogen source and last was the combination of ammonium chloride and potassium nitrate.

Table 3.39: Alkane degradation using different nitrogen sources

<table>
<thead>
<tr>
<th>Culture</th>
<th>Parameters</th>
<th>‘N’ Sources</th>
<th>NH₄Cl</th>
<th>KNO₃</th>
<th>Urea</th>
<th>NH₄Cl+KNO₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCMB-882</td>
<td>Degradation %</td>
<td>44.62</td>
<td>7.5</td>
<td>37</td>
<td>42</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CO₂ (µM)</td>
<td>43.40</td>
<td>9.4</td>
<td>46</td>
<td>44</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cells/ml</td>
<td>2.6 x10⁷</td>
<td>5 x10⁶</td>
<td>2.4x10⁷</td>
<td>2.6 x10⁷</td>
<td></td>
</tr>
<tr>
<td>MCM B-883</td>
<td>Degradation %</td>
<td>46</td>
<td>12</td>
<td>42</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CO₂ (µM)</td>
<td>50</td>
<td>10.5</td>
<td>47</td>
<td>43</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cells/ml</td>
<td>3.2 x10⁷</td>
<td>1 x10⁷</td>
<td>2.8x10⁷</td>
<td>2.8 x10⁷</td>
<td></td>
</tr>
<tr>
<td>MCM B-884</td>
<td>Degradation %</td>
<td>40.80</td>
<td>7.15</td>
<td>26.50</td>
<td>40.50</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CO₂ (µM)</td>
<td>46.5</td>
<td>8.5</td>
<td>23.8</td>
<td>42.35</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cells/ml</td>
<td>2.6 x10⁷</td>
<td>1.2 x10⁷</td>
<td>1.6 x10⁷</td>
<td>2.4 x10⁷</td>
<td></td>
</tr>
<tr>
<td>MCM B-885</td>
<td>Degradation %</td>
<td>52</td>
<td>5</td>
<td>36</td>
<td>39</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CO₂ (µM)</td>
<td>54</td>
<td>7</td>
<td>40</td>
<td>43</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cells/ml</td>
<td>3.5 x10⁷</td>
<td>8.4 x10⁶</td>
<td>3.0 x10⁷</td>
<td>3.2 x10⁷</td>
<td></td>
</tr>
<tr>
<td>MCM B-886</td>
<td>Degradation %</td>
<td>42</td>
<td>7</td>
<td>35</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CO₂ (µM)</td>
<td>38</td>
<td>9</td>
<td>44</td>
<td>42</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cells/ml</td>
<td>2.6 x10⁷</td>
<td>6.6 x10⁶</td>
<td>2.4 x10⁷</td>
<td>2.6 x10⁷</td>
<td></td>
</tr>
<tr>
<td>Consortium</td>
<td>Degradation %</td>
<td>56</td>
<td>22</td>
<td>48</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CO₂ (µM)</td>
<td>54</td>
<td>20</td>
<td>54</td>
<td>61</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cells/ml</td>
<td>3.8 x10⁷</td>
<td>2.0 x10⁷</td>
<td>3.8x10⁷</td>
<td>4.2 x10⁷</td>
<td></td>
</tr>
</tbody>
</table>
The experiment was carried out at 60°C (except for MCM B-884 for which temperature was kept 55°C). Biodegradation of alkanes was monitored in terms of change in CO₂ evolution, increase in biomass yield/ cell density and analysis of residual alkanes on GC. The results are shown in the table 3.40.

It was observed that all the isolates showed maximum alkane degradation when NH₄Cl was used as a nitrogen source. For consortium combination of NH₄Cl and KNO₃ was the ideal nitrogen source. The highest alkane degradation was shown by MCM B-885 (52%) when NH₄Cl was used as a nitrogen source. All cultures showed very low alkane degradation when KNO₃ was as a nitrogen source. The combination of NH₄Cl and KNO₃ did not cause any increase in the alkane degradation profile of all cultures. When urea was used the percentage of alkane degradation was slightly lower than that of NH₄Cl.

3.11 Degradation of crude oil

For crude oil degradation minimal medium with 1% crude oil was used. Individual cultures as well as their consortium were used as inoculums. Degradation of crude oil was assessed spectrophotometrically after 20 days of incubation. The results are shown in the table 3.37 and figure 3.33.

When consortium was used in the present study, the degradation of crude oil was 9.5% and under these conditions 35 ppm volatile fatty acids were produced. The productions of VFA were also observed during degradation of aliphatic and aromatic components of crude oil in two separate experiments.
Figure 3.33: Degradation of crude oil by individual isolate and consortium

Table 3.40: Degradation of crude oil monitored in terms of increase in the cell density and CO₂ production

<table>
<thead>
<tr>
<th>Organism</th>
<th>Cell Density</th>
<th>CO₂ (µM)</th>
<th>% Degradation</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCM B-882</td>
<td>8.2 x 10⁷</td>
<td>48 ± 4.0</td>
<td>6.05 ± 0.8</td>
</tr>
<tr>
<td>MCM B-883</td>
<td>7.9 x 10⁷</td>
<td>40 ± 5.0</td>
<td>6.40 ± 0.4</td>
</tr>
<tr>
<td>MCM B-884</td>
<td>4.8 x 10⁷</td>
<td>46 ± 2.3</td>
<td>6.6 ± 0.9</td>
</tr>
<tr>
<td>MCM B-885</td>
<td>7.8 x 10⁷</td>
<td>42 ± 4.5</td>
<td>6.7 ± 1.0</td>
</tr>
<tr>
<td>MCM B-886</td>
<td>6.8 x 10⁷</td>
<td>41 ± 3.8</td>
<td>6.5 ± 0.4</td>
</tr>
<tr>
<td>Consortium</td>
<td>2.2 x 10⁸</td>
<td>55 ± 5.4</td>
<td>9.5 ± 0.7</td>
</tr>
</tbody>
</table>
According to Bello (2007), the microorganisms first attacked the lower and higher hydrocarbon chains and those of middle length were attacked later in the course of incubation. It was observed that all the isolate could degrade 6.0-6.7% of crude oil. Satishkumar et al (2009) reported the degradation of crude oil for Bacillus sp., Corynebacterium sp., Pseudomonas sp. and mixed consortium. It was observed that these three cultures could degrade 45-67% of 1% crude oil provided. Mixed consortium could degrade up to 76%. Similar results were reported by Chhatre et al (1996) and Rahman et al (2004) produced. Lal and Khanna (1996) reported 58% degradation of Indian crude oil samples by mixture of Acinetobacter calcoaceticus and Alcaligenes odorans in a 15 day period. Ijah (1998) reported that bacteria and yeast isolates from tropical soils capable of degrading 52% and 69% of crude oil in 16 days, respectively, where the isolates primarily degraded the alkanes over specific carbon number ranges. Okoh et al. (2002) reported between 8.8 and 29% degradation of the heavy crude oil in soil microcosm by mixed bacterial consortium in 15 days.

3.11.1 Effect of pH on crude oil degradation

Minimal medium was used for the determination of optimum pH for crude oil degradation. Same pH range (as in case of alkanes i.e. 6-10) was used along with one percent crude oil in the medium. Incubation was carried out at 60°C (except for MCM B-884 for which temperature was maintained at 55°C). Biodegradation of alkanes was monitored in terms of change in CO₂ evolution, increase in biomass yield/ cell density and analysis of residual crude oil spectrophotometrically. The results are shown in the table 3.41. It was observed that each isolate could degrade crude oil over the entire range of pH used. It was found that MCM B-882 showed highest crude oil degradation (8.2%) in
comparison to other individual cultures. The evolution of CO$_2$ was maximum in case of MCM B-882 at pH 8.0. At highly alkaline pH 10, the MCM B-882 was found to be degrading 7.30% of crude oil in 20 days. Even remaining isolates were also able to degrade crude oil effectively at pH 10. In case of consortium the degradation was highest (9.70%) at pH 8.0.

Table 3.41: Crude oil degradation at various pH

<table>
<thead>
<tr>
<th>Cultures</th>
<th>Parameters</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>6.0</td>
</tr>
<tr>
<td>MCM B-882</td>
<td>Degradation (%)</td>
<td>6.20</td>
</tr>
<tr>
<td></td>
<td>CO$_2$ ($\mu$M)</td>
<td>36.0</td>
</tr>
<tr>
<td></td>
<td>Cells/ml</td>
<td>2.8 x10$^7$</td>
</tr>
<tr>
<td>MCM B-883</td>
<td>Degradation (%)</td>
<td>6.4</td>
</tr>
<tr>
<td></td>
<td>CO$_2$ ($\mu$M)</td>
<td>41.2</td>
</tr>
<tr>
<td></td>
<td>Cells/ml</td>
<td>2.4 x10$^7$</td>
</tr>
<tr>
<td>MCM B-884</td>
<td>Degradation (%)</td>
<td>6.4</td>
</tr>
<tr>
<td></td>
<td>CO$_2$ ($\mu$M)</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td>Cells/ml</td>
<td>3.5 x10$^7$</td>
</tr>
<tr>
<td>MCM B-885</td>
<td>Degradation (%)</td>
<td>6.8</td>
</tr>
<tr>
<td></td>
<td>CO$_2$ ($\mu$M)</td>
<td>42.14</td>
</tr>
<tr>
<td></td>
<td>Cells/ml</td>
<td>2.4 x10$^7$</td>
</tr>
<tr>
<td>MCM B-886</td>
<td>Degradation (%)</td>
<td>6.4</td>
</tr>
<tr>
<td></td>
<td>CO$_2$ ($\mu$M)</td>
<td>36.23</td>
</tr>
<tr>
<td></td>
<td>Cells/ml</td>
<td>2.4 x10$^7$</td>
</tr>
<tr>
<td>Consortium</td>
<td>Degradation (%)</td>
<td>7.80</td>
</tr>
<tr>
<td></td>
<td>CO$_2$ ($\mu$M)</td>
<td>46.20</td>
</tr>
<tr>
<td></td>
<td>Cells/ml</td>
<td>3.8 x10$^7$</td>
</tr>
</tbody>
</table>
3.11.2 Effect of temperature on crude oil degradation

Effect of temperature on crude oil degradation was evaluated in minimal medium containing 1% crude oil. The same temperature range used for the optimization of alkane degradation was selected here. The results are given in table 3.42.

Table 3.42: Crude oil degradation at various temperatures

<table>
<thead>
<tr>
<th>Cultures</th>
<th>Parameters</th>
<th>Temperature °C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>45</td>
</tr>
<tr>
<td>MCM B-882</td>
<td>Degradation %</td>
<td>7.4</td>
</tr>
<tr>
<td></td>
<td>CO₂(µM)</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td>Cells/ml</td>
<td>3.0 x10⁷</td>
</tr>
<tr>
<td>MCM B-883</td>
<td>Degradation %</td>
<td>8.45</td>
</tr>
<tr>
<td></td>
<td>CO₂(µM)</td>
<td>57.50</td>
</tr>
<tr>
<td></td>
<td>Cells/ml</td>
<td>3.9 x10⁷</td>
</tr>
<tr>
<td>MCM B-884</td>
<td>Degradation %</td>
<td>7.81</td>
</tr>
<tr>
<td></td>
<td>CO₂(µM)</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>Cells/ml</td>
<td>4.5 x10⁷</td>
</tr>
<tr>
<td>MCM B-885</td>
<td>Degradation %</td>
<td>8.3</td>
</tr>
<tr>
<td></td>
<td>CO₂(µM)</td>
<td>52.22</td>
</tr>
<tr>
<td></td>
<td>Cells/ml</td>
<td>3.8 x10⁷</td>
</tr>
<tr>
<td>MCM B-886</td>
<td>Degradation %</td>
<td>7.81</td>
</tr>
<tr>
<td></td>
<td>CO₂(µM)</td>
<td>57.56</td>
</tr>
<tr>
<td></td>
<td>Cells/ml</td>
<td>3.6 x10⁷</td>
</tr>
<tr>
<td>Consortium</td>
<td>Degradation %</td>
<td>10.00</td>
</tr>
<tr>
<td></td>
<td>CO₂(µM)</td>
<td>62.25</td>
</tr>
<tr>
<td></td>
<td>Cells/ml</td>
<td>4.5 x10⁷</td>
</tr>
</tbody>
</table>
Upon spectroscopic analysis of oil degradation it was observed that MCM B-882 showed approximately same degree of oil degradation (8.1 to 8.3%) at temperatures 50, 55 and 60°C. At 65°C, the degradation was found to be 4.4%. MCM B-883 and MCM B-885 showed maximum crude oil degradation (8.5% and 8.3%, respectively) at 45°C. There was no considerable difference in percentage of alkane degradation of MCM B-886 in temperature range of 45 to 60°C. In case of consortium the maximum crude oil degradation was observed at 60°C. There was no significant difference in oil degradation values at 55°C (10.2%) and 60°C (10.5%). At 65°C the consortium showed 4.5% crude oil degradation (table 3.42).

3.11.3 Effect of nitrogen sources on crude oil degradation

The effects of N sources on crude oil degradation by individual cultures and consortium were evaluated in terms of production of CO₂, increase in the cell density and spectrophotometric analysis of residual oil. Four different nitrogen sources which were used in the optimization of alkane degradation study were used individually for the optimization of crude oil degradation process in separate experiments. The results are summarized in the table 3.43.

When crude oil degradation was observed using different nitrogen sources it was found that NH₄Cl was the ideal nitrogen source for the crude oil degradation for all cultures. There was no considerable difference found in the degradation percentage when NH₄Cl or NH₄Cl+KNO₃ was used as a nitrogen source. It was also observed that the all the organisms could use urea as a nitrogen source effectively and their degradation values are comparable with NH₄Cl containing experiments. In case of consortium, interestingly the crude oil degradation was highest when ammonium chloride was used along with
potassium nitrate. Consortium showed 10.8% crude oil degradation using NH$_4$Cl-KNO$_3$ combination. Similarly, it showed 10.2 and 9.7% oil degradation when NH$_4$Cl and urea were used, respectively.

Table 3.43: Crude oil degradation using different nitrogen source

<table>
<thead>
<tr>
<th>Culture</th>
<th>Parameters</th>
<th>‘N’ Sources</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>NH$_4$Cl</td>
</tr>
<tr>
<td>MCM B-882</td>
<td>Degradation %</td>
<td>8.25</td>
</tr>
<tr>
<td></td>
<td>CO$_2$ (µM)</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>Cells/ml</td>
<td>4.2 x 10$^7$</td>
</tr>
<tr>
<td>MCM B-883</td>
<td>Degradation %</td>
<td>7.4</td>
</tr>
<tr>
<td></td>
<td>CO$_2$ (µM)</td>
<td>43.5</td>
</tr>
<tr>
<td></td>
<td>Cells/ml</td>
<td>4 x 10$^7$</td>
</tr>
<tr>
<td>MCM B-884</td>
<td>Degradation %</td>
<td>7.75</td>
</tr>
<tr>
<td></td>
<td>CO$_2$ (µM)</td>
<td>53.6</td>
</tr>
<tr>
<td></td>
<td>Cells/ml</td>
<td>4.2 x 10$^7$</td>
</tr>
<tr>
<td>MCM B-885</td>
<td>Degradation %</td>
<td>8.3</td>
</tr>
<tr>
<td></td>
<td>CO$_2$ (µM)</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>Cells/ml</td>
<td>4.5 x 10$^7$</td>
</tr>
<tr>
<td>MCM B-886</td>
<td>Degradation %</td>
<td>7.4</td>
</tr>
<tr>
<td></td>
<td>CO$_2$ (µM)</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>Cells/ml</td>
<td>4.2 x 10$^7$</td>
</tr>
<tr>
<td>Consortium</td>
<td>Degradation %</td>
<td>10.2</td>
</tr>
<tr>
<td></td>
<td>CO$_2$ (µM)</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>Cells/ml</td>
<td>5.2 x 10$^7$</td>
</tr>
</tbody>
</table>
Among cultures MCM B-882 showed highest degradation of crude oil (8.3%) when NH_4Cl used as a nitrogen source in the medium.

The optimum parameters for crude oil as well as alkane degradation are summarized in the following table 3.44

**Table 3.44: Optimum parameters for hydrocarbon degradation**

<table>
<thead>
<tr>
<th>Cultures</th>
<th>Hydrocarbon</th>
<th>N source</th>
<th>pH</th>
<th>Temperature °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCM B-882</td>
<td>Alkane</td>
<td>NH_4Cl</td>
<td>8.0</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>Oil</td>
<td>NH_4Cl</td>
<td>8.0</td>
<td>50</td>
</tr>
<tr>
<td>MCM B-883</td>
<td>Alkane</td>
<td>NH_4Cl</td>
<td>8.0</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>Oil</td>
<td>NH_4Cl</td>
<td>8.0</td>
<td>45</td>
</tr>
<tr>
<td>MCM B-884</td>
<td>Alkane</td>
<td>NH_4Cl</td>
<td>7.0</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>Oil</td>
<td>NH_4Cl+KNO_3</td>
<td>7.0</td>
<td>30</td>
</tr>
<tr>
<td>MCM B-885</td>
<td>Alkane</td>
<td>NH_4Cl</td>
<td>8.0</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>Oil</td>
<td>NH_4Cl</td>
<td>7.0</td>
<td>50</td>
</tr>
<tr>
<td>MCM B-886</td>
<td>Alkane</td>
<td>NH_4Cl</td>
<td>7.0</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>Oil</td>
<td>NH_4Cl</td>
<td>7.0</td>
<td>60</td>
</tr>
<tr>
<td>Consortium</td>
<td>Alkane</td>
<td>NH_4Cl+KNO_3</td>
<td>8.0</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>Oil</td>
<td>NH_4Cl+KNO_3</td>
<td>8.0</td>
<td>60</td>
</tr>
</tbody>
</table>

In the present investigation it was observed that all isolates were able to degrade alkanes effectively, at alkaline pH. Many alkaliphilic bacteria have been reported in the literature having potential of hydrocarbon degradation in the oil polluted fields. Yamahira et al (2008) isolated *Acinetobacter* sp. from petroleum contaminated hot water spring that can degrade n-alkanes (C13-C30) at pH 9.0. *G. thermodenirificans* isolated
from a deep subterranean oil-reservoir could degrade hexadecane (up to 55.6%) at 60°C and pH 7.0, in 10 days (Wang et al, 2006). In the present investigation MCM B-882, MCM B-885 and MCM B-886 were identified as *Aeribacillus pallidus* (renamed for *Geobacillus pallidus*). Most of the *Geobacillus* species (initially they were the member of *Bacillus* group) having ability to degrade alkanes has been isolated so far. *G. usenensis*, *G. subterraneus*, *G. stearothermophilus*, *G. thermoleovorans*, *G. kaustophilus* and *G. uralicus* can grow in slightly alkaline conditions (Popova et al, 2002). MCM B-883 identified as *Hydrogenophilus hirschi*, has been reported in the literature as thermophilic and alkali tolerant bacterium (Stohr et al, 2001).

Petroleum reservoirs are characterized by high temperatures, acidic or alkaline pH, high salt concentrations etc. Hydrocarbon biodegradation can occur over a wide range of temperatures. Thermophilic, mesophilic and psychrotrophic hydrocarbon-utilizing microorganisms have been isolated from oil fields. ZoBell (1969) and Traxler (1973) reported hydrocarbon degradation below 0°C. Mateles et al. (1972) reported hydrocarbon degradation at 70°C. Temperature can have a marked effect on the rates of hydrocarbon degradation. ZoBell (1969) found that hydrocarbon degradation was faster at 25°C than at 5°C. Atlas and Bartha (1972) reported that the effect of temperature on biodegradation of hydrocarbons depends on composition of a petroleum mixture. At low temperatures, the rate of volatilization of low-molecular-weight hydrocarbons (Some of which are toxic to microorganisms) is reduced. The presence of such toxic components was found to delay the onset of oil biodegradation at low temperatures. Thermophilic bacilli, geobacilli and species of *Hydrogenophaga* have been reported to be responsible for petroleum hydrocarbon degradation (Popova et al, 2002; Stohr et al, 2001).
Microorganisms need nitrogen and phosphorus for their growth. They should be present in the vicinity of hydrocarbons in the oil reservoirs for \textit{in situ} methanogenesis. In many cases the supply of nitrogen and phosphorus is dependent on their diffusion to the oil layer. In case of low level of hydrocarbons, nitrogen and phosphorus are probably not limiting to prevent establishment of an adverse C/N or C/P ratio. Like biological oxygen demand, nitrogen demand is important for biodegradation of hydrocarbons. In the present study ammonium chloride was found to be the optimum nitrogen source for alkane degradation. Ammonia, is the primary nitrogen source in oil reservoirs and can be taken up easily by the native bacteria (Head et al, 2003), potassium nitrate was found to be a weak nitrogen source for all isolates as percent degradation of alkanes as well as crude oil was less compared to other nitrogen sources.

The low level of degradation of crude oil in the present study is may be because, the degradation was carried out under anoxic conditions and therefore the oxygen limitation and elevated temperature could be the reasons for low oxidation rate of petroleum hydrocarbons. In the present study the results are in line with the previous reports. Dietzia sp. A14101, isolated from an oil reservoir model column inoculated with oil-field bacteria was able to utilize n-alkanes (C10-C17) from crude oil in 28 days. After 120 days crude oil was heavily biodegraded at 30°C. However in anaerobic conditions in the presence of nitrate the level of degradation of n-alkanes was very low (Bordtker et al., 2009). According to Tehran et al (2009) microorganism from soil were able to degrade 40% crude oil, provided with aeration and degradation was reduced to 15% in non-aerated conditions at room temperature (25-28°C). Similar results were obtained in the degradation of polyaromatic hydrocarbons. Benka-Coker and Ekundayo (1997) observed
that strains of *Serratia* and *Rhizopus* were able to degrade crude oil effectively if it was in lower concentration. According to Hassanuzzaman et al (2007) crude oil may have some toxic compounds which are lethal to the cells. In their study the population of *Pseudomonas aeruginosa* strain WatG decreases initially up to 50% because of the toxicity of the crude oil. This strain was able to degrade C36 (28%) and C40 (29%) from crude oil at 30°C in two weeks. The consortium showed better tolerance and utilization of the crude oil at the various concentrations. Crude oil degrading novel species of *Nocardiodes* was isolated by Schippers et al (2005) from crude oil sample. Hao et al (2004) isolated a thermophilic bacterium from Shengli oil field in East China which nonmotile and could grow up to 83°C. This strain could grow in the neutral to alkaline pH range and could effectively degrade crude oil. Meintanis et al (2006) isolated ten thermophilic hydrocarbon degrading bacteria from volcanic islands. Out of ten nine bacteria were phylogenetically affiliated to *Geobacillus* species and one isolate was affiliated with *Bacillus* species. All isolates were able to use crude oil as the sole carbon source and were found to degrade long chain crude oil alkanes in a range between 46.64% and 87.68% at 55°C in 10 days. Fukui et al (1999) reported approximately 10% degradation of crude oil using sulfate reducing bacteria which were tested previously for their ability to utilize alkanes and alkylbenzenes. In case of pure compounds like toluene the degradation by SRB was observed to be 80% in 30 days (Beller et al 1996). So and Young (1999) reported 91% degradation of n-alkanes by sulfate reducing bacterial strain AK01 in 44 days at 30°C.

However, crude oil degradation efficiencies observed at >50°C and under oxygen limiting conditions were always significantly lower. The crude oil degradation
efficiency of approximately 10% observed in the present investigation was, thus, considered encouraging and prompted us to undertake sand pack trials at 60°C and under oxygen limiting conditions.

3.12 Sand Pack Studies

The objective of this part was to demonstrate production of methane in sand pack studies by indigenous microbial populations from oil reservoirs. Methanogenesis at elevated temperature sand pack studies were conducted using microbial cultures isolated from formation water samples. Laboratory studies indicative of in situ production of biological methane were conducted in sand pack columns. The results of the sand pack studies have lead to the development of a feasible and cost-effective technology for the recovery of methane from residual crude oil. The columns were maintained anaerobically and incubated at 60°C. Sand pack columns provide an easy to construct model system to demonstrate in situ methanogenesis. Sand pack columns can be set in a variety of environmental conditions and are easily scaled up to large size for use with different field brines or oils. Sand pack systems could be flooded successfully at 1% salt and up to 60 °C with anaerobic conditions.

In the present study sand pack column trials were performed at 60°C and incubation period of 70 days. Instead of glass columns, one liter capacity serum bottles were used for the sand pack trials. Approximately 0.75 volumes of the bottles were filled with oil coated sand. Along with minimal medium consortium was inoculated using syringe so as to maintain anoxic conditions inside the bottles. After detection of metabolites of oil degradation after 30 days like H₂, CO₂ and volatile fatty acids,
methanogens were added in to the bottles. These metabolites in turn could act as substrates for the methanogens to produce methane. This experiment was performed in triplicates with three uninoculated controls. Sand pack studies are not exact but a little more than simulation of oil reservoir environment. Indirectly this type of study provided some clues whether this process was feasible or not in natural oil reservoirs.

The experimental set up of sand packed trials and the results are illustrated in figure 3.33 and table 3. 46. The production of methane was confirmed after 70 days of incubation. It was evident from the data that on average 2 ml methane was produced in all three sand packed bottles whereas in control bottles methane was not detected.
Table 3.45: H₂, CO₂ and CH₄ production in sand pack columns after 70 days.

<table>
<thead>
<tr>
<th>Bottle Code</th>
<th>Total H₂ in µl</th>
<th>CO₂ in µl/ml</th>
<th>Total CO₂ in ml</th>
<th>CH₄ in µl/ml</th>
<th>Total CH₄ in ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test 1</td>
<td>56</td>
<td>95.49</td>
<td>18.14</td>
<td>10.66</td>
<td><strong>2.02</strong></td>
</tr>
<tr>
<td>Test 2</td>
<td>50</td>
<td>83.55</td>
<td>15.45</td>
<td>10.53</td>
<td><strong>1.94</strong></td>
</tr>
<tr>
<td>Test 3</td>
<td>42</td>
<td>128.31</td>
<td>23.09</td>
<td>10.01</td>
<td><strong>1.80</strong></td>
</tr>
<tr>
<td>Control 1</td>
<td>ND</td>
<td>49.60</td>
<td>9.67</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Control 2</td>
<td>ND</td>
<td>44.30</td>
<td>8.41</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Control 3</td>
<td>ND</td>
<td>51.85</td>
<td>9.85</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND- Not detected

The selected consortium was able to convert crude oil in to methane along with methanogens. Therefore, Sand pack trial was successfully used for the study of *in situ* methanogenesis in oil reservoirs.

### 3.12.1 DGGE analysis of sand pack trials

The presence of inoculated bacteria after 70 days of incubation was confirmed by DGGE analysis. The bacterial DNA from the sand pack bottles was isolated using GenElute kit from SIGMA. The DNA was subjected to the nested PCR and 600bp amplicons were loaded on the gel. The DGGE profile was shown in the figure 3.34. In DGGE profile three bands were seen. These were eluted, reamplified and sequenced using automatic sequencer. Upon sequencing, SP-1 was identified as *Aeribacillus pallidus*, SP-2 and SP-3 were identified as *Hydrogenophilus hirschii*. The presence of two bands for *H. hirschii* is may be because of microheterogeneity of 16S rRNA. The DGGE analysis indicated the isolated cultures could adapt in simulated oil reservoir environment. Suthar et al (2008) reported that the sand pack columns are easy to construct and are inexpensive.
Most of the time a the problems associated with core 0flood studies like preservation of live cores, single usage, high capital investment, specialized equipment requirements etc. are not encountered in the sand pack column. This makes the sand pack column a suitable bench-scale technique for screening of microorganisms showing potential for oil recovery. According to Hitzman et al (2003) requirements of various nutrients and physic-chemical conditions can be easily adjusted in sand packs for the optimization of the process. In the present study indigenous microbial population was used, therefore the possibility is high that the consortium will adapt in the oil reservoir environment. This process might be the new technology to oil industry which is practical, cost effective, easily implemented, and is essential for increasing energy recovery.