Part I: Degradation of paraffin wax at high temperatures (55°-70°C)

Characterization of the paraffinic crude oil collected from oil wells with a history of paraffin deposition problems

The paraffinic crude oil used in this study was collected from the western oil fields of Gujarat as well as eastern regions (Digboi, Assam) of India. The Kalol region (Gujarat) has a history of paraffin depositions in oil well tubings with mechanical scraping being done almost every alternate day in some of the wells. The paraffinic fraction was extracted from the crude oil and characterized. This paraffinic fraction of the crude oil collected from Limbodara oil wells of Gujarat state of western India is shown in figure 4.1. In addition to the paraffinic crude oil, some paraffin wax deposits were also scraped from the oil well tubings and brought to the laboratory to study its paraffin content. This paraffinic-alkane finger print is shown in figure 4.2. The gas chromatographic analysis indicates the presence of alkanes ranging from hexadecane \((C_{16})\) to tetratriacontane \((C_{34})\). The relative percentages of the paraffins in this fraction are shown in figure 4.3. The alkanes heptadecane \((C_{17})\), octadecane \((C_{18})\), nonadecane \((C_{19})\), tetracosane \((C_{24})\), hexacosane \((C_{26})\) and heptacosane \((C_{27})\) each comprised 6% of the paraffinic fraction of the crude oil of Limbodara oil well. Tricosane \((C_{23})\) formed the maximum percentage (9%) of the paraffinic fraction with heneicosane \((C_{21})\) and docosane \((C_{22})\) at a close 8% (Figure 4.3). Triacontane \((C_{30})\) and nonacosane \((C_{29})\) were at 5% each in this crude oil as revealed by gas chromatography. The study of the paraffinic fraction of the crude oil collected from the Digboi refinery of Assam state in eastern India also revealed the presence of paraffinic-alkanes from octadecane to dotriacontane \((C_{32})\) (Figure 4.4).
Figure 4.1: Gas chromatogram depicting the alkane fingerprint of paraffinic crude oil collected from an oil well of Limbodara region that has a history of paraffin deposition related problems

Figure 4.2: Gas chromatogram depicting the alkane fingerprint of paraffinic wax scraped from an oil well with a history of paraffin deposition related problems in western India
Figure 4.3: Distribution of paraffinic-alkanes in the paraffinic fraction of crude oil collected from Limbodara oil field of Gujarat, India.

Figure 4.4: The alkane fraction of the crude oil sample collected from Digboi refinery in eastern India.

A commercial paraffin-wax had to be selected that would be representative of the paraffinic fraction of most of the crude oils of India. After analyses of the wax of different melting points, it was concluded that the alkane fingerprint of the wax with melting point of 58-60°C was most representative of the paraffinic crude oils under study. The gas chromatogram of Figure 4.5 shows the paraffinic-alkane distribution from eicosane to tetratriacontane in commercial wax. As evident from the paraffin wax...
distribution in figure 4.6, \( \text{C}_{22} \) to \( \text{C}_{28} \) forms a sizable fraction representing most paraffinic crudes of India. In addition to the paraffin distribution, the melting point of the selected wax was also slightly more than the cloud point of the oil wells of Limbodara (55°C). Since this wax was representative of most paraffinic crude oils, a bacterial strain that could efficiently degrade this wax at high temperatures, would be a potential strain for application to oil wells.

Figure 4.5: Gas chromatogram depicting the alkane fingerprint of commercial paraffinic wax (58°-60°C) selected to represent the paraffinic crude oils (of India)

Figure 4.6: The distribution of alkanes in commercial paraffinic wax (58°-60°C) selected to represent paraffinic crude oils

TERI University-PhD. Thesis, 2008
**Enrichment for thermophilic paraffin-wax degrading bacteria**

The selective enrichment of the paraffinic crude oil contaminated soil sample at 70°C, using commercial paraffin wax as the carbon source, yielded a stable thermophilic bacterial consortium that could survive high temperatures and utilize wax as the sole carbon source. The appearance of this consortium, with light microscopy and the electron micrograph of the cells, is shown in Figure 4.7.

![Gram stained cells and scanning electron micrograph of the bacterial strains obtained by enrichment using commercial wax as the sole carbon source](image)

In order to determine the degradation ability of the developed consortium, degradation studies were performed at 55°C since that was the cloud point of the paraffinic crude oil collected from oil wells with paraffin deposition problems of western India.

Monitoring the degradation of the representative paraffinic alkane, triacontane, by this consortium indicated that while cell multiplication was taking place throughout the 10 days of the study, a concomitant degradation did not occur during the first three days of growth. However, from the fourth day onwards, degradation was visible and 50% degradation of triacontane was observed in 10 days at 55°C. During this period, the bacterial cells increased from 1.5 x 10^4 CFU/ml on day zero (at the start of the experiment) to 2 x 10^6 on the 10th day (Figure 4.8).

Following the degradation of triacontane, the ability of the thermophilic consortium to degrade paraffin-wax (58-60°C) was studied. The gas chromatographic analysis of the degradation of commercial paraffin-wax by the thermophilic consortium is shown in figure 4.9. The extent of degradation of paraffin-wax was similar to that observed for triacontane, though the oxidation of all the paraffins was observed by gas chromatographic analysis.
Finally, the ability of the developed thermophilic consortium to degrade actual paraffinic crude oil was tested. With the crude oil collected from Limbodara oil well the consortium showed efficient utilization of the paraffin fraction in ten days. The degradation of all the constituent alkanes from hexadecane to triacontane was noted (Figure 4.10).

The purpose of the study was the utilization of the paraffinic-wax as the sole carbon source so co-metabolism was not considered as an alternative. Hence, in order to increase the degradation of paraffins, a variation in the nitrogen source was attempted. When 0.5% (w/v) of different nitrogen sources was added to the growth medium (containing 1g wax in 100 ml minimal salts medium), it was observed that the addition of tryptone, yeast extract and peptone did not enhance the degradation of wax while potassium nitrate did show a marginal increase (5%) in the extent of degradation of wax. With potassium nitrate already a component in the minimal salts medium, no complex nitrogen sources were added to the medium to enhance degradation by the consortium.

Figure 4.8: Growth and degradation of triacontane by the thermophilic bacterial consortium at 55°C
Figure 4.9: Gas chromatogram of the alkane fingerprint depicting the degradation of paraffin-wax (58-60°C) by the thermophilic bacterial consortium.

Figure 4.10: Gas chromatogram of the alkane fingerprint depicting the degradation of the paraffinic fraction of crude oil by the thermophilic bacterial consortium.

Having established the paraffin degrading potential of the thermophilic bacterial consortium, the culture was purified to obtain the constituent isolates. Based on the morphological distinction of the bacterial colonies on nutrient medium, 10 paraffin...
degrading bacterial strains were obtained. The ten strains were named TERI N1 to N5 and TERI H1 to H5. The average size of the bacterial cells (in microns) of TERI N1 was 3.35 μm, TERI N2 was 4.25, TERI N3 was 4.5, TERI N4 was 4.1 and TERI N5 was 3.4. The arrangement of the cells was in short chains, as well as singly and in pairs. The average size of the rods bacterial cells (in micrometers) from H1 to H5 was, 2.65 for H1, 18.6 for H2, 6.1 for H3, .45 for H4 and 4.05 for H5.

The degradation capability of the ten isolates was tested for commercial wax, paraffinic crude oil as also paraffinic alkanes as the carbon source. The results of these degradation studies indicated TERI N1 and N2 to be efficient degraders. The mean of three degradation experiments (with one gram of paraffinic crude oil as carbon source) indicated a degradation of 38.62% and 37.38% with TERI N1 and N2 respectively. Similarly, TERI H1 (33.46%), TERI H2 (34.02%), TERI H4 (35.1%) and TERI H5 (36.04%) also showed potential for the degradation of paraffinic crude oil (figure 4.11). The degradation of commercial paraffinic-wax (one gram of paraffinic wax in 100 ml medium) by the ten isolates indicated that the strains TERI N2 and TERI N4 show the highest degradation potential (figure 4.12). The strain TERI H5 also showed efficient utilization of the paraffinic wax. The gas chromatographic analysis, of the utilization of the individual paraffinic alkanes, showed that these strains were able to degrade all the alkanes of the paraffinic wax from heneicosane (C_{21}) to dotriacontane(C_{32}), efficiently (Figure 4.12).

![Graph showing degradation of paraffinic crude oil by bacterial isolates](image-url)
Since the bioavailability of hydrocarbons is a critical factor for efficient degradation, the emulsification ability of the isolated strains was also tested. The results of the crude oil biosurfactant assay indicated that the strain TERI N1 and TERI N2 were capable of emulsification of crude oil. The cell free supernatant when analysed for its interfacial tension using the DuNuoy’s ring method indicated a 21-point reduction in surface tension of TERI N1 as compared to the control (Table 4.1). The strain TERI N2 and the cell free supernatant of the consortium showed the same decrease in surface tension of 8 d/cm².

In addition to studying the functional abilities of the isolated strains, their tDNA banding pattern was also studied. Each band in the gel corresponded to the amplified DNA between two tRNA genes present within the genome of the isolate. Strains H1, H2, H3, H4 and H5 gave a similar banding pattern. The strains TERI N2, N4 and N5 also showed a similar banding pattern that in turn closely resembled that of TERI H1 to H5 based on tRNA spacer polymorphism. The banding pattern of TERI N1 and N3 however, was completely different from the rest of the isolates. So based on DNA fingerprinting, the 10 isolates were classified into 3 groups: (i) TERI N1 and TERI N3 (ii) TERI N2, TERI N4 and TERI N5 (iii) TERI H1 to TERI H5. One isolate
was selected from each of these groups based on earlier degradation results of paraffinic crude oil and paraffinic wax.

<table>
<thead>
<tr>
<th></th>
<th>Surface Tension (d/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL</td>
<td>66.95</td>
</tr>
<tr>
<td>TERI N1</td>
<td>45.90</td>
</tr>
<tr>
<td>TERI H1</td>
<td>61.69</td>
</tr>
<tr>
<td>TERI N2</td>
<td>58.40</td>
</tr>
<tr>
<td>Consortium</td>
<td>58.40</td>
</tr>
</tbody>
</table>

Table 4.1: Crude enzyme assay of the cell free enzyme for estimating biosurfactant production by the paraffin wax degrading bacterial isolates and estimation of surface tension of select isolates using Du Nuoy’s tensiometer.

The degradation of paraffin-wax by these selected isolates TERI N1, TERI N2 and TERI H5 were then compared. The results indicated that TERI N2 showed maximum degradation of all the constituent alkane fractions. Based on all these results, TERI N2 was selected for further studies. This strain was referred to as ‘TERI NSM’ in all further studies.

**Characterization of the selected thermophilic paraffin-wax degrading bacterial strain TERI NSM**

Cell morphology studies of this isolate by light microscopy and electron microscopy revealed a cell size of approximately 2-5 µm length. However, in addition to normal rod shaped morphology, the cells of strain TERI NSM showed the presence of long flexuous rods of variable length with some going up to 100µm at 70°C. The light microscopy photographs and scanning electron micrographs of these cells are shown in Figure 4.13. The occurrence of these flexuous thread shaped morphology was higher when the bacterial strains were repeatedly subcultured at 70°C than at 55°C. At 55°C, the elongated filaments were observed in LB medium as also when LB medium was supplemented with paraffinic alkanes eicosane (C₂₀) and triacontane (C₃₀) but no such elongated structures were noted in LB medium supplemented with wax. Similarly, no filamentous elongated of cells of TERI NSM was observed when
grown in MSM containing wax or MSM containing the paraffinic alkanes \( C_{20} \) and \( C_{30} \) as the carbon source. At 70°C, however, elongated flexuous rods were observed on repeated subculturing on MSM as well as LB medium containing wax. At 70°C, it was also noted that the rods of this strain sometimes appear curved. The length of the filaments was higher at 70°C compared to that of 55°C. While the length of the rods can extend easily up to 80 \( \mu \)m upon repeated sub-culturing at 70 °C (figure 4.13b), the length of the rods on LA (Luria Agar) at 55 °C has been recorded around 22 \( \mu \)m (figure 4.13d). However, at all times the bacterial shapes are a mixture of shorter rods (figure 4.13a and 4.13d) and the longer flexuous rods.

Figure 4.13: The cell morphology of the thermophilic bacterial strain TERI NSM using light microscopy (a and b) and electron microscopy (c and d).

The transmission electron micrographs of TERI NSM are shown in figure 4.14. The ultra structure prominently reveals the presence of terminal endospores in TERI NSM (Figure 4.14 b). However, following the protocol described by Ishige et al (for detecting wax crystals), no intracellular wax inclusions were observed in TERI NSM (Figure 4.14 d).

TERI University-PhD. Thesis, 2008
The selected paraffin degrading thermophilic strain, upon application to oil wells would be required to survive temperatures of upto 90°C and degrade the paraffins of crude oil at the cloud point of 55°C. Hence, the ability of the selected strain TERI NSM to survive temperatures of 90°C and grow at temperatures of 55 and 70°C were tested. A comparison of the growth of TERI NSM at these temperatures shows maximum growth at 55°C and least at 90°C. After 24 hours of incubation at 55°C, the total cell protein was at 1.85 mg/ml which decreased to 1.47 and 0.98 mg/ml in the next 24 hour intervals. The growth of TERI NSM at 70°C, on the other hand, showed maximum cell protein of 1.80 in 48 hours and at 90°C a maximum of 0.126 mg/ml. This shows that TERI NSM is unable to proliferate at 90°C. However, since this strain has only to survive a temperature of 90°C and degrade at 55°C it satisfied the criteria. The growth of the strain TERI NSM measured in terms of absorbance at 600 nm is shown in figure 4.15.

**Figure 4.14: Transmission electron micrograph of TERI NSM**
The strain TERI NSM could not grow in the presence of ampicillin, chloramphenicol, spectinomycin, streptomycin, tetracycline and vancomycin but could grow in the presence of nalidixic acid and Nystatin (Table 4.2). The ability of TERI NSM to grow in the presence of Nystatin is explained by the fact that it is an antifungal agent.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin (50 µg/ml)</td>
<td>-</td>
</tr>
<tr>
<td>Chloramphenicol (20 µg/ml)</td>
<td>-</td>
</tr>
<tr>
<td>Nalidixic Acid (15 µg/ml)</td>
<td>+</td>
</tr>
<tr>
<td>Spectinomycin (100 µg/ml)</td>
<td>-</td>
</tr>
<tr>
<td>Streptomycin (30 µg/ml)</td>
<td>-</td>
</tr>
<tr>
<td>Tetracycline (12 µg/ml)</td>
<td>-</td>
</tr>
<tr>
<td>Vancomycin (50 µg/ml)</td>
<td>-</td>
</tr>
<tr>
<td>Nystatin (1000 µg/ml)</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 4.2: The antibiotic profile of the paraffin degrading bacterial strain TERI NSM
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The paraffin degrading strain TERI NSM could grow in the temperature range of 45°C to 85°C and was capable of utilizing several sugars including glucose, mannose and starch (Table 4.3). However, it could not utilize the pentose sugar ribose as also Tween as a carbon source.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>TERI NS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram staining</td>
<td>+</td>
</tr>
<tr>
<td>Maximum growth temperature</td>
<td>85°C</td>
</tr>
<tr>
<td>Minimum growth temperature</td>
<td>45°C</td>
</tr>
<tr>
<td>Growth and utilization of:</td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>+</td>
</tr>
<tr>
<td>Mannose</td>
<td>+</td>
</tr>
<tr>
<td>Raffinose</td>
<td>+</td>
</tr>
<tr>
<td>Trehalose</td>
<td>+</td>
</tr>
<tr>
<td>Cellobiose</td>
<td>+</td>
</tr>
<tr>
<td>Galactose</td>
<td>+</td>
</tr>
<tr>
<td>Starch</td>
<td>+</td>
</tr>
<tr>
<td>Arabinose</td>
<td>+</td>
</tr>
<tr>
<td>Ribose</td>
<td>-</td>
</tr>
<tr>
<td>Mannitol</td>
<td>+</td>
</tr>
<tr>
<td>Xylose</td>
<td>+</td>
</tr>
<tr>
<td>Sucrose</td>
<td>+</td>
</tr>
<tr>
<td>Maltose</td>
<td>+</td>
</tr>
<tr>
<td>Lactose</td>
<td>+</td>
</tr>
<tr>
<td>Citrate</td>
<td>-</td>
</tr>
<tr>
<td>Catalase</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 4.3: The growth and biochemical characteristics of the bacterial strain TERI NSM

The paraffinic hydrocarbon utilization profile of TERI NSM indicated a preference for long chain alkanes (Table 4.4). While this strain could not utilize alkanes from octane to tetradecane it was capable of degrading paraffinic alkanes upto triacontane as revealed by its growth (measured in terms of absorbance and cell protein).
<table>
<thead>
<tr>
<th>Alkane</th>
<th>TERI NSM (Growth)</th>
<th>OD₆₀₀</th>
<th>Protein (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Octane</td>
<td>-</td>
<td>0.06</td>
<td>-</td>
</tr>
<tr>
<td>Nonane</td>
<td>-</td>
<td>0.063</td>
<td>-</td>
</tr>
<tr>
<td>Decane</td>
<td>-</td>
<td>0.096</td>
<td>-</td>
</tr>
<tr>
<td>Undecane</td>
<td>-</td>
<td>0.096</td>
<td>-</td>
</tr>
<tr>
<td>Dodecane</td>
<td>-</td>
<td>0.102</td>
<td>-</td>
</tr>
<tr>
<td>Tridecane</td>
<td>-</td>
<td>0.084</td>
<td>-</td>
</tr>
<tr>
<td>Tetradecane</td>
<td>-</td>
<td>0.109</td>
<td>-</td>
</tr>
<tr>
<td>Heptadecane</td>
<td>+</td>
<td>0.361</td>
<td>0.508</td>
</tr>
<tr>
<td>Octadecane</td>
<td>+</td>
<td>0.690</td>
<td>1.214</td>
</tr>
<tr>
<td>Nonadecane</td>
<td>+</td>
<td>0.561</td>
<td>1.350</td>
</tr>
<tr>
<td>Eicosane</td>
<td>+</td>
<td>1.039</td>
<td>2.762</td>
</tr>
<tr>
<td>Docosane</td>
<td>+</td>
<td>0.827</td>
<td>2.341</td>
</tr>
<tr>
<td>Tetracosane</td>
<td>+</td>
<td>1.003</td>
<td>3.220</td>
</tr>
<tr>
<td>Pentacosane</td>
<td>+</td>
<td>0.187</td>
<td>0.275</td>
</tr>
<tr>
<td>Hexacosane</td>
<td>+</td>
<td>0.639</td>
<td>1.523</td>
</tr>
<tr>
<td>Octacosane</td>
<td>+</td>
<td>0.308</td>
<td>0.716</td>
</tr>
<tr>
<td>Triacontane</td>
<td>+</td>
<td>0.301</td>
<td>0.708</td>
</tr>
</tbody>
</table>

Table 4.4: The alkane utilization profile of the thermophilic strain TERI NSM

The supernatant assay was carried out using paraffinic compound C₂₂ as the substrate. The results of gas chromatographic analysis show a prominent peak at the retention time of 25 min which is representative of C₂₂ (Figure 4.16a). However, at 60 min onwards (i.e., 60 min, 120 min, 150 min readings) there is appearance of another peak at 21 min and a smaller peak at 17 min in addition to the peak of C₂₂. These two peaks were the results of the extracellular activity of the enzyme when the C₂₂ compound was used as the substrate. In another experiment, eicosane was used as the substrate and the intermediate formation followed for five hours. It was observed that there was a rise of an intermediate at 4 hours, which was further degraded after 5 hours (4.16b).
Figure 4.16a: Crude enzyme assay with docosane as the substrate and the supernatant of TERI NSM grown with octacosane as the carbon source.

Figure 4.16b: Crude enzyme assay with eicosane as the substrate and the supernatant of TERI NSM grown with octacosane as the carbon source.
The cell wall fatty acid methyl ester (FAME) analysis of the strain TERI NSM was done at MIDI Labs. (USA). The cell wall fatty acids of TERI NSM grown on LB at 55°C revealed that it was largely composed of iso C15:0 and iso C17:0 fatty acids. The iso C15:0 fraction formed 47.85% and the iso C17:0 formed 23.9% of the fatty acid composition. Other fatty acids detected were iso C16:0 at 6.93%, iso C14:0 at 0.35%, 1.87% of anteiso C15:0 and 2.3% of iso C17:1 w5c. Besides these, C17:0 anteiso at 5.11% also formed a sizable fatty acid fraction. Based on the dendrogram cluster analysis technique, that produces unweighted pair matching based on fatty acid compositions to show the relatedness between organisms, TERI NSM showed similarity with *Geobacillus kaustophilus* and *G. thermocatenulatus* (graphically depicted in Figure 4.17).

Identification of the paraffin-wax degrading bacterial strain TERI NSM

**Partial 16s rRNA gene sequence**

Using universal primers that bind at the 5’ and 3’ ends of 16s rDNA of Eubacteria, a partial 538 bp fragment was obtained. The nucleotide sequence obtained was submitted to GenBank (Accession number EF199739, later amended to include full 16s sequence). The phylogenetic and molecular evolutionary analyses were conducted using MEGA version 3.1 (Kumar et al, 2004). The result of the partial sequence alignment is shown in Figure 4.18. The database homology showed that the
strain TERI NSM displayed a high level of similarity with the strains of the
*Geobacillus sp.* of Gram positive bacteria. The closest matches showing 100%
homology were strains of *Geobacillus kaustophilus* and *Geobacillus
stearothermophilus* while those showed a close 99% homology were *Geobacillus sp.
N60, G. lituanicus, G. thermoleovorans and Bacillus caldotenax.*
Since, the partial 16s rRNA gene sequence was unable to accurately reveal the
identity of the strain TERI NSM, a full gene sequence was done.

**Complete 16s rRNA gene sequence**
The complete gene sequence, when analysed by comparison with 16s rDNA
nucleotide sequences of GenBank, revealed a 100% identity to the 16s sequence of the
thermophilic *Geobacillus* strain N60 isolated from a volcanic island and to
*Geobacillus kaustophilus* strain HTA426 isolated from a deep sea sediment of the
Mariana Trench (Takami *et al*, 2004). The strain also showed 99% homology to
several thermophilic strains of the *Geobacillus* gp. It showed single nucleotide
difference with *Bacillus caldotenax, G. kaustophilus, a Bacillus sp. WPD616,
Geobacillus sp. STB2* and 2 nucleotide difference (99% homology) with *G.
kaustophilus* strain BGSC 90A1, *G. stearothermophilus BGSC9A19 and B.
thermoleovorans* and 4 nucleotide difference with Bacilli strains *B. vulcani* and *B.
caldolyticus*. The E value of all the strains was zero. The relatedness of TERI NSM
was to members of the family *Bacillaceae*, so, to determine its phylogenetic position,
a phylogenetic tree was constructed with homologous sequences of the databank
(Figure 4.19). The phylogenetic analysis places TERI NSM in close phylogenetic
proximity to the volcanic isolate *Geobacillus STB2*.

The strain TERI NSM was assigned to the *G. kaustophilus* sp. considering the results
of the 16s sequence and the supporting evidence provided by FAME analysis.
Figure 4.18: Phylogenetic tree showing the position of the strain TERI NSM among Geobacillus sp. based on the partial 16S rDNA sequence using the MEGA 3.1 software. Bootstrap values are shown at branch points.
Figure 4.19: Phylogenetic tree showing the position of the strain TERI NSM among *Geobacillus* *sp.* based on the complete 16S rRNA gene sequence using the MEGA 3.1 software. Bootstrap values are shown at branch points.
Results: Paraffin

Growth of *G. kaustophilus* TERI NSM on paraffinic hydrocarbons and paraffin-wax

Alkanes of carbon chain length of C18 (octadecane) and above are generally considered paraffinic (Addison, 1984) and are responsible for the paraffin deposition in oil wells. Hence, the alkanes C20, C25 and C30, were chosen to represent the paraffinic-wax in this study.

The growth of TERI NSM on the paraffinic hydrocarbons (C\textsubscript{20}, C\textsubscript{25} and C\textsubscript{30}) and paraffin-wax measured every 24 hours, in terms of increase in total cell protein is shown in Figure 4.20. While there was no lag phase, slow growth was noted within the first 24 hours. However, after 24 hours, the culture grew rapidly on eicosane. The growth was maximum between the 3\textsuperscript{rd} and 4\textsuperscript{th} day of study after which it began to decline. The growth of TERI NSM on pentacosane, however, showed a lag phase of nearly 48 hours, after which growth increased rapidly up to the 6\textsuperscript{th} day before entering the stationery phase and then started declining around the 8\textsuperscript{th} day. When grown on triacontane and wax however, TERI NSM showed a very slow but steady growth. The strain seemed to be in the growth phase even up to 15 days of growth when the remaining nutrients might be exhausting. The growth of *G. kaustophilus* TERI NSM on paraffinic hydrocarbons as the sole carbon source, however, showed that the growth and therefore the degradation of the paraffins was slow.

The degradation of the paraffinic alkanes when used as a mixture (0.1% w/v each of C\textsubscript{20}, C\textsubscript{25} and C\textsubscript{30}) showed more than 60% of the 3 alkanes being degraded in a week. On the 10\textsuperscript{th} day of incubation, 77% of the triacontane and 85% of pentacosane were degraded compared to 97% utilization of eicosane. Thus, *G. kaustophilus* TERI NSM shows a preferential degradation of eicosane over pentacosane and triacontane (Figure 4.21). Optimization of growth parameters was therefore required to increase the degradation of these paraffinic hydrocarbons.
Figure 4.20: Growth of *G. kaustophilus* TERI NSM on the paraffinic hydrocarbons C<sub>20</sub>, C<sub>25</sub>, C<sub>30</sub> and wax as the carbon source in shake flask conditions at 55°C (total cell protein)

Figure 4.21: Degradation of the paraffinic alkanes: eicosane, pentacosane and triacontane by *G. kaustophilus* TERI NSM at 55°C

TERI University-PhD. Thesis, 2008
Optimization of cultural parameters for enhanced degradation of paraffin-wax by *Geobacillus kaustophilus* TERI NSM

**Optimization of nitrogen source**
The degradation of paraffin-wax as the sole carbon source using different nitrogen sources indicated a preference of this strain for inorganic sources of nitrogen like potassium nitrate and ammonium chloride compared to complex nitrogen sources like yeast extract and tryptone (figure 4.22). The growth with yeast extract and tryptone might give TERI NSM a growth flux in the first 2-3 days of growth, however, the degradation of paraffin wax takes more than a week and hence potassium nitrate and ammonium chloride, which will provide sustained nitrogen to this strain until the wax is present, is desirable. Since potassium nitrate was already present in MSM, ammonium chloride was also incorporated into the medium.

![Graph showing degradation of individual paraffinic alkanes of wax at 55°C by G. kaustophilus TERI NSM as analysed by Gas chromatography](image_url)

Figure 4.22: Degradation of individual paraffinic alkanes of wax at 55°C by G. *kaustophilus* TERI NSM as analysed by Gas chromatography

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Inoculum optimisation

The quantum of bacterial cells in the growth medium is a determinative factor for the degradation of hydrocarbons. Different inoculum volumes of *G. kaustophilus* TERI NSM grown in LB (1 x 10⁷ CFU/ml) were added to the MSM containing paraffin-wax as the sole carbon source. As indicated in figure 4.23, rapid growth was observed within the first 24 hours of growth and the growth was proportional to the amount of inoculum, with 10% inoculum showing maximum growth and 1%(v/v) inoculum showing the least amount of growth as estimated with total cell protein. However, by the 5th day of incubation, the 10% (v/v) inoculum showed a decline in growth compared to the 5% inoculated culture. This observation hinted at the fact that, while the increase in inoculum upto 5% shows proportional increase in growth, an increase upto 10% does not show a proportional growth flux with aeration probably becoming a limiting factor. A pattern similar to that observed in the growth of TERI NSM was visible with the extent of degradation of paraffin-wax as well. While the degradation with 1% and 2% inoculum volume was similar at 34.5% and 35.5% respectively, the degradation 10%(v/v) inoculum was much less at 21%. The degradation using 5% (v/v) inoculum was, however, the maximum at 52%. This inoculum volume was then maintained for further experimentation.

Figure 4.23: The growth of *G. kaustophilus* TERI NSM measured as total cell protein on paraffin wax at 55°C with varying inoculum sizes

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The degradation of the various carbon fractions of paraffin-wax, as analysed by gas chromatography, is shown in figure 4.24. As is evident from these results, 5% inoculum showed maximum degradation of all the fractions of paraffin-wax. While the shorter chain alkanes were utilized in all the experimental sets (hinting at their preferential utilization over long chain alkanes), there was limited degradation of carbon fractions with chain length C25 and higher alkanes by the 10% inoculum set indicating the importance of aeration in hydrocarbon degradation. For further degradation studies, 5%(v/v) inoculum was used.

![Figure 4.24: The degradation of the different carbon fractions of paraffinic wax using different inoculum volumes as analysed by gas chromatography](image)

**Optimisation of the surfactant Tween**

The bioavailability of substrates is a major limiting factor in hydrocarbon degradation (esp. long chain alkane), *Geobacillus kaustophilus* TERI NSM was, therefore, grown with different concentrations of Tween and the extent of paraffin-wax degradation was estimated by comparing with the degradation of wax without the addition of Tween. No growth was observed in the experiment containing Tween but no wax as the carbon source, thereby confirming that TERI NSM does not utilize Tween as a carbon source. The growth of TERI NSM showed a lag phase in growth for nearly 48 hours.
hours where only wax (without the addition of Tween) was added, while rapid growth was seen within 24 hours in the Tween supplemented medium. However, the growth (as indicated by total cell protein) was higher in the medium containing Tween compared to the media containing higher concentrations of the surfactant. The degradation of paraffinic wax at the end of the experiment showed degradation of 46% where no Tween was added, 80 and 86% with 0.01% and 0.05% (v/v) Tween and 64% with 0.1%(v/v) Tween. This means that compared to the medium without the supplementation of Tween, the effective enhanced degradation was 34, 40 and 18% with the addition of 0.01, 0.05 and 0.1% (v/v) Tween. Figure 4.25 clearly shows the positive effect of Tween on the degradation of the various carbon fractions of paraffin-wax as analysed by gas chromatography. While concentrations of 0.05% and 0.1% Tween degraded more than 60% of the alkanes eicosane (C_{20}), heneicosane (C_{21}) and docosane C_{22}), the degradation of the alkanes higher than octacosane was not observed. Since the difference in the degradation of paraffinic wax with the supplementation of 0.01% and 0.05% Tween was not very huge, minimal salts medium was now amended to include 0.01% (v/v) Tween.

Figure 4.25: Gas chromatographic analysis of the degradation of different carbon fractions of paraffin wax, by G. kaustophilus TERI NSM, at 55°C in the presence of the surfactant Tween

**Optimisation of headspace**

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The effect of medium volume and thereby headspace on the degradation of paraffinic wax by *G. kaustophilus* TERI NSM showed different growth patterns at the four volumes tested. The growth of TERI NSM at 25 ml was higher and rapid compared to that of the others. The growth pattern in 50 ml and 75 ml did not show a lot of difference but was higher than that in 100 ml medium where agitation and consequently aeration was probably a limiting factor and growth though continuous, was slow (Figure 4.26). The degradation of paraffinic wax also followed a trend similar to that observed with growth. The degradation of paraffin-wax in 25 ml medium was complete, however, due to evaporation losses that accompany high temperatures, there was a dearth of medium indicating that the degradation could be increased if the medium volume was increased. The degradation of wax in 50 ml and 75 ml medium was similar at 64 and 70% degradation. However, the degradation of wax was least in the case of 100 ml medium at 48%. It was noticed that the medium volume showed higher evaporation losses at 55°C in the 25 ml flasks compared to the others. Since the degradation ability was nearly the same with 50 ml and 75 ml medium, 50 ml medium was selected as the ideal volume for further experiments.

![Graph showing growth and degradation](image)

**Figure 4.26:** The growth of *G. kaustophilus* TERI NSM with varying head space volumes of MSM with paraffin-wax as the carbon source at 55°C

*Assessment of the effect of substrate concentration on growth of TERI NSM and degradation of wax*

*TERI University-PhD. Thesis, 2008*
The growth pattern of the selected strain TERI NSM on different concentrations of paraffinic-wax showed no harmful effects of higher concentrations of substrate with growth being unaffected by increasing concentrations of wax (Figure 4.27). The degradation study, on the other hand, showed a 100% degradation of 0.01% paraffinic wax, and a degradation of 51.6 and 57% with 0.1% and 0.05% (w/v) wax respectively. Since, the degradation of wax did not increase on addition of 0.5% and 1% (w/v) wax, it remained wasted in the medium as the undegraded residue. On the other hand the complete degradation of 0.01% (w/v) wax indicates that there was a higher scope of degradation of wax compared to the nutrients present in the medium. Hence, for all future experiments, 0.1% (w/v) paraffins were used as the carbon source.

Figure 4.27: The growth of *Geobacillus kaustophilus* TERI NSM on different concentrations of paraffin-wax as the carbon source at 55°C
Degradation of paraffinic-wax and the representative paraffinic alkanes by *G. kaustophilus* TERI NSM

The growth of *Geobacillus* strain TERI NSM on paraffinic wax showed a lag phase for the first two days of growth with the degradation also being negligible during this period (figure 4.29). However, from the second day onwards growth and degradation began to rise steadily with approximately 21% and 60% of paraffinic wax being degraded on the 3rd day and at the time of maximum growth (in terms of total cell protein) on the 7th day respectively (Figure 4.28).

![Graph](image)

**Figure 4.28**: Degradation of paraffinic wax by *G. kaustophilus* TERI NSM at 55°C under shake flask conditions. (●) Total cell protein (mg/ml) — Degradation in the treated samples; (▲) Degradation in the control samples

At the end of 8 days, 90% of the paraffinic wax had been degraded. Gas chromatographic analysis indicated an efficient utilization of all the alkanes of the paraffin-wax by the 8th day (figure 4.29).
Figure 4.29: Degradation of the carbon fraction of paraffinic wax by *G. kaustophilus* TERI NSM at 55°C in eight days

*Geobacillus kaustophilus* TERI NSM was grown on the paraffinic alkane eicosane as the sole carbon source and growth monitored by measuring the total cell protein. The growth profile showed that there was a negligible lag phase of growth and therefore degradation was very rapid. The cells reached the stationary phase in 30 hours when 58% of eicosane was degraded. The consumption was rapid during the growth phase but slowed down a little as it reached the stationary phase, finally attaining a 96% degradation of eicosane in 72 hours. The growth pattern and the extra cellular extract at zero h, 30 h and 72 h is shown in figures 4.30.

The degradation of pentacosane, on the other hand, showed a small lag period after which growth proceeded rapidly but most of the degradation of pentacosane seemed to occur in the stationary phase. While there was a mere 10% degradation in 30 hours, the strain TERI NSM degraded 77% of the pentacosane in 72 hours. The growth of TERI NSM on C_{25} and the degradation of pentacosane when used as the sole carbon source by this strain is shown in figure 4.31.
Figure 4.30: Growth of G. kaustophilus TERI NSM in eicosane (C20) as the carbon source under shake flask condition at 55°C

- Total cell protein
- Eicosane utilization
- Untreated control

Figure 4.31: Growth of TERI NSM and degradation of pentacosane (C25) when used as the sole carbon source at 55°C

- Total cell protein (mg/ml)
- Degradation in the treated samples
- Degradation in the control samples

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The degradation of triacontane is shown in figure 4.32. The lag phase for triacontane degradation was more than that of eicosane and pentacosane. For 72 hours, while there seemed to be growth (based on the absorbance and total cell protein), no visible degradation occurred. However, after the lag phase rapid degradation took place with 84% of the triacontane being degraded in 12 days. The growth and degradation of triacontane, by *G. kaustophilus* TERI NSM, when used as the sole carbon source is shown in figure 4.32.

Figure 4.32: Growth of TERI NSM and degradation of triacontane (C₃₀) when used as the sole carbon source at 55°C. (— Total cell protein (mg/ml) — Degradation in the treated samples •••••• Degradation in the control samples)
Intermediate metabolites formed during the degradation of paraffinic alkanes that constitute paraffin-wax

The intermediates formed during the degradation of various paraffinic alkanes were studied by growing the strain *G. kaustophilus* TERI NSM on these alkanes as the sole carbon source and then derivatising the extracted metabolites. When eicosane, pentacosane and triacontane were used as the sole carbon source by TERI NSM, the GC-MS analysis indicated the presence of several monocarboxylic acids. The characteristic mass abundance (74) of monocarboxylic acids was noted in these chromatograms (Figures 4.33 a, b and c).

Figure 4.33: The analysis of the intermediate metabolites formed when *Geobacillus kaustophilus* TERI NSM was grown at 55°C in MSM containing (a) eicosane (b) pentacosane and (c) triacontane as the sole carbon source
Since, the appearance of monocarboxylic acids was noted on the degradation of paraffinic alkanes, the strain TERI NSM, was grown on the known oxidative intermediates of alkane degradation. A comparison of the metabolites formed on the degradation of octadecane (C), octadecanol and octadecanoic acid is shown in Figure 4.34. Octadecanoic acid methyl ester at a retention time (Rt) of 30.71 min was noted in all three chromatograms. Besides, this hexadecanoic acid methyl ester at 26.81 min and tetradecanoic acid methyl ester at 23.9 min also featured prominently in the chromatograms.

Figure 4.34: Comparison of the oxidative metabolites formed during the degradation of octadecane, octadecanol and octadecanoic acid by TERI NSM at 55°C

A comparison of the oxidative metabolites of formed during the degradation of eicosane (C₂₀) and octadecane is shown in Figure 4.35. It reveals an additional monocarboxylic acid, eicosanoic acid methyl ester at an Rt of 34.8 min which was not noted in the degradation products of octadecane. Besides, eicosanoic acid, other monocarboxylic acids like hexadecanoic acid, tetradecanoic acid etc were detected during the degradation of eicosane.
The metabolites formed during the growth of *G. kaustophilus* TERI NSM with eicosane, octadecane and their mono-oxidative metabolites hinted at the possibility of the monoterinal oxidation pathway being the major degradation pathway in this strain. To confirm this, TERI NSM was grown with a longer carbon chain alkane, octacosane and its oxidative metabolites, octacosanol and octacosanoic acid as the carbon source. The intermediate metabolites formed during this degradation are shown in figure 4.36. While the degradation of octadecane and eicosane had yielded monocarboxylic acids with carbon chain lengths of up to C18-acid and C20-acid, during octacosane degradation, methyl esters of the carboxylic acids docosanoic acid and tetracosanoic acid were detected. Tetracosanoic acid and docosanoic acid were also detected when TERI NSM was grown on octacosanol and octacosanoic acid. The growth of *G. kaustophilus* TERI NSM however, was always lesser on the alkanols and alkanoic acids, however, compared to the corresponding alkanes.
Figure 4.36: Intermediate metabolites formed during the growth of *G. kaustophilus* TERI NSM with octacosane (C\textsubscript{28}), octacosanol and octacosanoic acid at 55°C under shake flask conditions

Since, all the paraffinic alkanes that were studied for their intermediate metabolites so far, were even chain alkanes, TERI NSM was grown on an odd chain alkane, pentacosane (C\textsubscript{25}), as the sole carbon source. The oxidative metabolites formed during the degradation of pentacosane were compared with those formed during the degradation of eicosane to search for oxidation products that have not come as cell fatty acid contaminants (Figure 4.37). As pointed out in Figure 4.37, the odd chain carboxylic acids heptadecanoic acid methyl ester and pentadecanoic acid methyl ester were detected in the pentacosane grown culture but which were absent among the carboxylic acids detected in the eicosane grown culture. These two carboxylic acids however, were not detected in the initial stages of growth of TERI NSM on C\textsubscript{25} but were detected only from the third day onwards (Figure 4.38).
Figure 4.37: The metabolic intermediates formed during the growth of TERI NSM with pentacosane as the sole carbon source, depicted in comparison to the intermediates of eicosane degradation

Figure 4.38: The periodic analysis of the oxidative intermediates formed during the degradation of pentacosane by G. kaustophilus TERI NSM
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A similar pattern of appearance of the carboxylic acids was observed on monitoring the metabolic intermediates of eicosane degradation by TERI NSM. Eicosanoic acid (Rt 34.1 min, mass abundance 74) was detected from the first day of growth (Figure 4.39a) and was detected continuously after that. However, there was a marked shift in the pattern of appearance of shorter chain carboxylic acids. While there were a negligible number of carboxylic acids visible in the initial stages of growth, their frequency of appearance greatly increased by the 5th and 6th day of growth (could not be identified individually, but visible as metabolites with a mass abundance of 74), indicating the break down of the parent alkane into the carboxylic acid products (Figure 4.39b).

In addition to studying the intermediate metabolites produced during the degradation of paraffins at 55°C, the degradation of paraffins at 70°C was also studied and the same pattern of metabolite formation was detected. The metabolites formed when glucose was added as a carbon source in addition to paraffins also yielded monocarboxylic acids indicating that the monoterminal oxidation pathway was followed even if an additional carbon source was present.

Figure 4.39a: Intermediate metabolites of eicosane degradation (eicosanoic acid methyl ester at 34 min retention time) by TERI NSM at 55°C on consecutive days
The intracellular accumulation of carboxylic acids was also studied during the degradation of the paraffinic alkanes eicosane, pentacosane and triacontane as the sole carbon source by the strain *G. kaustophilus* TERI NSM. The profile of the oxidative metabolites thus formed is shown in figure 4.40. In addition to the cell wall fatty acids, some of the intermediates of the degradation of the paraffinic alkanes were also detected. The intracellular products of pentacosane degradation for instance show the presence of pentadecanoic acid and heptadecanoic acid, which are not present among the intracellular metabolites of eicosane and triacontane degradation.

Figure 4.39b: Intermediate metabolites (shorter carbon chain with mass abundance of 74) of eicosane degradation by TERI NSM at 55°C
Alkane hydroxylase gene detection in the thermophilic strain
*Geobacillus kaustophilus* TERI NSM

Of the three sets of primers tested, amplification could not be observed using the primer sets 1 and 2 but a PCR product of expected size of 330 bp was amplified using the third set of primers (Figure 4.41). The DNA sequence analysis of this amplicon revealed a homology with the cloned putative alkane hydroxylase genes and to the alkane hydroxylase of *Ralstonia sp.* The deduced amino acid sequence comparison with the NCBI protein databank entries revealed a varying degree of similarity (70-84%) to the putative alkane hydroxylases of uncultured bacteria. There was a 77% identity with putative alkane hydroxylase of *Ralstonia sp.* PT11. Besides these, the translated alkane hydroxylase gene fragment showed varying identities with alkane hydroxylases of *Acidisphaera sp.* C197 (55%), a *Marinobacter aquaeoli* strain VT8 (53%) and *Alcanivorax borkumensis* (53%). Among Pseudomonads around 50% identities, were *P. putida* (CAB51047), *P. aeruginosa* (CAC86944 and CAC86947) as also *P. chlororaphis* (CAB595525) and *P. mendocina* (EAV23052).

The phylogenetic analysis of the alkane hydroxylase gene fragment is shown in Figure 4.42.
Figure 4.41: Amplification of the partial alkane hydroxylase gene fragment of 330 bp from the genomic DNA of G. kaustophilus TERI NSM using the primers of set 3 designed by Kohno et al (2002). The 500 bp ladders are denoted as the marker lanes M. L3 and L4 lanes indicate the 330 bp amplicon before purifying the product and L1 and L2 lanes indicate the 330 bp alkane hydroxylase pure gene product.
Figure 4.42: Phylogenetic tree showing the position of the alkane hydroxylase gene of TERI NSM among closely related alkane hydroxylases using the MEGA 3.1 software. Bootstrap values are shown at branch points.
Application of *Geobacillus kaustophilus* TERI NSM to the paraffinic crude oil of Kalol oil fields of Gujarat (India)

The Kalol oil fields of Gujarat state of western India have history of paraffin deposition problems in the oil wells. The gas chromatographic analysis of the paraffinic fraction of this crude oil revealed an alkane carbon fraction from tetradecane to dotriacontane. While lower alkanes were present in this crude oil, the composition of paraffinic alkanes from octadecane (C\textsubscript{18}) to octacosane (C\textsubscript{28}) was very high (Figure 4.43), thereby imparting this crude oil its paraffinic characteristics. The pour point of this crude oil was 33°C and the cloud point was 39°C. The viscosity at 50°C was 28.43 with a negligible amount of volatiles.

![Degradation of paraffinic crude oil](image)

**Figure 4.43:** Degradation of the paraffinic crude oil of an oil field of Gujarat (India) by *Geobacillus kaustophilus* TERI NSM at 55°C under shake flask conditions

This oil (1 g crude oil added to 50 ml MSM) when treated with *Geobacillus kaustophilus* TERI NSM at 55°C under shaking conditions (180 rpm) showed an effective degradation of the paraffins of the crude oil (Figure 4.4). A 66% degradation of this crude oil was noted within a week. The emulsification of this paraffinic crude oil was evident during the growth of TERI NSM with the paraffinic crude oil as the sole carbon source (Figure 4.44)
The test of flow behavior of the treated and untreated crude oil using the fabricated apparatus, showed an outflow of 180 ml for the untreated crude oil as compared to the 200 ml of the treated crude oil under the same experimental conditions indicating a reduction in viscosity.

The treatment of the oil wells of Kalol by TERI NSM resulted in a reduced scraping frequency of the oil wells. Before treatment the paraffin-wax depositions had to be scraped from the oil well tubing every week, but after treatment with TERI NSM the scraping was not done for 4 months. The *G. kaustophilus* strain is believed to have formed a biofilm on the inner wall of the oil well tubing preventing the formation of the nucleation point for the paraffin-wax crystals. The viscosity of the crude oil from well KL89N decreased from 28.43 to 18.4 cSt and that of KL24N decreased from 41.36 to 5.44 cSt at 50°C. It was noted by the oil company that while earlier these oil wells had to be mechanically scraped every alternate day, after application of TERI NSM, the scraping was not required to be done for 6 months (Figures 4.45 and 4.46).
4.46). This roughly amounts to a saving of $1500 per week of the cost of mechanical scraping.

This study redeems the potential of this strain for mitigation of paraffin deposition problems in oil wells of the paraffin plagued oil wells of western India. The technology has now been implemented in other oil fields of Gujarat.

Figure 4.45. Treatment of oil well KL89N of Gujarat with \( G. \ kaustophilus \) TERI NSM

Figure 4.46. Treatment of oil well KL24N of Gujarat with \( G. \ kaustophilus \) TERI NSM
Part II: Biodegradation of Acidic Oily Sludge at Digboi refinery

Characterization of the acidic oily sludge

The acidic oily sludge at Digboi refinery had accumulated in the refinery premises over a century. This had resulted in the formation of a thick solid crust that did not allow percolation of petroleum hydrocarbons to leach down. The site was bereft of any vegetation and the sulphuric acid fumes being emitted from the sludge in the heat, made it difficult to work for long at the site. The sludge sample collected from Digboi refinery was analysed for several parameters like pH, water content, organic carbon and ash content as also the TPH and its constituents. The pH of the sludge varied from 1.5 to 3, due to the high quantities of sulfuric acid present in the sludge. The solvent extractable Total Petroleum Hydrocarbons (TPH) at 60% (w/w) formed the major portion of the acidic oily sludge (figure 4.47). The sludge also contained 14% water, 16% sediment/ash content and the remaining part was the organic carbon in acidic oily sludge. The composition of this acidic oily sludge is shown in Figure 4.47 and 4.48. The total petroleum hydrocarbons (TPH) of this sludge from Digboi contained 26% (w/w) alkane fractions with carbon numbers of alkanes ranging from C\textsubscript{14} to C\textsubscript{34} and 45% (w/w) aromatics in the solvent extractable hydrocarbon fraction, while the remaining 28% mainly formed the NSO containing fraction and asphaltenes (figure 4.48). The aromatic fractions included PAHs like pyrene, anthracene, fluoranthene, benzopyrene, phenanthrene and dibenzothiophene. Besides these identifiable, polycyclic aromatic hydrocarbons, several substitutes and derivatives were also present. The Gas chromatograms of representative samples of the alkane and aromatic fractions of the TPH are shown in Figures 4.49 and 4.50 respectively.
Figure 4.47: Composition of the acidic oily sludge of Digboi refinery

*Values are a mean of ten replicates

Figure 4.48: Composition of the total petroleum hydrocarbon in the acidic oily sludge

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*Values are a mean of ten replicates
Figure 4.49: Gas chromatogram showing the alkane fingerprint of the acidic oily sludge at Digboi refinery

Figure 4.50: Gas chromatogram showing the aromatic profile of the acidic oily sludge at Digboi refinery
Screening for acidic oily sludge degrading microbial strains

The enrichment protocol after several sequential transfers using acidic oily sludge as the sole carbon source yielded a stable consortium. The morphology of the consortium is shown in figure 4.51. The cell morphology indicated at the possibility of the consortium to be predominantly composed of yeast like cells.

Figure 4.51 Stained cells of the enriched consortium obtained from the acidic oily sludge contaminated soil

Alkane and Aromatic hydrocarbon Degradation by the developed consortium

This consortium was tested for its ability to degrade an alkane hydrocarbon (C$_{18}$) and an aromatic compound (Fluoranthene) present in the acidic oily sludge. As shown in Figure 4.52, this consortium was capable of transforming 85% of the octadecane (C$_{18}$) at pH 3 in a week. The oxidative transformation of fluoranthene, on the other hand, was less efficient at 50% (Figure 4.53). The degradation of the alkane and aromatic hydrocarbons of the acidic oily sludge were then tested using the consortium. The degradation of the alkane fraction was efficient with all alkanes, from octadecane to triacontane, of this fraction being efficiently degraded by the consortium (Figure 4.54). However, similar to the pattern of degradation of fluoranthene, the oxidation of the aromatic fraction was much lesser than that observed with the alkane fraction (Figure 4.55).
Figure 4.52: Degradation of octadecane in shake flask conditions by the microbial consortium enriched from the acidic oily sludge contaminated soil at pH3

Figure 4.53: Degradation of fluoranthene in shake flask conditions by the microbial consortium enriched from the acidic oily sludge contaminated soil at pH3

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Figure 4.54 Degradation of the alkane fraction of the acidic oily sludge by the microbial consortium at pH3 in a week under shake flask conditions.

Figure 4.55 Degradation of the aromatic fraction of the oily sludge by the microbial consortium at pH3 under shake flask conditions.
Having tested the hydrocarbon degrading ability of the consortium at pH3, it was purified on agar plates containing a minimal salts medium with acidic oily sludge as the sole carbon source and it yielded 8 isolates. These morphologically distinct looking isolates were also grown on Luria agar (LA) plates in order to study their cultural characteristics. The colonies at neutral pH were opaque, creamy, elevated and convex. Gram staining of these pure cultures showed morphology similar to that of yeasts.

In order to study the antibiotic sensitivity profile of the isolates they were plated onto LA plates containing different concentrations of various antibiotics (Table 4.5) and the growth was checked after 24 and 48 hours.

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<th>Antibiotic</th>
<th>Concentration</th>
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<td>Ampicillin</td>
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<td>100 µg/ml</td>
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<tr>
<td>Nystatin</td>
<td>100 µg/ml</td>
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Table 4.5: Antibiotic profile of the acidic oily degrading strains

All the eight isolates gave the same antibiotic profile. They were all capable of growth on all the antibiotics except Nystatin. It was not possible to differentiate among the isolates on the basis of their antibiotic profile but the fact that all of them were incapable of growth on the antifungal Nystatin, was strongly indicative of the isolates being fungi.

**Estimation of the degradation capability of the microbial isolates and selection of the most efficient acidic oily sludge degrading strain**

The eight isolates obtained were tested for their ability to degrade acidic oily sludge and its alkane and aromatic fractions. The ability of the 8 isolates to degrade the TPH of the acidic oily sludge ranged from 27% (w/v) to 77% (w/v) (Table 4.6). The degradation of the alkane and aromatic fractions of this sludge by the eight isolates is shown in figure 4.56 and 4.57. As evident from this figure, the isolates showed a variable transformation of the alkane fractions with TERI ASN1, TERI ASN4, TERI
ASN6 and TERI ASN8 showing better utilization of this fraction than the remaining strains. The transformation of the aromatic fraction showed that while TERI ASN3 was unable to oxidize the aromatic fraction of the acidic oily sludge; the isolates TERI ASN6 and TERI ASN were able to oxidize the aromatic hydrocarbons of the sludge (Figure 4.57). Based on the TPH degradation ability of the eight isolates, TERI ASN6 was selected for further studies.

<table>
<thead>
<tr>
<th>Isolates</th>
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<tr>
<td>TERI ASN 1</td>
<td>43.37 ± 3.4</td>
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<tr>
<td>TERI ASN 2</td>
<td>40.9 ± 2.8</td>
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<tr>
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<tr>
<td>TERI ASN 8</td>
<td>46.9 ± 5.1</td>
</tr>
</tbody>
</table>

Table 4.6: Degradation of the TPH of acidic oily sludge by the eight isolates under shake flask conditions at 30°C and pH3.

Figure 4.56 Utilization of the alkane fraction of the acidic oily sludge by the isolates obtained from the oily sludge degrading microbial consortium

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Identification and Characterization of the most efficient acidic oily degrading microbial strains

Preliminary results of Gram staining, antibiotic sensitivity testing and other biochemical tests had indicated that the selected microorganisms were yeasts. In order to confirm the identity of the two most efficient acidic oily degrading microbes they were sent to IMTech (Chandigarh, India) for 18s rDNA sequencing. The 18s sequences of these two isolates are now deposited in the NCBI database under the Accession numbers AJ 697749 and AJ 697750. The two strains were found to be of the same Genus and species. They were however, concluded to be novel strains and were therefore named Candida digboiensis with the species name following the place of discovery of the novel yeast species (Prasad et al, 2005).

The ability of the strain Candida digboiensis TERI ASN6 to degrade the various hydrocarbons that would be encountered in the heterogeneous oily sludge was tested by checking the growth of Candida digboiensis TERI ASN6 using different hydrocarbons as the sole carbon source at pH3 and pH7. The growth of C. digboiensis on each of these hydrocarbons, recorded in terms of the absorbance at 600 nm and total cell protein content (Biuret method) indicated that it could grow on all the tested hydrocarbons. While the growth on aromatic hydrocarbons was less, the growth on alkanes was much higher. Of the alkanes tested, the strain C. digboiensis
showed a much higher degradation of heptadecane and octadecane compared to the alkanes from octane to tetradecane and eicosane to triacontane (figure 4.58). This indicates a preference of TERI ASN6 for medium chain alkanes compared to short chain and long chain alkanes. The strain *C. digboiensis* also showed better growth on hydrocarbons at pH7 than at pH3 and was unable to utilize pristane. The growth of TERI ASN6, with alkane and aromatic hydrocarbons showed the same pattern that was observed during degradation studies. While TERI ASN6 was able to degrade the alkane fractions of the acidic oily sludge and model alkanes like octadecane efficiently, it was able to only oxidize fluoranthene and aromatic hydrocarbons. It was unable to degrade aromatic hydrocarbons as the sole carbon source. However, partial aromatic degradation was observed when additional carbon sources were available such as in total petroleum hydrocarbons where the alkane fraction was also present in addition to aromatic hydrocarbons.

![Figure 4.58](image)

**Figure 4.58** Growth of *Candida digboiensis* TERI ASN6 using various hydrocarbons as the sole carbon source in minimal salts medium at pH3 and pH7

TERI University-PhD. Thesis, 2008
Morphological characterization of Candida digboiensis TERI ASN6

During the growth and degradation studies, the strain TERI ASN6 seemed to show different morphologies, hence this trait was studied further. The composition of the medium directly influenced the growth and phenotype of Candida digboiensis TERI ASN6. It showed a yeast cell shaped morphology under some growth conditions and showed hyphal morphology under other growth conditions, indicating that this strain was ‘dimorphic’. The morphology of this strain was compared to that of two standard yeast strains. A comparison of the cell morphology of C. digboiensis TERI ASN6 with standard strains of Saccharomyces cerevisiae (MTCC 171) and Candida albicans (MTCC 227) is depicted in Figure 4.59.

It was observed that C. digboiensis TERI ASN6 exhibited yeast morphology in GYP medium at pH 7 (Figure 4.59e) but, produced pseudo hyphae when grown on the same medium at pH 3 (Figure 4.59f). The standard strains S. cerevisiae (MTCC 171) and C. albicans (MTCC 227) grew in the yeast form in this medium at pH 7 (Figure 4.59a and 4.59i, respectively) and pH 3 (Figure 4.59b and 4.59j, respectively).

When grown on LA pH 3, the cells of C. digboiensis TERI ASN6 showed pseudohyphal morphology (Figure 4.59h) while on LA pH 7, the cells were initially in the yeast form (Figure 4.59g) but after 24 hours of growth the cells switched to the pseudohyphal form. The cells of MTCC 171 maintained the yeast cell morphology on this medium (Figure 4.59c and 4.59d) while MTCC 227 cells were in yeast form at pH3 (Figure 4.59l) but showed the presence of pseudohyphal cells at pH 7(Figure 4.59k). These observations were confirmed by scanning electron microscopy studies.

The yeast cell morphology of TERI ASN6 on GYP pH 7 (Figure 4.60a) and the pseudohyphal morphology on the same medium at pH3 (Figure 4.60b) were confirmed by SEM studies. Similarly, the pseudophyphal morphology of TERI ASN6 on LA pH7 after 24 hours of growth (Figure 4.60c) and on LB pH3 (Figure 4.60a) were also observed with electron microscopy studies.

The pattern of emergence of the hyphae from the growing colonies of C. digboensis TERI ASN6 on LA at pH7 is shown in figure 4.61. The colony morphology of TERI ASN6 on LA where there is considerable growth of the colony before the emergence of the hyphae from the margins, is shown in contrast with the colony on acidic oily sludge.
Figure 4.59 Cellular morphology of Candida digboiensis TERI ASN6 growing on non-hydrocarbon liquid media. The first row depicts cell morphology on Glucose yeast extract medium at pH7; second row depicts morphology on Glucose yeast extract medium at pH3; third row depicts morphology on Luria Bertani medium at pH 7 and fourth row shows cell morphology on Luria Bertani medium at pH 3. The first column shows morphology of S. cerevisiae MTCC 171, the second column depicts the cell morphology of C. digboiensis TERI ASN6 and the third column shows the cell morphology of C. albicans MTCC 227.

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Figure 4.60 Scanning electron micrographs showing the cell morphology of Candida digboiensis TERI ASN6 growing on non hydrocarbon media. (a) yeast morphology on Glucose yeast extract peptone medium at pH7 (b) pseudophal formation in Glucose yeast extract peptone medium at pH3 (c) pseudohyphae formation on Luria Bertani Agar medium at pH 7 (d) pseudohydphae formation in Luria Bertani Agar medium at pH 3
When grown on MSM with hydrocarbons as the carbon source, the cells of *C. digboiensis* TERI ASN6 were in the mycelial form. The cells were not observed in the yeast form in this medium with any hydrocarbon source. While the strain *S. cerevisiae* MTCC 171 could not grow on MSM with hydrocarbons as the sole carbon source; *C. albicans* MTCC 227 showed reduced growth with the cells being in a distorted yeast form. The growth of *C. digboiensis* TERI ASN6 on MSM containing eicosane, fluoranthene or crude oil as the carbon source showed a variation in the morphological pattern. The pattern of mycelial emergence from the edges of the colonies was uniform when grown on crude oil (figure 4.62c) unlike the mycelial pattern of *C. digboiensis* TERI ASN6 on eicosane (Figure 4.62a) and fluoranthene (Figure 4.62b) as carbon sources. The growth of this yeast strain on acidic oily sludge however, showed distorted colony structure (Figure 4.62 d) when inoculated from rich medium.

The standard strain *S. cerevisiae* MTCC 171 did not show any dimorphic behaviour under the tested conditions while *C. albicans* MTCC 227 formed pseudohyphae in LA medium at pH 7 and 37°C temperature. The morphological behaviour of *Candida*
C. *digboiensis* TERI ASN6 remained the same at incubation temperatures of 30°C and 37°C if the same medium was used.

Figure 4.62  Colony morphology of Candida *digboiensis* TERI ASN6 growing on different media. (a) Mycelial growth on Minimal salts medium with eicosane as the sole carbon source (b) Mycelial growth on Minimal salts medium with fluoranthene as the sole carbon source (c) Growth on Minimal salts medium with crude oil as the sole carbon source (d) Distorted colonies of *C. digboiensis* TERI ASN6 on Minimal salts medium with acidic tar sludge as the sole carbon source.

**Degradation of hydrocarbons by the selected yeast strain**

*Candida digboiensis* TERI ASN6

The ability of the strain TERI ASN6 to degrade the various hydrocarbons of the acidic oily sludge was assessed. The even chain alkane eicosane (C₂₀) and the odd chain alkane heneicosane (C₂₁) were selected to represent the alkane fraction of the oily sludge. The intermediates formed during the growth of TERI ASN6 on the polycyclic aromatic hydrocarbons pyrene and phenanthrene were studied to represent the aromatic fraction of the oily sludge and Dibenzothiophene as a representative of the fraction comprising the NSO containing hydrocarbons.
Degradation of eicosane and heneicosane at pH 3 by TERI ASN 6

Candida digboiensis TERI ASN6 was capable of degrading the even chain alkane eicosane when used as the sole carbon source. When 0.1% (w/v) eicosane was taken as the carbon source, there was a 70% degradation from 0.05 g to 0.015g in 9 days (Figure 4.63). The oxidative transformation as revealed by gas chromatography also revealed a similar oxidation, indicating that the entire transformed parent compound eicosane was further degraded. The degradation of the even chain alkane, eicosane, by C. digboiensis revealed a rapid growth of this strain within the first 48 hours of growth with the total cell protein increasing nearly ten fold during this period. A slow degradation of eicosane had also begun within the first 48 hours of growth. However, as the growth reached its maximum at around 96-120 hours of growth, nearly 40% of the eicosane had been degraded. The alkane utilization continued as the cells reached the stationary phase, and utilized the eicosane as the sole carbon source.

Figure 4.63 The degradation of eicosane by C. digboiensis TERI ASN6 at pH3 under shake flask conditions
As revealed by the growth of TERI ASN6 on eicosane as the sole carbon source (Figure 4.63), there was a negligible lag phase with a simultaneous degradation of eicosane visible within 24 hours. *Candida digboiensis* was able to efficiently degrade the odd chain alkane heneicosane as the sole carbon source. The rapid growth in the first few hours after inoculation where no significant degradation of C21 was visible could be the acclimatization phase of the strain with the growth flux coupled to some of the nutrient medium carried over from the inoculum. However, from 48 hours to 120 hours of growth the extent of degradation increased considerably, with the growth peaking during this period. In the next 72 hours, however, while the growth stabilized and the cells entered a stationary phase, degradation was rapid with a sizable population of the yeast cells utilizing the heneicosane as the sole carbon source.

The growth of TERI ASN6 on heneicosane also showed a similar pattern of growth, with a negligible lag phase. However, while the growth phase showed a rapid increased of total cell protein, no degradation of heneicosane was observed for the first 48 hours of growth. After the initial lag in degradation however, a rapid decline of the alkane was noted with a 70% degradation of heneicosane (from 0.05g to 0.015g) in eight days of growth (Figure 4.64).

Figure 4.64 The degradation of heneicosane (C21) as the sole carbon source by *C. digboiensis* TERI ASN6 at pH 3 under shake flask conditions

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The degradation of pyrene revealed that *C. digboiensis* TERI ASN6 was not able to efficiently degrade it. There was an initial flux of growth due to the presence of the inoculum medium and probably initial oxidation but this was not translated into degradation by TERI ASN6. Gas chromatographic analysis also indicated oxidative degradation of the parent compound pyrene but not significant degradation. This behaviour of *C. digboiensis* indicates that while it is capable of efficiently utilizing alkanes as the sole carbon source, it is only able to carry out oxidative transformation of polycyclic aromatic hydrocarbons when supplied as the sole carbon source.
Detection of intermediate metabolites of hydrocarbon degradation

GE EI-MS analysis of the methylated products of eicosane degradation showed a peak at 26 min with a molecular ion at m/z 270. This ion is consistent with the molecular ion of the methyl ester of hexadecanoic acid and shows the same retention time as that of the standard. Typical diagnostic fragments with m/z 74, 87, 143 and 227 were also detected for both the metabolite and the authentic standard. Similarly other peaks were obtained at 30 minutes and 24 minutes with molecular ions at 298 and 242 respectively. These ions are consistent with the molecular ions of octadecanoic acid methyl ester and tetradecanoic acid (Figure 4.65) methyl ester respectively with identical retention times and fragment patterns as their respective controls.

![Mass spectrum of one of the metabolites of eicosane degradation, tetradecanoic acid methyl ester.](image)

The intermediate metabolites formed during the degradation of long chain alkanes, octacosane and triacontane, were also studied. The carboxylic acid methyl esters of docosanoic acid and hexacosanoic acid were detected (Figure 4.66).
Figure 4.66 Comparison of the intermediate metabolites produced during the degradation of octacosane and triacontane by Candida digboiensis TERI ASN6

The metabolites formed during the degradation of odd chain alkanes, tricosane (C\textsubscript{23}) and pentacosane (C\textsubscript{25}), were also determined. During the degradation of these odd chain alkanes, carboxylic acids tricosanoic acid, heptadecanoic acid and pentadecanoic acids were detected (Figure 4.67) based on the comparison of retention time and mass spectra with standard carboxylic acids.

In order to remove the ambiguity caused by the interference of cell wall fatty acid methyl esters, the intermediates formed during the degradation of the odd chain alkane pentacosane was compared with that of the even chain alkane octacosane (Figure 4.68).

Since the study of the intermediates formed during the degradation of alkanes hinted at the possibility of the monoterinal oxidation pathway being adopted by C. digboiensis TERI ASN6; it was grown with octadecanol and octadecanoic acids, which are mono-carboxylation products of alkane degradation (Figure 4.69 a,b).

While no products of the subterminal oxidation pathway were detected during the alkane degradation by C. digboiensis, the growth of this strain on the dicarboxylic acids, dodecanedioic acid and tetracosanedioic acid, did show the presence of dicarboxylic acid methyl ester intermediates (Figure 4.70) on the basis of mass abundance and mass fragmentation pattern. However, these dicarboxylic acid methyl esters could not be identified. Nevertheless, while monoterinal oxidation pathway would be the principal alkane degradation pathway, a minor contribution of the dicarboxylic acid pathway cannot be ruled out.
Figure D4.67: Comparison of the intermediate metabolites produced during the degradation of tricosane and pentacosane by *Candida digboiensis* TERI ASN6

Figure 4.68: Comparison of the intermediate metabolites produced during the degradation of an odd chain alkane (C25) and an even chain alkane (C28) by *C. digboiensis* TERI ASN6
Figure 4.69a Intermediates formed during the growth of Candida digboiensis on octadecane, octadecanol and octadecanoic acid.

Figure 4.69b Magnified view of the shorter chain carboxylic acids produced during the growth of C. digboiensis TERI ASN6 on octadecane (C18), octadecanol (C18-OH) and octadecanoic acid (C18-COOH)

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Figure 4.70 Intermediates formed during the growth of C. digboiensis on dicarboxylic acids (C12 and C24-dioic acids) at pH3
Oxidative transformation of polycyclic aromatic hydrocarbons found in the acidic oily sludge

The growth of *Candida digboiensis* TERI ASN6 in the presence of pyrene yielded the oxygenated intermediates of pyrene. The GC MS analysis of the intermediate products of pyrene oxidation showed pyrenol as the oxidized products. In two separate analyses of pyrene metabolites, 1-pyrenol and 2-pyrenol were detected (Figure 4.71). The detection of both these products could be explained by the fact that 1-pyrenol and 2-pyrenol are non enzymatically convertible. While the oxidation of pyrene by TERI ASN6 could be confirmed by the detection of pyrenol, no further degradation products were detected.

Figure 4.71 Mass spectrum of the oxidative intermediate metabolite, pyrenol, detected during the growth of *C. digboiensis* TERI NSM on pyrene at pH3
Identification of the phenanthrene oxidation products showed a peak at 27 min in GC EIMS with a molecular ion at m/z 194. This ion is consistent with the molecular ion of oxidation products of phenanthrene (Figure 4.72). On the basis of molecular weight, mass abundance and mass spectrum, it could be phenanthrol or phenanthrene oxide. However, because of the absence of an authentic standard the exact identity of the intermediate could not be established. But the detection of this oxidative metabolite confirms that TERI ASN6 predominantly follows the aromatic oxidation pathway known for non lignolytic fungi. However, a trace amount of the phenanthrene conjugate phenanthrene quinone was also detected (Figure 4.73).

Figure 4.72 Mass spectrum of the metabolite of phenanthrene oxidation formed during the growth of C. digboiensis TERI ASN6 on phenanthrene
Figure 4.73 Mass spectrum of the intermediate phenanthrene dione (9,10 phenanthrene quinone) formed during the growth of C. digboiensis TERI ASN6 on phenanthrene as the carbon source

The growth of C. digboiensis TERI ASN6 with the sulfur containing heterocyclic compound dibenzothiophene (DBT) (representative of the NSO fraction) revealed the presence of metabolites formed during the process of DBT desulfurization. The desulfurizing metabolites DBT sulfone and hydroxybiphenyl of the 4S pathway were detected (Figure 4.74 and 4.75). However, no intermediates of DBT degradation were detected during the growth of TERI ASN6 with DBT. This was a surprising finding, considering that oxidation products of pyrene and phenanthrene were detected.
Figure 4.74 The intermediate DBT sulfone detected during the growth of *C. digbioensis* with DBT.

Figure 4.75 Detection of hydroxyl biphenyl during the growth of *C. digbioensis* with DBT.

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Detection of the cytochrome P450 enzyme system in *C. digboiensis*

The degenerate primers Helix 1 and HR2 corresponding to the conserved Helix and heme binding regions were used. After the RT PCR reaction the PCR product of about 450 bp (Figure 4.76) was purified and the sequence submitted to NCBI’s GenBank (Accession no. DQ846834). The deduced amino acid sequence of the PCR product was highly similar to the corresponding region of the CYP52 family of *Yarrowia lipolytica* and *Candida* yeasts (about 40 to 60% identity). The phylogenetic analysis shown in figure 4.77 was conducted using MEGA version 3.1 (Kumar, Tamura and Nei, 2004)

![450 bp](image)

Figure 4.76 Amplification of the partial gene fragment (L) of the Cytochrome P450 enzyme using the degenerate primers based on the Helix and Heme binding regions of P450 in yeasts. The marker lane (M) indicates the 500 bp ladder.
Figure 4.77: Phylogenetic tree based on the partial Cytochrome P450 sequence analysis with the sequences of closely related genes of other species. Bootstrap values are shown at branch points.
Bioremediation of the acidic oily sludge at the dumping site at Digboi refinery by *Candida digboiensis* TERI ASN6

The bioremediation study was conducted on site at Digboi refinery. The indigenous flora had not been adequate to stimulate bioremediation. Both the feasibility and full scale bioremediation study was done within the refinery premises.

**Study of carrier material for storage and transport of *C. digboiensis* TERI ASN6 for field application**

The agro residue, corn cob, was tested for its efficacy to serve as the carrier material for field application. The CFU count of TERI ASN6 in corn cob was $6.8 \times 10^8$ CFU/g substrate, at the start of experiment. After incubation at 30°C, the count was $7.1 \times 10^8$ after seven days, $6.5 \times 10^8$ after two weeks, $5.1 \times 10^8$ after three weeks and $4.7 \times 10^8$ CFU/g carrier at the end of the month. The survival of TERI ASN6 at 4°C was checked monthly by monitoring the CFU counts. The count at the beginning of the experiment was $6.7 \times 10^8$ CFU/g of formulation and for three months the count was $5.2 \times 10^8$, $3.8 \times 10^7$ and $1.6 \times 10^7$ respectively. The carrier was found to have a water holding capacity of nearly 660% (Mishra, 2002, thesis) which would enable the retention of moisture to support the yeast culture during transport to the bioremediation site.

**Feasibility study for the bioremediation of the acidic oily sludge contaminated soil**

Of the four treatments employed for the feasibility study, bioaugmentation of the acidic oily sludge contaminated soil with *Candida digboiensis* TERI ASN6 and nutrients resulted in the maximum degradation of oily sludge. The concentration of TPH of the contaminated soil using this treatment reduced from 160g TPH/kg soil to 28.81 g/kg soil in 135 days indicating 82% degradation as compared to a 56.67% degradation of TPH in the plot treated with *C. digboiensis* TERI ASN6 after neutralization of the acidic oily sludge with lime (Fig. 4.78). The plot treated with nutrients alone resulted reduction of TPH from 190.25 gTPH/kg soil to 144.02 g TPH/kg soil revealed 24.30 % degradation. In control plot only 17.80 % reduction of TPH was recorded in 135 days (Figure 4.78). The pH of the soil at treatment plots was monitored during the experiment but no significant change was observed (pH ranged from 2.5 to 4). Based on the results of the feasibility study, a full scale study of bioremediation was conducted with the treatment consisting of *C. digboiensis* TERI ASN6 and nutrients.
Bioremediation of acidic oily sludge contaminated soil

The full-scale bioremediation study showed that TPH in soil of Plot A (Figure 4.79) at the beginning of the study was 163.15 g TPH/kg of soil. The application of *C. digboiensis* TERI ASN 6 and nutrients revealed that the TPH had reduced to 52.24 g TPH/kg soil in 71 days at a rate of 1.6 g TPH/kg soil per day and further reduced to 11.08 g TPH/kg of soil in 175 days at a rate of 0.49 g TPH/kg soil per day. In Plot B the TPH in the acidic oily sludge at the beginning of the study was 221.57 g TPH/kg of soil. Bioaugmentation with *C. digboiensis* TERI ASN 6 resulted in the reduction of TPH to 37.63 g TPH/kg of soil in 71 days, indicating a degradation of 2.59 g TPH/kg soil per day. The TPH in soil was then further reduced to 10.03 g TPH/kg of soil in 175 days exhibiting a degradation rate of 0.27 g TPH/kg soil per day (Figure 4.79). In Plot C, at zero day, the TPH was 184.06g TPH/kg of soil which reduced to 40.53 g TPH/kg of soil in 71 days and 7.96 g TPH/kg of soil in 175 days, revealed a degradation rate of 2.02 g TPH/kg soil per day for the first 71 days and a degradation of 0.31g TPH/kg soil per day from the 72nd day to 175 days by *C. digboiensis* TERI ASN6. However, in the control plot D, the TPH concentration in soil reduced from 205 to 165.50 g TPH/kg soil in 175 days.

![Figure 4.78 TPH contamination in soil during different stages of bioremediation. Soil samples were collected from different treatment plots. TPH from soil samples was extracted by using solvents and concentration of TPH in soil was quantified.](image-url)
Survival of the introduced Candida digboiensis TERI ASN6 at the bioremediation site

The population of TERI ASN6 was monitored on the antibiotic supplemented LA plates (pH3) and mycelia formation by colonies at low pH was observed. The randomly picked colonies of TERI ASN6 from these plates were studied and the pseudohyphal cells typical of TERI ASN6 were observed. These randomly picked colonies were also positive for the assimilation of melibiose. The population of TERI ASN6 at the beginning of the bioremediation experiment was $2.3 \times 10^8$ CFU/g of soil in plot A, $2.5 \times 10^8$ CFU/g soil in plot B and $1.9 \times 10^8$ CFU/g of soil in plot C. However, at the end of the experiment the cell count had decreased to $3.1 \times 10^6$ in plot A, $4.6 \times 10^6$ CFU/g soil in plot B and $8.9 \times 10^6$ CFU/g in plot C while in the untreated plot, the strain *C. digboiensis* TERI ASN6 was not detected.