CHAPTER-3

MATERIALS AND METHODS
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Materials and Methods

3.1. Description of the sites and climatic condition

Five crude oil contaminated sites namely Amguri, Borhulla, Gelaky, Lakowa, Rudrasagar of upper Assam were selected for the research work. Visual characteristics of the sites are specified in figure 5. Amguri, Gelaky, Lakowa and Rudrasagar come under Sivsagar district of upper Assam, situated between 94.25° and 95.25° longitude east and 25.45° and 27.15° Latitude north with an elevation of 86.6 metres above the sea level. Amguri is located at 26.58°N 93.07°E. It has an average elevation of 74 metres (242 feet). Total Area of contaminated sites was nearly 14,500 Sq. Gelaky drill site had a total area of nearly 14,500 Sq M. Lakowa drill site is situated between Latitude 27.0166° and longitude is 94.8333°. Lakowa has a number of oil wells and crude oil affected areas including certain tea gardens. Rudrasagar had already drilled out 182 wells of which 61 wells are flowing oil and gas wells and 10 are operating Effluent Injection wells. Present production of oil is about 580 M³/day. There are more than 20 wells in the Borhulla oilfield, apart from several abandoned wells. Borhulla drill site is in Jorhat district. Jorhat is situated between 26.75° North and 94.22° East with an elevation of 116 metres above sea level. Annual temperature ranges from 8°C in winter to maximum 35°C during summer in both the district of Assam. Both Sivsagar and Jorhat are highly humid with average annual rainfall of 94″.
Figure 5: Map and nature of crude oil contaminated sites of upper Assam.

Amguri, Gelaky, Lakowa, Rudrasagar of Sivsagar district and Borhulla of Jorhat district.
Soil sampling

Crude oil contaminated soil samples were collected randomly from twenty five different spots in each site. Top soil, sub soil and soil samples to a depth of 30 cm from the surface of the contaminated areas were collected by inserting a borer. Soil samples from each site were thoroughly mixed together to represent a single sample and were kept in polythene zipper bags and stored at 4º C in laboratory. Biological characterization of each sample was carried out within 7 days of sampling.

Chemicals

Chemicals, bacteriological media, staining reagent and solvents were purchased from Himedia Laboratory Pvt. Ltd. India. All the chemicals and solvents were of analytical grade.

3.2. Physical and chemical characteristics of crude oil contaminated soil

Chemical analysis of crude oil contaminated soil includes determination of soil pH, moisture, conductivity, estimation of essential elements, heavy metals, oil content, different fractions of crude oil like wax, resin, asphalt and separation of aliphatic and aromatic hydrocarbon and further Gas chromatographic analysis of aliphatic and aromatic hydrocarbon.

Determination of soil pH

pH of contaminated soil samples was determined in 1:2.5 soil-water suspension was done using automatic glass electrode pH meter, Eutech Model pH510.

Moisture

Moisture content was analyzed by gravimetric methods. For this 5 g of contaminated soil samples were dried in a moisture free oven at 60º C till a constant weight was obtained and the difference of weight was the moisture content present in the soil sample.

Conductivity
Conductivity of contaminated soil samples was determined in 1:5 soil-water suspension using Digital conductivity meter (M/S Icon, India).

**TPH (Total petroleum hydrocarbon)**

Total petroleum hydrocarbon content in the contaminated soil was estimated by a standard method adapted in NEIST-Jorhat (Saikia et al., 2008).

**Method of extraction**

- Contaminated soil sample (5g)
- Taken in a cellulose extraction thimble
- Soxhlet extraction with chloroform (8 hours at 60-70ºC)
- Concentrated the extracted oil

Then the extracted oil was expressed as percentage

**Resin and wax determination**

- Crude oil (5g)
- 150ml n-heptane conical flask
- Heated under refluxed condition (100ºC for 1h)
- Stoppered the flask with a ground glass stopper
  - (without treatment)
- Filtered the content through whatman42 filter paper
- The filtrate was used for estimation of resin and wax.

**Resin**

Resin was estimated according to Hubbered and Stanfield (1948). The filtrate of the crude oil after separation as mentioned above was absorbed with 200g of silica gel and heated the content in water bath for 1 h with continuous stirring and allowed to absorb
all the resin in to the silica gel. Then the content was cooled and kept for overnight. The residue after filtration was washed with toluene: methanol (90:10) mixture until the silica gel becomes free from resin and the solvent fraction was evaporated in boiling water bath. The percentage of resin content in the crude oil was calculated.

**Wax**

The wax content in crude oil was determined by adopting the procedure UOP 46/85. After separation of resin the filtrate was heated in an oven and treated with 5ml of H₂SO₄. Then the content was cooled down to the room temperature. The residual asphalt gets separated from the liquid. The wax solution was decanted and washed with warm water and ammonium hydroxide solution for several times to remove the acid. The n-heptane was evaporated to get the crude wax. Then the crude wax was dissolved in ethylene chloride (CHCl₃) and cooled down to-32°C. Filtered through cold filter funnel and wax was collected. Washed the filter funnel with hot n-heptanes and collected in a weighed flask. Evaporated the n-heptanes and dried. The percentage of wax content in the crude oil was determined from the weight after drying.

**Asphalt**

The oil extracted from soil is mixed with petroleum ether (40-60º C) then filter with Whatman 42 filter paper. The residue is the asphalt.

**Separation of aliphatic hydrocarbon**

It is done by using column chromatography technique where solvent used is petroleum ether (40-60ºC). Column is prepared with activated alumina. Crude oil is mixed with petroleum ether, filtered and the filtrate is used to elute the column wait up to fully absorption of the filtrate by activated alumina. Then the column elutes with petroleum ether and collect 200ml aliphatic hydrocarbon.
Separation of aromatic hydrocarbon

After collection of aliphatic hydrocarbon fraction, the column was eluted with benzene, methanol mixture (3:1) followed by collection of 50-100 ml aromatic hydrocarbon.

Estimation of essential elements and heavy metals in soil

Digestion of soil sample was done by adding 5 ml of nitric acid, 3 ml of perchloric acid and 3 ml of hydrofluoric acid to 0.5 g of soil and heated in platinum crucibles for 4 hours at 95-100º C inside a fume hood. Residues obtained from soil sample after digestion was dissolved in 25 ml de ionized water and filtered in to 25ml volumetric flask The filtered sample was analyzed for nitrogen(N), Potassium (K), magnesium (Mg), calcium (Ca), iron (Fe),Cadmium (Cd), Chromium (Cr), Copper(Cu), Lead (Pb)and Manganese (Mn) by Atomic absorption spectrometer Model: Analyst -100 (PerkinElmer) using the technique Flame (Air- Acetylene) in Analytical chemical division, NEIST-Jorhat.

Determination of available organic carbon(C) and phosphorus (P) was performed as follows.

Organic carbon

Organic carbon was estimated by Jackson`s potassium dichromate oxidation (1972). In short to 0.5 g soil sample 10 ml of K2Cr2O7. After mixing 20 ml of concentrated H2SO4 was added, allowed to stand for 20 min. 200 ml of water and 10 ml of 85% phosphoric acid , 0.2 g of NaF and 0.1 ml diphenylamine were then added to the incubating mixture in that order. The color developed by the diphenylamine was titrated with ferrous ammonium sulphate. The end point was based on the color which changed from a dull green to a dark blue and finally to a brilliant green.
Phosphorus

Available phosphorus was determined by using Bray and Kurtz (1945) method as follows.

**Bray’s solution:** 0.03 N NH₄F was prepared by dissolving 2.77g of NH₄F in 2.5 liter of 0.025 N HCl.

**Ammonium Molybdate reagent:** A) 40.5 ml H₂SO₄ was gently added to 109 ml of distilled water by continuous stirring. 12.5 g of ammonium molybdate was added to 100 ml hot water. Then 100 ml of ‘B’ and 25 ml of ‘A’ was mixed together and volume was made up to 250 ml with distilled water.

**Metol reagent:** A) 23 g of potassium metabisulphate or potassium disulphate or potassium pyrosulphate and 1 g of Na-sulphate was dissolved in 60 ml water.

B) 0.2 g of metol dissolved in 10 ml water. Solution ‘A’ and ‘B’ was mixed and the volume was made up to 100 ml with distilled water. The solution was filtered and the first 20 ml of solution was discarded. The remaining filtrate was kept in a brown bottle.

**Sodium acetate solution:** 0.85 g sodium acetate was dissolved in 250 ml of distilled water.

**Procedure**

In 10 g soil, 25 ml of Bray’s solution was added. After shaking well it was filtered. Then 1 ml or 2ml of the filtrate was added to 2ml of ammonium molybdate reagent and 1ml of metol reagent and kept exactly for 10 minutes. Then 4ml of sodium acetate was added and make up to 20ml with distilled water. Colorimetric reading was taken after 5 minutes at 578nm.

3.3. Biological characteristics of crude oil contaminated soil

3.3.1. Estimation of Most Probable Number (MPN) of microorganisms

Most probable number (MPN) of microorganisms was estimated by the standard method of serial dilution agar plate technique (Alexander, 1965). Determination of nitrogen fixing bacteria was done by using the medium developed by Bezbaruah *et al.*, (1994).
Chemolithotrophic sulphur oxidisers were determined using thiosulphate agar medium. Medium developed by Skinner (1971) was used to enumerate the cellulose degraders and general type bacteria were determined by using soil extract medium. For estimation of Phosphatase, Dehydrogenase and Urease enzyme activities standard method of Bremner and Tabatabai (1969), Casida (1977) and Smith and Chalk (1980) were used.

**Preparation of media for plate count**

**Soil extract medium**

Soil extract medium is prepared by dissolving 1 kg of normal soil in 2.5 liter distilled water and then filtered. To 1000 ml of soil extract 2 g glucose, 0.5 g K₂HPO₄ and 20 grams of agar are added.

**Skinner’s medium**

To 1000 ml of distilled water 1 g (NH₄)SO₄, 0.1 g MgSO₄, 2 g NaCl, 0.1 gCaCl₂, 13 g K₂HPO₄, 7 g KH₂PO₄, Cellulose suspensin(2%/w/v), 1 g Yeast extract, 0.5 g Cysteine hydrochloride are added.

**Thiosulphate medium**

To 1000 ml of distilled water 6 g Sodium thiosulphate, 0.1 g K₂HPO₄, 0.1gmNH₄Cl, 0.2 g Sodium bicarbonate, 20 g Agar are added.

**N₂ free medium**

**Under A:** To 1000 ml distilled water 5g K₂HPO₄, 2 g MgSO₄, 1 g CaSO₄, 0.2 gFeSO₄, 0.2 g MnSO₄ and 0.1 Potassium molybdate are added.

**Under B:** To 900 ml distilled water 10 g Sucrose, 3 g Calcium carbonate and 100 ml media from (A) are added.

All these four different types of media were autoclaved at 121°C for 20 minutes. For isolation of sulphur oxidizes petriplates were autoclaved. Each soil sample was diluted from
10<sup>-1</sup> to 10<sup>-8</sup>. One ml of each dilution (10<sup>-3</sup>-10<sup>-8</sup>) were plated in triplicate and to each plate 15 ml of thiosulphate medium was poured. For counts of general type bacteria sterile soil extract medium was poured in petriplates containing one ml of each dilution. Since Skinner’s medium and Nitrogen free medium do not contain agar, hence for isolation of cellulose degraders and nitrogen fixers both the media were sterilized in test tubes. Soil dilution used was same as prepared for isolation of sulphur oxidizers and general type bacteria. 1 ml of each dilution (10<sup>-3</sup>-10<sup>-8</sup>) was added to sterilized test tubes containing 5 ml skinner’s medium and another test tube containing 5 ml nitrogen free medium in triplicate.

3.3.2. Estimation of soil enzymatic activity

Phosphatase, Dehydrogenase, cellulase, Qualitative and quantitative urease tests were performed as follows:

**Phosphatase enzyme activity**

To 1 g soil, 4 ml of acid buffer, neutral buffer and alkaline buffer was added separately. After shaking well, 1 ml of PNP (p-nitropheny phosphate) solution was added in to each flask containing soil samples and the flasks were shaken for a few seconds to mix the content and incubate at 37°C for 1 hour. After 1 hour incubation, 1 ml of CaCl<sub>2</sub> solution and 0.5M NaOH solution was introduced. The extract was filtered over whatman no-40 filter paper and the color developed was read calorimetrically at 410 nm.

**Dehydrogenase activity**

Dehydrogenase activity estimated according to Casida (1977). To 1 gram of soil 0.1 g CaCO<sub>3</sub> and 1 ml of 1% 2, 3, 5-TTC (Triphenyl tetrazolium chloride) solution are added and incubated at 30°C for 24 hours. After 24 hours incubation 5 ml methanol is transferred to the slurry and centrifuged at 10,000 rpm and optical density is read at 485 nm.
Urease activity

5 g of soil samples were taken in flasks of equal dimension and shape. The soil samples were moistened with 2 ml distilled water and 2 ml of urea (40L⁻¹) solution and incubated at 30 °C for 24 h in air tight condition. Volatilizing nitrogen in the flasks was trapped in a vial containing 2 ml of acid KMNO₄ (0.5 M H₂SO₄ + 0.2 M KMNO₄) according to Smith and Chalk (1980) and analysed through Kjeldahl distillation according to Bremner (1965).

3.4. Isolation, characterization, screening and identification of isolates

Serial dilution pour plate method was used for isolation of bacterial isolates. Total 50 bacterial strains were isolated from crude oil contaminated soil of Amguri, Borhulla, Gelaky, Lakowa and Rudrasagar drill site using 2 free medium, Soil extract medium and Thiosulphate agar medium. By following standard methods adopted at NEIST, Jorhat, the bacterial strains were classified from the pure culture. Morphological and biochemical characterization of the isolates were performed with three replicates. (Cappuccino and Sherman, 1983). Colony morphology of the bacterial isolates, their pigmentation, staining reaction was performed. For biochemical characterization different biochemical tests such as Starch hydrolysis, Nitrate reduction, Gelatin liquefaction, Citrate utilization, Triple sugar iron phosphate, MR-VP tests were performed.

3.4.1 Morphological characterization of isolates

Colony appearance

Size, shape, color, texture, elevation of the colony, type of margin, consistency and translucent or opaqueness were determined for each isolated strains.

Gram reaction

It is done to differentiate bacteria in to two principal group: gram-positive and gram-negative. For gram staining 24 h nutrient agar slant cultures of the isolated strains are taken. Using sterile
technique, a thin smear of bacterial culture is prepared. The reagents used are Crystal violet, Gram’s iodine, 95% ethyl alcohol and safranin.

### 3.4.2. Biochemical characterization

#### Nitrate reduction test

Bacteria were inoculated in Trypticase nitrate broth. Then each isolate was gently inoculated in the medium to distribute it throughout the tube. Then tubes were incubated for 24 to 48 h at 37ºC. Following incubation of the organisms, an organism’s ability to reduce nitrates to nitrites is determined by the addition of two reagents: Solution A, which is sulfanilic acid, followed by solution B, which is di-methyl-alpha-naphthylamine. Following reduction, the addition of solutions A and B will produce an immediate cherry red color.

#### Indole production test

Pure bacterial culture must be grown in sterile tryptophan or peptone broth for 24-48 h before performing the test. Following incubation, add 5 drops of Kovac’s (isoamyl alcohol, para-Dimethylaminobenzaldehyde, concentrated hydrochloric acid) reagent to the culture broth. A positive result is shown by the presence of a red or red-violet color in the surface alcohol layer of the broth. A negative result appears yellow.

#### MR-VP (Methyl red-Voges- Proskauer test)

4 ml of MR-VP medium was prepared by dissolving 0.1 g of methyl red in 300 ml of 95% ethyl alcohol which was then diluted to a total volume of 500 ml with distilled water. The agar tubes were inoculated with the test bacterial isolate and incubated at 37ºC for 48 h. If the reagent turns red, which is due to accumulation of acidic product of fermentation of glucose, it indicates a positive test. For VP test, 0.5ml of 5% alpha napthyl certain was added to another half of the 2 ml culture.
The tube was shaken thoroughly and allowed to stand for 5-10 minutes. Positive test was indicated by the appearance of a pink color which is due to formation of acetone.

**Citrate utilization test**

The Simmons agar media comprised of Ammonium dihydrogen phosphate 1.0 g, Dipotassium phosphate 1.0 g, Sodium chloride 5.0g, Sodium citrate 2g, Magnesium sulphate 0.2 g, Agar 15 g, Brom thymol blue 0.08 g. Simmons agar slants were streaked inoculated with the bacterial isolates and incubated at 37º C. After 7 days of incubation blue color appearance in the tube indicated the utilization of citrate as the sole carbon source by the test bacteria.

**Gelatin liquefaction**

Nutrient broth containing 12% gelatin was inoculated with the test bacteria in the tube and incubate at 20 to 22º C for up to 48-72 h. After incubation the tubes were chilled in a refrigerator along with un-inoculated control. Failure of the test culture to solidify due to hydrolysis of gelatin is indicative of the positive result.

**Starch hydrolysis**

Nutrient agar plate supplemented with 0.2% soluble starch was spot inoculated in the culture plate with the test bacteria. After incubation for 2 days at 37º C the plate was flooded with iodine solution. A positive test was indicated with clear haloes around the colony.

**Triple sugar iron phosphate**

The culture media comprised of Beef extract 3.0 g, Yeast extract 3.0g, Peptone 15g, Proteose-peptone 5.0g, Lactose 10 g, Saccharose 10g, Dextrose 1 g, Ferrous sulphate 0.2 g, Sodium chloride 5.0 g, Sodium chloride 5.0 g, Sodium thiosulphate 0.3 g, Phenol red 0.024 g, Agar 12 g and distilled water 1 liter. The slants were inoculated using straight needle. The needle with inoculums was inserted vertically down to the bottom of the tube, then lifted and streaking was done on the surface of the slant.
The tubes were incubated at 37ºC for 24 h. The positive result was indicated by the appearance of red violet color in the tube.

### 3.4.3. Growth of the isolates at different physiological conditions

(a) **pH**

Sterile nutrient broth of different pH was prepared using NaOH and HCl for alkaline (pH 9) and acidic (pH 3) range respectively. 1% of inoculums of each isolate was provided to the medium of each pH range and incubated in shaker at 120 rpm, at 28ºC for 12h. The optical density measured in UV Spectrophotometer (Rayleigh UV-1601) gave the growth efficiency.

(b) **Temperature**

Each isolate was grown in sterile nutrient broth (pH 7) in test tube and incubated at 4ºC, 25ºC, 40ºC in a shaker at 120 rpm. The presence or absence of growth was defined in terms of optical density at 660nm.

(c) **Salt**

Sterile nutrient agar with salt concentration of 0.5M, 1.0M, 1.5 and 2.5 were prepared using NaCl and each isolate was inoculated using a loop, then incubated at 28ºC and growth of each organism was observed after 24h.

### 3.4.4 Screening of hydrocarbon degrading bacteria

The ability of the isolates to grow in liquid mineral media (LMM) (Ramsay *et al*., 1991) containing crude oil was tested to identify potential strains for hydrocarbon degradation. The 50 strains preserved in nutrient agar slants were inoculated in conical flasks containing LMM supplemented with 5% (v/v) crude oil as sole source of carbon. For this, a random pool of each culture was transferred to three flasks each containing 50 ml of LMM amended with 5% crude oil and incubated in shaker at 150 rpm at 28ºC. Experiment was conducted in triplicates.
LMM was prepared in conical flasks by dissolving NaNO\textsubscript{3}-4g/L, Na\textsubscript{2}HPO\textsubscript{4}-3.67g/L, KH\textsubscript{2}PO\textsubscript{4}-1.75g/L, MgSO\textsubscript{4}.7H\textsubscript{2}O-0.2g/L, CaCl\textsubscript{2}.2H\textsubscript{2}O-0.05g/L, FeSO\textsubscript{4} 0.001g/L, Trace element-1% in distilled water and the final pH of the medium was adjusted to pH 7 and autoclaved at 121° C for 20 minutes (Ramsay \textit{et al.}, 1991). Change of the culture media into muddy color and oil film disruption within the incubation period indicated that out of 50 nos. of isolates, 5 nos. of bacterial strains 3SG, 4SG, 18ML, 22ML and 26ML were able to grow in LMM supplemented with 5% crude oil (v/v) while the other 45 strains could not grow in LMM containing crude oil.

**Growth conditions of the strains**

3SG, 4SG, 18ML, 22ML and 26ML were maintained in mineral slants supplemented with 0.5% n-hexadecane. A random pool of each culture was transferred to 50 ml of LMM with 1% n-hexadecane as the sole carbon source in a conical flask and incubated in shaker at 150 rpm for 24 h at 28° C and each culture broth was used in remaining experiments. Two hydrocarbon degrading bacterial strains \textit{Pseudomonas aeruginosa} N3 and N4 (Saikia \textit{et al.}, 2007) were also maintained in n-hexadecane slants and used to reclaim the respective drill sites.

Then concentration of crude oil was increased up to 10-50% in LMM and growth of the tested organisms was observed up to period of 360h. Bacterial growth at the expense of hydrocarbon was established by recover of the remaining concentration of crude oil present in culture medium and determining cell number in culture media at various time intervals (0,72h, 120h, 240h and 360h). CFU/ml of each strain was checked by removing 100µl culture from all the experimental and control flask.
3.4.4.1. Estimation of quantity of crude oil recovered from media

Crude oil was recovered from the whole volume of each flask by shaking three times with 10 ml toluene. The mixture was centrifuged at 4000 rpm for 5 minutes. The organic phase was transferred to a fresh tube and the remaining amount of residual crude oil was transferred to a pre weighed beaker and the volume of extracted oil was deducted from the previously weighed beaker.

The percentage (%) of degradation was calculated as follows:

Weight of residual crude oil = Weight of beaker containing extracted crude oil – Weight of empty beaker.

Amount of crude oil degraded = Weight of crude oil added in the media – Weight of residual crude oil

Percentage (%) of degradation = Amount of crude oil degraded / Amount of crude oil added in the media x 100

CFU method

Increase in bacterial cell number at different hours (0, 72, 120, 240 and 360h) was assessed using CFU method. For this 100 µl of each culture grown in LMM containing different crude oil concentrations (w/v) were taken out and diluted serially up to $10^{-1}$ to $10^{-9}$. After that 100 µl of each serially diluted culture was spread over MMA (Mineral media containing agar) and incubated for 24h at 28°C. $\log_{10}$ CFU/ml was estimated for each culture after 24 h of incubation.

3.4.5 Identification of screened bacterial strain

Genotypic analysis

Molecular identification of the screened bacterial strains was done by sequence analysis of their 16s rDNA genes. Genomic DNAs of the 5 bacteria were extracted by using Gene Elute TM Bacterial genomic DNA extraction kit (Bangalore Genei, India). The genotypic analyses
of the 5 bacterial strains were carried out by using BOX-AIR1 primer (5'-CTACGGCAAGGCGACGCTGACG-3'). A 10-µL PCR product together with 500 (Bangalore Genei, India) bp marker was separated using 1.5% agarose gel stained with ethidium bromide in 1× TAE. A photograph of the gel was taken by gel documentation system (Syngene, Cambridge, UK).

3.5. Study on bacterial degradation of known hydrocarbons

3.5.1. Media and supply of model hydrocarbons

The n-heptane, n-dodecane, n-hexadecane, naphthalene, anthracene and fluoranthene (purity 99%) used in this study are purchased from Sigma-Aldrich. LMM was supplemented with individual hydrocarbons to achieve final concentrations of 200ppm (v/v) aliphatic hydrocarbon and 200ppm aromatic hydrocarbon. For this, LMM saturated with, n-heptane, n-hexadecane and n-dodecane individually were prepared by adding 1ml of each hydrocarbon in conical flask containing 50ml LMM followed by filtration and autoclaving. The stock solution (200ppm) of naphthalene, anthracene and fluoranthene were prepared in (Dimethyl sulfoxide (DMSO) and was sterilized by Millipore micro syringe filter assembly (0.45 µm pore size).

Enrichment culture

Previously prepared bacterial inocula (5ml) of 26ML and 3SG were used to inoculate 45ml of LMM containing individual aliphatic and aromatic hydrocarbon. After growth was visualized, 5 ml of enrichment culture were transferred to flasks containing fresh LMM with individual hydrocarbons and incubated in shaker at 150 rpm for 24 hours at 28ºC. Subsequent identical transfers were performed for three successive transfers in the respective aliphatic and aromatic hydrocarbon containing media. A series of conical flasks containing LMM amended with n-heptane, n-hexadecane, n-dodecane, naphthalene, anthracene and fluoranthene without
inoculum was considered as control. The cultures were incubated for 240h at 28°C with constant shaking at 150 rpm.

3.5.2. Utilization/ bacterial degradation of aliphatic and aromatic hydrocarbon

Bacterial growth associated with aliphatic and aromatic hydrocarbon was verified by demonstrating cfu method. Degradation of hydrocarbon in the culture broth was determined using gas chromatographic analysis. Cultures were analyzed for the increase in bacterial cell number at various hours (0, 24, 48, 72, 96, 120, 144, 168, 192, 216 and 240 h). For this 100 µl of each culture were taken out and diluted serially up to $10^{-1}$ to $10^{-10}$. After that 100 µl of each serially diluted culture were spread over MMA (Mineral media agar) and incubated for 24h at 28°C. Log$_{10}$ CFU/ml was estimated for each culture after 24 h of incubation.

3.5.3. Assessment of hydrocarbon degradation by GC analysis

After 240h of cultivation, each hydrocarbon remained in media was extracted from the total volume of each flask by shaking three times with 10 ml toluene. The mixture was centrifuged at 4000 rpm for 5 min. The organic phase was transferred to a fresh tube. The extent of different hydrocarbon biodegradation was checked by quantifying left over hydrocarbons in the biodegraded extracts by gas chromatographic analysis.
### Table 2: Physical and chemical properties of the aliphatic and Polycyclic aromatic hydrocarbons

<table>
<thead>
<tr>
<th>Properties</th>
<th>n-Heptane</th>
<th>n-Dodecane</th>
<th>n-Hexadecane</th>
<th>Naphthalene</th>
<th>Anthracene</th>
<th>Fluoranthene</th>
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<tbody>
<tr>
<td>M.F</td>
<td>C\textsubscript{7}H\textsubscript{16}</td>
<td>C\textsubscript{12}H\textsubscript{26}</td>
<td>C\textsubscript{16}H\textsubscript{34}</td>
<td>C\textsubscript{10}H\textsubscript{8}</td>
<td>C\textsubscript{14}H\textsubscript{10}</td>
<td>C\textsubscript{16}H\textsubscript{10}</td>
</tr>
<tr>
<td>M.M (g/mol(^{-1}))</td>
<td>100.20</td>
<td>170.33</td>
<td>226.44</td>
<td>128.17</td>
<td>178.23</td>
<td>202.26</td>
</tr>
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<td>Appearance</td>
<td>Colourless liquid</td>
<td>Colorless liquid</td>
<td>Colourless liquid</td>
<td>White solid crystals</td>
<td>Colorless</td>
<td>Yellow to green needles</td>
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<tr>
<td>Density (mg/mL(^{-1}))</td>
<td>679.5</td>
<td>780.8</td>
<td>773</td>
<td>1.14 g/cm(^3)</td>
<td>1.25 g/cm(^3) at 19.85 °C, solid; 0.969 g/cm(^3) at 220 °C, liquid</td>
<td></td>
</tr>
<tr>
<td>M.P</td>
<td>-91 to 90 °C</td>
<td>-10 to 9 °C</td>
<td>17 to 19 °C,</td>
<td>80.26 °C</td>
<td>218 °C</td>
<td>110.8 °C</td>
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<td>B.P</td>
<td>98-99 °C</td>
<td>214-218 °C</td>
<td>271-291 °C</td>
<td>218 °C</td>
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<tr>
<td>Solubility in water/Solvent</td>
<td>Approximately 30 mg/L</td>
<td>Water: none</td>
<td>Methanol: 0.908g/liter</td>
<td>Hexane: 1.64/L</td>
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<td></td>
</tr>
</tbody>
</table>
3.6. Study on crude oil degradation potential of the isolates under soil condition

Preparation and application of bacterial inocula

Strain 26ML and 3SG preserved in n-hexadecane slants are inoculated in two separate 250 ml conical flasks containing sterilized succinic acid medium and incubated in shaker at 150 rpm for 24 hours at 28º C. The medium was prepared by adding 4g succinic acid; 6.0g K$_2$HPO$_4$, 3.0g KH$_2$PO$_4$, 0.2g MgSO$_4$7H$_2$O, 1.0g NH$_4$SO$_4$ and Agar in 1000 ml distilled water (pH7). For degradation study, presterilized petriplates were filled with 40g of crude oil contaminated soil collected from Amguri, Borhulla, Gelaky, Lakowa and Rudrasagar drill sites and applied bacterial inocula according to Saikia., et al., (2009). For this, 48h old (log phase) culture of each bacterium was diluted with distilled water (1:10) and 20ml from each culture was applied on each contaminated soil. This incubation period produced 6×10$^{-7}$ cfu ml$^{-1}$ and hydrocarbon degradation potential was evaluated at 1 month interval for a period of 1 year. Experiment was done in triplicates. Petriplates filled with contaminated soil without treatment is considered as control. Degradation of crude oil of each treated and untreated soil was determined in three replicates.

3.7. Study on effect of bacterial treatment on seed germination and growth of plants grown in crude oil contaminated soil.

Seed germination study

Effect of crude oil as well as bacterial treatment on germination of seeds of four different types of plant species viz., *Vigna unguiculata* (Long Bean), *Zea mays* (Maize), *Vigna catjung* (French bean) and *Cicer arietinum* (Gram) was studied. The seeds were introduced in contaminated soil after 3 months of treatment with 26ML and 3SG individually when the concentration of crude oil in soil was below 14%. The entire experiment involves two controls. One is
introduction of seeds to contaminated soil without treatment and other one is introduction of seeds to uncontaminated soil. Before introduction of seeds to the contaminated soil surface sterilization was done for *V. unguiculata*, *Z. mays*, *V. catjung* and *C. arietinum*.

**Surface sterilization**

**Method**

1. Seeds are rinsed in 95% alcohol for 10 seconds to remove waxy material and trapped air. Drain off the alcohol.

2. Mercuric chloride, sodium hypochlorite or hydrogen peroxide solutions were added in sufficient volumes to immerse the seeds completely. Swirl the contents gently to bring the seeds and sterilant into contact. After 3-5 min, drain off the sterilant.

3. Seeds were rinsed with at least six changes of sterile water. After the sixth rinse, sufficient water was poured in to submerge the seeds and was left in refrigerator to imbibe.

After surface sterilization 8 seeds of each plant species were introduced in contaminated soil samples treated individually with 26ML and 3SG. Rate of seed germinated in treated and untreated soil was recorded after 15 days of seed introduction. The percentage germination in each treatment was calculated using the formula

\[
\text{Percentage germination} = \frac{\text{Numbers of seed germinated} \times 100}{\text{Numbers of seed sown}}
\]

**Plant growth study**

After 15 days of germination seedlings were uprooted and growth study was done by estimating plant dry biomass and development of root length and shoots height. Root length (cm) and shoot height (cm) were measured using a meter rule. Biomass of plants was determined by measuring the dry matter content of the plant after oven drying the plant in an oven at 60º C to a constant weight using a weighing balance.

Screening of plant species

Three sets of pots filled with crude oil contaminated soil are taken. Two sets of pots filled with crude oil contaminated soil are treated individually with 26ML and 3SG and allowed to acclimatize for 3 months. After that, 10 numbers of three months old tree species, *Anthocephalus indicus*, *Azadirachta indica*, *Bambosa*, *Gmelina arborea*, *Michelia champaca*, *Tectona grandis*, *Terminalia chabula*, *Terminalia arjuna*, *Thevetia peruviana* and *Saraca indica* are planted. Pots belong to third set are kept without treatment. The survival rate of the plant species was recorded. Out of 10 tree species only 7 species were found to show growth in crude oil contaminated soil after bacterial treatment. Out of these seven plant species four plant species viz., *Azadirachta indica*, *Gmelina arborea*, *Tectona grandis* and *Thevetia peruviana* belong to the family lamiaceae, verbenaceae, malieaceae and Apocynaceae respectively were found to be shown good growth and adaptability in soil with 15-32.8% crude oil after bacterial treatment. In the present study *Thevetia peruviana* was chosen for phytoremediation study.

3.8.1. Determination of physiological parameters of *Thevetia peruviana* (Pers.) K. Schum

Physiological parameters of *Thevetia peruviana* grown contaminated soil treated with strain 26ML and strain 3SG were studied with respect to plant survival rate, plant height, no. of leaves, dry biomass of different parts of plants, rate of transpiration and photosynthesis at 1 month interval from 0h to up to a period of 1 year. Plant height was measured from the soil
level to the terminal leaf using a meter rule. Number of leaves was determined by visual counting of the number of leaves per seedling per treatment. After 1 year of plantation the plants were uprooted and leaf number and dry biomass of different plant parts were recorded. While, rate of transpiration and photosynthesis was measured by Infra red gas analyzer TPS-2Version 2.0, USA at 1 month interval for 1 year. In case of *Thevetia peruviana* planted in contaminated soil under control condition physiological and biochemical study were not done as no plants survive up to 1 year of plantation. However, *Thevetia peruviana* planted in soil with less contamination (15.0-15.1% oil) minimum growth and survival rate was observed up to 3 months of plantation.

3.8.2. **Estimation of biochemical parameters (quantification)**

**Estimation of chlorophyll**

Chlorophyll was extracted using di-methyl sulphonic oxide (DMSO) method (Hiscox and Israelstam-1979)

**Method of extraction**

Fresh leaves (100mg) in test tube

\[ \downarrow \]

Added 5 ml of DMSO

\[ \downarrow \]

Heated at 65ºC (Until leaf disks were completely colorless).

\[ \downarrow \]

The suspended solutes were decanted off to a clear test tube.

**Estimation**

Absorbance at 645 nm and 663 nm against a DMSO blank was recorded. Using the absorption coefficient the amount of chlorophyll was determined by using Arnon’s method, 1949.
Calculation

\[
\text{Chla (mg/g)} = \frac{[12.7(\text{A663}) - 2.69 (\text{A645})] \times V}{(1000 \times W)}
\]

\[
\text{Chlb (mg/g)} = \frac{[22.9(\text{A645}) - 4.68(\text{A663})] \times V}{(1000 \times W)}
\]

\[
\text{Chltotal (mg/g)} = \frac{[20.2(\text{A645}) - 8.02(\text{A663})] \times V}{(1000 \times W)}
\]

Where, \( V \): Final volume of chlorophyll extract in DMSO, \( W \): Fresh weight of tissue extracted

Estimation of carbohydrate

Carbohydrate content of leaves of *Thevetia peruviana* was estimated by Anthrone method

Materials

a. Anthrone Reagent: 200mg anthrone was dissolved in 100ml of ice cold 95% H\(_2\)SO\(_4\).

b. Standard Glucose: Stock –100mg glucose was dissolved in 100ml water.

c. Working standard: 10 ml of stock diluted to 100ml with distilled water. It was stored refrigerated after adding a few drops of tolune.

Method of Extraction

Plant sample (100mg) in a boiling tube

Added 5 ml of 25N HCl

Kept in boiling water bath (3h)

Cool to room temperature

Neutralized with sodium carbonate until effervescence ceases

Make up the volume up to 100ml

Centrifused

Supernatant was collected
Estimation

- 0.5 and 1ml aliquots was taken for analysis.
- The standards were prepared by taking 0, 0.2, 0.4, 0.6, 0.8 and 1ml of the working standard. ‘0’ serves as blank.
- Make up the volume to 1ml in all the tubes including the sample tubes by adding distilled water
- Then 4mL of anthrone reagent was added.
- It was heated for eight minutes in a boiling water bath.
- It was cooled rapidly and read the green to dark green colour at 630nm.
- A standard graph was drawn by plotting concentration of the standard on the X-axis versus absorbance on the Y-axis.
- From the graph amount of carbohydrate was calculated from the sample tube.

Calculation

\[
\text{Amount of carbohydrate in 100 mg of sample} = \frac{\text{mg of glucose x 100}}{\text{Volume of test sample}}
\]

3.9. Analysis of different plant parts to assess the accumulation pattern of metals.

After 1 year of plantation concentration of metals accumulated by different plant parts of *Thevetia peruviana* grown in both treated and untreated crude oil contaminated soil was estimated. Plants were uprooted from both treated and untreated contaminated soil. Different parts (roots, shoots and leaves) of *Thevetia peruviana* were sterilized with alcohol (95%) to avoid metal present in soil.
**Digestion of plant sample**

Nitric-perchloric acid digestion was performed, following the procedure recommended by the AOAC (1990). Leaves shoot and root parts of each plant species were analyzed separately for heavy metal contents. 1 g of pre-sterilized plant materials was taken in digestion flasks and 5 ml of HNO₃ was added and digested at 100º C temperature until a grey-white ash was obtained. The ash sample was allowed to cool for 30 minutes and then 5ml of perchloric acid was added. The solution was evaporated to dryness. Residues obtained from both soil sample and plant sample after digestion were dissolved in 25 ml de ionized water and filtered in to 25ml volumetric flask. The filtered sample was analyzed for metals Mg, Ca, Fe, Cd, Cr, Cu, Pb and Mn by Atomic absorption spectrometer Model: Analyst -100 (PerkinElmer).

**3.10. Evaluation of physical, chemical and biological change of the soil after treatment with efficient hydrocarbon degrading bacteria.**

Soil physicochemical and biological changes were studied after uprooting the plants from both treated and untreated contaminated soil. Soil from each pot were collected, kept in polythene zipper bags and stored at 4ºC. Physicochemical and biological characteristics of the soil were determined as described in section 3.2 and 3.3.

**Statistics**

Student’s t test was performed to evaluate the effects of the reclamation process on the crude oil contaminated soil. Analysis of variance (ANOVA) was performed to evaluate the significant differences and post test Duncan’s multiple range test (DMRT) was used to compare the significant differences at p<0.05. The analysis was performed by using SPSS software.