3 Chemicals and glassware

All the chemicals used in present investigations were of analytical research (AR) grade, procured from Hi-media laboratories, Qualigens chemicals, Loba chemicals (Mumbai). Glassware used was cleaned with 6 N HCl and K$_2$Cr$_2$O$_7$ followed by rinsing with distilled water. Distilled water was used for preparing growth medium and all reagents. Substrate used for the experiment involved petrol, diesel and crude oil which were collected from various locations. Petrol and diesel was collected from Taloda petrol station (district Nandurbar, Maharashtra). Crude oil was collected from oil refinery (Ankleshwar Gujarat).

3.1 Sample collection

Sample collection was accomplished from soil from various locations. These included the “Satpuda Mountain ranges” in Maharashtra with moderate environmental conditions having temperature between 29$^\circ$C and 36$^\circ$C. This sampling site was chosen due to its rich content of carbon, nitrogen and micronutrients like chloride, calcium, potassium, copper, magnesium, sodium, zinc and iron, with near neutral pH, and organic matter. Such geomorphologic characteristics, special climatic conditions and nutrient rich locations were expected to enrich the inherent micro flora. Samples were also collected from garden soil.

3.2 Isolation and maintenance of bacterial cultural

Isolation was carried out by serial dilution of petroleum contaminated soil and garden soil. Each dilution aliquot was individually spread on nutritionally rich nutrient agar plate (NA) and incubated for 48 hours at 28$^\circ$C. Distinct colonies were re-streaked individually on NA plate. Various strains were selected and their morphological and colony
characteristics were studied. The isolated strains were preserved on nutrient agar at 4°C for further used for investigating degradation of hydrocarbons like petroleum oil (petrol), diesel oil and crude oil.

3.3 Comparative reference strain

A standard strain of *Pseudomonas desmolyticum* NCIM 2112 was procured from National Chemical Laboratory (NCL) for comparative study. On account of its ability of diesel oil. The aim was to investigate the biodegradation of other hydrocarbons by the strain at laboratory level and accomplish a parallel investigation with the strains isolated.

3.4 Screening of petroleum hydrocarbon degrading isolates

Screening of petroleum hydrocarbon that is petrol, diesel oil and crude oil degraders was carried out by growing each culture in carbon deficient medium that is Bushnell Haas medium (BH) containing gL⁻¹ magnesium sulphate 0.20, calcium chloride 0.02, monopotassium phosphate 1, dipotassium 1, ammonium nitrate 1, ferric chloride 0.05. Trace element solution in a concentration of 1ml L⁻¹ included (gL⁻¹) EDTA, 5.0, ZnSO4.7H2O, 2.2; CaCl2, 5.45; MnCl2.6H2O, 5.06; H3BO3, 0.05; FeSO4.7H2O, 4.79; NH4Mo, 24.4; CoCl2.6H2O, 1.6; CuSO4 . 5H2O, 1.57 was also added to the above medium. The pH was adjusted to 7±0.2. By enrichment technique carbon source in enrichment medium was replaced by petroleum hydrocarbon in various concentrations. Initially suboptimal level of oil was added in the enrichment medium ranging as 0.1μl, 0.2μl, up to 0.5μl per 100 ml of BH medium. This was followed by supplementing BH medium with increasing levels of oil till 5 ml. Isolates were individually added in 10 ml saline followed by serial dilution. 10⁻³ and 10⁻⁴ dilutions were used further. This medium was enriched for seven days on rotary shaker at 120 rpm at room temperature. On
observing turbidity the presence of organisms was confirmed by staining. The isolates so obtained were transferred by spreading on BH agar plates.

3.5. Preliminary detection method of biodegradation

Catechol is an intermediate product obtained during degradation of a petrol, diesel oil and crude oil compounds by microorganisms (Collins, LD 1997). The metabolic rate of the substrate was assessed by quantification of the key hydroxyl metabolite catechol that accumulated in aqueous spent medium. Detection of catechol was done by 4- amino antipyrine reagent (Michaud, L. 2004). The yellow color of the reagent turned reddish brown at pH 10 in presence of catechol with absorbance maxima of 510 nm.

3.6 Characterization of isolates

Petroleum hydrocarbon degrading isolates were characterized by using 16S rRNA gene sequencing and fatty acid methyl ester [FAME] analysis (Disha chemical from Ahmedabad).

3.6.1. Preliminary identification

On basis of enrichment method six isolates were isolated from petrol polluted soil and garden soil. Out of these, two isolates were finally screened on account of their potent ability of utilizing petrol, diesel oil and crude oil as carbon and energy source. The two isolates were subjected to various biochemical tests as per Bergey’s manual of systematic bacteriology. The preliminary morphological attributes were studied in the form of colony characters and Gram character. The isolates were further characterized based on cultural characteristics, at MTCC, Chandigarh.
3.7 16S rRNA gene sequencing

3.7.1 Genomic DNA isolation

Genomic DNA was extracted from pure cultures using HiPurATM Plant Genomic DNA Miniprep Purification Spin Kit (Hi-Media) according to the manufacturer’s instructions. The isolates were grown overnight in 10 ml of trypticase soya [TS] broth. The cultures were pelleted at 10,000 rpm after 10 minutes at 40°C using Sigma 3K30 refrigerated centrifuge [Sigma, CA, USA] and were suspended in 400 µl lysis buffer [PL] [DS0016] and 20 µl RNase A solution, vortexed vigorously for 1 minute and incubated at 65°C for 10 minutes. Precipitation buffer [PS] [DS0017] in quantity of 130 µl was added to the lysate, mixed and incubated in ice for 5 minutes. 500 µl of lysate was added to preassembled HiShredder [DSCA01] column seated in 2 ml collection tube, centrifuged at 14,000 rpm for 2 minutes; flow-through was transferred to a new 2 ml collection tube without disturbing the cell debris pellet, binding buffer [BB] [DS0018] in an aliquots of 675 µl was added and mixed by pipetting. The lysate was transferred to HiElute Miniprep Spin column [DBCA02] placed in 2 ml collection tube, centrifuged at 8,000 rpm for 1 minute and the elute was discarded. A 500 µl wash buffer [WSP] [DS0019] was added to HiElute Miniprep Spin column and centrifuged for 1 minute at 8,000 rpm. The flow-through was discarded and added to 500 µl wash buffer to the HiElute Miniprep Spin column and centrifuged for 3 minutes at 14,000 rpm to dry the column. The binding column was placed into 1.5 ml micro centrifuge tube and 100 µl of elution buffer [ET] [DS0040] was directly pipetted onto the centre of the column. The column was incubated for 5 minutes at room temperature and centrifuged for 1 minute at 10,000 rpm and
genomic DNA was eluted. DNA quality was checked by obtaining a single and sharp band in comparison to DNA ladder λ DNA HindIII digested [Fermantas, Vilnius, Lithuania] on 0.75% agarose gel [Sigma- Aldrich, MO, USA] prepared in working 1× TAE buffer consisting tris base, 242 g; glacial acetic acid, 57.1 ml; 0.5 M EDTA [pH 8.0], 100 ml; de-ionized water [18.2 Ω], up to 1000 ml. 1X TAE buffer was further prepared for use by diluting 50 times with de-ionized water [18.2 Ω] and stained with 0.5 μg/ml ethidium bromide. This work was accomplished by NCCS, Pune as per the above protocol.

3.7.2 Sequencing

The amplification of 16S rRNA gene was performed using the primers 27f (5’-AGAGTT TGA TCC TGG CTC AG-3’) and 1492r (3’-ACG GCT ACC TTG TTA CGACTT-5’) (Weisburg et al, 1991). PCR reaction mixture comprised of 200 μM dNTPs [Fermentas, Vilnius, Lithuania], 50 pmol each primer, 1× PCR buffer, 1 U Taq DNA polymerase [Promega, WI, USA], and 100 ng genomic DNA. The thermocycling procedure involved an initial denaturation at 94°C for 4 minutes, followed by 35 cycles of 94°C for 1 minute, 52°C for 1 minute, and 72°C for 2 minutes, and final extension at 72°C for 8 minutes. The PCR products were analyzed on 1.2% agarose gel and their size estimated using 1 kb DNA ladder [Fermentas, Vilnius, Lithuania]. The amplified band corresponding to ~1500 bp was excised using a sharp blade and eluted employing PureLinkTM Quick Gel Extraction Kit according to the manufacturer’s instructions [Invitrogen, CA, USA]. Gel slice of ~400 mg containing DNA band was placed into a 1.5 ml polypropylene tube, 1.2 ml Gel Solubilization [GS1] buffer was added, and incubated at 50°C for 15 minutes and mixed after every 3 minutes to ensure gel dissolution. After the gel dissolution it was
incubated for an additional 5 minutes. Quick Gel Extraction column was then placed into 2 ml wash tube and the dissolved gel piece was loaded onto the column, centrifuged at 10,000 rpm for 1 minute. The flow-through was discarded and the column was positioned back into the wash tube. 500 μl GS1 buffer was added to the column, incubated at room temperature for 1 minute and centrifuged at 10,000 rpm for 1 minute. The flow-through was discarded and the column was placed back into the wash tube. 700 μl W9 wash buffer was added with ethanol to the column, incubated at room temperature for 5 minutes, centrifuged at 10,000 rpm for 1 minute and the flow-through was discarded. It was centrifuged at 10,000 rpm for 1 minute to remove the residual W9 wash buffer and the wash tube was discarded and the column was placed into a 1.5 ml recovery tube. 50 μl warm [60-70°C] TE buffer was added to the column center, incubated for 1 minute at room temperature, centrifuged at 10,000 rpm for 2 minutes. The column was discarded and the purified 16S rRNA gene product was stored at -20°C. Sequencing of the purified 16S rRNA gene was done using four sequencing primers as stated in table 1 and Big-Dye Terminator Cycle Sequencing Kit [Applied Biosystems, CA, USA]. The PCR reaction of 5 μl included 1 μl 5X sequencing buffer, 1 μl Big-Dye Terminator premix, 1 μl primer [5 pmol] and 2 μl purified PCR product [~30 ng/μl]. Thermal cycling conditions consisted of an initial denaturation at 96°C for 3 minutes, followed by 30 cycles of 94°C for 10 s, 50°C for 40 s and 60°C for 4 minutes. The unincorporated dye terminators were removed using Montage SEQ96 Sequencing Reaction Cleanup Kit [Millipore, MA, USA]. The purified PCR products were transferred to 96 well injection plates for sequencing on 3130x Genetic Analyzer [Applied Biosystems, CA, and USA]. These procedures have been followed at NCCS, Pune and have been stated here accordingly.
### Table 6. Primers for amplification and sequencing

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Primer</th>
<th>Sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S</td>
<td>27f</td>
<td>AGA GTT TGA TCC TGG CTCAG</td>
<td>Weisberg et al. (1991)</td>
</tr>
<tr>
<td></td>
<td>68 5r</td>
<td>TCT ACG CAT TTC ACC GCT AC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>926f</td>
<td>AAA CTC AAA GGA ATT GAC GG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1492r</td>
<td>TAC GGY TAC CTT GTT ACG ACT</td>
<td></td>
</tr>
</tbody>
</table>

### 3.7.3 Sequence assembly and phylogenetic analysis

The sequence data obtained were assembled and analyzed using DNA sequence assembling software SEQUENCER™ 4.10.1 [Gene Codes Corporation, MI, and USA]. The sequences were searched as nucleotide query in nucleotide database of NCBI (http://www.ncbi.nlm.nih.gov/) to find the related sequences. Phylogenetic tree was constructed after aligning all acquired and related sequences with Clustal W software (Thompson et al, 1997), using the neighbor-joining method in MEGA 5 and the Kimura 2 parameter model with 1000 bootstrapped replications (Tamura et al, 2004). The protocol has been mentioned here accordingly.

### 3.7.4 Submission of isolates to Gene bank

The 16S rRNA gene sequence of *Acinetobacter lwofii (AHJ6)* and *Pseudomonas aeurigonsa (AHJ8)* was submitted to a gene bank (http://www.ncbi.nlm.nih.gov/) under accession No.JN834008 and JN834006 respectively.

### 3.7.5 Whole-cell Fatty Acid Methyl Ester [FAME] analysis

Chromatography [GC] was carried out using the Sherlock Microbial Identification System [MIDI, Inc. Newark, DE, USA]. The bacterial cultures were grown in triplicate
on trypticase soya agar for 24 hours at 28°C and fatty acid derivatives were separated from cell wall by saponification and extracted with hexane and methyl tertiary butyl ether. Extracts were analyzed on a GC 689 [Agilent Technologies, USA] fitted with Ultra 2 phenyl methyl silicone fused silica capillary column 25× 0.2mm [Agilent Technologies, USA], using hydrogen as the carrier gas, nitrogen as the “make up” gas and air to support the flame. GC oven was programmed from 170 to 270°C at 5°C rise per minute with a 2-minutes holding at 300°C. Fatty acids were identified and quantified by comparison of retention time and peak area obtained and compared with the fatty acid standards. Qualitative and quantitative differences in fatty acid profiles were used to compute the distance for each strain relative to other strains in the Sherlock bacterial fatty acid ITSA1 aerobe reference library. This part of the work was accomplished Disha chemicals Ahmedabad.

3.8 Growth kinetics in presence of petroleum, diesel oil and crude oil degrading isolates

Growth rate of the isolates was investigated in presence of petroleum, diesel oil and crude oil and it was monitored up to four days. For this BH medium was used in presence these substrate. Growth kinetics was studied in presence of this substrate using shake flask at 600 nm. Initially 0.01 optical density inocula were added and 0.2 ml substrate was supplied. After interval of 4 hours the absorbance was measured for a period of 4 days.

3.9 Antibiotic susceptibility test

Sterile Mueller Hinton Agar medium was used for studying the antibiotic sensitivity of the organism with a view to analyze the resistance power of the organism against various antibiotics. The motive was to check the chances of presence of plasmids as antibiotic
resistance character was mostly exhibited by plasmid bearing organisms. The medium with depth of 4 mm approximately was used for the test. Pure cultures of all isolates were used as inocula. 3-4 identical colonies of each isolate were selected & transferred separately into 5 ml Yeast Extract Mannitol Broth & incubated at 35°C for 2-8 hours till light to moderate turbidity developed. The turbidity was adjusted to yield a uniform suspension containing 10^5- 10^6 cells / ml. A sterile non toxic cotton swab on a wooden applicator was dipped into the standardized inocula. The entire agar surface of the plate was streaked with the swab thrice, turning the plate at 60° angle between each swabbing. The inocula were allowed to dry for 5-15 minutes. The discs of different antibiotics were properly placed on the inoculated agar surface using aseptic technique. The set was run in duplicate. The discs were placed with centers at least 24 mm apart. The plates were incubated at 37°C & examined after 14-19 hours or later as per the necessity. The zones representing complete inhibition were measured & the diameter of the zones were recorded in millimeters. The resistance, sensitivity and intermediate response of the organism to the various antibiotics was recorded.

3.10 Quantification of petrol, diesel oil and crude oil

The degradation of petrol, diesel oil and crude oil as a whole was expressed as the percentage of petrol, diesel oil, crude oil degraded with respect to the amount of the remaining fractions in the appropriate abiotic control samples (external standard technique). Combined areas under resolved peaks and the Unresolved Complex Mixture (UCM) were integrated to represent Total Petroleum Hydrocarbon. The biodegradation efficiency (BE) based on the decrease in the total concentration of hydrocarbons was evaluated by using the following equation:
\[ BE(\%) = 100 - \frac{(As \times 100)}{Aac} \]

where As = total area of peaks in each sample, Aac = total area of peaks in the appropriate biotic control, BE (\%) = efficiency of biodegradation.

3.11 Spectroscopic studies

The petroleum hydrocarbons petrol, diesel oil and crude oil were characterized with the help of UV Visible spectrophotometer and FTIR.

3.11.1 UV-visible spectrophotometric and Colony forming unit studies

Petroleum hydrocarbons petrol, diesel oil and crude oil, using a sterile pipette, 5 ml of nutrient broth were transferred into a bottle and aseptically inoculated with a loopful of pure stock culture of isolate and incubated at 37\(^{\circ}\)C. Five percent (v/w) of the inocula were transferred into another bottle and incubated at 37\(^{\circ}\)C. The samples were taken sequentially withdrawn further in a cuvette at 6 hours intervals beginning from zero and their corresponding absorbance were measured at 620 nm. Microbial inoculum (0.1 ml) was used to inoculate the polluted and control soil samples for degradation studies. UV-visible spectrophotometer [Shimadzu Model UV-MINI 1240] was used for measuring the absorbance. The colony forming unit was obtained by dilution method. This method based on the principle that when material containing bacteria are cultured every viable bacterium develops into a visible colony on nutrient agar medium. Serial dilutions were made in multiples of ten. Dilutions were later prepared by transferring a known volume of the dilution to second dilution blank and so on. Once diluted, the specified volume of the dilution sample (1ml or 0.1ml) from various dilutions was added to sterile Petri plates to which molten and cooled (45 \(^{\circ}\)-50 \(^{\circ}\)C) suitable agar medium was added. The colonies were counted on a Quebec colony counter. The number of organisms
developed on the plates after an incubation period of 24-48 hours per ml was obtained by multiplying the number of colonies obtained per plate by dilution factor, which was the reciprocal of the dilution. To facilitate calculations, the dilution is written in exponential notation.

The population of isolates was calculated using the relationship:

\[
\text{Colony forming unit (cfu)} = \frac{\text{Number of colonies} \times \text{Dilution factor}}{\text{Volume of inoculum used}}
\]

3.11.2 FTIR analysis

The functional groups present in degradation pattern of substrate petrol, diesel oil and crude oil were determined by FTIR. After the required time for degradation by the two isolates \textit{(Acinetobacter lwoffii AHJ6 and Pseudomonas auerigonsa AHJ8)} the medium was extracted with hexane. The residue was subjected to FTIR analysis using FTIR spectrophotometer Perkin Elmer Spectrum Version 10.03.06 spectrum. The two spectra were recorded in 4000/cm to 650/cm range.

3.12 Gravimetric method

Petroleum, diesel oil and crude oil degrading capacities of the isolates \textit{(Acinetobacter lwoffii (AHJ6) and Pseudomonas auerigonsa (AHJ8))} were monitored on the basis of gravimetric analysis. The aim was to study the feasibility and viability of the process under the prevailing conditions and optimize the process for the isolates. The gravimetric method was experimented as per the protocol of Udema and Antai (1988). Gravimetric method was also used for extraction of residual petroleum, diesel oil and crude oil after degradation by the isolates.
3.12.1 Treatment of samples

Samples were of petroleum oil was treated with thorough mixing soil. Because it should be properly mixed with soil. Treatment of the samples was carried out as per the method of Abu and Ogiji.

**Group A** involved samples of 20 g sterilized soil mixed with 1 ml (0.85 g) petrol, diesel oil and crude oil and 0.1 ml *Acinetobacter lwofii* (AHJ6) and *Pseudomonas aeurigonsa* (AHJ8)

**Group B** involved samples of 20 g sterilized soil mixed with 1 ml (0.85 g) petrol, diesel oil and crude oil and 0.1 ml distilled water.

**Group C** involved samples of 20 g sterilized soil mixed with 1 ml distilled water and 0.1 ml cultures of *Acinetobacter lwofii* (AHJ6) and *Pseudomonas aeurigonsa* (AHJ8)

Groups B and C served as control. The soil samples in each group were treated. In group A two samples each of sterilized soil were taken and mixed with petrol, diesel oil and crude oil and inocula was added. In group B two samples each of sterilized soil were taken and mixed with petroleum oil (petrol), diesel oil and crude oil and distilled water was added. In group C two samples each of sterilized soil were mixed with distilled water and inoculated. Group B and C served as controls. Similar protocols were followed for soil containing diesel oil and crude oil.

3.13 Petroleum hydrocarbon oil degradation studies

The ability of isolates to degrade petrol, diesel oil and crude oil was demonstrated in terms of reduction in the quantity of petrol, diesel oil and crude oil. The rate of utilization was monitored on the first day (day zero) of the study and subsequently at 3 day intervals for 27 days. Carbon tetrachloride was employed as extractant. Treated sample at the
interval of three days was analyzed for the quantity of residual oil using the methods of Udema and Antai. Each of the 2 g soil treated samples were mixed with 8mL carbon tetrachloride, placed in separating flask, shaken vigorously for 3 minutes and allowed to settle for 5 minutes. The liquid phase was separated by allowing the mousse (oil- carbon tetrachloride) to pass gradually through a funnel fitted with filter paper (Whatman No.1). Anhydrous sodium sulphate was spread on the filter paper to remove the moisture in the mixture. Further liquid phase was collected in 50 ml pre–weighed Pyrex beaker containing the extract, and was placed in an oven, allowed to cool to room temperature and weighed to determine the quantity of oil by difference. The percentage of oil degraded at the interval of three days was determined from the equation

\[
\text{Percent of oil degraded} = \frac{\text{weight of oil degraded}}{\text{original weight of oil degraded}}
\]

Original weight of oil degraded was determined as original weight incorporated minus weight of residual oil obtained after evaporating the extractant.

3.13.1 Detection of hydroxyl metabolite catechol by TLC

Catechol is an intermediate product obtained by degradation of a petrol, diesel oil and crude oil compounds by microorganisms (Collins, L.D. 1997). The metabolic rate of the substrate was assessed by quantification of the key hydroxyl metabolite catechol that accumulated in aqueous spent medium. Detection of catechol was done by 4- amino antipyrine reagent (Michaud, L 2004). The yellow color of the reagent turned reddish brown at pH 10 in presence of catechol with absorbance maxima of 510 nm. The acidified spent medium was extracted three times with diethyl ether (1:3 volumes). The extract was dried over anhydrous sodium sulfate and evaporated to dryness. TLC aluminium sheets (Merck TLC Silica gel 60F25K Germany) 5X10 cm were used for
separation after the extraction of catechol with mobile phase that is benzene: methanol: acetic acid (8:2:0.1).

3.13.2 Detection of hydroxyl metabolite catechol by HPLC

The acidified spent medium was extracted three times with diethyl ether (1:3 volumes). The extract was dried over anhydrous sodium sulfate and evaporated to dryness. The residue obtained was dissolved in methanol and further characterized by HPLC (Shimadzu LC8A, Japan) equipped with a 250 x 4.6 mm C18 phenomex column. The elution scheme was methanol-water (70:30) with the flow rate of 1 ml minute⁻¹. This part of the work was accomplished with the courtesy of Shree Industrial Training and Research Centre, Jalgaon.

3.14 GC and GC-MS analysis

3.14.1 Sample preparation

For gas chromatographic analysis samples were prepared according to the methods of Michaud et al., (2004). After the incubation period, 5 ml of the cultures were extracted with two 20 ml volumes of n-hexane as a solvent by using separating funnels to remove cellular material. The residues were transferred to tarred vials and the volume of each extract was adjusted to 100 ml by adding n-hexane. The vials were kept at 4°C until gas chromatographic analysis.

3.14.2 GC analysis

For detection of petroleum hydrocarbon that is petrol, diesel oil and crude oil, a gas chromatography [GC] was performed using Shimadzu, GC model 2014 equipped with a capillary column TR-WAXMS [30 m X 0.25 mm internal diameter] and a flame ionization detector [FID]. The spectra obtained were compared with the spectra for
standard petrol, diesel oil and crude oil. One micro liter of petrol oil, diesel oil and crude oil extract was injected through split injection port with a split ratio of 10:1 using 10 μl syringe [SGE analytical, Australia]. Helium was used as carrier gas with flow rate of 15 ml minute⁻¹. The column oven temperature was programmed from 280°C for 40 minutes, increased at a rate of 8°C per minute to 360°C, and held at this temperature for 6 minutes. The temperature of injector and detector was 230°C and 240°C respectively.

3.15 GC-MS analysis

For identification of hydrocarbons present in petrol, diesel oil and crude oils obtained from residual oil remaining in the broth, a coupled gas chromatography mass spectrophotometry [GC-MS] was performed using Perkin Elmer gas chromatograph model Auto system XL with turbo mass GC+ equipped with an SGE forte GC capillary column BP20, [30 m X 0.250 μ internal diameter X 0.25 μ] [Australia] and mass spectrophotometer. The mass spectra obtained were compared with the National institute of standards and technology [NIST] mass spectra library. One μl of petrol, diesel oil and crude oil extract was injected by split injection with a split ratio of 10:1 using 10 μl syringe [SGE analytical, Australia]. Helium was used as carrier gas at a flow rate of 1 ml/min. The column oven temperature was programmed from 120°C for 2 minute, increased at a rate of 20°C/min up to 230°C, and further held at this temperature for 10 minutes. The temperature of injector and detector was 225°C and 230°C respectively.

3.16 Optimization for petroleum degradation

With a view to enhance increase the bacterial degradation potential of petroleum hydrocarbons detailed investigation of composition of the specific medium, physical and nutritional parameters need was obligatory. For this purpose, the physical and nutritional
parameters were optimized. For any fermentation procedure, the degradation performance of organisms was affected by numerous variables. So the physical parameters including temperature and pH, and biochemical parameters like inoculum concentration, substrate concentration, organic and inorganic nitrogen, carbon, metals, and phosphate requirements were optimized. The motive of optimization was to maintain the nutrient concentrations within the optimum range, to prevent any alteration in the metabolism due to overfeeding or underfeeding of key nutrient and to maintain the bioprocess in a more economical state optimization was carried out. Various strategies have been developed to control the nutrient concentrations during bacterial degradation of petrol, diesel oil and crude oil. All these parameters were optimized on the basis of bacterial growth rate measured in terms of absorbance at 620 nm and colony forming unit (cfu).

3.16.1 Growth and bacterial degradation in different media

To sustain the optimum growth a suitable media had to be selected for degradation of petrol, diesel oil and crude oil. Various media were used to check the potency of degradation. These included Mineral base medium containing gL⁻¹ NH₄Cl, 0.5g; NaH₂PO₄, H₂O, 0.5g; KH₂PO₄ 0.5g; MgSO₄.7H₂O, 0.5g; NaCl, 4.0g, trace element solution 1ml and BH medium. Growth and degradation were determined by absorbance at 620 nm and simultaneous determination of the colony forming unit (CFU).

3.16.2 Influence of petrol, diesel oil and crude oil concentration

The influence of varying concentrations of petrol, diesel oil and crude oil ranging from 0.2 to 2.5 % was carried out to check the optimum growth of isolate. The experiments were run in BH medium with respective substrates containing 2 percent inocula and
incubated at 30 °C on rotary shaker at 120 rpm. The growth rate of the isolate was measured in terms of absorbance at 620 nm and colony forming unit (CFU) in presence of the respective substrates.

3.16.3 Influence of nitrogen sources on petrol, diesel oil and crude oil degradation

Ammonium ferrous sulphate, ammonium oxalate, triammonium citrate, ammonium sulphate, ammonium acetate, urea, ammonium ferrous citrate, ammonia, malt, peptone, casein, yeast extract, beef extract were used as the nitrogen sources for investigating their effect on growth of the two isolates in presence of 0.1 ml petrol, diesel oil and crude oil as substrate respectively. The nitrogen sources were added in concentrations of one percent. All the flasks were incubated at 37°C for four days under shaking conditions on rotary shaker at 120 rpm. The bacterial growth was determined by measuring growth at absorbance 620 nm and determining the colony forming unit (CFU) for the respective substrates.

3.16.4 Influence of phosphorus sources

Various phosphate sources like sodium hydrogen phosphate, sodium dihydrogen phosphate, potassium dihydrogen phosphate and dipotassium hydrogen phosphate were used separately for the investigation. They were added in media at concentration of one percent containing respective substrate. All the flasks were incubated at 37°C for four days under shaking conditions on rotary shaker at 120 rpm. The bacterial growth was determined by measuring growth as absorbance at 620 nm and determining the colony forming unit (CFU) in presence of the respective substrates.
3.16.5 Influence of various metal sources

Various metal sources like MnCl₂, FeSO₄, CuCl₂, AgNO₃, and HgCl₂ and ZnSO₄ were added separately at 2 ppm concentrations in medium. Their effect on growth of the two isolates in presence of one percent petrol, diesel oil and crude oil as substrate was investigated. All the flasks were incubated at 37°C for nine days under shaking conditions on rotary shaker at 120 rpm. The bacterial growth was determined by measuring growth as absorbance at 620 nm and by determining the colony forming unit (CFU) in presence of the respective substrates.

3.16.6 Influence of carbon source

Various carbon sources like glucose, fructose, lactose, and maltose were separately added in one percent concentrations. Their effect on growth of the isolates in presence of 1% petrol, diesel oil and crude oil as substrate was investigated. All the flasks were incubated at 37°C for nine days under shaking conditions on rotary shaker at 120 rpm. The bacterial growth was measured as absorbance at 620 nm and by determining the colony forming unit (CFU) in presence of the respective substrates.

3.16.7 Influence of inoculum concentration

Inoculum concentration is another economically important factor since level of inocula required for commercial fermentation is co-related to the cost of product. Degradation study was influenced by the level of inocula. The influence of inocula on growth in presence of petrol, diesel oil and crude oil were determined. Inocula in concentrations ranging from 1-5 percent were used for the media containing the respective substrate and were incubated overnight on rotary shaker at 37°C at 120 rpm.
3.17 Scale up of biodegradation of petroleum hydrocarbons

After optimizing all parameters, the optimized protocol implemented at shake flask level was applied for biodegradation experimentation in bioreactor. This was accomplished to check the performance of organism and to confirm the validity of various parameters optimized at shake flask level. For fermentative degradation in presence of substrates petrol, diesel oil and crude oil, shake flask process was scaled up to 5L capacity in bioreactor [Murhopye Scientific Co., India, Model LF-5]. The reactor was sterilized along with optimized medium and all optimized parameters were incorporated. The medium was separately inoculated with 3 percent [v/v] inoculum of *Acinetobacter lwofii* and *Pseudomonas aeruginosa* independently and incubated at room temperature for 48 hours. The pH was adjusted to 6.8, with constant agitation of 70 rpm, dissolved oxygen content maintained at 30 percent saturation value by manually adjusting the airflow rate. All optimized parameters were incorporated for investigation degradation potential.
3.17.1 Biochemical changes during biodegradation in fermentor

During above course of biodegradation samples were withdrawn after every 24 hours up to nine days to check the pH profile, growth, biomass, protein, and residual petrol, diesel oil and crude oil concentration. The residual sugar, viscosity, residual phosphate and residual nitrogen were also determined in supernatant. Degraded residual petrol, diesel oil and crude oil were determined by GC. Degradation of petrol, diesel oil and crude oil were detected by anti pyrene agent.

3.17.2 Studies on pH profile

The pH profile during fermentation and degradation of petrol diesel oil and crude oil was determined by pH probe mounted on headspace of fermentor and was noted down as indicated on pH controller.

3.17.3 Estimation of growth and biomass

For growth measurement, absorbance was measured at 620 nm on UV-VIS spectrophotometer [Shimadzu UV Mini 1240] against uninoculated broth as blank, the biomass concentration and growth were evaluated using a calibration curve. Weight of biomass was determined after centrifugation of broth at 10000 rpm for 20 minutes and the sediment was dried down to constant mass.

3.17.4 Measurement of viscosity and density

Sample withdrawn from fermented broth were subjected for viscosity measurement using Ostwald viscometer and uninoculated medium served as reference. Viscosity and density were expressed as centipoises and gcm⁻³ respectively.
3.17.5 Estimation of phosphate–phosphorus concentration

The purpose underlying the experiment was to establish a co-relation between phosphate utilized by the organisms for cellular growth and degradation of petrol, diesel oil and crude oil. The concentration of phosphate–phosphorus was determined by measuring the absorbance of the colored solution formed by the reaction of phosphate with 2,4 paranitrophenol reagent and ascorbic acid at 660 nm according to Olsen method (Olsen, et al, 1954). The phosphate–phosphorus concentration was evaluated using a calibration curve and expressed in ppm.

3.17.6 Estimation of residual sugar

The purpose underlying the experiment was to establish a co-relation between sugars added to sugar utilized by the organisms for cellular growth and degradation of petrol, diesel oil and crude oil. The residual sugar from fermented broth was estimated by dinitrosalicylic acid [DNSA] method (Miller, 1959) and expressed as mg/ml.

3.17.7 Estimation of protein

Protein is the important component present in cell. During the degradation of petrol, diesel oil and crude oil protein concentration were released. The concentration of protein was determined by measuring the absorbance of the colored solution formed by Folin–Ciocalteau reagent measured by absorbance at 750 nm according to Lowry method (Lowry, O.H., 1951). The protein concentration was evaluated using a calibration curve and expressed in milligram.

3.17.8 Percentage degradation of petrol, diesel oil and crude oil by GC analysis.

Degradation of petrol, diesel oil and crude oil in the broth was monitored at regular intervals of 24 hours after extraction by hexane. These residual substrates petrol, diesel
oil and crude oil were analyzed by GC. GC analysis was experimented as per the routine protocol used earlier.

3.18 Applicability of the process in natural soil environment

The present experimentation was accomplished with a motive to determine the efficiency of isolates to remediate diesel, petrol and crude oil in contaminated cultivable soils and thereby investigate the effect of the process on growth of plants. The purpose underlying the experiment was to determine whether the process was liberating any toxic degradation products which when present in soil would adversely affect plant growth. Soil samples were packed into experimental pots and contaminated with diesel, petrol and crude oil at the concentrations of 1ml to 5ml. The soil samples were then laid in the open air for one week to facilitate evaporation of the unstable components in diesel, petrol, and crude oil sample. The low molecular weight components were evaporated. Wheat and groundnut seeds with similar biomass were transplanted into the pots, along with an unplanted control. The experiment was divided into three sets. In the first set 25 ml of bacterial suspension was mixed with the planted soil. The second set included 25ml suspensions of \textit{Pseudomonas aeruginosa} and \textit{Acinetobacter lwoffii} only. Un-planted and un-inoculated soil samples served as control. All the pots were placed in the open air for 30 days.

3.18.1 Component analysis

Diesel, petrol and crude oil contaminants of every soil sample in the pots were estimated at the beginning of the experiment. Likewise residual diesel, petrol and crude oil were also estimated after a period of 60 days. Diesel, petrol and crude oil contaminants were extracted (5 g dry soil, 30ml of carbon tetrachloride) and evaluated by gravimetric
method. The components of the residual substrates were analyzed using Gas Chromatography (GC). Biodegradation of diesel oil, petrol, and crude oil was monitored by quantitative gas chromatographic analysis by means of a Shimadzu Gas chromatogram (GC-204) equipped with a SE-54 capillary column (25 m X 0.32mm) and flame ionization detector (FID). Helium was used as the carrier gas (30 ml min-1). The oven program included temperature of 40°C (4 minutes), increased to 200°C (5 minutes) at a rate of 80 °C minute⁻¹. Injector temperature was 200°C while detector temperature was 280°C. The individual parameters of the fluorescein diacetate hydrolysis reaction were studied to optimize the assay used for estimation in soil samples. These factors included effect of pH, amount of soil, amount of substrate, time of incubation, optimum temperature of incubation, choice of solvent for terminating the hydrolysis reaction and preparation of suitable standards. The results from each parameter were recorded. The use of fluorescein esters as a measure of enzyme activity has been noted as per Kramer and Guilbault (1963).

3.18.2 Method for enzyme detection.

Soil enzymes the important component of soil, play an important role in the mineral circulation of carbon, nitrogen, phosphorus, etc., as well as the transformation of organic matter in soil. Some important edaphic metabolic processes which include the decomposition of organic input and the detoxification of xenobiotics are usually catalyzed by soil enzymes. Soil enzymes, which can reflect the strength and direction of various biochemical processes, are also in the central position in the soil environment and are typically taken as a sensitive indicator of the physicochemical properties of soil (Dick R.P., 1997, Wick B. Kuhne R.F., Vlekp.L.G.,1998). Moreover, soil enzymatic activities,
which can display the overall level of soil biological activity as well as the transforming
course of soil nutrients are important indices to measure the fertility level of soil
(Zhang,Y.H.,2007). Soil enzymes are also closely related to the amount of petroleum
degrading microorganisms, participating directly or indirectly in the degradation of
petroleum contaminants. The determination of fluorescein diacetate hydrolysis (FDA
hydrolysis) was carried out by the methods described by Schnürer and Rosswall (1982).
1 g of dry soil sample was placed in a triangular flask of the volume of 25 cm$^3$
followed by addition of 10 cm$^3$ phosphate buffer (pH = 7.6) and 0.1 cm$^3$ of FDA (2 mg·cm$^{-3}$,
acetone). After incubation for 2 h at 25 °C, to the mixture within the triangular flask 5
cm$^3$ of acetone were added to terminate FDA hydrolysis. Absorbance of the solution
obtained by filtering the above mixture was determined photometrically at 490 nm. The
activity of FDA hydrolase was expressed as the amount of fluorescein (in μg/ml) released
by 1 g of dry soil after 2 h.

4.0 Plasmid isolation

Plasmid Genomic DNA was extracted from pure cultures using HiPurATM Plasmid
DNA Miniprep Purification Spin Kit (Hi-Media) according to the manufacturer’s
instructions. 30 ml of rich medium (LB, YT, or Terrific Broth) containing the appropriate
antibiotic was inoculated either with a single colony of the or with 0.1-1.0 ml of a small-
scale liquid culture grown from a single colony. The culture was incubated at the
appropriate temperature with vigorous shaking until the bacteria reached logarithmic
phase. 500 ml of LB, YT, or Terrific Broth medium (prewarmed to 37°C) containing the
appropriate antibiotic was inoculated with the late-log-phase culture in a 2-liter flask. The
culture was incubated for approximately 2.5 hours at 37°C with vigorous shaking (300
cycles/minute on a rotary shaker). For relaxed plasmids with low or moderate copy numbers, 2.5 ml of 34 mg/ml chloramphenicol solution was added. The final concentration of chloramphenicol in the culture needed to be 170 µg/ml. For high-copy-number plasmids, chloramphenicol need not be added. The culture was incubated for a further 12-16 hours at 37°C with vigorous shaking (300 cycles/minute on a rotary shaker). An aliquot (1-2 ml) of the bacterial culture was added to a fresh micro centrifuge tube and stored at 4°C. The remainder of the bacterial cells was harvested from the 500 ml culture by centrifugation at 2700g (4100 rpm in a rotor) for 15 minutes at 4°C. The supernatant was discarded. The open centrifuge bottle was allowed to stand in an inverted position. The bacterial pellet was re-suspended in 200 ml of ice-cold STE. The bacterial cells were collected by centrifugation as described above. The bacterial pellet was stored in the centrifuge bottle at -20°C. DNA from the 1-2-ml aliquot of bacterial culture was set aside. The mini preparation plasmid DNA was digested with the appropriate restriction enzyme(s) and agarose gel electrophoresis was accomplished to ensure that the correct plasmid had propagated in the large-scale culture. The frozen bacterial cell pellet was allowed to thaw at room temperature for 5-10 minutes. The pellet was re-suspended in 18 ml (10 ml) of alkaline lysis solution. 2 ml of a freshly prepared solution of 10 mg/ml lysozyme was added. 40 ml of freshly prepared alkaline lysis solution II was added. The top of the centrifuge bottle was closed and the contents were mixed thoroughly by gently inverting the bottle several times. The bottle was incubated for 5-10 minutes at room temperature. 20 ml of ice-cold alkaline lysis solution III was further added. The top of the centrifuge bottle was closed and the contents were mixed gently well by swirling the bottle several times (there should no longer be two). The crude plasmid DNA was
purified either by column chromatography precipitation with polyethylene glycol or by equilibrium centrifugation in CsCl-ethidium bromide gradient.