3.1. Screening and isolation of a hydrocarbon degrading bacteria by enrichment culture technique.

3.1.1. Sample collection

Sediment samples were collected from the Munakkal beach (Trichur dist, Kerala) located on the west coast of India immediately after the major event Tsunami in 2004. The site mentioned here experienced impact of Tsunami and hence the sediment deposits after tsunami were subjected to screening of potential bacteria that has ability to degrade hydrocarbons. The collected sediment samples were immediately transported to laboratory under refrigerated conditions and analyzed for bacterial composition and subsequent isolation.

3.1.2. Isolation and screening of hydrocarbon degrading bacteria

Ten gram of sediments was added to 90 ml of sterilized sea water and homogenized by placing the flasks on an orbital shaker at 150 rpm for
1 hour. This homogenate was used as inoculum for plating purposes. 100ml of Zobell Marine Broth (ZMB) prepared in a 250 ml conical flask was inoculated with 1 ml of the prepared sediment homogenate solution and incubated at 37°C for 24h in a shaker at 150 rpm. Enrichment technique using Bushnell - Haas medium (BH) (Appendix 1) supplemented with 1% v/v hydrocarbon substrates was used for the isolation of hydrocarbon degrading bacteria associated with sediment. Bombay High Crude oil Samples obtained as gift by Bharat Petroleum Corporation, Cochin, India were used as substrates for the study. The crude oil used in the enrichment medium contained equivalent mixture of octane, cyclohexane, hexane, benzene, toluene, ethyl benzene, xylene besides other fractions in the crude oil. The culture obtained from Zobell Marine broth was transferred to the enrichment medium and incubated at 37°C for one week in a shaker at 150 rpm. After incubation 1 ml of the culture from primary enrichment medium was transferred to a fresh Bushnell– Hass medium containing the same hydrocarbon mix and incubated for one more week. After second enrichment, 0.1 ml of the culture broth was plated on Zobell Marine agar medium supplemented with same hydrocarbons and incubated at 24 h at 37°C.

Those colonies which were capable of utilizing the given hydrocarbons as sole source of carbon in the medium were isolated, purified and stocked in a phosphate buffer (50Mm KH₂PO₄/K₂HPO₄, pH 7.2) containing 20%( v/v) glycerol at −20°C. Permanent stock cultures were made by lyophilization. Working cultures were maintained by sub culturing on mineral salt agar slants containing crude oil at 1%(v/v)
Materials and Methods

concentration, at intervals of 10 days. The isolate which showed maximal
degradation had ability to utilize most of the hydrocarbons tested was
selected as the potential strain and used in the study.

3.1. 3. Inoculum preparation and inoculation

A loop full of the culture was transferred from the agar slope into 5ml
of ZMB medium taken in a boiling tube and incubated for 16 h at 37°C at
150 rpm in an orbital shaker. Later using this preculture as inoculums 50 ml
ZMB medium taken in an Erlenmeyer flask was inoculated (1% (v/v)) and
incubated until the Optical Density (OD) reached 1.00. This culture was used
as inoculum for further studies unless otherwise mentioned.

3.2. Identification of the selected bacterial strain

The selected bacterial strain was identified based on its
morphological and biochemical characteristics as outlined in Bergeys
Manual of Systematic Bacteriology (Buchanan and Gibbons, 1974).
Molecular ribotyping was also done towards confirmation of the identity of
the strain.

3.2.1. Molecular ribotyping

3.2.1.1. Isolation of genomic DNA (Sambrook et al., 2000)

1) Mid-log phase culture of the bacteria (40 ml) were taken in a sterile
oakridge tube and centrifuged at 5000 rpm for 10 min at 4°C.
2) The supernatant was discarded and the pellet blot dried.
3) The cell pellet was dissolved in 8.75 ml of TE buffer.
4) To the content, 50 µl of Proteinase K (10 mg/ml) and 10 % SDS (1 ml) were added, mixed gently, and incubated at 37°C for 1 hr.

5) To this equal volume of phenol-chloroform mixture (1:1) was added, mixed gently, and kept for 10 min. at 4°C.

6) The contents were centrifuged at 10,000 rpm for 10 min. at 4°C, and the supernatant was transferred to a fresh sterile tube using sterile cut tip.

7) The steps 5 and 6 were repeated three times.

8) The DNA was precipitated by adding 0.1 ml of 5 M sodium acetate (pH 5.2) and 20 ml of isopropanol.

9) The precipitated DNA was washed gently with 70 % ethanol.

10) The prepared DNA was dissolved in 1 ml of TE buffer.

3.2.1.2. Agarose gel electrophoresis (Sambrook et al., 2000)

The agarose gel electrophoresis was done in order to check the quality of the DNA prepared.

1) Agarose gel with a concentration of 0.8 % (w/v) was prepared.

2) 10 µl of the DNA sample was loaded on to the gel and electrophoresed at 80 V until the migrating dye (Bromophenol blue) had travelled two-thirds distance of the gel. Lambda DNA cut with E.coR1 and HindIII (Bangalore Genei) was used as markers.
3) The gel was stained in a freshly prepared ethidium bromide solution (0.5 mg/ml) for 20 min.

4) The gel was viewed on a UV transilluminator, and image captured with the help of Gel Doc system (Biorad).

3.2.1.3. Ribotyping

Ribotyping was performed using universal primer pair for 16S rDNA. A portion of the 16S rRNA gene (1.5 kbp) was amplified from the genomic DNA (Reddy et al., 2000; 2002a, 2002b; Shivaji et al., 2000). The sequences of forward (16SF) and reverse (16SR) primers were used for amplifying 16S rDNA were as follows:

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>16F (5’-AGTTTGATCCTGGCTCA-3’)</td>
<td>(Shivaji et al., 2000)</td>
</tr>
<tr>
<td>16R (5’-ACGGCTACCTTGTTACGACTT-3’)</td>
<td>(Reddy et al., 2002a, 2002b)</td>
</tr>
</tbody>
</table>

(All chemicals were procured from SIGMA -ALDRICH, USA; PCR reactions were carried out in a Biorad thermal cycler under the following conditions standardized in our laboratory).

**PCR mix composition**

- 1.5mM MgCl$_2$ 200µmol
- Deoxy nucleotide mix 200µmol
- Primer 0.1µ mol each
- Taq polymerase 1unit
- Tris-HCl -pH 8.3 100mM
KCl 500 mM

Deionized water 6 μl

**PCR conditions**

1. Initial denaturation - 94°C (90 sec.)
2. Denaturation - 94°C (30 sec.)
3. Annealing - 56°C (30 sec.)
4. Primer extension - 72°C (2 min.)
5. Repeat step 2, 3 and 4 for 34 times
6. Final extension - 72°C (10 min.)
7. Hold - 4°C (5 min.)
8. End

**3.2.1.4. DNA sequencing**

Nucleotide sequence of the amplicon was performed after checking the concentration in a micro-volume spectrophotometer (Thermo Scientific Nano Drop™ ND-2000) and purification using Nucleospin purification column (Macherey-Nagel). Sequencing was done in an ABI 3730xl cycle sequencer. Forward and reverse sequences obtained were assembled and contigs were generated after trimming the low quality bases.

**3.2.1.5. Phylogenetic tree construction.**

The nucleotide sequences of the partial gene sequences of the 16sRNA subjected to multiple sequence alignment and homology search
Materials and Methods

using multiple sequence alignment software Clustal W and bioinformatics tool BLAST of NCBI. Based on maximum identity score, first few sequences were selected and a dendrogram was constructed.

3.3. Characterization of bacteria with potential for degradation of crude oil and its fractions.

3.3.1. Antibiotic sensitivity

Antibiotic sensitivity profile of the bacterial strain was determined using the octadiscs of Himedia in which 8 antibiotic discs were incorporated in one test (Octadisc. Combi-69, Octadisc.T Combi-61, Octadisc. Combi-1 Octadisc-G-V111-plus, Octadisc-G-V1 minus). The strain was evaluated against 27 different antibiotics. Log phase bacterial cultures were inoculated by spread plating on the Zobell Agar and the antibiotic discs were carefully placed over the inoculum and incubated at 37°C for 24 h and checked for the sensitivity.

3.3.2. Enzyme profile of the culture

Enzyme profile of the selected strain was determined as described below.

3.3.2.1. Protease

The proteolytic activity was measured according to Nitkowski et al., (1977). The proteolysis medium consisted of 0.3 % beef extract, 0.5 % peptone, 3% NaCl and 1.5 % agar in distilled water. Casein was provided as substrate for protease in the form of diluted skimmed milk to the medium such that the final concentration of milk was 1.5 %. The medium containing
skimmed milk was swirled gently and poured into petriplates, which were subsequently dried at room temperature. A loop full of bacterial culture was spot inoculated on the solidified skimmed milk agar plate and the plates were incubated at room temperature (28 ± 2°C) for 72 h. An uninoculated media was kept as control. Formation of a clear halo around the bacterial colony was considered as positive for casein hydrolysis.

3.3.2.2. Lipase

Lipase assay was done according to Kim and Hoppe, (1986). The Zobell Marine agar medium was supplemented with 1% (w/v) Tween 80 (sorbitol monooleate) as lipase substrate, autoclaved and poured into sterile petriplates. A loop full of bacterial culture was spot inoculated on to the top of agar plates and incubated at room temperature (28 ± 2°C) for 72 h. An uninoculated media was kept as control. Appearance of dense opacity around the colony was considered as positive indicating production of extracellular lipase.

3.3.2.3. Alpha amylase

Alpha amylase was assayed according to Kim and Hoppe, (1986). To the Zobell Marine agar medium 1% of soluble starch (w/v) was added, autoclaved and poured to pre sterilized petriplates. Bacterial culture was spot inoculated on the solidified agar plates and incubated at room temperature (28 ± 2°C) for 72hours. After incubation the plate was flooded with Iodine reagent. Iodine reagent reacts with starch and forms a blue coloured complex. A clearing zone around the bacterial colony is an
indication of extracellular alpha amylase production. An uninoculated media was kept as control.

3.3.3. Optimization of growth conditions.

Various process variables namely incubation temperature, pH, sodium chloride concentration, and carbon sources, that influence growth were optimized by growing the bacterium in Zobell Marine broth at different conditions as detailed below. 100ml of Zobell Marine broth prepared in 250 ml conical flasks was inoculated with mid-log phase culture (01 OD @1% v/v) and incubated for a total period of 48 h at 28 ±2³C in an orbital shaker at 150 rpm unless otherwise specified. At the end of incubation samples were drawn and assayed for growth in terms of OD at 600nm in a UV–Visible spectrophotometer (Shimadzu-Japan). Uninoculated Zobell Marine broth was used as control. All the experiments were conducted in triplicate and analyzed statistically using Zigma plot.

3.3.3.1. Incubation temperature

Optimum temperature for maximum growth was evaluated by incubating the inoculated media (ZMB) at various temperatures, viz 25³C, 30³C, 37³C, 40³C, 50³C and 55³C, for 24 h at 150 rpm in an environmental shaker.

3.3.3.2. Sodium chloride concentrations

Effect of sodium chloride on growth was studied by incubating the media (ZMB) supplemented with different concentrations of sodium chloride
Chapter 3

viz. 100mM, 200mM, 300mM, 400mM, 500mM, 600mM, 700mM, 800mM, 900mM and 1 M.

3.3.3.3. **pH**

To find the optimum pH for growth, the bacteria were grown in media (ZMB) prepared with different pH ranging from pH 2 to 12.

3.3.3.4. **Carbon sources**

3.3.3.4.1. **Carbohydrates as carbon sources.**

Various carbohydrates were tried as source of carbon for growth of bacteria. Mineral salt medium supplemented with starch, sucrose, cellulose, maltose, lactose, dextrose, galactose, sorbitol, manitol and fructose at 100mM concentration was used for the study.

3.3.3.4.2. **Organic solvents as source of carbon.**

Various organic solvents namely acetone, benzene, toluene, ethyl benzene, chloroform, dichloromethane, octane, pentane, heptanes, hexane, decane, ethanol, methanol, propanol, phenol and cyclohexane were tried as source of carbon by the bacteria towards determining the ability of the bacteria to utilize them. The solvents were added at a concentration of 10% and 50% (v/v) to the mineral salt medium.

3.3.4. **Growth curve**

Growth curve of the bacteria was studied using Zobell Marine Broth as growth medium. The prepared medium (100 ml) was inoculated with mid-log phase culture (01 OD @1% v/v) in a 250 ml conical flask and incubated for a total period of 48 h at 28 ±2°C in an orbital shaker at 150
Materials and Methods

rpm. At regular intervals samples were drawn and assayed for growth in terms of OD in a UV–visible spectrophotometer (Shimadzu-Japan). Uninoculated Zobell Marine broth was used as control. All the experiments were conducted in triplicate.

3.3.5. 2,6-dichlorophenol indo phenol (DCPIP) as an indicator of biodegradation of hydrocarbons by bacteria

Aerobic biodegradation of hydrocarbons was studied using DCPIP as redox indicator. The principle of using DCPIP is that during the microbial oxidation of hydrocarbons, electrons are transferred to electron acceptors such as O₂, sulphates and nitrates. When an electron acceptor such as DCPIP is incorporated into the culture medium, it is possible to ascertain the ability of the microorganism to utilize hydrocarbon substrate by observing the colour change of DCPIP which is blue in colour (oxidized) to colourless (reduced). This technique has been employed in several works (Hanson et al., 1993). BH medium was taken in 100 ml screw capped vial and aliquots of 100µl of different solvents viz. acetone, pentane, heptanes, hexane, cyclohexane, benzene, toluene, xylene, dichloromethane, propanol and methanol were added to the medium and inoculated with bacteria (01 OD @1% v/v)) and kept under shaking for 24 h. Concentration of DCPIP added was 27mg/ml. All the bottles were kept in darkness and the colour change was observed. All the experiments were conducted in triplicate.

3.3.6. Tolerance and accumulation of metals by bacteria.

Tolerance to various metals and their accumulation in cells of bacteria were evaluated using 0.1M of mercuric chloride, cadmium sulphate, copper
Chapter 3

sulphate, lead nitrate, zinc sulphate and sodium meta arsenate by addition of
the same in mineral salt medium (B.H.) supplemented with sucrose as the
source of carbon. The pH of the medium was kept as 9. Inoculated with
bacteria (01 OD @1% v/v)) and kept under shaking at 150rpm for 24 h at
37°C. The controls were made without adding any metals. The growth of
the cells were measured in a U.V–visible spectrophotometer at OD600
nm.(Higham et al.,1985).

Accumulation of arsenic, mercury and cadmium by bacteria was
studied by growing the bacteria in the corresponding medium containing
these metals for 24h. After incubation, the cells were harvested by
centrifugation at 1000 rpm in a centrifuge and washed thrice in physiological
saline. The harvested cells were dried in a hot air oven at 100°C and their dry
weight was weighed. The dried cells were digested by adding 2ml HNO₃,
slightly heated, vortexed thoroughly to mix well and then made up to 50 ml
with deionized water. The contents were analyzed by Inductively Coupled
Plasma -Atomic Emission Spectroscopy (IC-AES).

3.3.7. Accumulation of L-citrulline capped ZnS:Mn nanoparticles
by bacteria Lysinibacillus fusiformis BTTS10 by fluorescence
microcopy

Accumulation of L-citrulline capped ZnS:Mn nanoparticles by the
bacteria Lysinibacillus fusiformis BTTS10 was analyzed by fluorescence
microscopy. Different concentrations of (250µl, 500µl, 1000µl, 2000 µl and
4000 µl) the nanocolloids were added into 10 ml nutrient broth, inoculated
with mid-log phase cells of the bacterium and incubated at 37°C in a rotary
shaker with a speed of 150 rpm for 24 h. *Salmonella typhymurium* (no autofluorescence) was taken as control. The growth was measured in a U.V -visible spectrophotometer at OD$_{600}$ nm. The experiment was conducted in triplicate. The cells were harvested by centrifugation at 1000 rpm in a centrifuge, washed in physiological saline and observed under Fluorescence Microscope. Bacterium grown in the medium lacking nano particles was used to find whether the bacteria have the capacity to produce auto fluorescence.

3.3.8. Bio surfactant production

Bio surfactant production by the bacterium was studied by drop collapse assay (Jain *et al.*, 1991). This assay relies on the destabilization of liquid droplets by surfactants. 4 drops of oil was placed on a clean glass slide. 100μl of supernatant of the culture grown in hexadecane, benzene, and crude oil independently was added on to a drop of the hydrocarbons and observed the drop. If the liquid contains surfactants, then the drops collapse because the force or interfacial tension between the liquid drop and the hydrophobic surface is reduced. The stability of drops is dependent on surfactant concentration and correlates with surface and interfacial tension. One drop was kept as control without adding sample supernatant.

3.4. Biodegradation of crude oil and its fractions with bacteria under submerged culture conditions.

3.4.1. Biodegradation of crude oil

Biodegradation was carried out in modified Bushnell and Haas (BH) medium supplemented with 0.2M sodium chloride. The pH was
adjusted to 9.5 with 1N NaOH and Nitrogen and Phosphorus solutions were prepared separately and autoclaved at 121°C for 15 min.

100 ml of B.H medium supplemented with 1% crude oil as sole source of carbon was taken in a 250 ml of conical flask and inoculated with 1 gm wet weight of 24 h old culture of bacteria. The experimental set up was done in triplicate and one control. The inoculated flasks were incubated at 37°C and at 150 rpm in a rotary shaker. Samples were drawn after incubation for 72, 120 and 168 h and the extent of biodegradation was determined by estimating the growth of bacteria and total residual hydrocarbons in the flasks gravimetrically and variation in pH besides analysing the intracellular and extracellular products of biodegradation. 1 ml pristane was added as an internal standard.

3.4.2. Saturates, Aromatics, Resins and asphaltene (SARA) separation of crude oil

Crude oil was fractionated by silica gel column chromatography the various components were separated (Mishra et al., 2001). The total petroleum hydrocarbons TPH extracts were dissolved in 10 ml pentane and the insoluble fraction (asphaltene) was removed using Whatman filter paper and weighed. The soluble fraction was loaded on top of silica gel G (60-120 mesh) column (2cm x30 cm) activated at 80°C and eluted with solvents of different polarities. The alkane fraction was eluted with 100 ml of hexane; aromatic fraction was eluted with 100ml benzene, and finally NSO (non saponifiable organic acid) fraction was eluted with methanol and chloroform (100 ml each). The methanol and chloroform
fractions were evaporated and weighed to get the weight of NSO compounds.

3.4.3. Extraction of residual total petroleum hydrocarbons

Extraction of the residual hydrocarbon was conducted according to the method of Mishra et al., (2001). At the end of incubation, residual crude oil was extracted twice from the experimental flask with 100ml of hexane, methylene chloride and chloroform respectively. Each time the solvent was evaporated at 60°C in a vacuum evaporator. The amount of residual hydrocarbon recovered was determined gravimetrically in an analytical electronic balance (Sartorius Element ETL602). The biodegradation efficiency (BE), based on the decrease in total weight of crude oil was evaluated by the following expression

\[
BE = \left( \frac{M_0 - M}{M_{C0} - M_C} \right) \times 100 \quad \text{Eq. 1}
\]

Where,

- \(M_0\) = the weight of crude oil sample before inoculation,
- \(M\) = weight of crude oil of sample after inoculation,
- \(M_{C0}\) = weight of crude oil of control,
- \(M_C\) = weight of crude oil of control after the same treatment as that of sample without inoculation.

After gravimetric quantification, the extracts were suspended in 5ml hexane and dried by passing through sodium sulphate and analyzed by FTIR and a gas chromatograph (SIMDIS Analyser, VARIAN CP-3800. Gas Chromatogram). The Gas chromatograph fitted with CP-7562
column. The column was 10m x 1.2µm. The detection was by flame ionization at a temperature range of 35°C to 450°C. Helium was used as carrier gas. The column pressure was 2.5 psi.

**3.4.4. GC-MS analysis of biodegradation products.**

The biodegradation products after solvent extraction were analyzed using high resolution GCMS -Agilent 7890. The column used was 30m x 25mm x 0.25µm. The temperature of the programme was 40°C isothermal time, heating up to 250°C with a heating rate of 40°C /min. Helium was used as carrier gas with a flow rate of 1.2ml /min and the molecules were scanned from 50-550(m/z). The most relevant peaks were identified on the basis of mass spectra data bases (NIST). Pristane was added as internal standard.

**3.4.5. Estimation of pH during biodegradation of crude oil by**

*L.fusiformis* BTTS10

The pH of the growth medium was measured at intervals during the course of biodegradation to study the change in pH during biodegradation of crude oil as described by Olajide *et al.*, (2010).

**3.4.6. Dry biomass determination.**

After the extraction of residual crude oil, dry biomass was determined by centrifugation of the aqueous phase at 1000rpm for 10 min. The cell pellet was washed with 10ml acetone: hexane 3:1 to remove adherent hydrocarbon. The pellet was then resuspended in 10ml hexane

54
and centrifuged again and dried in an oven at 80°C overnight for determination of dry mass (Verma et al., 2006).

3.5. Factors affecting biodegradation of hydrocarbons

Various factors affecting biodegradation was studied and analyzed statistically and mean values were used as results.

3.5.1. Effect of agitation on biodegradation

The effect of agitation on biodegradation of crude oil was studied by incubating the inoculated media taken in a conical flask on a rotary shaker at different rpm (100, 110, 120, 130, 140, and 150). Control was maintained by incubating the inoculated media without agitation. The biodegradation was studied by gravimetric analysis of residual crude oil. The medium preparation, inoculation, incubation and gravimetric analysis were performed as described in the section 3.4.1.

3.5.2. Effect of sodium chloride on biodegradation.

The effect of sodium chloride concentration on biodegradation of crude oil was studied by incubating the media with different concentrations of sodium chloride viz. 0.1M, 0.2M, 0.3M, 0.4M, 0.5 M, .6 M, .7M .8M, .9M and 1M in the BH medium at 150rpm for 5 days in an environmental shaker. The medium preparation, inoculation, incubation and gravimetric analysis are as described in the section 3.4.1.

3.5.3. Effect of inoculum concentration on biodegradation

Optimum concentration of inoculum that gives maximum degradation of crude oil was studied by adding different concentration of
inoculum (1%, 2%, 3%, 4% and 5% w/v) prepared as mentioned in the section 3.1.3. The medium preparation, inoculation, incubation and gravimetric analysis were done as described in section 3.4.1.

3.5.4. Effect of pH on biodegradation

To find the effect of pH on biodegradation bacteria were grown in media prepared with different pH ranging from pH 6 to 12. Growth was determined by measuring the turbidity at 600nm in UV-visible spectrophotometer (Shimadzu, Japan) after 4 days of incubation in an orbital shaker at 150 rpm. The medium preparation, inoculation, incubation and gravimetric analysis were done as described in section 3.4.1.

3.5.5. Biodegradation of crude oil in a biometric system

Biodegradation of crude oil in a biometric system was studied using biometric flasks. Four, 250 ml biometric flasks were taken; one flask was taken as control. 100 ml Bushnell-Haas(BH) medium added with 1% crude oil as sole source of carbon was taken in a conical flask and inoculated with 1 gm wet weight of mid-log phase cells of bacteria. The side arm of biometric flask was filled with 10 ml of 0.1 M KOH. The experiment was done in triplicate and a control was maintained. All the flasks were incubated at 37°C and 150 rpm in a rotary shaker. Samples were drawn after incubation for 72, 120 and 168 h and carbon dioxide (CO₂) was measured by taking the sample out using a pipette from the side arm of the flask. Evolution of CO₂ during the growth of bacteria utilizing crude oil as sole source of crude oil was estimated by colorimetric titration. CO₂ produced during the microbial activity was
Materials and Methods

captured by 0.2 M KOH solution (10 ml) located by the side of the biometric flasks. To the KOH solution, 1 ml of 0.5 M barium chloride solution and 3 drops of 1% phenolphthalein indicator were added. The residual KOH was titrated with 0.1 M standard HCl solution.

The amount of CO₂ produced was computed using the equation
\[ \text{CO}_2 \text{ generated (mg)} = (V_B - V_A) \cdot \text{MCO}_2 = 2 \cdot \text{M HCl} \cdot \text{CF}, \quad \text{Eq.2} \]

Where, \(V_B\) and \(V_A\) are the volume of HCl (0.1M) used to titrate the blank and the treatment in ml, respectively;

\text{MCO}_2 is the molar mass of carbon dioxide in g/mol. \text{M HCl} is the molar concentration of HCl standard solution in mol/L; and \text{CF} is the correction factor for acid/base molarity (\text{M HCl}/\text{M KOH}). All the experiments were conducted in triplicate and analyzed statistically.

3.6. Biodegradation of Toluene (aromatic compound)

Biodegradation of toluene was studied using the B.H medium taken in sterilized screw capped brown bottles was added with 0.5% toluene (v/v) and inoculated with 500 mg wet weight of 24 h old culture. The growth of the cells utilizing aromatic hydrocarbon as sole source of hydrocarbon was measured by studying the increase in turbidity just after the inoculation and during the subsequent days of incubation. The rate of biodegradation was determined by analyzing the degraded sample by GC-MS.

3.7. Biodegradation of crude oil and its fractions with immobilized whole cell biomass
Biodegradation of crude oil and its fractions was studied with whole cell biomass immobilized on petiole cuttings of aquatic weed *Eichhornia crassipes* which is quite common on backwaters, and polystyrene beads.

### 3.7.1. Immobilization of viable bacterial cells on *Eichhornia* petiole

*Eichhornia crassipes* petiole was used for immobilization of bacteria. After washing in Tween 20 and in distilled water three times, the petioles were cut into small cubes of approximately 1 cm$^3$, washed again in distilled water, dried in an oven at 100°C for 1 h, and then sterilized by autoclaving. Log phase cells grown in the mineral salt crude oil medium for 24 hours at 37°C in a rotary shaker at 120 rpm were harvested by centrifugation at 10,000 rpm. The pellet obtained was washed in Mineral salt medium phosphate buffer (pH. 10) and 1gm (wet weight) cells were added to 50 ml of ZMB medium which was used as immobilization medium. 1gm of sterilized *Eichhornia* petiole was added to the above medium and kept for incubation at 37±2°C for 24 h at 60 rpm in a shaker. The immobilized petioles were pelleted by centrifugation at 1000 rpm and washed in sterile distilled water. Immobilized petioles were used for cell viability testing, SEM analysis, and biodegradation studies.

### 3.7.1.2. Enumeration of Viable bacterial cells immobilized on *Eichhornia crassipes* petiole

58
To assess the number of viable bacterial cells attached to petioles, 3 cuttings were washed thrice in 5ml physiological saline and then 1gm of cutting was suspended in physiological saline. The cuttings in suspension were disintegrated with a vortex mixer for 1 min. Successive decimal dilution were made from the supernatant and 0.1ml of the dilutions were spread on nutrient agar medium. The numbers of CFUs were calculated on a regular interval of 45 days for 225 days to study the viability of bacteria immobilised on Eichhornia petiole (Rajeev et al., 2001). All the experiments were conducted in triplicate and analyzed statistically and mean values were taken as result.

3.7.1.3. Estimation of total protein content of viable bacterial cells immobilized on Eichhornia petiole

Total protein content in the immobilized petioles were quantified using three petiole cuttings which were recovered, washed thrice in 5ml physiological saline and suspended in 5ml distilled water in a test tube. 5ml of 1M NaOH was added and mixed in a cyclomixer. The contents were boiled by keeping the tubes in a boiling water bath and after which they were rapidly cooled under tap water. The protein released by alkali lysis of the cells were measured by Bradford method (1976) using Bradford kit (Biogene,USA). The samples were diluted to 100ml with 0.15N NaCl. One ml of Bradford reagent was added, vortexted and allowed to stand for 2 minutes and absorbance was read at 595 nm. Quantification of protein was done by comparing with a standard curve plotted with BSA as standard and was expressed as microgram/ml. (Beena, P.S. 2010).
3.7.1.4. Scanning Electron Microscopic (SEM) examination of immobilized viable bacterial cells

Immobilized cells were examined by Scanning Electron Microscopic (SEM) method. For SEM, the cuttings were suspended in 10ml of B.H medium and stirred at 500 rpm for 1 min in order to wash off the non-attached cells. Washed cuttings were taken with sterile forceps and placed in fixative (buffer phosphate, pH 7.2 and 1% v/v glutaraldehyde) in phosphate buffer (pH7.2) at room temperature for 1 h. Washed in the same buffer, post fixed in 1%w/v osmium tetroxide for 1 hr, dehydrated in graded alcohol(50,75,90,100.), and then mounted. The petiole cuttings were finally observed in a scanning electron microscope (JEOL 6390LA).

3.7.2. Crude oil biodegradation by immobilized viable bacterial cells

Biodegradation of crude oil by viable bacterial cells immobilized on petioles of *Eichhornia* was carried out in artificial sea water (ASW) with 1% (w/v) nitrogen and phosphate solution (N &P solution) to stimulate biodegradation (Foght *et al.*, 1989). The composition of ASW included per litre of distilled water:

- NaCl, 23.4g
- KCl, 0.75g
- MgSO₄.7H₂O, 7g
- CaCl₂.2H₂O, 0.67g
- FeSO₄.7H₂O, 0.001g

Nitrogen and Phosphorus (N&P) solution contained (per litre):

60
Materials and Methods

K$_2$HPO$_4$  70 g  
KH$_2$PO$_4$  30 g  
NH$_4$NO$_3$.  100 g

ASW and the N&P solutions were prepared separately and autoclaved at 121°C for 15 min. Final pH was adjusted using 1M NaOH.

1% crude oil was added to 100 ml of ASW medium taken in a 250 ml Erlenmeyer flask and inoculated with 10 g of petiole cuttings containing immobilized viable bacterial cells. The flask was incubated at 37°C and 150 rpm in a rotary shaker. The experiments were done in triplicate. Samples were drawn after incubation for 72, 120 and 168 h and the rate of biodegradation was monitored by estimating the pH of the medium, total viable bacterial counts, and total residual hydrocarbons by gravimetric analysis.

3.7.3. Immobilization of viable bacterial cells on polystyrene beads

Immobilization of whole cells of viable bacteria was done on polystyrene beads which are commercially available as a packaging material according to the method of Nagendra Prabhu and Chandrasekaran, (1995). Polystyrene beads of 4-5 mm diameter were autoclaved at 121°C for 20 min during which time the beads collapsed and reduced in size (Brydson, 1982). Pre treated beads of uniform size (0.0084 cc) were selected for immobilization. 50 cc (21.786 gm) sterilized beads were used for immobilization. Log phase cells were grown in the mineral salt crude oil medium for 24 h at 37°C in a rotary shaker at 120 rpm. Cells
were harvested by centrifugation at 10,000 rpm, pellet was washed in Phosphate buffer (pH. 10) and 1gm (wet weight) cells were added to 50 ml immobilization medium. L.B medium was used as immobilization medium. Immobilization was performed by growing the cells along with pretreated polystyrene beads in an immobilization medium. The cells were attached to the support matrix and colonized the surface as a biofilm during their growth. The immobilized polystyrene beads were separated by filtration on sterilized filtration unit, washed in sterile mineral salt medium, and used for cell viability testing, SEM analysis, and biodegradation studies.

3.7.3.1. Enumeration of viable cells immobilized on polystyrene beads

Enumeration of viable cells immobilized on polystyrene beads was done as described in section 3.7.1.2 mentioned above.

3.7.3.2. Estimation of total protein content of cells immobilized on polystyrene beads

Estimation of total protein content of viable bacterial cells immobilized on polystyrene beads was estimated as described in section 3.7.1.3. mentioned above.

3.7.3.3. Scanning Electron Microscopic (SEM) examination of viable bacterial cells immobilized on polystyrene beads
Scanning Electron Microscopic (SEM) examination of viable bacterial cells immobilized on polystyrene beads was done as described in section 3.7.1.4 mentioned above.

3.7.4. Crude oil biodegradation by viable bacterial cells immobilized on polystyrene beads

Biodegradation of crude oil by viable bacterial cells immobilized on polystyrene beads was carried out in artificial sea water (ASW) with nitrogen and phosphate solution to stimulate biodegradation (Foght et al., 1989). The protocol adopted for biodegradation studies is as that described under section 4.2 mentioned above.


3.8.1. Extraction of asphaltene from crude oil

Asphaltene was precipitated from paper and washed with hot heptanes. The filter paper was placed in the extractor and extracted with n- heptanes till clear. Discarded the extract and the filtrate was then refluxed with 30-60ml of toluene till the asphaltene were dissolved completely. It was then transferred into a glass beaker and dried at 100± 10 for 30 min. (Tavassoli et al., 2011).

3.8.2. Asphaltene biodegradation.

Asphaltene biodegradation was studied in a biometric flask.100 ml. of modified BH mineral salt medium was taken in a pre sterilized biometric flask. To this 200 mg of asphaltene and 1% (w/v) of 18h of old culture were added and incubated for 14 days in an orbital shaker at 37°C
and 150 rpm. The side arm of biometric flask was filled with 10 ml of 0.1 m KOH. All flasks were incubated at 37\(^{\circ}\) C and 150 rpm in a rotary shaker. At 72, 120 and 168 h of incubation the samples were drawn for measuring CO\(_2\) from the side arm of the flask. Evolution of CO\(_2\) during the growth of bacteria utilizing asphaltene as sole source of carbon was estimated by colorimetric titration. CO\(_2\) produced during the microbial activity was captured by 0.20 M KOH solution (10 ml) located by the side arm of the biometric flasks.

To the KOH solution, 1 ml of 0.5 M barium chloride solution and 3 drops of 1% phenolphthalein indicator were added and the residual KOH was titrated with 0.1 M standard HCl solution. The amount of CO\(_2\) produced was obtained through the equation

\[
\text{CO}_2 \text{ generated (mg) = } (V_B - V_A) \cdot M CO_2 = 2 \cdot M \text{ HCl} \cdot CF.
\]

Where, VB and VA are the volume of HCl (0.1 M) used to titrate the blank and the treatment in ml, respectively.

\(M CO_2\) is the molar mass of carbon dioxide in g/mol M HCl is the molar concentration of HCl standard solution in mol/L; and CF is the correction factor for acid/base molarity (M HCl/M KOH).

At definite time intervals the growth of the bacteria was studied by measuring the reduction in pH and the number of CFU for 24 days. The biodegraded sample was extracted with hexane and subjected to GC analysis. FTIR analysis of both the control and biodegraded sample was also performed.

**3.9. Genetic study of biodegradation**
3.9.1. Isolation of Plasmid.

Isolation of plasmids from the bacteria that degraded crude oil was done using alkaline extraction procedure (Birnboim and Doly, 1979)

**Materials**

- LB medium
- TE buffer *(APPENDIX-2)*
- NaOH/SDS solution
- Potassium acetate solution
- 100% ethanol
- 70% ethanol

1. Inoculated 5ml LB medium with single bacterial colony grown in the presence of crude oil (overnight).
2. Transferred 1.5 ml of the saturated culture to a centrifuge tube and pellet the cells by spinning 20sec.
3. Resuspended the pellet in 100μl TE buffer and let sit 5min at room temperature.
4. Added 200 μl NaOH/SDS solution, mix by tapping tube with finger, and placed on ice for 5min.
5. Added 150μl potassium acetate solution and vortex at high speed for 2 sec to mix. Placed on ice for 5 min.
6. Spinned 1 min in microcentrifuge to pellet cell debris and chromosomal DNA.
7. Transferred supernatant to a fresh tube, mix it with 0.9 ml of ethanol, and kept for 2 min at room temperature to precipitate nucleic acids.

8. Spinned at room temperature to pellet the plasmid DNA.

9. Removed supernatant, wash pellet with 1 ml of 70% ethanol and dry pellet.

10. Resuspended the pellet 20μl TE buffer, resuspended DNA was used for further studies.

3.9.1.1. Agarose gel electrophoresis (Sambrook et al., 2000)

The agarose gel electrophoresis was done to visualize the plasmids

a) Agarose gel with a concentration of 0.8 % (w/v) was prepared for electrophoresis.

b) 10 μl of the DNA was loaded on to the gel and electrophoresed at 80V until the migrating dye (Bromophenol blue) had travelled two-thirds distance of the gel. Lambda DNA cut with E.coR1 and HindIII (Bangalore Genei) was used as the marker.

c) The gel was stained in a freshly prepared 0.5 mg/ml ethidium bromide solution for 20 min.

d) The gel was viewed on a UV transilluminator, and image captured with the help of Gel Doc system (Biorad).

3.9.2. PCR amplification of aromatic dioxygenase gene.
Primer of the subunits of type D iron sulfur multi-component of aromatic dioxygenase gene was purchased from sigma Aldrich.USA. It was then amplified using plasmids isolated from the bacteria in a PCR. The PCR conditions are as follows.

<table>
<thead>
<tr>
<th>Primer pair</th>
<th>Proteins targeted</th>
<th>Sequences</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>sulfur multi-component of aromatic dioxygenases.</td>
<td>16R5’ GCCACTTCCATGCACCCCATCCA-3’.</td>
<td></td>
</tr>
</tbody>
</table>


**PCR mix composition**

- **Plasmid** 100ng
- **1.5mM MgCl₂** 200 µmol
- **Deoxy nucleotide mix** 200 µmol
- **Primer** 0.1mM each
- **Taq polymerase** 1 unit
- **Tris-HCl pH 8.3** 100mM
- **KCl** 500 mM
- **Deionized water** 6 µl

**PCR conditions**

1. **Initial denaturation** - 94°C (90 sec.)
2. Denaturation - 94°C (30 sec.)
3. Annealing - 65°C-75°C (30sec.)
4. Primer extension - 72°C (2 min.)
5. Repeat step 2, 3 and 4 for 34 times
6. Final extension - 72°C (10min.)
7. Hold - 4°C (5 min.)
8. End

3.9.3. Transformation of *E.coli* DH5α with plasmid isolated from oil degrading bacteria

Plasmid isolated from the oil degrading bacteria BTTS was used to transform *E.coli* DH5α to characterize the properties of the plasmid.

3.9.3.1. Competent cell preparation

*E.coli* DH5α was used as the host cell for transformation. A single colony of *E.coli* DH5α was inoculated in 5ml Luria Bertani (LB) broth and incubated with a constant shaking of 150 rpm at 37°C overnight. 1%(v/v) of overnight culture was inoculated into 50ml LB broth and incubated at 150rpm at 37°C until the OD was 0.4 to 0.6. The cells were harvested by centrifugation at 10000 rpm for 10 min at 4°C and the pellet was suspended in 10 ml of ice cold 0.1 M CaCl₂ and incubated in ice for 30 min. The cells were harvested again by centrifugation at 4°C for 5 min at 7000 rpm. The pellet was resuspended in 1ml of 0.1 M CaCl₂. This was
aliquoted as 80 µl fractions and 20 µl chilled glycerol was added and stored at -80 until use (Sambrook et al., 1989).

3.9.3.2. Transformation of *E.coli* DH5α with Plasmids

Transformation of *E.coli* DH5α with plasmids coding for hydrocarbon degradation was performed adopting the methods of Sheikh et al.,(2003); Fujii et al.,(1997) and Trevors, (1986).10 µl of plasmid was added to 100µl of competent cells and incubated in ice for 40 min. Then the cells were given heat shock for 40 sec at 42°C in a water bath then the cells were plunged quickly on ice for 5 min. 250 µl of LB medium was added to the transformed cells and incubated at 37°C of 1hour. 50 µl of the cells were plated on LB agar plates. The agar plates were incubated in an inverted position by keeping 1 % BTEX in the lid of the plates and the plates were sealed firmly by paraffin film and kept for incubation for 24 hours. The colonies which developed on the agar plates in presence of solvent were selected as the transformed cells. The control was done in the absence of any solvent. The experiments were conducted in triplicate. Transformed colonies were further utilized for plasmid isolation to confirm transformation.

3.9.4. Curing of plasmid from *Lysinibacillus fusiformis* BTTS10

Curing of plasmid DNA was done as suggested by Hardy (1993). The bacterial isolates were grown in100ml Zobell Marine broth medium containing the hydrocarbon of preference. The cells were collected from the late exponential phase and added to media containing different concentrations of ethidium bromide ranging from 100µg/ml to 1mg /ml.
This was incubated at $37^\circ$C for 24 h. The highest concentration of ethidium bromide showing the growth of bacteria was selected. Thereafter the broth was homogenized by vortexing and loop full of the broth was sub cultured on Zobell Marine agar plates (control) and also on BH agar medium (supplemented with hydrocarbon). The colonies that failed to grow on the above medium were identified by replica plating (Sambrook et al., 1989) and were considered as cured.