4. RESULTS

4.1. Physico-chemical parameter of water samples

Crude oil contaminated water and soil samples were collected from Tiruchengode, Mallur, Pallipalayam in Namakkal, Salem and Erode districts of Tamil Nadu. Among the sample area, the soil and water samples collected from Tiruchengode have high concentration of crude oil content. Due to nature of this place the samples obtained from Tiruchengode was chosen for further studies and analysed for physico-chemical characteristics such as pH, total solids, total suspended solids, total dissolved solid, dissolved oxygen, BOD, COD, alkalinity and total hardness and the results are represented in table 4.1. The pH of the water sample was 8.4. Total solids (TS) recorded were 10160 mg/l. The total suspended solids (TSS) and total dissolved solids (TDS) present in water samples were 10020 and 140 mg/l. The concentration of dissolved oxygen (DO) was 100 mg/l, BOD was 420 mg/l and COD was 8520 mg/l in the water samples. Total hardness of 450 mg/l in the water was may be due to presence of calcium and magnesium content. The chloride and salinity were also analysed, they were 331.90 and 599.45 mg/l respectively.

4.2. Microbial diversity in crude oil contaminated water and soil

The total heterotrophic bacterial (THB) population was estimated in crude oil contaminated water and soil samples and the results are given in Table 4.2 and plate 4.1. Higher THB populations of the water samples in Tiruchengode, Pallipalayam and Erode areas were 80 x 10^4, 69 x 10^3 and 70 x 10^4 CFU/ml respectively. Similarly the soil samples contain the THB about 190 x 10^5, 190 x 10^3 and 80 x 10^4 CFU/g respectively. The maximum level bacterial populations in the soil samples obtained from Salem and Mallur areas were 60 x 10^4 and 70 x 10^4 CFU/g respectively.
4.3. **Bacterial genera in crude oil contaminated water and soil**

There are 124 morphologically different bacterial strains were obtained from the contaminated water and soil samples. The isolated strains were identified belonged to the genera *Micrococcus* spp., *Bacillus* spp., members of *Enterobacteriaceae*, *Pseudomonas* spp., *Alcaligenes* spp., *Moraxella* spp., *Acinetobacter* spp., *Vibrio* spp. and *Actinobacteria* spp. (Fig 4.1). Among the bacterial genera *Bacillus* spp. was found to be higher which was 48%.

4.4. **Primary and secondary screening of crude oil degrading bacteria**

Primary screening by 2, 6, dichlorophenol indophenol (DCPIP) was carried out for taking out the effective crude oil degrading bacterial strains among isolated. As a pattern observed in the assays, DCPIP quantification demonstrated a tendency through time, but it was not as significant as the DCPIP concentration reduction attributable to oil biodegradation by bacterial strains. These assays presented a DCPIP absorbance decrease, which indicates a positive interaction of the bacteria in oil degradation, since all “Inoculum” assays yielded a faster biodegradation. From this study, 10 effective bacterial strains namely TW2, PB15, EW4, EW6, MSS4, SS1, SSM2, SSM8, SSM9 and SSM10 were selected (Table 4.3 and 4.4; Fig 4.2). These strains were selected based on the growth and intensity of the DCPIP colour.

Further, crude oil degrading ability was confirmed by secondary screening through the gravimetric analysis. Most of the researchers used this method as a quantitative analysis to select crude oil degrading microbes under lab scale level. From this study, there are 5 effective bacterial strains namely EW4, EW6, SS11, SSM2 and SSM10 were selected based on percentage of degradation among selected in the primary screening (Table 4.5). There five strains showed significant crude oil degradation about 50, 65, 35, 35 and 35% respectively.
4.5. Identification of effective bacterial strains

4.5.1. Phylogenetic analysis

Among the five bacterial genera, EW4, EW6 and SSM2 has efficient crude oil degrading ability in the form of individual and mixed bacterial consortium. These three strains were purified by repeated streaking on nutrient agar (Plate 4.2) and processed for identification by 16s rDNA sequencing. PCR amplification of 16S rDNA gene followed by sequence was made and the results are given in plate 4.3 to 4.5. The nucleotide sequences were submitted and accession numbers have been received for all the strains from genbank database (Table 4.6).

4.6. Fungal diversity in crude oil contaminated water and soil

Maximum fungal populations in crude oil contaminated water and soil samples collected from Tiruchengode, Pallipalayam and Erode areas were $19 \times 10^3$, $1 \times 10^4$ and $3 \times 10^4$ CFU/ml in water and $11 \times 10^4$, $3 \times 10^4$ and $2 \times 10^4$ CFU/g in soil samples respectively. In these areas the soil sample contains higher bacterial populations when compared to water. When compared to those areas the fungal strains were found to be less in soil samples collected from Salem and Mallur. They were $2 \times 10^4$ and $3 \times 10^3$ CFU/g respectively (Table 4.7).

4.7. Generic distribution of fungi in crude oil contaminated water and soil samples

Totally 50 fungal isolates were obtained from crude oil contaminated water and soil and identified (Plate 4.6). About 33.3% of Aspergillus niger, 22.2% of Aspergillus flavus, 22.2% of Aspergillus fumigatus, 11.1% of Rhizopous sp. and 11.11% of Penicillium sp. were observed (Fig 4.3).

4.8. Primary and secondary screening of crude oil degrading fungi

Primary and secondary screening of crude oil degrading fungi was carried out and the results are given in figure 4.4 and plate 4.7. Among 50
fungal isolates, the *Aspergillus fumigates* (PN1) alone grown significantly in mineral salts medium with crude oil as a substrate. Hence, this strain PN1 was used as potent crude oil degraders for further studies. In primary screening, *A. fumigatus* (PN1) showed crude oil degradation in very extreme level when compared to the bacteria. The fungal strain *A. fumigatus* (PN1) degraded about 77.9% of crude oil within 60 hrs of incubation period. In subsequent study of secondary screening the strain PN1 attained about 84.4% crude oil degradation in liquid medium within 60 hrs of incubation period.

**4.9. Cultural characteristics**

**4.9.1. Carbon source utilization test**

The bacterial strains (EW4, EW6, SS11, SSM2 and SSM10) that show the maximum degradation in secondary screening was further subjected to increase the degradation rate in way of various carbon sources amendment in aqueous medium. Totally there are 6 different analytical grade carbon sources namely glucose, fructose, maltose, dextrose, sucrose and starch were selected for optimization process. In which the bacterial strains EW6 and SSM10 were utilize glucose and fructose primarily. All the bacterial strains utilized dextrose and starch as carbon source predominantly (Table 4.8).

**4.9.2. Antagonistic activity and consortium formation**

Antagonistic activity was carried out to make bacterial consortium and the results are given in table 4.9. Antagonism means a relationship between two organisms in which one is inhibiting the growth of the other (or both). In natural ecosystem, certain bacteria are used as antagonists, it will suppress (inhibit) the growth of other bacteria. In the field of bioremediation process the most of the results substantiated microbial consortia was suitable for complete remediation of many kind of environmental pollutants compare to single strain. Before forming microbial consortia antagonistic relationship of each strain with other strains was
important. In this antagonistic study the SSM10 had an antagonistic activity against all the other four strains. Due to nature of this strain, SSM10 was mislaid from effective strains and formed consortium using other four stains. Similarly, SS11 strains also had an antagonistic activity against EW4.

**4.9.3. Consortia formation**

Based on the antagonistic activity, seven different consortiums were made for crude oil remediation. Mutual metabolic activities in bacterial consortia during degradation of organic pollutants may be an interesting phenomenon that is widespread in nature and that involves two mechanisms: (i) complementation of metabolic deficiencies, in which the degrading bacteria are fastidious and depend on secondary strains that provide essential growth factors or nutrients as presented above for the degrading strain; (ii) associated metabolism, in which cross-feeding with metabolites from the degradation pathway occurs between members of the consortium. Among the consortium, the combination, EW4 + EW6 + SSM2 removes about 54% of crude oil which was maximum when compared to other consortium (Table 4.10).

**4.10. Crude oil degradation in MSM amended with coir waste as an absorbent**

In this study the bacterial consortia EW4 + EW6 + SSM2 alone removes oil content in aqueous media up to 54% with dextrose (1%) as a carbon source after 2nd day. The same bacterial consortium with coir waste as amendment removed up to 95.5% crude oil in aqueous media. There was a slight crude oil removal was noted after 12th day of incubation. The coir waste absorbed crude oil content which was floating on the surface of the medium. Once crude oil accumulated on coir wastes, the absorbent providing sufficient atmospheric contact to the bacterial species. Thus, the
bacterial consortium could degrade the oil content at higher level. This result in the figure 4.5 clearly indicates that advantage of coconut coir pith used to absorb oil in oil spill situations and it will enhance the biodegradation. Coconut coir pith floats upon water and remains afloat even when saturated with oil. Coconut coir pith absorbs oil, coolants, solvents and other oily materials that float on water.

4.10.1. Adsorption kinetics

Adsorption kinetics describes that the relationship between the equilibrium adsorption capacity of adsorbate adsorbed onto adsorbent and the equilibrium concentration of adsorbate in liquid. The adsorption capacity of coir waste increased rapidly at increasing time, and then slowly approached equilibrium. In order to establish the best-fit model, the experimental equilibrium data were fitted into two isotherm models, namely Langmuir (Langmuir, 1918) and Freundlich (Freundlich, 1906) equations.

**Table 4.11. Comparison of first and second order adsorption**

<table>
<thead>
<tr>
<th>Kinetic models</th>
<th>Parameters</th>
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<tbody>
<tr>
<td></td>
<td>R&lt;sub&gt;1&lt;/sub&gt;</td>
<td>0.89607</td>
</tr>
<tr>
<td>Pseudo-first order</td>
<td>K&lt;sub&gt;1&lt;/sub&gt;</td>
<td>0.00654052</td>
</tr>
<tr>
<td></td>
<td>q&lt;sub&gt;e&lt;/sub&gt;</td>
<td>0.296</td>
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<tr>
<td></td>
<td>R&lt;sub&gt;2&lt;/sub&gt;</td>
<td>0.9611</td>
</tr>
<tr>
<td>Pseudo-second order</td>
<td>K&lt;sub&gt;2&lt;/sub&gt;</td>
<td>0.2792688</td>
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The values of Langmuir equation constants are listed in Table 4.11. The correlation coefficient (R<sup>2</sup>) of Langmuir equations for the adsorption of crude oil by coir waste in the aqueous medium was high (R<sup>2</sup> > 0.89607), which suggested that the model was suitable for describing the crude oil adsorption system (Fig.4.6). Hence, the correlation coefficient (R<sup>2</sup>) of Freundlich
equations was in the range of 0.9611. This showed that the model was very suitable for describing the crude oil adsorption using coir waste.

Current results showed that both Langmuir and Freundlich models can be used to fit the equilibrium data. However, Freundlich model gave slightly better fitting at adsorption using coir waste, with $R^2$ values of 0.9611. This implies that the Freundlich model may be relatively more suitable to predict the adsorption of coir waste onto crude oil absorption.

**4.11. Effect of carbon sources, nitrogen sources, pH and temperature on degradation of crude oil**

The potential crude oil degradation was assessed based on the process development with bacterial consortia when cultured on a basal mineral medium using suitable carbon source. The carbon source utilization test shows that the three potent strains were utilized starch and dextrose at maximum for their growth. In the optimization using minimal media, the consortia EW4 + EW6 + SSM2 had about 44% of crude oil degradation when starch (1%) used as a carbon source on 10th day. But the same consortia exhibited maximum crude oil degradation which was 54.4% by incorporating dextrose at 1%. Hence, dextrose was selected as a significant substrate to enhance the crude oil degradation for further studies.

Fungal strain PN1 also presented notable degradation (77.9%) of crude oil when dextrose (1%) was used as a carbon source. The effect of carbon sources on crude oil degradation clearly indicates that the bacterial consortia (EW4 + EW6 + SSM2) and the fungal strain PN1 were utilized dextrose for their growth and faster crude oil degradation. Hence, 1% dextrose was finalized as optimum for further processes (effect of nitrogen source, pH and temperature).

Bioremediation by optimal conditions within contaminated aquifers are often found to be limited by the availability of nutrients, including
nitrogen. Consequently, microorganisms those are capable to degrade contaminants as well as fixing molecular nitrogen as their sole nitrogen source. The nitrogen deficient environments would be favourable for promoting *in situ* bioremediation. Here, five different nitrogen sources namely peptone, beef extract, yeast extract, casein acid hydrolysate and casein enzyme hydrolysate were used for optimization of nitrogen source. In the study carried out with bacterial consortium, about 72.22% of crude degradation was achieved using yeast extract as a nitrogen source at 35°C incubation. The other nitrogen substrates namely peptone, beef extract, casein acid and casein enzyme hydrolysate removed 50, 24.4, 67.7 and 51.1% of crude oil respectively (Fig 4.7).

The pH and temperature are the two important environmental factors which influences the biodegradation of crude oil. The pH plays a crucial role for the physiological performances of the bacterial cell. It helps to transport various micro nutrients and minerals across the cell membrane aiming at maximizing the degradation process. The bacterial consortia was inoculated in the minimal media and incubated at different pH's namely 3, 5, 7, 9 and 11. The maximum degradation activity of 72.22% was observed at 35°C in the medium at pH 7 (Fig.4.8). The other pH notably insignificant on crude oil degradation.

For the temperature optimization process, bacterial consortia were inoculated in minimal media containing crude oil and processed at different temperatures (15, 25, 35, 45 and 55°C). The optimum temperature on crude oil degradation was found to be 35°C showing 78.8% of degradation (Fig.4.9). The temperature 35°C and pH 7 was found to be optimum for the degradation of crude oil by the bacterial consortia made.

The optimization of environmental conditions, each and every parameter depends other parameters. This study shows that 1% of the yeast
extract and 1% dextrose produced maximum degradation (72.22%) at 35°C and pH 7. However, the degradation percentage slightly decreased when the pH and temperature was changed to below or above optimum, when used the same carbon and nitrogen source. This study clearly proves that pH and temperature had a significant role in environmental conditions due to degradation crude oil contaminants. Based on these results the further remediation strategies were carried out using dextrose and yeast extract since they play a suitable carbon and nitrogen source respectively. Also pH 7 and temperature 35°C was appropriately maintained.

4.12. Biodegradation of crude oil in contaminated water through a bioreactor with optimized condition

The study was conducted in a bioreactor to evaluate the degradation ability in crude oil polluted water using bacterial consortium with optimized environmental parameters. The bioreactors were continuously stirred (by impellers) at 800 rpm for 10 days experimental period. Sterile air was supplied to the bioreactors from the air compressor through hoses running in and out of them. They were sealed with Teflon to prevent the ingress of atmospheric air and were operated at room temperature (30°C) throughout the experimental period. In these study four different sets of self-designed bioreactor was continuously operated for a period of 10 days and the amount of total crude oil content was analyzed at every 12 hrs (Fig 4.10). Colour change and odour of the effluent was observed, when the process was completed on 10th day with individual and bacterial consortium (Plate 4.8).

**Bioreactor setup A:** This set up was processed using 10 litters of sterile crude oil contaminated water, 1% dextrose and 1 x 10^8 CFU/ml of bacterial consortium. The results were clearly showed that the decline of crude oil content in every 12 hrs time intervals. In the process on 7th day, the results demonstrated that there was a 95.22% of the oil degradation (Fig 4.10 and
Thus, it was noticed that increasing in the operation period of bioreactor up to 10 days, the oil content were removed up to 98% in bioreactor and the effluent was changed black to straw yellow in colour.

The physic chemical parameters of crude oil contaminated water was tested and the results are given in table 4.12. The pH level of the crude oil contaminated water was decreased from 8.4 to 7.2 after bacterial treatment followed by sand filtration. The temperature of the untreated effluent was 29°C and treated effluent hasn’t showed any major changes.

The level of total suspended solids consecutively decreased from 10020 to 1115 mg/l at the end of sand filtration. The TSS was highly decrease in before (10,020 mg/l) and after the bioreactor treatment processed crude oil contaminated water (1025 mg/l). Similarly the total dissolved solids decreased from 140 to 90 mg/l. The amount of total solids was decreased from 10160 to 1115 mg/l. The BOD of bacterial consortium treated was 20 mg/l whereas in effluent it was untreated 420 mg/l. Other parameters namely COD, hardness, salinity and chloride were also found decreased.

**Bioreactor setup B:** In this setup about 10 litters of sterile crude oil contaminated water was processed with 10 x 10⁷ CFU/ml of bacterial consortium. There is no carbon source was amended in this study. The results revealed that there was 95% of crude oil degradation within 10 days period (Fig 4.10 and 4.11).

The physic chemical parameters of crude oil contaminated water was investigated and the results are given in table 4.12. The pH level was decreased in the oil contaminated water from 8.04 to 6.4 when bacterial consortium alone incorporated. The temperature of the untreated effluent was 29°C and treated effluent hasn’t showed any major changes. The level of total suspended solids consecutively decreased up to 1825 mg/l after the sand filtration. Similarly the total solids, total dissolved solids were found to
be decreased from 10160 to 1935 mg/l and from 140 to 110 mg/l respectively. The BOD of untreated effluent was 420 mg/l. It was drastically decreased to 120 mg/l. The other parameters in this study were reduced significantly when compared with untreated water.

**Bioreactor setup C:** This set up was performed in 10 litters of unsterile crude oil contaminated water incorporated with 1% of dextrose and $10 \times 10^7$ CFU/ml of bacterial consortium. The results of this set up showed that there was a 90% of the crude oil removed within 10 days (Fig 4.10 and 4.11).

Physico-chemical parameters were analysed before and after treatment. The physical natures of the treated water are given in table 4.12. The pH of the crude oil contaminated water after treatment was decreased from 8.04 to 6.4. The temperature of the untreated effluent was 29°C and there is no major change noted after treatment. The TSS was 10,020 mg/l recorded before treatment. After bioreactor treatment the amount of TSS was reduced up to 2000 mg/l. Similarly the total dissolved solid was decreased from 140 to 120 mg/l after bacterial treatment. There was considerable levels of total solid was reduced (from 10160 to 2120 mg/l). The BOD in bacterially treated was 90 mg/l. In untreated it was found to be 420 mg/l.

**Bioreactor setup D:** In the setup D, about 10 litters of unsterile crude oil contaminated water was processed with $1 \times 10^8$ CFU/ml of bacterial consortia. The results of this set up represented that after treatment there was 1263 ppm of crude oil content was remaining. Here, about 80% of the oil was degraded within 10th day (Fig 4.10 and 4.11).

The physic-chemical characteristics of the treated water assessed and the results are given in table 4.12. The pH level of the crude oil contaminated water was decreased from 8.04 to 6.4 when bacterial consortium alone incorporated. The temperature of the untreated effluent was 29°C and treated effluent hasn’t showed any major changes. Total suspended solids
were consecutively decreased from 10,020 to 1,985 mg/l after the sand filtration. Similarly the total dissolved solids were decreased from 140 to 115 mg/l. In the case of total solids, the level was decreased from 10160 to 1800 mg/l. The BOD in bacterially treated water was 320 mg/l. In untreated effluent it was 420 mg/l.

Among four setup made, the setup A was gave a 98 % degradation of crude oil. This result clearly shows that the sterilization and carbon source were not very much influencing the degradation process using bacterial consortia. The unsterilized effluent gave a 95 and 80% of degradation. The degradation difference was occurred mainly due to because of antagonistic activity of bacterial consortia and natural micro flora in crude oil contaminated water.

4.13. Removal of crude oil from contaminated soil through different column treatment using aerobic bacterial consortium

The individual and bacterial consortium on the remediation of crude oil in contaminated soil was examined over a period of one month using various columns packed with soil. In this study there are four different manual soil column setups were equipped. First three columns were run with synthetic water enriched with individual bacterial strains (EW4, EW6 and SSM2) and the fourth column was run with mixed bacterial consortium.

Crude oil degradation was carried out in contaminated soil with dextrose (1%) as a carbon source. The initial crude contamination in soil was 7000 ppm. During this study, every 12 h, the decreased oil contents was analysed in the effluent obtained from four set up columns and the results are given in Fig. 4.12.

The influence of bacterial consortium (Treat A) on the crude oil degradation was significant in the contaminated soil treated through the column study. In the treatment A, the residual crude oil content in the
effluent obtained from the column was 1432 ppm. There was notable oil content was observed when the samples collected in the subsequent days. On final day, the amount of residual oil was noted about 247 ppm.

In the column treated with the bacterial strain *Bacillus* sp. EW4 (Treat B), the residual oil content was noted about 1284 ppm on 1st day. When the day was increased the solubility of the oil content was reduced. On final extract, the oil content was found that 290 ppm. Similarly the *Bacillus* sp.EW6 (Treat C) was delivered degraded crude oil content about 1004 and 214ppm on 1st and final day respectively. When compare to EW4, the EW6 attributed a maximum level of crude oil degradation. However, compared to the above strains the *Bacillus* sp. SSM2 (Treat D) showed less amount of crude oil degradation. On 1st day the residual oil was noted about 668 ppm. By using this strain the degradation rate also decreased when the process day was increased.

After 30 days experiment, the column set up was dismantled and treated soil was collected, dried and measured the total crude oil content by the same toluene extraction method (Fig.4.13). The soil colour and pH was significantly changed in the treated soil (Plate 4.9). The colour of the soil was turned dark black to brownish. The pH range was elevated from 8.4 to 7.4. The total crude oil removal in the treated soil by *Bacillus* sp.EW4 was 73%, Similarly, about 84% of crude oil was removed in the soil treated by *Bacillus* sp. EW6. There was lower (56%) crude oil degradation was recorded in the soil treated by *Bacillus* sp.SSM2. In the case of bacterial consortium attained a maximum level of degradation which was 93%. The results clearly showed that bacterial consortium could be a notable contestant on the crude oil degradation in contaminated samples.

The water and soil samples after treatments were extracted with dichloromethane. The extracts were taken and analyzed in GC-FID for quantitative of total hydrocarbon, GC-MS used for identification of unknown compounds.

4.14.1. GC-FID and GC-MS

The extracts of the treated crude oil contaminated water and soil were analysed with GC-FID and GC-MS for the detection of residual crude oil, secondary compounds and their toxic nature (Plate 4.10 to 4.16). The chromatogram showed that several peaks at the retention times between 1.67 to 24.52 minutes. Before analyzing GC-MS the GC-FID used to identify the quality of the sample and several peak variations. To identify the percentage of crude oil removal attained based on the area spotted in GC-FID spectrum. Here, the bacterial consortia (set up A) removed maximum percentage (99.97%) crude oil in contaminated water after bioreactor treatment. Crude oil degradation in contaminated soil also the bacterial consortium showed a maximum percentage (96.90%) of removal when compared to the individual strains (Table 4.13 and 4.14).

The results of GC/MS analysis confirmed that the capability of the bacterial consortium to degrade crude oil. Lower cases peaks from decane (C_{10}) to pentadecane (C_{15}) were detected in the crude oil at the beginning of the incubation experiment and low-molecular alkanes (<C_{10}) were not detected, either because these components were rapidly volatilized or biodegraded. After treatment the residual oil showed a sharp decrease of component abundance in the range from decane (C_{10}) to pentadecane (C_{15}), thus confirming that the bacterial consortia was capable to degrade a broad range of petroleum hydrocarbons within 30 days, including crude oil derivatives.
The GC-MS spectra listing the compounds detected by GC/MS analysis of extracts of the various soil and water samples treated with bacterial consortium obtained from the soil column and bioreactors, only the main compounds from the hit lists of probability-based matching (PBM) search are given.

The compounds detected by GC/MS analysis of the soil column extracts treated by bacterial consortium and individual strains. The data reflects the fact that the soil used in this investigation was sampled from the dumping area of a refinery where the initial pollutants were of very diverse composition, i.e., a mixture of crude oil, masut, diesel, middle distillates, heavy distillates, kerosene, etc. The untreated soil and water sample contained a large variety of straight-chain hydrocarbons and their methyl derivatives (both those with even and odd numbers of C atoms), many of which persisted during the treatment.

However, many of the aromatic hydrocarbons found in the untreated soil, mainly substituted polycyclic aromatic hydrocarbons, were not detected in the samples of treated soil, thus showing lower persistence than the alkanes. The absence of volatile components may indicate that the contamination was caused by heavier oil fractions, but is most likely caused by the loss of lighter components by evaporation and biodegradation. According to a previous investigation, the \( n \)-alkanes \( \leq C20 \) disappear very quickly, leaving behind isoprenoid structures; this was confirmed by the presented data.

At the start of the process, the soil sample contained a large variety of straight-chain hydrocarbons and their methyl derivate. Many of these compounds, in particular branched alkanes, were also detected after the treatment. Several new compounds were found at the end of the experiments, including mainly unsaturated \( n \)-alkenes and different derivate
of cycloalkanes. It was very difficult to establish which compounds originated exclusively from the spilled oil and which were of natural origin, but data clearly showed that the number of organic compounds extracted from the soil decreased during the treatment process.

4.15. Bioremediation of crude oil in contaminated soil through a soil column with biosurfactant

4.15.1. Preliminary determination and production of biosurfactant

Biosurfactant producing ability of the selected isolates was screened. In this study, the bacterial strains *Bacillus* sp. (EW4) KJ600629, *Bacillus* sp. (EW6) KJ600630 and *Bacillus* sp. (SSM2) KJ600631 and a fungi *A. fumigates* (PN1) was used as efficient strain to produce biosurfactant based on the results of emulsification index, foaming index and oil spread assay (Plate 4.19). The range of oil displacement activity by the bacterial isolates was ranged from 1.3 - 2.5 cm (Fig 4.14). The strain SSM2 exhibit lowest activity of 1.1cm and the highest activity of 2.5 cm were scored by the strain EW6.

The emulsification index was carried out by comparing biosurfactant (cell free supernatant CFS) with synthetic surfactants (SDS and Triton X 100) using different hydrophobic phases (petrol, diesel, kerosene, used engine oil and fresh engine oil). The CFS emulsifies 50% of petrol and fresh engine oil, 23% of kerosene and used engine oil and 16 % in diesel (Fig 4.15; plate 4.17). The synthetic surfactants gave a very significant emulsification rate of 63% when compared to the biosurfactant. However, the literature says that the synthetic surfactants are toxic to environment and natural ecosystem and cannot be applicable.

4.15.2. Optimization of carbon source on the production and extraction of biosurfactant

Now a day the carbon sources play an important role on microbial activity. But they needed suitable carbon sources for production of efficient
biosurfactant which will be helpful to enhance the product quantity and also the potentiality. Here, glucose, dextrose, fructose, starch, cellulose and sucrose were used as a carbon sources for biosurfactant production to increase the yield of biosurfactant. The dextrose promotes higher oil removal (97.7%) from the medium and showed, 2 cm clear zone in oil spread assay (Fig 4.16).

Several natural sources were used as an alternative nutrient source for the production of biosurfactants. These sources gave a fine and efficient production of biosurfactant (Fig. 4.17). The cotton seed acted as greater efficient on the production of biosurfactant than the analytical grade dextrose. Dextrose was produced around 0.5g/l, but when cotton seed used as a substrate with the bacterial consortia and also with fungal strains, yielded up to 3g/l. Hence, the cotton seed was used for production of biosurfactant in a lab scale (1000 ml flasks) using continuous stirring with magnetic stirrer (Plate 4.18). After incubation period the biosurfactant was extracted by solvent extraction method using separating funnel.

4.15.3. Characterization of the biosurfactant

The preliminary analysis demonstrated that the composition of biosurfactant which was extracted from A. fumigates (PN1) as a glycoprotein, consists 70% of protein and 15% of carbohydrates. In the case of bacterial consortia based biosurfactant contains 85% of protein and 20% of carbohydrates. The agar double diffusion test revealed that the appearance of precipitation lines between the bacterial biosurfactant and the ionic compounds used. No lines formed between the biosurfactant produced by A. fumigatus (PN1) and the ionic compounds.

4.15.4. Ionic nature of the biosurfactant

Compound in which molecules are held by ionic bonds; with opposite charges (cations +ve charges and anions –ve charges) are called ionic compounds. Depending on their charge characteristics the surface-active
molecules can be categorized as anionic, cationic, zwitterionic (ampholytic) or non-ionic. Under the experimental conditions, this test results showed that the non-ionic character of the fungal biosurfactant and anionic character of the bacterial biosurfactant.

4.15.5. **Microbial toxicity test**

Significant property of many biosurfactant that has not been reviewed in-depth, may certain antimicrobial property. Other medical relevant of biosurfactants include their role as anti-adhesive agents to pathogens, making them useful for treating many diseases and as a therapeutic agents. The antimicrobial property of biosurfactants to cell surfaces caused determination in the integrity of cell membrane and also breakdown in the nutrition cycle. The results of well diffusion method clearly reveals that, the biosurfactant produced from *A. fumigates*(PN1) and bacterial consortium had there is no antibacterial properties against tested bacteria (*Rhizobacteria* sp. (PGPR) *Azotobacter* sp. (Nitrogen fixers), *Azospirillum* sp. (Nitrogen fixers) and *Pseudomonas* sp.(Phosphate solubilize), *Bacillus* sp. (Phosphate solubilize). These compounds are non-toxic in nature and it cannot affect any natural soil beneficial micro flora.

4.15.6. **Stability study of bacterial biosurfactant: Effect of temp, pH and NaCl concentration**

The good emulsification stability is critical for biosurfactant to be promising in different environmental conditions and also for industrial applications (Plate 4.20 and 4.21). The effect of pH, salinity and temperature on the emulsification activity was checked using cell-free supernatant against crude-oil (Fig 4.18).

The emulsification activity of cell-free supernatant was carried out with six different hydrocarbon sources at various pH from 2 to 12. This suggested that the activity of this biosurfactant is limited to acidic pH. The
highest emulsification ($E_{24}$) activity was 83.3 % at pH 7. When increased the pH 8, the $E_{24}$ also increased up to 95% in biosurfactant produced by bacterial consortium.

The results obtained from the present work suggests that the bacterial consortium was moderately halophilic showed a maximum emulsification ($E_{24}$) activity about 83.3 and 73.3% at 2 and 4% of NaCl concentrations respectively.

The temperature was one of the critical parameter that has been controlled in bioprocess. The result in the present study revealed that there was no notable changes on biosurfactant activity obtained when the temperature reached to highest 70, 100 and 120°C and obtained $E_{24}$ about 83.3, 66.6 and 73.3% respectively. This was clearly indicates that biosurfactant was the thermostable in different environmental conditions. The competence of the biosurfactant produced from strain for emulsification of crude-oil indicates that it can be applied as a potent tool to highly crude oil contaminated places.

**4.15.7. Stability study of fungal biosurfactant: Effect of temp, pH and NaCl concentration**

The effects of pH, salinity and temperature on the emulsification ($E_{24}$) activity was checked by using cell-free supernatant of fungal culture against crude-oil (Fig 4.19). In the pH ranges from 2 to 12, the emulsification activity of cell-free supernatant was carried out with six different hydrocarbon sources. This suggested that the emulsification activity of this biosurfactant is limited to acidic pH. The highest emulsification ($E_{24}$) activity was 83.3%. At the same time when increased the pH up to 8, the $E_{24}$ showed 95% in biosurfactant produced by *A. fumigatus* (PN1).

The results obtained from the present work suggests that the biosurfactant was moderately halophilic showed a maximum emulsification activity about 95 and 75% at 2 and 4% of NaCl concentrations respectively.
The result in the present study revealed there was no notable changes of biosurfactant activity was obtained when the temperature reached to highest 70, 100 and 120°C. The \( E_{24} \) was showed about 83.3, 68 and 74% respectively. This was clearly indicates that biosurfactant was the thermostable in different environmental conditions. The competence of the biosurfactant produced from strain for emulsification of crude-oil indicates that it can be applied as a potent tool to remediate crude oil contaminated places.

4.15.8. Effect of biosurfactant on the removal of crude oil from contaminated sand under batch mode condition

Biosurfactants were used to emulsify the crude oil and enhance the water solubility, decreasing surface tension and increasing the displacement of oil substances from soil particles. Satisfactory results were obtained from crude oil contaminated sand by the cell-free broth (crude biosurfactant) from *A. fumigatus* (PN1) and bacterial biosurfactant with the maximum removal rates of 80 and 95% respectively when compared to the control (distilled water) (Plate 4.22). This method was used to identify the efficiency of the biosurfactant on artificially contaminated sand. This study revealed a good emulsifying property of both bacterial and fungal biosurfactants. It can be a efficient technique to emulsify the naturally polluted soil or sand.

4.15.9. Remediation strategies of crude oil contaminated soil using biosurfactant and synthetic surfactant through column study

Column study was performed to remediate the crude oil from contaminated soil. The surfactant emulsified soil column extracts were collected periodically at every 12 hrs treatment. The total oil contents of the extracts were examined by toluene extraction method followed by spectrophotometric analysis at 420 nm. In this study thirteen different soil column setups were equipped. The different types of liquid phase (emulsifiers) were passed through the column using peristaltic pump.
4.15.9.1. Effect of bacterial biosurfactant on crude oil removal from contaminated soil through column studies

Crude oil emulsification was carried out in contaminated soil using various concentrations (0.5, 1.0 and 1.5%) of bacterial biosurfactant and the results are given in Fig. 4.20. The initial concentration of crude oil in soil was 7000 ppm. In control the maximum solubility of crude oil was 635 ppm at 12 hrs interval and thereafter decreased rapidly. In case of bacterial surfactant at 1.5% the emulsification was noted as maximum from 967 to 195 ppm during the treatment process. The emulsification rate was increased when the concentration of the biosurfactant increased. In this case the bacterial biosurfactant highly compete one with synthetic surfactant when increased the concentration to 1.5%. After treatment the soil were dried and extract the total residual oil content. Based on the results of toluene extraction method the crude oil emulsification in the treated soils were 57, 68 and 43% by 0.5, 1.0 and 1.5% of the bacterial biosurfactant respectively.

4.15.9.2. Effect of fungal biosurfactant on crude oil removal from contaminated soil through column studies

The crude oil emulsification was carried out in contaminated soil using different concentrations (0.5, 1.0 and 1.5%) of biosurfactant produced by the fungal strain *A. fumigates* (PN1). Fig 4.21 shows the emulsification of crude oil in every 12 hrs treatment. In control the maximum solubility of crude oil was 635 ppm and thereafter decreased rapidly. The fungal surfactant at 0.5%, the emulsification was showed from 1123 to 435 ppm. When the concentration of the biosurfactant was increased the emulsification rate also increased. In this case the fungal biosurfactant when the concentration was increased to 1.5% the emulsification efficiency were decreased vigorously. Fungal biosurfactant showed less efficiency on
crude oil emulsification when compared to the bacterial biosurfactant. After treatment the soil were dried and extract the total residual oil content. Based on the results of toluene extraction method the emulsification of crude oil content in the treated soils were 5.2, 53 and 2.9 % by adopting 0.5, 1.0 and 1.5% of the fungal biosurfactant respectively.

4.15.9.3. **Effect of synthetic surfactants on crude oil removal from contaminated soil through column studies**

The crude oil emulsification was carried out in the column packed with contaminated soil using two different synthetic surfactant (SDS and Triton X 100) at various concentrations (0.5, 1.0 and 1.5%). Fig 4.22 shows the emulsification of crude oil in every 12 hrs treatment. The initial concentration of crude oil in soil was 7000 ppm. In control the maximum solubility of crude oil was 635 ppm and thereafter decreased rapidly.

In the case of SDS at 1% was emulsified as a maximum level of crude oil from 1769 to 780 ppm. When the concentration of the SDS surfactant were increased the emulsification were highly increased. In this case, the SDS was emulsified crude oil content significantly from the contaminated soil when compared to the fungal biosurfactant. However, the bacterial biosurfactant efficiency was high when compared to the SDS. After treatment the soil were dried and extract the total residual oil content. Based on the results of toluene extraction method the crude oil was emulsified in the soil up to 22, 26 and 14 % by using 0.5, 1.0 and 1.5% of the SDS respectively (Fig. 4.22).

The emulsification of crude oil by triton X 100 was noted from 1581 to 267 ppm at 1.5% (Fig. 4.23). While the concentration of the synthetic surfactant were increased the emulsification were highly increased. Comparative of all surfactants, the triton x100 showed a very good emulsification activity in the oil contaminated soil. After treatment the soil
were dried and extract the total residual oil content. Based on the results of toluene extraction method the crude oil contents were degraded up to 46, 78 and 2.9% by 0.5, 1.0 and 1.5% of the SDS respectively. Among all the emulsifiers the triton x100 showed 78% of crude oil emulsification in contaminated soil through the soil column process (Fig. 4.24). However, the chemical surfactants were also having high emulsifying activity compared to the biosurfactant, but the synthetic surfactant is very toxic to the environment and also it’s having the high foaming activity.

4.15.9.4. Statistical significance between various emulsifiers

Correspondingly both chemical surfactants showed higher emulsifying activity than the biosurfactant. Significance between various concentrations biosurfactant and synthetic surfactants was analysed using SPSS package Version 16.0 and the results are given in table 4.15 to 4.17. In this study, the significance between biosurfactant and synthetic surfactant found to be very less. However, SDS and Triton X 100 emulsified crude oil at 5% significant level. Analysis of significance shows that there was no significant difference (p > 0.05) for both bacterial surfactant and fungal surfactant. Hence, the bacterial surfactant when increase the concentration competes with chemical surfactant at 5% significance level. The significance between two chemical surfactants was very less, at 1% significant only present in high concentration of SDS. Compared to SDS the triton X100 were gave a high significance when compare with bacterial biosurfactant. In the overall study bacterial biosurfactants have a great emulsification activity at compare to fungal biosurfactant. It also forms a more or less equal emulsification to chemical surfactants.

4.15.9.5. Instrumentation analysis

The most important to identify the percentage of removal based on the area spotted in GC-FID spectrum. Here the bacterial consortia removed
maximum percentage in contaminated soil in biosurfactant treatment. The quantitative result of GC-FID clearly proves biosurfactant highly compete with synthetic surfactant in emulsification of crude oil contaminated soil (Plate 4.23 to 4.31a, b). In case of crude oil degradation in contaminated soil using synthetic surfactant shows a maximum percentage (98.37%) of removal compared to the single strains. But the fungal biosurfactants remediated higher than the synthetic surfactant (98.96%). Bacterial biosurfactant also emulsified crude oil in greater percentage (95.98%) (Table 4.18).

In the biosurfactants study the extractions of soil samples after degradation of 10 days were analysed by GC-MS. The results of the GC-MS clearly indicates that the removal efficiency of oil components in the samples with biosurfactant were higher than in the blank sample and also most compete with synthetic surfactants. Comparing the GC-MS spectrum for control (distilled H₂O) with biosurfactant, which were left stagnant after 10 days, 32 peaks were shown in the treated soil sample (control) spectrum while the treated with biosurfactant showed only 14 peaks in the GC-MS. That is branched alkanes, alkene, carotane and alkylnaphthalenes and was thoroughly degraded.

Gas chromatography of the saturate fractions from the soil column indicated that oil content was degraded better with the help of biosurfactant. The hydrocarbons involved in the saturate fraction were identified in the range of C₁₂ to C₃₅ in treated with synthetic surfactant. The biodegradation, the aliphatic fraction analysed showed that chromatographic profiles of this fraction had a different degradation pattern. Treatments bacterial biosurfactant and fungal biosurfactant showed a preferable removal of C₁₂ to C₂₉ compounds, as compared with the relative accumulation of saturates above C₂₉ at the end of the treatment period. Moreover, in treatment with
synthetic surfactants a slight different chromatographic profile was observed, in this case there was a greater extent of consumption of C\textsubscript{19} to C\textsubscript{23} compounds than in treatment.

4.16. Toxicity study of the treated water and soil

4.16.1. Phytotoxicity assay

The germination index, of the relative seed germination and the onion root tip assay was used to evaluate the toxicity. As a germination index value of 80\%, it indicates the phytotoxicity of biosurfactant and also indicates that the biosurfactant solutions tested with remediated sample did not have any inhibitory effect on seed germination and root elongation. The results clearly show the leaf growth and the elongation of secondary roots occurred under all biosurfactant treated soil samples. The 90\% of the plant growth was inhibited by synthetic surfactants (Table 4.19 to 4.32; plate 4.32). This study clearly proved that the chemical surfactants were highly toxic to the plants.

Thus it is very essential to test the toxicity of the treated and untreated crude oil contaminated water and soil on the agricultural seeds. This is the common methods employed to study phytotoxicity are monitoring of seed germination and plant growth. Effect of crude oil contaminated water and soil on the seedlings of green gram is the main and primary toxicity study to assess the toxic nature of hydrocarbon molecules. The seeds of green gram showed a higher percentage of germination in distilled water and, treated crude oil contaminated water and soil. At the same time the length of the shoot and germination was found to be lower in the untreated water and soil. There is no growth was recorded in the study performed with synthetic surfactant.

There was a remarkable performance in the germination percentage of green gram seeds under biologically treated crude oil contaminated water to
about 83.66 - 81.31 % when compared to that of the Control (Group I) on 10th day, respectively. Further, biologically treated wastewater did not show any inhibitory effect on seed germination. Moreover adversely reduced the seed germination as in green gram in untreated crude oil contaminated water.

The reduction in germination percentage in untreated water might have been due to presence of high concentration of hydrocarbon and other toxic organic compounds that cause a range of cellular toxicities. Although the osmotic potential of the wastewater was not recorded, it is also possible that the presence of high amount of salts and organic compounds in untreated wastewater reduces the availability of water thereby resulting in reduced germination. This aspect is further supported by the fact that the presence of high salts in water or soil reduces the germination and early growth of plants by salt-induced osmotic stress.

4.16.2. Genotoxicity assay

The Allium oschyaninii test has been used by many researchers mainly as a bio indicator of environmental pollution. In this study, the potential cytotoxic and genotoxic effects of soil column extracts, treated soil and bioreactor treated water on Allium oschyaninii were evaluated (Plate 4.33). Results showed that there was a linear relationship between macroscopic and microscopic parameter. In macroscopic observation found that Allium oschyaninii root growth was decreased. It indicates that the samples were toxic; our results showed other aberrations, also induction of sticky chromosomes, bridges and disturbance of spindle fibers at different stages of mitotic division in the onion root cells.

Plant systems have a variety of well-defined genetic endpoints including alterations in ploidy, chromosomal aberrations and sister chromatid exchanges. Reduction in the mitotic activity might be due to inhibition of DNA synthesis or a blocking in the G2-phase of the cell cycle,
preventing the cell from entering mitosis. That exposure of root tips to high concentrations of the hydrocarbon has led to inhibition of DNA synthesis.

Several types of aberrations were noted in some specific phases of chromosomes. Here, mainly two types of aberrations were illustrated that is structural aberrations (gaps, breaks, dicentric chromosomes, ring chromosomes) and numerical aberrations (polyploidy (4n), hypodiploidy (<46chr), hyperdiploidy (>46chr).

The frequencies of chromosomal aberrations increase with chemically treated crude oil polluted soil. The differences among the concentrations have been significant, when compared with untreated control. The most frequent aberrations are break, gap and multiple breaks (Plate 17). The reduction in the mitotic activity increases when the concentration has been increased from 0.5 to 1.5% of synthetic surfactant.

Based on the previous reports related to this assay, the extracts from chemical surfactants were inhibiting the root growth compare to biosurfactant. Based on the microscopic assessment, the root tips from chemical surfactants were had a changes in morphological aberrations (Plate 4.10). In microscopically A. oschyaninii, a chromosome aberration was occurred and also a growth restriction was found. Most of these aberrations are lethal, which can cause genetic effects, either somatic or inherited. Based on these potential results of genotoxicity study comparison with the control (untreated soil and water) and the treated soil and water shows more over equal efficiency to normal fertilizer soil and water. This study revealed that very moderate aberration only present in treated with remediated water, since these type of aberration was present in normal plants also, when compared with untreated crude oil contaminated water the onion was not grown well and spoiled within two to three days and also changes in morphological aberrations, It indicates treated soil doesn’t have genotoxicity.
The phytotoxicity and genotoxicity assay provides a rapid, economical deadliness assay that has been used to measure the response of the green gram and *A. oschyaninii* to chemical agents in treated with various treated contaminated soil and water. The test appears to be less sensitive and more variable than the other bioassay. Also it was found sensitive to toxic components of crude oil and was used successfully to monitor oil residues toxicity during bioremediation, but appeared less sensitive or too variable in some other circumstances.