CHAPTER 5

5. RESULTS

5.1 Isolation and Identification of Bacterial Strains

In the present study, 169 bacterial strains in total were isolated from the sediment and water sample collected during various periods from Ennore creek, Chennai. Out of which 95 strains are from sediment and 74 strains are from water sample. The bacterial inoculum is shown in Figure 1 and the bacterial density of the strains during different time period is tabulated in Table 1.

Isolation was carried out with the aim of getting efficient biosurfactant producing strains. Hence, all the 169 strains were tested for their ability to produce biosurfactant in the culture medium. The inoculated medium in which the surface tension was reduced significantly was chosen for further screening process.

5.2 Screening of Biosurfactant Producing Bacteria

Among the 169 strains, only 26 strains were selected based on their biosurfactant production in the culture medium and they are subjected to further screening tests like drop collapse test, hemolytic assay, oil displacement capacity, and emulsifying property to identify the most efficient strain.

5.2.1 Drop Collapse Test

In drop collapse test, all the 26 strains showed positive results, whereas 13 strains showed very good activity (Figure 2A and Table 2). Each drop of culture media collected from these 13 strains was collapsed within 1 min of placing it in on the oil coated glass surface. The good activity indicated the presence of biosurfactant in the culture media.
5.2.2 Haemolytic Assay

All the 26 strains were streaked onto the blood agar plate in order to determine its haemolytic activity and also to find the type of haemolysis caused. All the strains tested showed positive results for haemolysis, among which, 15 strains showed $\alpha$-haemolysis and 11 strains showed $\beta$-haemolysis (Figure 2B).

5.2.3 Oil Spread Method

Similar to the drop collapse test, the oil spread method showed positive results for all strains tested, whereas 9 strains showed very good activity in displacing the oil placed on the glass surface. The formation of clear zones was very quick for these 9 strains. This zone of displacement proves the production of biosurfactants (Figure 2C and Table 2).

5.2.4 Surface Tension Measurement

Biosurfactant production was also measured in terms of surface tension reduction which was carried out in the tensiometer. Out of the 26 strains, only 2 strains, namely, ES39 and EW20 showed the reduction of surface tension below 40 dynes/cm (Table 2).

5.2.5 Emulsification Index

The emulsification index is an important parameter in judging the efficacy of biosurfactant produced. The emulsification index was calculated in terms of percentage. The emulsification index stability designates the strength of a surfactant. It was shown that (Table 2) the strain ES39 had the highest emulsification index among all the other strains tested. It had the highest emulsification index of 72% in the culture medium. The emulsification index stability determines the strength of a surfactant.

Thus, the strain ES39 showed positive results for all the above mentioned screening test, and it was also found to have the highest emulsification index and surface tension below
40 dynes/cm. Hence, the strain ES39 was selected as the candidate species for further process.

5.3 Morphological and Biochemical Identification of the Microbe

For the identification of bacterial species, morphology of the isolate, its biochemical and physiological characteristics were observed. Gram staining was performed to know whether the isolate was Gram (+)ve or Gram (-)ve. Oxidase reaction test, gelatin hydrolysis test, catalase test, urea, citrate, nitrate, starch, and indole tests were performed to get an idea about the biochemical activities of the strain. The results are tabulated in Table 3.

The strain showed negative in gram staining test, it was rod shaped, short, creamish in color, had an undulated margin, concave, found irregular in shape, and opaque. By means of biochemical identification, it showed positive results for tests like oxidase, catalase, urea and citrate, whereas negative for carbohydrate, gelatin, DNAase, nitrate, starch and indole test.

From the morphological and biochemical identification, the strain showed a high similarity to *Pseudomonas*. However, due to the intrinsic limitations, the morphological and biochemical test can only provide a preliminary identification, hence, to confirm the results obtained, 16s rDNA analysis was performed.

5.3.1 16s rDNA Sequencing

The final identification of the bacterial strain was accomplished by combining the alignment results of the 16s rDNA sequence. A similarity search for the nucleotide sequence of the test isolates was done using BLAST search at NCBI. Alignment of the 16s rDNA gene sequence of the bacterium with sequence obtained from BLAST search revealed similarity to *Pseudomonas putida*. The results are shown in Figure 3. The sequence of 377 bases are as follows:
5.4 Plasmid Curing

Result of the plasmid analysis revealed that, *P. putida* was found to harbor two plasmids of molecular weight of 4.2 and 3.8 kb.

When wild strains as well as plasmid cured cells were inoculated in the MSM with 0.5% corn oil as a sole carbon substrate (Figure 4), it was found that, wild cells produced rhamnolipids at a concentration of $3.65 \pm 0.11$ g/l and the plasmid cured cells could produce rhamnolipids at a concentration of $3.78 \pm 0.16$g/l. The emulsification index of wild cells was found to be 72.42 %, whereas, the plasmid cured cells had better emulsification index of 73.35%. The results are tabulated in Table 4. Values with the same superscript in the table are not significantly different at $P > 0.05$ level according to Duncan’s multiple range test.

The results show that the rhamnolipids concentration and the emulsification index was slightly higher in the plasmid cured cells when compared to the normal wild cells.

5.5 Characterization of Biosurfactant Produced

The biosurfactant produced was extracted from the culture medium and are then characterized. Extraction and quantification of the biosurfactant was carried out to determine the yield of the biosurfactant. Biochemical and spectral analyses were conducted to find out the composition and nature of the biosurfactant.
All the strains which were streaked onto the blue agar plate developed blue halos around the colonies, indicating the production of biosurfactants. If the biosurfactants secreted by the microbes growing on the plate are of glycolipid in nature, they form dark blue halos (Siegmund and Wagner, 1991). Thus, in the present study, the development of blue halos around all the colonies confirmed that the biosurfactant produced by them are all glycolipid in nature.

5.6 Optimization Conditions for the Production of Biosurfactants

5.6.1 Environmental Factors

Biosurfactant production can be enhanced at an optimized pH and temperature, therefore, optimization of different environmental conditions for the strain ES39 was carried out to achieve the highest production of biosurfactant. Hence, the bacterial strains were inoculated at different temperature and pH to find the suitable requirement of the strains. Normal as well as cured cells were taken for all the optimization studies. For all the optimization procedures, values with the same superscript in the table are not significantly different at $P > 0.05$ level (Duncan’s multiple range test).

5.6.1.1 pH and Temperature

The bacterial inoculums containing normal as well as cured cells were maintained in a range of temperatures starting from 25°C to 50°C to determine the best one required for the production of biosurfactants. The results (Table 5) show that the highest concentration of $3.89 \pm 0.11$ g/l of rhamnolipids was produced at 35°C. The concentration of rhamnolipid was found to increase with increase in temperature and beyond 35°C, the concentration decreased. Hence, it was chosen as the optimal temperature.
The pH of the media has also influence on the production of biosurfactants. Hence, a range of pH was evaluated with the aim of selecting the most suitable one for the highest production by ES39 strain.

For the estimation of optimal pH, bacterial inoculum containing normal and cured cells were maintained at 35°C and the pH of varied range (5.0–7.5) were evaluated. From the results (Table 5), it is proven that pH 7.0 was the optimal pH required for the production of biosurfactant. At pH 7.0, the rhamnolipid concentration was found to be $3.72 \pm 0.12$ g/l which was the highest and beyond which the concentration was found to decline. Values with the same superscript are not significantly different at $P > 0.05$ level according to Duncan’s multiple range test.

The results show that with the increase in the pH and temperature the production of biosurfactants also increased up to a level beyond which the rate decreased. The level in which the production was found to be at the peak was recorded as the optimal, beyond the optimal, the rate of production gradually decreased.

In the present study, the optimal temperature was found to be 35°C and pH was 7.

### 5.6.1.2 Incubation Time

Rhamnolipid concentration was investigated at various incubation period and the results were properly recorded. The various incubation time investigated for the study are 1, 3, 5, 7 and 9 days. Maximum rhamnolipid concentration was found at the 5th day of incubation period, beyond which, the concentration of rhamnolipid was found to decrease. The rhamnolipid concentration recorded was $4.26 \pm 0.06$ g/l at the 5th day. Hence, the incubation period for maximum production was taken as 5 days (Table 6).
5.6.1.3 Inoculum Size

The size of bacterial inoculum was also said to influence the rate of rhamnolipid production. Biosurfactant production is a growth associated process, parallel relationships exist between growth, substrate utilization and the production of biosurfactant. Thus, the measurement of inoculum size is an important parameter to be observed. The concentration of rhamnolipid was investigated at various inoculum sizes such as, at 1%, 3%, 5%, 7% and 9%, respectively. It was found that the maximum concentration of 4.85 ± 0.57 g/l was found at 7% inoculum size, beyond which the reduction in concentration was noted (Table 6). Hence, 7% was selected as the optimal inoculum size required for the maximum biosurfactant production.

Similar to the temperature and pH, the concentration of rhamnolipid increases with increase in incubation time and inoculum size up to a level beyond which there is a reduction. The level where the concentration was found to be the maximum was taken as the optimal, thus, the optimal incubation period was 5 days and inoculum size was 7% for the efficient production of biosurfactant.

5.6.2 Nutritional Factor

Nutritional factors like carbon source, nitrogen source, availability of multivalent cations etc. affects the production of biosurfactant like all other biological activities. These occur through the effects on cellular growth (Al-Araji et al., 2007). Therefore, it is important to find out the best one to attain the maximum activity. Results of investigation on different carbon and nitrogen source have been discussed below.

5.6.2.1 Carbon Source

The quality and quantity of biosurfactant produced are greatly affected and influenced by the nature of carbon substrate (Raza et al., 2007; Rahman and Gakpe, 2008). In order to increase the yield of biosurfactant, different carbon substrates were evaluated. The ability of \textit{P. putida}
ES39 to utilize various types of carbon sources were tested using five different oils, namely, corn oil, coconut oil, sesame oil, sunflower oil and kerosene, respectively.

Among all the carbon sources tested, corn oil was found to produce higher rhamnolipid concentration when compared to other sources. The cured cells of ES39 was found to produce $4.02 \pm 0.17$ g/l of rhamnolipid which was the highest, whereas the least production was observed in sesame oil. The results are tabulated in Table 7. Hence, corn oil was selected as the optimal carbon source required for the maximum production by ES39 strain.

5.6.2.2 Nitrogen Source

Microorganisms can utilize a variety of nitrogen sources for their growth and activity but maximum activity can be achieved only with some specific nitrogen sources. In the present study, five different nitrogen sources, namely, peptone, yeast extract, ammonium chloride, sodium nitrate and potassium nitrate were investigated for their efficacy.

Use of different nitrogen sources showed that maximum amount of rhamnolipid concentration was achieved in sodium nitrate ($\text{NaNO}_3$) followed by yeast extract (Table 7). The cured cells of $P. \text{putida}$ showed $4.22 \pm 0.17$ g/l of rhamnolipid concentration when grown on medium containing sodium nitrate as the nitrogen source. Though all the medium showed the presence of rhamnolipid, the maximum was found in the medium containing $\text{NaNO}_3$. The lowest rhamnolipid in ammonium chloride. Thus, sodium nitrate ($\text{NaNO}_3$) was found to induce highest biosurfactant production by ES39 strain when compared to the other source, hence, $\text{NaNO}_3$ is selected as the optimal nitrogen source required for the enhanced biosurfactant production.
5.7 Extraction and Purification of Biosurfactants

Preliminary biochemical characterization of the biosurfactant showed that the surfactant is of glycolipid in nature, further purification of biosurfactant was performed by column chromatography followed by TLC.

Rhamnolipid biosurfactants produced by *Pseudomonas putida*, using corn oil as the carbon source was purified and characterized. The crude extracts of biosurfactant produced by *P. putida* was recovered by solvent extraction method. When the substrate utilized was corn oil, a partially purified viscous honey colored rhamnolipid product was obtained.

Purification of the extracted products was done by different chromatographical methods. The rhamnolipid components were separated by silica gel column chromatography. Twenty-milliliter fractions were collected from the column, concentrated and was then analyzed by Thin Layer Chromatography (TLC) in order to monitor the separation of the components. The distinct rhamnolipid spots on preparative TLC plates were then checked further for purity by High Performance Liquid Chromatographical analysis (HPLC).

5.7.1 Thin Layer Chromatography

The partially purified rhamnolipid separated by silica gel column chromatography was collected and the collected fraction was analyzed by TLC in order to monitor the separation of the components. The biosurfactant sample produced two spots on the TLC plates. This indicated that the sample was composed of congeners having one or two molecules of sugar which influenced their movement. These fractions showed TLC spots at Rf values 0.21 and 0.68. The lower spot having 0.21 Rf value consists of dirhamnolipids, while the higher spot Rf 0.68 consists of monorhamnolipid. The crude rhamnolipids contained both monorhamnolipids and dirhamnolipids (Figure 5).
5.7.2 High Performance Liquid Chromatography

The mixture of two distinct rhamnolipid spots (Rf 0.21 and 0.68) were then checked further for purity by HPLC. Two components were observed in retention times of 2.4 and 3.1 minutes. The HPLC chromatogram of the blank sample was shown (Figure 6) for the comparison of this result.

HPLC chromatogram of the mixture of rhamnolipid spots having Rf values 0.21 and 0.68 were found using a shimodgu Vp series 1 socrutic Lc 10 AT pump with UV-visible SPD 10 AVP in detector including Rheodyne injector. The column was C18 Phenomenex Gemini (250 × 4.6 5μ).

5.7.3 Fourier Transform Infrared Spectroscopy

FTIR spectroscopy is used to determine the functional groups and chemical bonds present in the biologically active sample of the biosurfactant (Das et al., 2008).

Through the FT-IR spectrum of the pure biosurfactant produced by Pseudomonas putida (Figure 7), the presence of a rhamnolipid structure, which is composed of rhamnose rings and long hydrocarbon chains, is clearly indicated by the absorbance bands at the wave numbers of 3448.1 cm\(^{-1}\), 2934.1 cm\(^{-1}\), 1638.7 cm\(^{-1}\), 1089.58 cm\(^{-1}\) and 991.714 cm\(^{-1}\). The strong absorbance at 1638.7 cm\(^{-1}\), which is considered to be the characteristic peak of biosurfactants must be assigned to the C–H stretching vibrations of the hydrocarbon chain positions. However, the strong absorbance in the range of 1300–991.714 cm\(^{-1}\) indicated the presence of bands formed between carbon atoms and hydroxyl groups in the chemical structures of rhamnose rings.
5.8 Bioaugmentation Study

5.8.1 Recovery of Hydrocarbons from Refinery Sludge

The feasibility and efficacy of the biosurfactants in recovering hydrocarbons from oily sludge and contaminated petrol bunk soil was evaluated in the present study. The sludge from KRPL and soil from petrol bunk was used for the study.

Initially, the Total Petroleum Hydrocarbon (TPH) content of the original sludge sample and the petrol bunk soil was estimated and also their physico-chemical characteristics were analyzed. Estimation of the TPH content of the original sludge sample revealed that an amount of 750 ± 20 g/kg TPH was present in the sludge sample (Figure 8). The physico-chemical characteristics of the sludge and petrol bunk were analyzed in order to identify the components present in it. Determination of TOC values give us an idea about the gross measure of all forms of organic carbon during the bioremediation. The various elements analyzed are represented as mg/kg (Table 8).

To evaluate the recovery of hydrocarbons by biosurfactants, sludge collected from KRPL and soil sample from petrol bunk were added to the mineral medium at different concentrations and then inoculated with ES39 strain to see the efficacy of the strain in recovering hydrocarbon from the added sludge. For the present study, whole culture as well as the culture supernatant of ES39 was used in order to determine the role of microbes in the recovery. The inoculated media containing the samples and strain were incubated and left undisturbed for 20 days. After 20 days of incubation, residual TPH content found in each sludge concentration was noted, and the hydrocarbon recovery was estimated after decanting the culture media. Results of the recovery were tabulated (Tables 9–12).

Various concentrations of sludge used are 5%, 7.5%, 10%, 12.5% and 15%, respectively. The results showed that, with the increase of sludge concentration in the culture
media, the residual TPH content decreased in whole culture as well as in culture supernatant. This shows that the biosurfactants produced by the microbes acts on the sludge thereby degrading it and recovering hydrocarbons. The lowering of TPH content clearly indicated the recovery of hydrocarbons in the sample. The residual TPH content of the whole culture was 172±12.40 g/kg at 5%, 164 ± 13.12 g/kg at 7.5%, 151 ± 12.00 g/kg at 10%, 138 ± 11.40 g/kg at 12.5% and 125 ± 17.22 g/kg at 15% sludge concentration. The residual TPH content was found to be lowest at 15% sludge concentration. Also, calculations revealed that maximum recovery of hydrocarbons at 84% was observed at 15%. Since the recovery of hydrocarbon is indirectly proportional to the residual TPH content of the culture media, it is concluded that the efficiency of recovery of hydrocarbon was maximum at 15% sludge concentration. The above results prove that though the strain could recover hydrocarbons from all sludge concentrations, the percentage of recovery was found to be maximum at 15%.

The use of culture supernatant instead of whole bacterial culture showed comparatively lower removal than the whole culture. But even in this case, residual TPH content was found minimum only at 15% sludge concentration indicating maximum recovery of hydrocarbon at this particular concentration.

Results of the petrol bunk soil revealed similar results to that of sludge. The residual TPH content was lowest at 12.5% of sludge concentration. The amount of residual TPH at 12.5% concentration was 87 ± 1.46 g/kg. Similar to the sludge sample, with the increase in sludge concentration, the TPH content was found to decrease indicating the process of degradation taking place. It was also found that the residual TPH content increased above 12.5% concentration.
From the above results it can be concluded that using whole bacterial culture instead of supernatant was the best way for the removal of hydrocarbon from the refinery sludge as well as from the artificially contaminated soil.

5.9 Anti-cancer Activity
The cytotoxic activity of biosurfactant produced by the strain ES39 was evaluated using human cancer cell lines. Three cell lines namely, HeLa (cervical carcinoma cells), MCF7 (breast cancer cell) and VERO (normal cell-Monkey kidney) cells were chosen for the study. After the treatment period, the cell lines are assessed for their viability.

Control as well as the cancer cell lines were treated with different concentrations of biosurfactants and the cell viability corresponding to each concentration was noticed and recorded. The cell viability of both the cancer cell lines was found to decrease in a dose dependent manner. The IC$_{50}$ value for each of the cell line was found to vary. In the MCF7 cell line, around 50% of cell mortality occurred at 3.12 µg/ml concentration, whereas in HeLa cell line, 50% mortality occurred at 1.56 µg/ml, which was the lowest level of concentration used. As the concentration increased, the viability of cell was found to decrease for both the cancer cell lines and it reached almost to 0% at 100 µg/ml concentration. The results are tabulated in Tables 13–15. Microscopic view of the cells showing degeneration at different concentrations can be observed in Figures 9–11.

The most prominent anti-proliferative effect was found to be against the cervical cancer (HeLa) cell line because it showed drastic reduction in the cell viability when compared to the MCF7 cell lines. The results clearly portray that as the concentration of doses increases the viability of the cell decreases thereby inhibiting the proliferation of cells. Thus, it is clear that the biosurfactant produced by ES39 strain serves to be a potent anti-cancer agent.
From the IC\textsubscript{50} values, it is clear that the biosurfactant was most effective against HeLa cell lines as it could bring about 50\% mortality even with the minimal concentration of rhamnolipid. In case of VERO cell line, the rate of mortality was minimal. IC\textsubscript{50} value of VERO cell line was found to be 25 \(\mu\text{g/ml}\), and even at 100 \(\mu\text{g/ml}\) of concentration 20\% of the cells was found to be alive. This in fact is an important constraint with regard to the treatment of cancer cells, where the effect of the compound given should be minimal to the normal cell lines. Therefore, in the present study, the viability of VERO cell lines proved that the biosurfactant produced by the strain ES39 is an efficient anti-cancer agent.

### 5.10 Immunomodulatory Activity

In the present study, the immunomodulatory effect of the rhamnolipids against bacterial infection was evaluated by infecting the fishes artificially with \textit{Aeromonas hydrophila} and the changes in the blood parameters were assessed after 21 days of study period.

The percentage of hemoglobin and RBC count of the treated fishes were found to increase in a dose dependent manner. In case of both normal and cured rhamnolipid, the count was found more at 100 ppm concentration. It is well known that, increase in RBC count obviously increases the volume of the packed cell. Similarly, the leukocyte count was also found to increase with the increase in rhamnolipid concentration indicating the positive response of the immune system. The percentage of survival rate also increased in a dose dependent manner and the maximum survival rate was found at 100 ppm concentration of cured rhamnolipid (Table 16).

Findings of the present study clearly indicates that, biosurfactants triggered the immunomodulatory defense mechanism in fishes. Also, the percentage of blood parameters and the survival rate of fishes was maximum with cured rhamnolipid when compared to the normal rhamnolipid.