CHAPTER 8
SUMMARY AND CONCLUSION

The current research work dealt with the isolation of biosurfactant-producing bacterial isolates from hydrocarbon-contaminated areas. After designing an appropriate consortium, the biosurfactants produced by the isolates were applied to facilitate the rapid biodegradation of polycyclic aromatic hydrocarbons like, naphthalene, anthracene and pyrene. Experiments were also conducted to check the applicability of the biosurfactants for enhanced oil recovery operations. The salient findings of the research have been described below:

- The bacteria were isolated from different hydrocarbon-contaminated zones such as refinery site, petroleum depot, petrol bunk and automobile workshop. Serial dilution and plating led to isolation of 23 different bacterial isolates.
- The isolates were screened for the production of biosurfactants through a series of qualitative tests that followed after they were grown in MSM. The tests included oil spreading technique, blood agar haemolysis test, drop collapse test, CTAB agar plate test, tilted glass slide test, emulsification index and determination of surface activity. The results of the screening tests indicated that seven of the isolates (RT3, RT7, RT9, RT10, RT16, RT19, and RT21) showed promise in producing significant quantities of extracellular biosurfactants.
- The percentage reduction in surface tension of the cell-free broth after 24 hours, ranged from 49% to 62%, among the seven isolates.
Morphological features of the selected strains were observed. The standard biochemical tests were performed that included Gram Staining Reaction, Motility Test, Starch Hydrolysis Test, Casein Hydrolysis Test, Methyl Red – Voges Proskauer Test, Nitrate Reduction Test, Oxidase Test, Catalase Test, Citrate Utilization Test, Indole production Test and Spore Forming ability Test. From the tests, it was concluded that RT7, RT19 and RT21 were *Bacilli*. RT9 and RT16 were *Pseudomonas* isolates. The tests proved inconclusive for RT3 and RT10.

Sequencing using 16S rRNA technique helped in the complete identification of the seven isolates. They were identified as:

- RT3: *Acinetobacter calcoaceticus*
- RT7: *Bacillus subtilis*
- RT9: *Pseudomonas aeruginosa*
- RT10: *Rhodococcus terrae*
- RT16: *Pseudomonas aeruginosa*
- RT19: *Bacillus siamensis*
- RT21: *Bacillus subtilis subsp. inaquosorum*

*Bacillus siamensis* (RT19) has been reported to produce biosurfactant for the first time, to the best of knowledge. Similarly, the subsp *inaquosorum* of *B. subtilis* is also noted for the first time, in literature.

Since *B. siamensis* is novel for the production of biosurfactant, this isolate, RT19, was made part of all microbial combinations. Of the two *Bacillus subtilis* isolates, the better isolate, in terms of higher reduction of surface tension and better emulsification index was chosen. This isolate was *B. subtilis* subsp. *inaquosorum* (RT 21). Of the two
*P. aeruginosa* isolates, the better isolate (RT 9) was chosen. Hence, different combinations of RT9, RT19 and RT21 were formulated for development of microbial consortia.

- CONS 1: RT19 + RT21
- CONS 2: RT19 + RT9
- CONS 3: RT19 + RT21 + RT9

The role of plasmid DNA, if at all, in the production of biosurfactants was checked. Plasmid DNA was extracted from each of the seven isolates. Agarose gel electrophoresis of the samples did not show the presence of plasmid DNA in any of the lanes. It was thus concluded that the genes responsible for biosurfactant synthesis were present in the chromosomal DNA, itself, where it is thought to be more stable.

After the seven isolates were separately grown in MSM supplemented with petrol-diesel admixture, the biosurfactant was extracted from the fermentation broth. The appearance of the biosurfactant from the various isolates ranged from an oily appearance to a viscous yellow product to a viscose honey-brown colored material.

Stability studies were performed using the 24-hour cell-free culture broth.

- When the effect of temperature on the surface activity was studied over a range of 4°C to 121°C, it was observed that the surface tension of the cell-free broth gradually took a dip. The observed change of surface tension among the isolates was between 2% and 33%. With respect to emulsification index, the effect of temperature had an opposite effect. With an increase in temperature, the emulsifying effect also increased between 1% and 32%.
When the effect of pH on the surface activity was studied over a range of 2 to 12, it was observed that the surface tension of the cell-free broth was nearly a constant. The observed negligible decrease of surface tension among the isolates was between 0% and 7%. With respect to emulsification index, the effect of pH had a different effect. With an increase in pH, the emulsifying effect also increased between 3% and 36%.

When the effect of salinity on the surface activity was studied over a range of 0% to 20% by addition of sodium chloride, the surface tension values showed a similar trend as was with the pH. Surface tension remained practically a constant, the change being 1% to 5.5%, among the isolates. With respect to emulsification index, as the salinity rose, the emulsifying effect decreased between 5% and 43%.

To compare the effect of chemical and biosurfactants on remediation of laboratory-made contaminated soil, sand-pack column are employed to determine the efficiency of the isolated biosurfactant in releasing the residual (commercial) engine oil. The difference in performance between an aqueous solution of crude biosurfactant and an aqueous solution of sodium dodecyl sulphate (separately poured on to column) was studied. To assess the influence of temperature on biosurfactant-induced oil recovery, the entire sequence of experiments was carried out at 30°C, 50°C and 70°C. Invariably for all the cultures, it was observed that higher temperatures led to better recovery of residual oil.

At a temperature of 30°C, the percentage of oil recovery ranged from 81.6% to 86.3% using the biosurfactant solutions from the three microbial isolates. The range increased to 83.5% to 91.2% at 50°C. A further rise in oil recovery was observed at 70°C; the percentage of oil recovery ranged from 87.6% to 92.9%
across the various microbial isolates. The general trend is that surface tension decreases with the increase of temperature.

- At 30°C, 50°C and 70°C, the chemical surfactant SDS yielded a recovery of 90.6%, 92.9% and 95%, respectively.
- In a separate study, the average percentage of recovery of residual engine oil by biosurfactants produced by the consortium, CONS-3 was 89.2%. The recovery using the biosurfactant sourced from the consortium was slightly better than the individual isolates.

- An attempt was made to use plant wastes and used oil as a sole source of carbon to be included in the production medium for the extracellular secretion of biosurfactants by the selected isolates. Four novel substrates were used in fermentation experiments: garlic peel, jackfruit seed coat, plantain pith and used engine oil. When the strain RT19 was inoculated in MSM containing the different substrates as the sole carbon source, they portrayed significant reduction of surface tension. The reduction of surface tension using garlic peel (72%), jackfruit seed coat (67%), plantain pith (43%) and used engine oil (40%) was comparable to reduction induced by glucose (58%), orange peel (46%) and apple pomace (55%), that have been documented by previous researchers. All the four carbon sources were then evaluated for reduction of surface tension using the three selected microbial specimens, viz., RT9, RT19 and RT21. In the case of *P. aeruginosa* (RT 9), the highest reduction of surface tension (68%) resulted when used engine oil was incorporated as the sole carbon source, in the production medium. For *B. siamensis* (RT 19) and *B. subtilis subsp. inaquosorum* (RT 21), the corresponding values were 72% and 74%, when garlic peel was used (in both the cases).
Additionally, when the consortium, CONS-3, was grown using garlic peel as the sole carbon source, the amount of biomass and biosurfactant produced was found to be higher than that produced by the individual isolates using the same carbon source.

The three isolates were then grown using the selected carbon source. Growth and biosurfactant production kinetics was studied using Logistic Model and Logistic-incorporated Leudeking-Piret Model, respectively.

- When *P. aeruginosa* (RT9) was grown in the presence of used engine oil as the sole carbon source, the maximum amount of biomass of 4.63 g/L was attained after 108 hr. The maximum amount of biosurfactant yield of 2.14 g/L was attained after 120 hr. From the Logistic Model, the estimated values of the parameters were: coefficient of carrying capacity ($\mu_m$) = 0.0792 /hr, initial biomass concentration ($X_0$) = 0.1299 g/L and maximum biomass concentration ($X_m$) = 3.0025 g/L. Biosurfactant production kinetics was studied using Leudeking-Piret Model. The estimated values of the parameters were: $\alpha = 0.9045$ g / g biomass and $\beta = 0.0033$ g / g biomass. hr.

- When *B. siamensis* (RT19) was grown using powdered garlic peel as the sole carbon source, the maximum amount of biomass of 3.09 g/L and biosurfactant of 0.64 g/L was attained after 96 hr. Using the Logistic Model, the estimated values of the parameters were: coefficient of carrying capacity ($\mu_m$) = 0.0882 /hr, initial biomass concentration ($X_0$) = 0.149 g/L and maximum biomass concentration ($X_m$) = 3.0794 g/L. Using Leudeking-Piret Model for biosurfactant production kinetics, the estimated values of the parameters were: $\alpha = 0.8988$ g / g biomass and $\beta = 0.2322$ g / g biomass. hr.
• When *B. subtilis* subsp. *inaquosorum* (RT21) was grown using powdered garlic peel as the sole carbon source, the maximum amount of biomass of 5.57 g/L and biosurfactant of 1.18 g/L was attained after 120 hr. Using the Logistic Model, the estimated values of the parameters were: coefficient of carrying capacity ($\mu_m$) = 0.0645 /hr, initial biomass concentration ($X_0$) = 0.2343 g/L and maximum biomass concentration ($X_m$) = 5.298 g/L. Biosurfactant production kinetics was studied using Leudeking-Piret Model. The estimated values of the parameters were: $\alpha = 1.0034 \text{ g / g biomass}$ and $\beta = 0.3565 \text{ g / g biomass. hr}.$

- The biosurfactants extracted from the three isolates were characterized using Thin Layer Chromatography, FTIR Spectroscopy and NMR Spectroscopy. The chromatograms and spectral results proved that the structure of the extracted biosurfactants was in tandem with the available literature and controls tested.

- Three polycyclic aromatic hydrocarbons were chosen to study their extent of degradation – Naphthalene, Anthracene and Pyrene. Since the biosurfactants secreted by the isolates help in increasing the bioavailability of the PAH, they were added as the sole carbon source during degradation studies, which were conducted separately for the three PAHs.

- Naphthalene was added as the sole carbon source at a final concentration of 20 mg/L. The concentration of naphthalene in the uninoculated flask was practically, a constant, over a 20-day period. Among the three individual isolates, *B. siamensis* (RT19) degraded 63% naphthalene after 20 days. This was on par with the proven PAH degrader *B. subtilis* BMT4i that could degrade up to 63.5% during the same period. Among the consortia, CONS-3 could enable the highest biodegradation of 84% in 20 days. This was followed by CONS-2 (77.5%) and CONS-1 (68.5%) in 20 days. Biodegradation of naphthalene
was found to follow first-order kinetics and the biodegradation rate constants were determined.

- Anthracene was added as the sole carbon source at a final concentration of 200 mg/L. Over a 20-day period, there was a highly negligible decrease of anthracene concentration in the uninoculated flask ensuring that the PAH is not lost or degraded on its own. Among the three individual isolates, *B. subtilis* (RT21) showed the highest of 67% degradation after 20 days. The positive control culture *B. subtilis* BMT4i was able degrade 70.8% in the same period. When it came to consortia, it was CONS-3 that was able to facilitate the biodegradation of 84.4% of anthracene in 20 days. This was followed by CONS-2 (82.6%) and CONS-1 (78.6%), both in 20 days. Biodegradation of anthracene was found to follow first-order kinetics and the biodegradation rate constants were determined.

- Pyrene was added as the sole carbon source at a final concentration of 200 mg/L. During the 20-day period, pyrene concentration in the uninoculated flask was largely unchanged. Among the three individual isolates, *B. siamensis* (RT19) degraded 97.5% PAH after 20 days. The positive control culture *B. subtilis* BMT4i was able degrade nearly 98% after 20 days. It was interesting to note that the consortium CONS-3 was able to facilitate the 100% biodegradation of pyrene in 16 days. However, near-complete biodegradation of pyrene was achieved by the other two consortia after 20 days. Biodegradation of pyrene was found to follow first-order kinetics and the biodegradation rate constants were determined.

- It was found that the consortium, CONS-3 was invariably, the most effective in degrading the three selected PAHs, compared against individual isolates and the proven PAH degrader, *B. subtilis* BMT4i (MTCC 9447).