CHAPTER 5

SUMMARY AND CONCLUSIONS

Around 300 microorganisms were isolated from enriched petroleum-contaminated soil and sludge samples. These isolates were further screened for biosurfactant producers by employing three screening methods and eleven promising strains were short-listed. Initially drop collapse method was employed for screening in which 11 isolates exhibited positive result. These eleven isolates were promising as they reduced the surface tension of culture medium to values below 42 mN m^{-1}. The eight isolates among these 11 isolates produced the biosurfactants which were more effective than SDS as they were capable of reducing the surface tension to below that of 1% SDS solution. Two strains, BS-161R and BS-223, were found to be potent as they were able to reduce the surface tension of culture medium to the value below of 27 mN m^{-1}. Only two isolates, BS-161R and BS-243, showed β-hemolysis on blood agar among the 11 isolates.

All of the tested strains exhibited good emulsification activity against *N*-hexadecane, hexane, xylene, mineral oil, olive oil and castor oil suggesting that the biosurfactants produced can act as emulsifiers and this property would be useful in various applications like biodegradation of hydrocarbons or other water-immiscible substrates as well as in enhanced oil recovery. But few of them, even though possessing good surface tension lowering property, were unable to form good stable emulsions. From the analysis of screening methods it can be concluded that the direct screening methods like drop collapse assay and surface tension measurement were more reliable than indirect methods like hemolytic activity and emulsification capability because of the probability of getting more false positives and/or negatives in indirect methods due to their poor correlation with surface tension reduction property. The direct surface tension measurement is final confirmation method to assess surface tension lowering property, however, the measurement of surface tension was inconvenient to use for screening of a large number of isolates as it was time consuming and required large volume of sample for analysis. Thus sequential screening was carried out to short list the isolates and finally based on surface tension measurement, 11 promising isolates were fished out. Furthermore, low values of CMC were recorded for the crude biosurfactant produced by these strains ranged between 25 and 190 mg L^{-}

[1]. From an environmental perspective, possessing low values of CMC and high capacity to reduce surface tension suggest their possible use to promote remediation of contaminated subsurface environments by increasing the hydrocarbon mobility. Besides exhibiting surface active properties, all of the 11 strains exhibited flocculating activity and among them, three isolates BS-2, BS-161R and BS-243 were found to be potent.

The strains were identified up to species level by morphological, physiological, biochemical characteristics and 16S rDNA sequence analysis. The isolated microorganisms belonged to diverse genera including *Bacillus, Brevibacillus, Pseudomonas, Ochrobactrum, Kocuria, Nocardia* and *Microbacterium*. Two new microorganisms, namely, *Ochrobactrum* sp. and *Microbacterium* sp. were identified as new additions to the list of biosurfactant producing microorganisms, while *Microbacterium* sp. exhibiting flocculating activity was identified as new addition to the list of bioflocculant-producers.

The crude biosurfactant produced from *P. aeruginosa* BS-161R had an excellent heat tolerance which could withstand at higher temperatures and had broad pH stability. These findings suggest that the robust characteristics of the crude biosurfactant which are beneficial for applications carried out under extreme conditions of temperature and pH, such as in oil recovery and in the bioremediation of a polluted environment. The strain, BS-161R, was capable of producing rhamnolipids in presence of both water-soluble and water-insoluble carbon sources. However, glycerol was found to be little more effective at promoting the slight increase in the production of rhamnolipids. The bio-glycerol which is a by-product of biodiesel production acted as a cheap raw material for rhamnolipid production. The optimum concentration of carbon source was found to be 25 g L^{-1}, while sodium nitrate 4 g L^{-1} was found to be suitable nitrogen source. The maximum production of rhamnolipids was observed at C:N ratio of 15 and C-to-Fe ratio of 28350. The optimum pH and temperature for the highest yield of rhamnolipids ranged between pH 7.5 and 8, while temperatures of 34 and 36°C. The agitation speed also influenced the production of rhamnolipids as the yield of rhamnolipids was gradually increased with speed and reached maximum at 200 rpm. After optimization of nutritional and environmental conditions the production of rhamnolipids was increased from 0.369 to 3.312 g L^{-1} which is approximately 9.0 fold. The optimum medium for *Pseudomonas aeruginosa* BS-161R consisted of the

following components (g L^{-1}): $MgSO_4$, 0.2; $CaCl_2$, 0.02; KH_2PO_4, 1.0; K_2HPO_4, 1.0; NH_4NO_3, 1.0; $FeCl_3$ .001, $NaNO_3$, 4.0 and bio-glycerol, 25 while the optimum environmental conditions were follows as: pH 7.5, temperature, 35°C and agitation speed, 150 rpm.

Thin-layer chromatography of the crude biosurfactant produced from *P. aeruginosa* BS-161R revealed the presence of three major spots. These compounds were purified successfully from crude biosurfactant using silica gel column chromatography. The chemical structure of purified compounds was deduced successfully as phenazine-1-carboxylic acid (PCA), rhamnolipid-1 (RL-1) and rhamnolipid-2 (RL-2) based on the ^{1}H and ^{13}C NMR, FT-IR and mass spectral data analyses.

Crude extracts of biosurfactant producing isolates, except BS-323, BS-206 and BS-207, exhibited broad-spectrum antimicrobial activity. Among all the isolates, crude extract of BS-161R showed good activity against all indicator strains. Purified rhamnolipids and PCA from isolate BS-161R exhibited a broad spectrum antimicrobial activity against a wide range of pathogens, including both Gram-positive and -negative bacteria and *Candida albicans*. Rhamnolipid-1, which is a precursor of rhamnolipid-2, showed more antimicrobial activity than rhamnolipid-2. The least MIC value was recorded against *Escherichia coli* for rhamnolipid-1 at 16 µg mL^{-1}, while rhamnolipid-2 exhibited highest activity against *Escherichia coli* (64 µg mL^{-1}) and PCA exhibited highest activity against *Klebsiella planticola* at 16 µg mL^{-1}. The anti-proliferative activity of purified rhamnolipids and PCA was evaluated against a panel of human cancer cell lines in culture, since no published toxicity data on purified rhamnolipids was available. The rhamnolipid-2 exhibited more cytotoxic activity as compared to rhamnolipid-1 and exhibited least IC_{50} against MDA-MB-231 cell line at a concentration of 54 µM. The PCA exhibited the most efficient cytotoxic potency against A549 (IC_{50}, 7 µM), followed by MCF-7 (IC_{50}, 9.2 µM), HepG2 (IC_{50}, 11.1 µM) and HeLa (IC_{50}, 30 µM), among the cell lines tested.

Silver nanoparticles were successfully synthesized using an eco-friendly approach by using a reverse microemulsion technique in which purified rhamnolipid mixture (RL-1 and RL-2) acted as stabilizing agent where sodium borohydride acted as a reducing agent. The synthesized nanoparticles were characterized by UV-visible spectroscopy, transmission electron microscopy,

and energy dispersive X-ray spectroscopy (EDS). These formed nanoparticles had a sharp adsorption peak at 410 nm, which is a characteristic surface plasmon resonance of the silver nanoparticles. The nanoparticles were mono-dispersed, with an average particle size of 15.1 nm (σ = ±5.82 nm) and spherical in shape. The EDS analysis revealed the presence of elemental silver signal in the synthesized nanoparticles. The FTIR and EDS results indicated that the possible capping ligands were rhamnolipids. The formed silver nanoparticles exhibited good antibiotic activity against both Gram-positive and Gram-negative pathogens and *Candida albicans*, suggesting their broad-spectrum antimicrobial activity.

The culture supernatant of *Pseudomonas aeruginosa* isolate BS-161R was used for green synthesis of silver nanoparticles with silver nitrate at room temperature. It was proposed that the reduction of the silver ions may plausibly be due to the protein component contributed by the enzyme nitrate reductase, since nitrate reduction is the phenotypic biochemical property of this culture. The nanoparticles formed were stabilized probably by the capping of rhamnolipid present in the culture supernatant as revealed by FT-IR spectral analysis. The UV-visible spectrum showed a surface plasmon resonance peak at around 430 nm, which is characteristic of silver nanoparticles. The silver nanoparticles formed were spherical in shape with an average particle size of 13 nm, crystalline in nature, and the particle surface was anionic; these properties were confirmed by TEM, XRD and zeta potential analysis. The silver nanoparticles had a broad spectrum antimicrobial activity against various Gram-positive and Gram-negative bacteria and different *Candida* species.