Chapter 5

Summary and Conclusions

Biosurfactants have gained considerable attention in recent years due to their unique properties like very high surface activities even at low concentration, higher biodegradability, lower toxicity, and effectiveness at extremes of temperature, pH and salinity in comparison to chemical surfactants. Biosurfactants are mainly classified according to their chemical structure and their microbial origin. The main classes of biosurfactants are lipopeptides, glycolipids, phospholipids and polymeric biosurfactants.

Several features and biological activities have been reported for lipopeptides, mainly for iturinA and surfactin. They have been described as antibiotics, antiviral and antitumor agents, immunomodulators or specific toxins and enzyme inhibitors. Surfactin has been found to interact with artificial and biomembrane systems. Several biological activities have been attributed to surfactin including the induction of ion channels formation in lipid bilayer membranes, the inhibition offibrin clot formation and haemolysis, the inhibition of cyclic adenosine monophosphate (cAMP), the inhibition of platelet and spleen cytosolic phospholipase A2 (PLA2) and antimicrobial, antiviral and antitumor activity against Ehrlich's ascite carcinoma cells. According to the differences in their amino acid sequences, different types of surfactins (A, B and C) have been identified. Surfactin C was found to enhance the activation of prourokinase (plasminogen activator) and the conformational change in plasminogen, leading to increased fibrinolysis in vitro and in vivo.

Investigations suggest that the membrane barrier properties are likely to be damaged in the areas where surfactin oligomers interact with the phospholipids, at concentrations much below the onset for solubilisation. Such properties can cause structural fluctuations that may well be the primary mode of the antibiotic action of this lipopeptide. Surfactin type peptides that can rapidly act on membrane integrity rather than other vital cellular processes may perhaps constitute the next generation of antibiotics. Lipopeptide biosurfactants produced by several bacteria exhibit insecticidal activity against fruit fly Drosophila melanogaster and hence are promising to be used as biopesticides. Fengycins are also reported to possess antifungal activity and therefore may be employed in biocontrol of plant diseases.

Cyclic lipopeptide (CLP) are stable over a wide pH range (7.0- 12.0) and heating them at high temperature does not result in any loss of their surface-active property. They show good emulsion formation capability with vegetable oils and demonstrated excellent compatibility and stability with commercial laundry detergents favouring their inclusion in laundry detergents formulation. Bacterial lipopeptides constitute potent non-toxic, nonpyrogenic immunological adjuvant when mixed with conventional antigens.

The rhamnolipid biosurfactant, mostly produced by the genus *Pseudomonas* is known to possess potent antimicrobial activity. Further, no adverse effects on humans or the environments are anticipated from aggregate exposure to rhamnolipid biosurfactants. Some microbial extracellular glycolipids induce cell differentiation instead of cell proliferation in the human promyelocytic leukemia cell line. Glycolipids have also been implicated with growth arrest, apoptosis and the differentiation of mouse malignant melanoma cells.

Biosurfactants also play a major role in petroleum extraction, transportation, upgrading and refining and petrochemical manufacturing. These surfactants are used as emulsifiers, foaming agents, solubilizers, wetting agents, cleansers, antimicrobial agents, mediators of enzyme action, in insect repellents, antacids, bath products, acne pads, anti dandruff products, contact lens solutions, baby products, mascara, lipsticks, toothpaste, dentine cleansers to mention but a few.

In spite of their very promising applications in health, industrial sectors as well as in soil bioremediation processes only few of them are produced for commercial applications. The major drawback being the high production and recovery costs. However, low cost raw materials, new microorganisms, are being explored, to reduce the cost of production and increase the yield of the biosurfactant product. Use of inexpensive substrates, such as agricultural residues, by products or waste materials that contain carbohydrates could lower the production costs of biosurfactants.

The present work was undertaken to primarily use low cost agro based substrates for biosurfactant production and work out the economics of the production of biosurfactants on commercial scale. The significant conclusions drawn from this work are summarized in four categories.

(a) Justification of the use of different low cost agro based substrates as potential source for biosurfactant production,
(b) Growth of *Bacillus Subtilis*, biosurfactant production and downstream processing for recovery
(c) Identification and characterization of biosurfactant
(d) Process economics.

5.1 Low cost agro based substrates as potential sources for biosurfactant production

- India is the second largest producer of rice in the world, looking at the total rice production in India, the quantity of rice bran available would be huge. Approximately 10-15 kg of rice bran is generated per 100 kg of raw rice processed during polishing process in a rice mill. Rice bran if processed within 24 hrs can be used for the extraction of rice bran oil for edible purpose. However, extraction of rice bran oil within 12-24 hr of rice bran production is not always feasible at local rice mills, therefore most of the rice bran is utilized only for cattle feed. Almost 9 million tons of rice bran is produced in India of which ~ 5 million tons is processed for oil and the rest is used partly as cattle feed but mostly wasted, decayed and discarded.
- Molasses, a by-product of sugar manufacturing is a rich source of carbohydrates, is a solution of sucrose, some glucose, fructose and other organic and inorganic matter in water. 100 T of sugarcane gives 10-11 T of sugar and 3-4 T of molasses. In India molasses is primarily used to obtain ethanol nearly 90% of molasses produced is consumed by industrial alcohol manufacturers and remaining 10% for various other uses like potable liquor. Molasses is quite a controversial resource and its availability is subject to numerous government regulations.
- Considerable amount of starch is released during the processing of potato to obtain potato chips, the effluent waters from such processing units contains high concentration of starch, and protein. Almost five tons of water is needed per ton of potato processed. The projected growth of such potato processing units is at a rate of 7 – 8% annually hence there will be a corresponding increase in waste water which if not valorised will only enhance the BOD of the neighbourhood water bodies. Thus waste water from potato processing industry, rice bran, and molasses are potential resources that can be utilized as a cheap source of carbon for fermentation using appropriate microorganism for biosurfactant production.

- Agro based substrates and carbohydrate containing wastes such as wash waters containing potato starch from potato wafer processing industry, molasses and rice bran, were used as substrates for the production of biosurfactant by fermentation using the organism *B.subtilis* MTCC 2423.

5.2 Growth of *B.subtilis* , biosurfactant production and downstream processing

- Surfactin was the biosurfactant that was targeted to be produced in this investigation. It is known that the microorganism *B.subtilis* synthesises surfactin by fermentation of carbohydrate sources. Hence, the strain of *B.subtilis* MTCC 2423 was procured from Institute of Microbial Technology (IMTECH), Chandigarh and was grown in nutrient media using standard procedures.
- Literature specifies that fermentation of potato starch by *B.subtilis* MTCC 2423 yields surfactin. Hence, to establish the fermentation procedure, potato starch containing effluents were first chosen as the substrate, while the growth pattern of *B.subtilis* and sugar consumption pattern were studied, no attempt was made to identify in detail the product characteristic, the only product parameter studied was the surface tension reduction pattern in the fermentation broth which indicated the synthesis of the biosurfactant.
- Three different sources of potato starch were utilized as the carbon source for the production of biosurfactant. Soluble starch procured from Hi media, wash liquid of potato chips obtained during domestic preparation of potato chips and starch containing effluent from Jabson's food industry. Each test sample contained 2% (w/v) starch.
- Fermentation experiments were carried out for varying time intervals, optimized amounts of micronutrients were added to the media. Drop collapse test gave a positive result indicating the presence of surfactant synthesized in the fermentation broth by *B.subtilis* MTCC 2423
- Surface tension measurements showed that in the initial 24 hours of fermentation there was a sharp decline in the surface tension from 70 dynes/cm to ~ 34 dynes/cm, thereafter the decline in surface tension was gradual and was influenced by addition of mineral salts. Surface tension reduction– time plots were nearly identical for all the three media studied. These results helped in establishing the operating procedures for growth of *B Subtilis*

- Addition of mineral salts showed enhanced reduction in surface tension reaching a low of 27 dynes/cm in 48 hours. This was attributed to the fact that addition of mineral salts such as magnesium, manganese and calcium to an agro substrate enhance the yield of biosurfactant production, while mineral salts such as potassium, ammonium and sodium are responsible for rapid growth of bacterial population in the medium, addition of iron enhances both the yield as well as the microbial growth.
- The effect of starch concentration (1.5 - 3%) on biosurfactant production was investigated for all the three potato starch media used. Reduction in the surface tension was nearly identical in media containing initial starch concentration of 2%, 2.5% and 3.0% (w/v). Surface tension reduction was marginally less when the medium contained 1.5% starch indicating substrate deficiency.
- Black strap molasses (2% w/v) was the other substrate used. Molasses contains several compounds other than sucrose such as minerals, vitamins and organic compounds that are useful for the fermentation process. Hence, fermentation was carried out without the addition of nutrients. Microbial growth in molasses media showed about 10^9 colony forming units (cfu) after 48 hours of fermentation, this was less than that obtained with potato starch as substrate.
- Rice bran fermentation media was slurry of uniform consistency (2 % w/v). Maximum cell growth was 10^7cfu/mL. The growth of *B Subtilis* on rice bran substrate was solely due to inherent nutrients present in the media without addition of nutrients. This aspect is certainly supportive of the use of rice bran as a fermentation media for growth of *B subtilis* and production of biosurfactant.
- Critical micelle dilution, a measure of the biosurfactant concentration or the dilution necessary to reach the CMC at which the surface tension starts to increase was determined for the biosurfactant produced using all the three substrates. It is a particularly useful index when the surfactant is likely to be used in applications where surfactant micelles play a critical role as in enhanced oil recovery. Results show that the biosurfactant produced in the media is significant because the surface tension does not abruptly rise even on 100 times dilution. When the dilution was further increased to 1000 times there was a sharp rise in the surface tension values.
- The microbial growth pattern in industrial fermentations are important not only to know the factors affecting the growth but also to develop strategies for scale

up and control of the fermentation system. Sigmoid growth curve models, the three parameter, Logistic model and Gompertz model were fitted to the experimental data of growth of *B Subtilis* during fermentation of potato, molasses and rice bran substrates. The maximum growth value reached A, maximum specific growth rate μ_{max} and lag time λ were determined.

Substrate	Model					
	Logistic			Gompertz		
	Parameters					
	A	μ_{max}	λ(hrs)	A	μ_{max}	λ(hrs)
Potato Effluent	19.815	1.105	10.927	21.07	1.003	9.288
Rice Bran	11.898	0.8538	3.852	12.641	0.8049	3.041
Molasses	17.604	0.9189	4.1499	18.067	0.9026	3.42

The growth curve of *B Subtilis* in different substrates for different micro nutrients amounts shows almost similar specific growth rates for the three substrates but the lag times are markedly different. Lag phase of *B Subtilis* in potato starch was almost three fold longer than that observed for rice bran or molasses. This large increment is attributed to concentration of nutrients, which were naturally present in substantial amount in rice bran and molasses but had to be added externally during fermentation of potato starch. An increase in the enzyme synthesis rate shortens the lag phase therefore presence of micronutrients aided the enzyme synthesis rate that led to decline of the lag period in rice bran and molasses as substrates.

- Foam fractionation was used as a downstream processing technique to concentrate the biosurfactant prior to its recovery by acid precipitation. Three stage foam fractionation was found sufficient for concentration of surfactin. It was observed that the net yield was in the order rice bran> molasses > potato. The recoverable yields of surfactin turn out to be 2.985 g/kg for rice bran and 1.904g/kg for molasses accounting for 69.2% of the product recovered in foamate from rice bran and 68% recovered in the foamate in case of molasses.
- The total yield of the surfactin from rice bran and molasses varied widely in spite of the fact that during fermentation both rice bran and molasses were prepared utilizing 2 % (w/v) as substrate in the initial media. The total carbohydrate content was higher in rice bran than in molasses fermentation media. The total carbon content (w/w) in rice bran was 15.9 % higher than that in molasses. Moreover, higher iron and

magnesium content in rice bran also favoured high yields. Rice bran therefore had all the key nutrients that were necessary for the good yield of surfactin.

5.3 Identification and Characterization of the biosurfactant

- Identification and characterization of the surfactin product was done by determining the chemical moiety and chemical groups by using TLC, FTIR, and NMR. Molecular weight and the peptide sequence were determined using ESIMS/MS, while DLS was used to estimate of the size of surfactin micelles formed in solution.
- TLC of the product revealed the lipopeptide nature of the biosurfactant since observation under UV lamp gave a band, further when the plate was developed with iodine vapours yellow coloured stain was also observed at similar height indicating the presence of peptide moiety in the same molecule again confirming its lipopeptide nature. The retention factor R_f was found to be 0.525 and 0.52 when using rice bran and molasses as substrate respectively these values correspond with reported data for surfactin.
- The IR spectra of the fermentation broth as well as the concentrates obtained by foam fractionation revealed the enhancement of intensity of various bands with a corresponding increase in concentration of the product. All the peaks that benchmark surfactin were identified in the spectra of the third foamate.
- NMR sample was prepared by hydrolyzing the product, NMR spectra revealed the presence of a fatty acid side chain. However, amide shifts corresponding to all of the amino acids present and α-carbon proton shifts were not visible indicating that perhaps hydrolysis of each peptide bond of the heptapeptide amino acid sequence in surfactin molecule did not take place. However, shifts corresponding to presence of Glutamic acid(1), Leucine(2), Leucine(3), Valine(4), Aspartate(5), Leucine(6),Isoleucine(7) were observed in the spectra.
- HPLC of the product obtained by the fermentation of rice bran and molasses was conducted using 3.8 mM trifloroacetic acid (20%) and acetonitrile (80%) as the mobile phase with the objective of judging the effectiveness of foam separation as a concentration and purification technique as well as identifying the product. The intensity of the peaks at specific retention times progressively increased with an increase in number of stages of foam fractionation, some minor peaks totally disappeared in the final concentrate, indicating that there is an increase in the purity as well as concentration of the product during the multistage foam fractionation recovery process. The major peaks identified in the HPLC spectra of the product

obtained by fermentation of rice bran and molasses were close to that reported for commercially available surfactin although the relative intensities were different.

- Positive ion ESI-MS was used to determine the molecular weight. The prominent $[M+H]^+$ peaks were observed at m/z 963.3, 994.3, 1022.5, 1036, 1044.3,1050, and 1075.3 for surfactin obtained from rice bran The fragmentation behaviour of two isoforms 1075.8 and 1022.5 was further investigated by performing MS^2 spectra. The MS^2 showed that the fragmentation route of 1075.8 and 1022.5 were similar and was dominated by a common ion peak at m/z 685.7 which corresponds to peptide component of surfactin. The fragmentation results indicate that the product is a cyclopeptide with varying number of methylene groups attached to the aliphatic chains. Both the homologs, at m/z 1076 and 1023, show similar peptide component confirming the similarity in the peptide moiety. ESI-MS for surfactin obtained from molasses with m/z values similar as that observed for surfactin from rice bran with predominance of m/z at 1076. In both surfactin samples obtained by fermentation of rice bran and molasses using BS there is predominance of surfactin of molecular weight 1076, with carbon chain length of 19.
- DLS studies were carried out to determine the surfactin micelle size. A broad, bimodal size distribution was observed ranging from 40 to 1000 nm. The lower range typically 75-85 nm corresponds to micelle size while larger sizes 300-350 nm correspond to micellar aggregates formed by intermicelle hydrogen bonds Addition of univalent K^+ ions and bivalent ion Ca^{2+} prominently affected surfactin micelle sizes and its distribution showing two separate size distributions instead of one broad size distribution.
- In the presence of the univalent K^+ ions a low intensity distribution is observed between 40 nm and 90 nm while a high intensity distribution is observed between 300 nm and 750 nm but in the presence of the bivalent Ca^{2+} ions width of the primary size range was between 25 and 50 nm while the second distribution was intense and narrow between 200-400 nm. The addition of univalent cations leads to a decrease in surfactin cmc value with more fatty acid chains getting assembled in the micelle core favouring aggregation at a concentration above cmc. With divalent cations the two charged residues of surfactin form a ‘claw’ and thus induce a tighter molecular packing and compressed structure of surfactin micelles. Therefore the size of micelle aggregates available upon binding of two univalent potassium ion on single surfactin molecule was relatively larger compared with the surfactin micelles formed in presence of calcium ion.

- Scale up of surfactin production from 200 ml sample size to 1000 ml sample size a fivefold increase in volume resulted about 1.4 times higher yield of surfactin. The yield obtained on scale up was 6.02 g/kg for rice bran in comparison 4.3 g/kg that was obtained during fermentation carried out with working volume of 200 ml.
- A process was developed and the tentative total time required for the various steps of the process spans 100 hours for a batch irrespective of its size. The fermentation time is the dominant factor that dictates the overall production and scheduling scheme. A Gantt chart in the overlapping mode considering one batch of product every day was constructed.
- The economics is worked out on the basis of 20 kg of surfactin production per year considering rice bran as the major raw material and that almost 70% surfactin can be recovered. The cost estimation was performed considering Bharuch and Ankleshwar region in Gujarat as the main site for production. On setting the market selling price of Surfactin at Rs 20 per mg the payout period turned out to be 2.1 years.
- The successful commercialization of surfactin production depends largely on bioprocess economics. Utilization of cheap and readily available agro waste such as rice bran, molasses and potato process effluent in this work for bacterial growth and subsequent biosurfactant production using *Bacillus subtilis* MTCC 2423 is a step forward in the process of reduction in the production cost of surfactin. The high yield of surfactin obtained with rice bran suggests that it holds promise for being utilized in large scale production of surfactin with proper implementation of three stage foam fractionation recovery process coupled with acid precipitation. Foam fractionation process utilized for recovery of surfactin provided benefits such as efficient recovery in foam, less maintenance cost as no high pressure device is utilized, no moving assembly required, no clogging of filter media as is the case with other filtration separation processes and easy viability of being clubbed with upstream part for continuous product recovery. In addition to its promise this work also renews interest in foam fractionation as an effective concentration and separation technique. Synthesized Surfactin had a high molecular weight of ~ 1075 and exhibited exceptional surface tension reduction property from around 70 down to 27 dyne cm^{-1}.

5.4 Scope for future work

The present work of surfactin production utilizing rice bran as primary source of carbon and as substrate was attempted along with novel three stage foam fractionation

concentration and recovery process coupled with acid precipitation of surfactin from the foamate resulted in ~70% recovery of surfactin. The residual broth contains all biodegradable material and hence its disposal would not be an environmental problem. The COD and the BOD values for the residual supernant broth that contained ~30% of the total surfactin not recovered by foam fractionation was 69mg/L and 23 mg/L respectively.

The residual 30% of surfactin in the supernant could be used for bioremediation as there are reports suggesting the use of lipopeptide type of biosurfactant surfactin for bioremediation activity (Mercade et al. 1996). This aspect needs to be further explored. The other option is to concentrate the residue using appropriate membrane separation technique to enhance the recovery and the yield of surfactin.

Biosurfactants have considerable use in effective therapeutics, especially at time when drug resistance among causal organisms for many life-threatening diseases is on the rise. In spite of the immense potential of biosurfactants in biomedicine, their usage still remains limited, possibly due to their high production cost, scarce knowledge of their molecular mode of action and an unclear toxicity towards the human organism. Further investigation on human cells and natural microbiota needs to be carried out to validate the use of biosurfactants in biomedical and health-related areas.

The ability to obtain reasonably pure products from fermentation requires several extraction and purification steps. These steps are made simpler by the use of pure carbon sources, such as oleic acid and other alkanes. However, the uses of such pure carbon sources are expensive; as a result other agricultural resources such as sunflower oil and soybean are becoming important as cost effective resources.

Recent trend is to explore frying oils and other waste oils as main carbon sources, which would also be an environmental solution for recycling these waste products. Such resources would be reasonably inexpensive and could cut the cost of large scale production. The downside however, would be that these highly complex oil sources would be quite problematic when it comes to product purification. Generally, the ability to remove all impurities of these carbon sources to leave highly purified BS or BE presents a challenge. Future work, therefore, needs to be focused on the production of microbial surface active compounds using inexpensive carbon substrates with the highest

yields possible, combined with cost effective downstream processing methods. By focusing on these areas, microbial surface active compounds would become more attractive as possible alternatives to commercial surfactants

Future biosurfactant research is expected to be more focused on economization of the biosurfactant production process, particularly through development of novel recombinant hyperproducers. The potential use of these hyperproducers in addition to novel cost-effective bioprocesses throws the real challenges and offers tremendous opportunities for making industrial production of biosurfactants a success story. A judicial and effective combination of different strategies might, in the future, lead the way towards large-scale profitable production of biosurfactants

Suggestions for future activity in this field could be summarized as follows:-

(a) There is a need to develop various applications for bioremediation, biomedical, therapeutic drugs, anticancer agents and other high end applications of surfactin.

(b) Bioprocess economics need to be addressed in order to make use of biosurfactant viable commercially in competition with chemical surfactants.

(c) Various strains are observed to produce biosurfactant and even genetic modifications by chemical methods, radiation techniques should be employed to develop hyper producing strains which can increase the yield and thereby reduce the production costs.