Chapter 3

Materials and Methods

This Chapter highlights the agro based materials used for the production of biosurfactant and the experimental methodology to produce, recover and concentrate the surfactant. The different techniques used for the characterization of the surfactant are also detailed.

3.1 Materials

3.1.1 Agro based wastes as substrate

Three different agro based wastes, potato chips manufacturing process effluent, black strap molasses and waste from the rice polishing industry were used as substrates for biosurfactant production.

Effluent water obtained from washing of potato in a potato chips manufacturing industry although high in carbohydrate content is usually discarded as a waste stream. Such potato process effluent stream from *Jabsons Foods (A division of Jabsons Cottonseed India Pvt Ltd)* Gujarat, a company involved in production of various food products was utilized as a substrate for the production of biosurfactant. Black strap molasses produced as side product of the sugar manufacturing industry was also utilized as a substrate; it was procured from a local sugarcane processing industry *Ganesh Sugar Industry*, Vataria. Figure 3.1 shows the molasses as a dark brown viscous liquid.

Fig. 3.1: Blackstrap molasses from Ganesh sugar industry, Vataria

Rice bran generated at the time of polishing brown rice was collected in dry solid powdery form. Rice bran has to be processed early just after removal from brown rice for producing rice bran oil for edible purposes hence, its business is limited and not possible at all rice mills. Rice bran has its application as cattle feed, which is of low commercial value. Rice bran collected from *Gayatri rice mill*, Olpad, Gujarat was the third substrate utilized in this work. Figure 3.2 shows the sample of rice bran.

Fig. 3.2: Rice bran collected from Gayatri rice mill, Olpad

3.1.2 Microorganism

B. subtilis MTCC 2423 was used as the bacterial culture for the production of biosurfactant. It was procured from Institute of Microbial Technology (IMTECH), Chandigarh in freeze dried form, in a sealed glass vial and was grown under optimum favorable conditions in a nutrient medium and preserved on nutrient agar (Himedia) slants at 4^0C.

3.1.3 Mineral salts

Mineral salts, KH_2PO_4 (min 99.5% assay, Merck Specialties Ltd.), Na_2HPO_4 (> 99% assay, Merck Specialties Ltd.), $(NH_4)_2HPO_4$ (>99% assay, Merck Specialties Ltd.), $MgSO_4$ (≥ 99% assay, Merck Specialties Ltd.), $FeSO_4$ (≥ 99.0% assay, S.D.Fine Chem. Ltd) and $CaCl_2$ (fused, >98% assay on dried substance, Merck Specialties Ltd.) were added in requisite amounts to potato process effluent as nutrients to facilitate the growth of the microorganism.

3.1.4 Miscellaneous Chemicals used

Numerous chemicals were used for a variety of analytical and characterization techniques. They include the following with reference to the analytical procedures:-

- **Phenol sulfuric acid test:** Total sugar was determined using phenol sulfuric acid test wherein Phenol of GR grade (SD Fine Chem Ltd) and sulfuric acid of AR grade (SD Fine Chem. Ltd) were used.
- **Enumeration of Colony Forming Units (CFU):** Nutrient agar plates (Himedia) and sterile distilled water was utilized for the serial dilutions during the enumeration of the colony forming units.
- **Thin layer chromatography:** Solution of butanol (AR grade, SD Fine Chem. Ltd), acetic acid (AR grade, SD Fine Chem. Ltd) and double distilled water was utilized as the mobile phase. Silica gel 60 plates (Merck Specialities) deposited on aluminum was utilized as stationary phase. The detection of band after TLC was performed by exposing to Iodine vapours and with UV tests.
- **FTIR:** Dried KBr (99.9% assay) was used for pelletizing the sample for FTIR analysis.
- **HPLC:** Trifluroacetic acid and acetonitrile of HPLC grade were utilized as solvent for the mobile phase. C_{18} column (5µm, Merck) was utilized for chromatography.
- **^{1}H-NMR:** DMSO-d_6 (Deuterated DMSO) of NMR grade was utilized as solvent for ^{1}H-NMR test and hydrochloric acid (S.D.Fine Chem. Ltd) was used for hydrolysis of sample before carrying out NMR.
- **ESI-MS:** Methanol as solvent (AR grade, SD Fine Chem Ltd) was used to prepare sample for the Electro spray ionization mass spectrometry.
- **DLS:** Dynamic light scattering samples were prepared in tris buffer (Aminomethane- Merck Specialities Ltd) and pH was adjusted using HCl (SD Fine Chem. Ltd). $CaCl_2$ (S.D.Fine Chem Ltd) and KCl (S.D.Fine Chem Ltd) were used as salts to detect micelle size in presence of counterions.

3.2 Methods

3.2.1 Inoculum preparation and culture conditions

B.subtilis MTCC 2423 bacterial culture was preserved and stored as per the recommendations of MTCC. The freeze dried culture from the sealed vial was activated by transferring it to nutrient agar plates as per standard procedure and was incubated at 30° C for 24 hours. A loop full of culture from the colony formed on nutrient agar plate was further transferred to nutrient agar slants, incubated at 30° C for 24 hours and stored at 4°C in a refrigerator. The preserved nutrient agar slants of *B.subtilis* culture were used for inoculum preparation. A loop full of culture from nutrient agar slant was transferred to 100 ml nutrient broth (NB) medium in a 250 ml Erlenmeyer flask. This was grown overnight at 30°C ($OD_{600\ nm}$ 0.8-0.9) and at 150 rpm on a rotary incubator shaker. The grown culture (Figure 3.3) was further utilized at 2% (v/v) as inoculum for all set of fermentations.

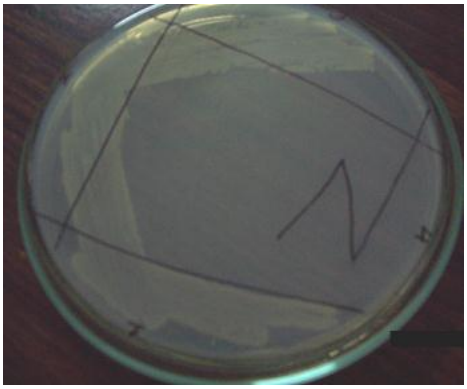


Fig.3.3: Petri dish with *B.subtilis* MTCC 2423 culture grown on nutrient agar (Himedia)

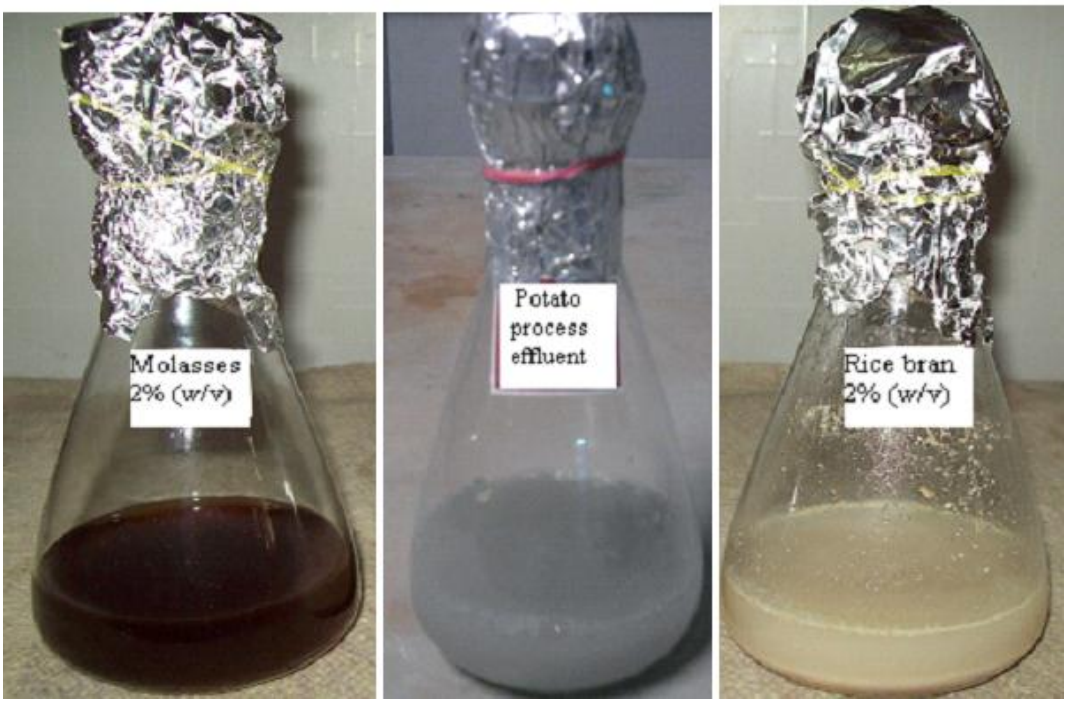
3.2.2 Substrate preparation and analysis

Three sources of potato starch were utilized as carbon source. 2% (w/v) soluble starch procured from Himedia, starch containing wash liquid of potato chips prepared domestically and potato process effluent generated after washing of the potatoes in potato processing industry. Insoluble were removed by filtration. The filtered samples were stored at 4°C in refrigerator. Fermentation media was prepared by heating the samples till boiling and gradual addition of different mineral salts KH_2PO_4, Na_2HPO_4, $(NH_4)_2HPO_4$, $MgSO_4$, $FeSO_4$, and $CaCl_2$ to the filtered medium. pH of the media was 6.4 which was further adjusted to 7.0.

Black strap molasses was preserved at 4°C, and was used without the addition of mineral salts as nutrients since initial tests revealed that it already contained some minerals.

Media was prepared by diluting molasses with double distilled water to obtain a 2% (w/v) solution. pH of the media was 6.8 and was left unaltered.

Rice bran was preserved below 4°C since it has a tendency to go rancid. Rice bran was diluted using double distilled water the solids present in the media were not removed to obtain 2% (w/v) slurry of uniform consistency. However, the rice bran media was heated with constant stirring up to 70°C for 10 min for solubilization of the starch. The pH of the medium was adjusted to 7.0 prior to sterilization. For all the three substrates used the media was transferred to three 500 ml Erlenmeyer flasks with working volume of 200 ml in each flask. Media was further sterilized in an autoclave at 15 psi, 121 °C for 15 minutes. Fermentation media prepared from substrates of different origin are shown in Figure 3.4.



(a) (b) (c)

Fig. 3.4: Substrates utilized for biosurfactant production (a) Molasses, (b) Potato process effluent and (c) Rice bran

The elemental content of molasses was determined using Inductive Coupled Plasma (ICP), Perkin Elmer (USA), Atomic absorption spectroscopy (AAS), Shimadzu – AA7000 (Japan) and EDTA titration method. CHNS analyzer (Leco-True Spec) was used for carbon, hydrogen, nitrogen and sulfur analysis. In case of rice bran, the sample was subjected to microwave digestion in nitric acid for 15 minutes and this digested

sample was utilized for the determination of elemental composition in rice bran. The elemental analysis of rice bran was also determined by ICP, AAS, EDTA titration method and carbon, hydrogen, nitrogen and sulfur content was determined using CHNS analyzer.

3.2.3 Experimental procedure

- ***Submerged fermentation***

Submerged fermentation of all the three substrates was carried out by inoculation with nutrient broth (NB), inoculum, 2 % (v/v). Fermentation was carried out in three sets, in 500 ml Erlenmeyer flask with 200ml as working volume in a refrigerated shaking incubator of Zexter Lab Solutions at 150 rpm, 30°C incubation temperature for fermentation time up to 72 h. Samples were collected at definite time intervals and centrifuged to collect the supernatant for analyses of surface tension reduction and other measurements. Figure 3.5 shows the schematic diagram from inoculum preparation up to fermentation and further centrifugation.

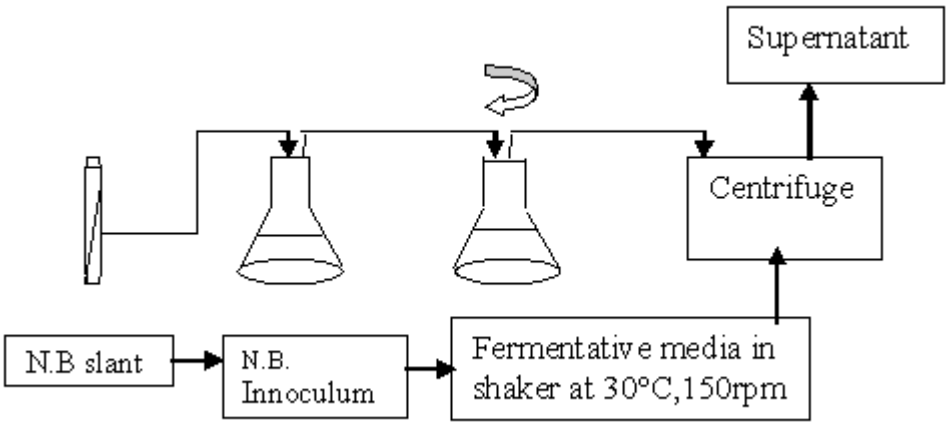


Fig. 3.5: Schematic diagram of the production process

- ***Recovery of biosurfactant***

Fermented broth from all the substrates were centrifuged at 12000g for 10 min after stipulated fermentation time to obtain complete cell free supernatant. The supernatant collected was subjected to foam fractionation. Foam fractionation of cell free supernatant was carried out in three stages in a fractionating column of height 35 cm, inside diameter 2.4 cm. Air was bubbled at 60 cc min^{-1} from the bottom of the column through a sinter into 25 ml of supernatant solution. The column assembly is shown in Figure 3.6. The dry foam collected after foam fractionation was utilized further for various analyses as well as for the recovery of biosurfactant via acid precipitation using 6N HCL. Samples

were kept overnight at 4ºC after acid addition for precipitate formation and then were centrifuged at 10000g for 10 min. The precipitates of biosurfactant obtained were dried in an oven below 60°C and the dry precipitates were weighed to obtain the yield.

- ***Analytical procedures***

This section discusses the details of the measurement techniques utilized in various analyses such as CFU (Colony Forming Units), carbohydrate content, surface tension measurement etc

Fig. 3.6: Foam fractionation column

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- ***Measurement of the colony forming units (CFU)***

The enumeration of cells was done in terms of CFU (colony forming units) using the drop plate technique. The nutrient agar plates were prepared by pouring 20mL of sterilized media aseptically in each sterilized (anumbra) plate. Serial dilutions up to 10^{-10} using sterile distilled water were prepared for samples collected at defined time intervals. 20µL drop of different dilution sample was put in each sector of the eight sector nutrient agar plate and then the plates were kept for incubation at 30°C for 18-24 hours. Colony forming units were counted and multiplied with the relative dilution factor for CFU counting. Sampling was done at regular time intervals and the fermented samples collected were diluted serially for CFU analysis with sterile distilled water. The nutrient agar plate with colony forming units is shown in Figure 3.7.

- ***Analysis of total sugar content.***

Total sugar content of the fermented samples collected at various time intervals were analyzed using phenol-sulfuric acid test (Charalampopoulos et al, 2002). It was assumed that the acid hydrolysis converted polysaccharide structure into glucose monomer units. For analysis 2 ml of the sample was taken and diluted depending on carbohydrate content. To the above sample, 5µl phenol and 5 ml concentrated (90%) sulfuric acid was added and kept for 10 minutes at room temperature. A yellow-orange colour developed due to reaction of carbohydrate with phenol. The test tubes were allowed to cool down to room temperature and absorbance was measured on a UV-VIS spectrophotometer (Shimadzu 260) at 490 nm. The total sugar concentration was determined from the absorbance value using standard curve of concentration versus absorbance (Appendix 1). The standard curve was generated using glucose of varying concentrations and the absorbance was measured at 490 nm.

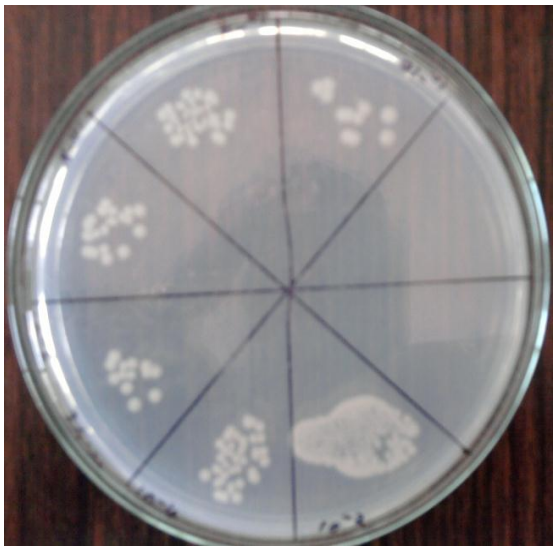

Fig.3.7: Colony forming units in each sector for different serial dilutions grown in nutrient agar medium (Himedia)

- ***Surface tension measurement***

The surface activity of the biosurfactant present in the fermented cell free supernatant was determined by measuring surface tension using a Kruss Tensiometer by Du Nuoy ring method. Fermented broth samples were centrifuged at 10000g for 10 minutes to obtain cell free supernatant. Reduction in surface tension of the fermented samples at regular intervals up to 72 hours of fermentation time was determined. The surface tension of 10-times and 100-times diluted broth samples collected at various time intervals up to 72 hours were also measured for all the samples.

3.2.4 Biosurfactant Characterization

This section discusses the different techniques, TLC, FTIR, NMR, HPLC, ESI-MS used for characterization of the biosurfactant.

- ***Thin layer chromatography for separation and extraction of biosurfactant***

Foam collected from third stage of foam fractionation after its disintegration was subjected to Thin Layer Chromatography (TLC) to obtain biosurfactant in pure form. The components of the liquid were separated on silica gel 60 plates (Merck) using solvent system of n-butanol-acetic acid-water in the optimized proportion of 12 : 3 : 5 respectively. Silica gel plates were prepared for TLC under similar conditions. The plates after chromatography were allowed to dry and further the detection of the components on TLC plate was done by UV and by development under iodine vapors. All the plates were run under similar solvent and at atmospheric conditions.

- **Fourier transform infrared spectroscopy (FTIR)**

Fourier transform infrared spectroscopy (FT-IR) was used to determine the material characteristics of the crude biosurfactant and the concentrated fractions after foam fractionation. Utilizing this technique, functional groups were identified that provided clue on the chemical nature of the biosurfactant. The KBr technique was adopted and the crude biosurfactant in the supernatant of fermented broth as well as the foam fractionated concentrated samples were ground with dried KBr and palletized. Infrared absorption spectra were recorded on a FT-IR (8400S Shimadzu) spectrometer with a spectral resolution of 4.0 cm^{-1} and wavelength range of 400-4000 cm^{-1}. All measurements consisted of 16 scans.

- **Nuclear magnetic resonance spectrometry (NMR)**

The H-NMR was carried out for the determination of structure of the biosurfactant synthesized during fermentation using *B.subtilis* MTCC 2423. Sample preparation involved collecting supernatant after centrifugation of the fermented broth of substrates at 10000g for 10 minutes. and subsequent concentration and purification using foam fractionation, the collapsed foam was acid precipitated and precipitates were recovered by centrifugation at 12000g for 8 min. The supernatant was discarded and precipitates were dried. The dried precipitates were dissolved in 2 ml methanol and transferred to a

5ml glass vial. Methanol was used as medium for transferring the precipitates of centrifuge tube in to the glass vial. Solvent methanol was evaporated leaving the biosurfactant alone in glass vial.

The hydrolysis of the biosurfactant was carried out in glass vial by addition of 2 ml, 6N HCl to the precipitates and the vial was sealed from the top by heating and bending the glass. The sealed vial was kept at 90°C for 24 h in an air oven to complete the acid hydrolysis. Three different samples were prepared in this manner; Sample (a) was based on rice bran fermentation, Sample (b) was on molasses fermentation while Sample (c) was prepared by direct acid precipitation of supernatant of rice bran without any purification by foam fractionation.

Fig.3.8 Glass vials prepared for hydrolysis with (a) rice bran (b) molasses fermented sample and (c) crude biosurfactant

Vials were broken from the top after hydrolysis for all the three samples and contents were recovered in a flat glass dish and dried in a vacuum drier below 70°C. The hydrolyzed sample after drying was dissolved in dimethyl sulfoxide (DMSO) of NMR grade for analysis. Figure 3.8 shows the photograph of the sample in sealed glass vials prepared prior to performing hydrolysis.

- **High pressure liquid chromatography (HPLC)**

High pressure liquid chromatography was performed on samples obtained from fermentation experiments. HPLC was carried out to compare the improvements in the relevant and important peaks of biosurfactant obtained during different stages of foam fractionation recovery process.

The liquid obtained after the disintegration of the foam was analyzed by reverse phase HPLC (Agilent Technologies-1200 series) fitted with C_{18} (5 µm Merck) 250x 4.6 mm column. Mobile phase for HPLC consisted of 3.8 mM trifluroacetic acid (20%) and acetonitrile (80%) as solvents. The elution rate for HPLC was fixed to 1 ml min^{-1} and the absorbance was monitored at 205 nm wavelength.

- **Electrospray ionization mass spectrometry (ESI-MS)**

Electrospray ionization mass spectrometry (ESIMS) was carried out in positive electrospray ionization mode on API 2000 Applied Biosystem (MDS SCIEX). The foam fractionated samples were further purified using TLC and the biosurfactant was scrapped off from the silica gel plates. The biosurfactant from the scrapped silica was subsequently extracted using optimized chloroform (c): methanol (m) mixture containing c and m in the ratio of (65:35) (v/v) respectively while the silica waste settled down in the extraction process was carefully removed from the solvent system. The biosurfactant recovered after solvent evaporation was again dissolved in pure methanol and was subjected further to the ESI-MS analysis.

- **Dynamic Light Scattering (DLS)**

DLS was performed to get an idea of the micelle sizes in biosurfactant solution. Tris buffer (aminomethane-0.05 mol/L) was prepared by dissolving the required quantity of tris (aminomethane) in distilled water. The pH of the prepared buffer was adjusted to 8.5 by the addition of 0.1 mol/L HCl. Biosurfactant separated by acid precipitation of the foam fractionated cell free broth of *Bacillus subtilis* MTCC 2423 grown on rice bran medium was dissolved in the Tris buffer solution for DLS measurements (on a DLS spectrophotometer Nano-ZS, Malvern Instruments Ltd. UK) at 90° angle and 25° C. The presence of univalent K^+ and bivalent Ca^{2+} ions on surfactant micelle size was also determined by adding KCl and $CaCl_2$ to the prepared tris buffer and biosurfactant solution.