

REVIEW ARTICLE

# Methods for investigating biosurfactants and bioemulsifiers: a review

Surekha K. Satpute<sup>1</sup>, Arun G. Banpurkar<sup>2</sup>, Prashant K. Dhakephalkar<sup>3</sup>, Ibrahim M. Banat<sup>4</sup>, and Balu A. Chopade<sup>1</sup>

<sup>1</sup>Department of Microbiology, University of Pune, Pune 411007, Maharashtra, India, <sup>2</sup>Center for Advanced Studies in Materials Science and Condensed Matter Physics, Department of Physics, University of Pune, Pune 411007, Maharashtra, India, <sup>3</sup>Division of Microbial Sciences, Agharkar Research Institute, Pune 411004, India, and <sup>4</sup>School of Biomedical Sciences, University of Ulster, Coleraine, BT52 1SA, Northern Ireland, UK

## Abstract

Microorganisms produce biosurfactant (BS)/bioemulsifier (BE) with wide structural and functional diversity which consequently results in the adoption of different techniques to investigate these diverse amphiphilic molecules. This review aims to compile information on different microbial screening methods, surface active products extraction procedures, and analytical terminologies used in this field. Different methods for screening microbial culture broth or cell biomass for surface active compounds production are also presented and their possible advantages and disadvantages highlighted. In addition, the most common methods for purification, detection, and structure determination for a wide range of BS and BE are introduced. Simple techniques such as precipitation using acetone, ammonium sulphate, solvent extraction, ultrafiltration, ion exchange, dialysis, ultrafiltration, lyophilization, isoelectric focusing (IEF), and thin layer chromatography (TLC) are described. Other more elaborate techniques including high pressure liquid chromatography (HPLC), infra red (IR), gas chromatography-mass spectroscopy (GC-MS), nuclear magnetic resonance (NMR), and fast atom bombardment mass spectroscopy (FAB-MS), protein digestion and amino acid sequencing are also elucidated. Various experimental strategies including static light scattering and hydrodynamic characterization for micelles have been discussed. A combination of various analytical methods are often essential in this area of research and a numbers of trials and errors to isolate, purify and characterize various surface active agents are required. This review introduces the various methodologies that are indispensable for studying biosurfactants and bioemulsifiers.

**Keywords:** Biosurfactant; bioemulsifier; screening; purification; analysis; detection; characterization

## Introduction

Biosurfactants (BS) and bioemulsifiers (BE) are amphiphilic molecules mainly produced by microorganisms including bacteria, yeast, fungi. They possess both hydrophilic and hydrophobic moieties and are able to display a variety of surface activities that, among other roles, help solubilize hydrophobic substrates (Desai and Banat, 1997; Kokare et al., 2007; Kokare, Chopade, and Mahadik, 2009). These compounds have been the subject of increased interest as potential replacements for synthetic surfactants and are expected to have many potential future industrial and environmental applications (Banat, Makkar, and Cameotra, 2000). BS are generally the low molecular weight compounds mainly consisting of glycolipids and some short chain lipopeptides

while high molecular weight polymeric and lipopeptides surface active agents are described as BE. All have some surface active properties leading to the reduction of surface tension (SFT) and interfacial tension (IFT). Among the low molecular weight BS, rhamnolipids and surfactin have been studied extensively (Perfumo et al., 2009; Smyth et al., 2009a,b). Interest in research into and application of both BS and BE is gaining increased momentum due to their environmental friendly nature and lower toxicity in comparison to synthetic surfactant (Shete et al., 2006; Perfumo et al., 2009). Diverse functional properties namely, emulsification, wetting, foaming, cleansing, phase separation, surface activity and reduction in viscosity of crude oil, makes it feasible to utilize them for many application purposes (Kosaric, Gray, and

Address for Correspondence: B.A. Chopade, Director, Institute of Bioinformatics and Biotechnology (IBB) and Professor of Microbiology, University of Pune, Pune 411007, Maharashtra, India. E-mail: directoribb@unipune.ernet.in

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Cairns, 1987). There are several screening methods known for detection of BS/BE producers. These methods includes haemolysis of erythrocytes (Carrillo et al., 1996; Banat, 1993), aximetric drop shape analysis (ADSA) (Van Der Vegt et al., 1991), cell surface hydrophobicity (Rosenberg, Gutnick, and Rosenberg, 1980), drop collapse (Bodour and Miller-Maier, 1998), oil spread (Morikawa, Hirata, and Imanaka, 2000), tilted glass slide (Persson and Molin, 1987), blue agar plate method (Siegmond and Wagner, 1991), emulsification activity (Ellaiah et al., 2002), agar plate method (Morikawa, Ito, and Imanaka, 1992) and direct colony chromatographic (TLC) technique (Matsuyama, Sogawa, and Yano, 1987). Several conventional (solvent extractions, acid precipitation, filtration, centrifugation) as well as more sophisticated methods such as ion exchange, adsorption-desorption are known for their purification while others such as TLC, HPLC, GC-MS, NMR, FAB-MS are used for their characterization and analytical (Makkar and Cameotra, 1998). Various experimental strategies such as static light scattering, microscopic hydrodynamic studies are crucial for characterization of size and shape of micelles.

Several authors have briefly described the different screening, recovery and characterization methodologies for both BS and BE (Banat, 1995a,b; Bodour and Miller-Maier, 2000; Makkar and Cameotra, 2002; Youssef et al., 2004; Maneerat 2005; Muthusamy et al., 2008). This review however aims to provide a quick glance into the main screening methodologies employed to obtain microbial cultures producing BS and BE and the main techniques employed for their purification, concentration, characterization and analysis.

### **Important terms related to surfactants/emulsifiers**

The terminology associated with surfactants has a long history, where soaps were used as general surfactants in chemical and detergent industrial application in several terminologies were associated with such industries as follows.

**Surfactant:** This is a surface active molecule that tends to adsorb at surfaces or interfaces by modifying the chemical interaction of liquids at the surface boundary. These amphiphiles (hydrophilic and hydrophobic) are classified as nonionic, anionic, cationic, or amphoteric which is dependent on their molecular structures.

**Emulsifier:** These are the groups of surface active molecules that can form emulsion of two immiscible liquids. These compounds are not necessary to reduce the SFT. Therefore, surfactant can have both SFT reduction and emulsification activity. However, emulsifier may just bind water insoluble substrates together to form an emulsion.

**Amphiphilic molecule:** It is a molecule with hydrophilic head and hydrophobic tail which accumulate at surface, interfaces and reduces forces of repulsion between unlike phases leading to the mixing of immiscible phases (Bodour and Miller-Maier, 2000).

**Surface tension (SFT):** It is the force per unit length exerted by a liquid in contact with a solid or another liquid. It can also be considered as a measure of the free energy per unit area associated with a surface or an interface. Among the

known liquids a water has highest SFT value of 72 dyne/cm or mN/m which would be reduced upon the addition of surfactant.

**Interfacial tension (IFT):** It is an intermolecular attractive force held within the molecules in a liquid. A liquid with low IFT are more easily emulsified.

**Critical micelle concentration (CMC):** It is the initial value of minimum SFT and the term micelle was denoted by McBain (1913), where surfactant molecules form micelles in solution. Below CMC value, surfactant molecules are loosely integrated into the water structure known as a monomer. Whereas, at CMC value, the surfactant-water structure aggregate to form micelles (spherical/lamellar form). CMC leads to abrupt change in the physical properties of SFT (solution), conductivity, viscosity, density, osmotic pressure, turbidity, and chemical shifts (Margaritis, Zajic, and Gerson, 1979). CMC values of nonionic micelles depend on lipophilic and hydrophilic groups, whereas for ionic micelles, length of lipophile and charge are crucial. Each surfactant has its own individual CMC value.

**Inverse/reverse micelles:** This type of micelle is seen in the nonpolar solvent system where, hydrophilic head groups of a surfactant molecule are exposed to the surrounding solvent which is energetically unfavorable, which results in a water-in-oil system. Under such conditions, the hydrophilic groups are sequestered in the micelle core and the hydrophobic groups protrude away from the centre. These inverse micelles are proportionally less likely to form an increasing headgroup charge, since hydrophilic sequestration results in a highly unfavorable electrostatic interactions.

**Hydrophilic and lipophilic balance (HLB):** Griffin (1949, 1954) denoted this term for nonionic surfactants, as the relative simultaneous attraction of an emulsifier for two phases of an oil/water system (Attwood and Florence, 1987). This property is represented by an arbitrary scale of 0-20, wherein the most hydrophilic materials have highest number. The HLB scale denotes the ability of surfactant to form emulsions of water-in-oil or oil-in-water by comparing with surfactants of known HLB values and properties. For example, the HLB scale can be constructed by assigning a value of 1 for oleic acid and a value of 20 for sodium oleate. Further using a range of mixtures of these two components in different proportions, intermediate values can be obtained. Emulsifiers with HLB values less than 6, favor stabilization of water-in-oil emulsification, whereas emulsifiers with HLB values between 10 and 18, have opposite effect and favor oil-in-water emulsification.

**Contact angle (CA):** Depending upon the SFT value, a well defined angle is obtained for the contact of a liquid on a solid surface. It is the angle between the tangent to solid-vapor interface and the line of solid-liquid interface. When liquid completely wets the solid surface, CA in such cases is zero.

**Emulsion:** It is a colloidal dispersion of one liquid in another (oil/water) which is often stabilized with the surfactant. Emulsions are not truly stable but may be metastable.

**Microemulsion:** A colloidal dispersion of one liquid droplet in another liquid (oil/water) in microscopic form leads to a

microemulsion formation. It is stabilized with surfactants and co surfactants thermodynamically, where the size of droplet is about 10–100 nm diameter. This structure allows them to decrease the IFT and form emulsions as well as micro-emulsions (Goma, Pareilleux, and Durand, 1973).

### Functional properties of surfactant/emulsifier

The location and size of the hydrophilic and hydrophobic functional groups determines the property of a surfactant which consequently determines the practical application of surfactants in various industrial applications related to detergents, oil recovery, cosmetics, food, pharmaceuticals, agriculture, mining (Desai and Banat, 1997; Jagtap et al., 2009).

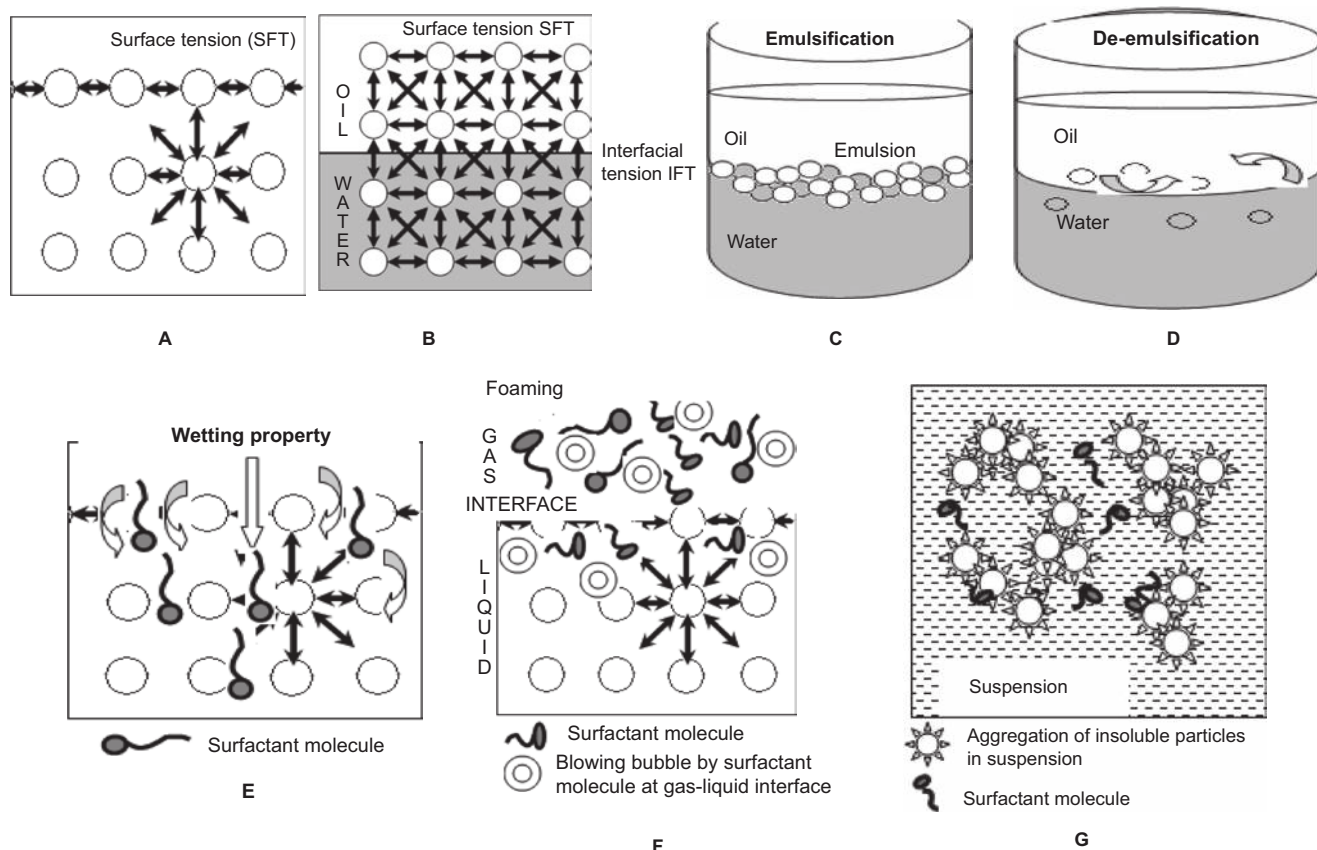
**Surface activity:** SFT and IFT are the important properties of surfactant (Figure 1A and B). Molecules of water droplet are held together by cohesive forces. These strong intermolecular attractive forces build the tension on the surface, i.e. called as SFT (Figure 1A). SFT of the DW is 72 mN/m and when surfactant is added to it, this SFT value is reduced. One BS unit is defined as the amount of surfactants forming 1 cm<sup>3</sup> of oil displaced area (Thaniyavarn et al., 2003). Surfactin produced by *Bacillus* sp. is the most effective BS reducing water SFT from 72 to 27 dynes/cm (Cooper and Goldenberg, 1987; Banat, 1993).

**Emulsification:** Dispersion of one liquid into another (as microscopic droplets) leading to the mixing of two immiscible

liquids (Figure 1C). It represents a micellar solubilization; however, the resultant solubilized particles are much bigger.

**De-emulsification:** This process breaks emulsions through the disruption of stable surface between the bulk phase and the internal phase (Figure 1D) which is important in oil production processes, where natural emulsifying agents hinders the production processes (Bosch and Axcell, 2005). De-emulsion is achieved by disturbing the thermodynamic conditions at the interface. Equipment used in petroleum industry mainly suffers from the corrosion therefore, before downstream oil processing, de-emulsification action are important. Different industries such as mining, food, nuclear fuel reprocessing, cosmetics and pharmaceuticals are dependent on this property (Kosaric, Gray, and Cairns, 1987). De-emulsification capabilities of mixed bacterial population were usually tested by using a kerosene-water and petroleum-oil emulsion system, where up to 96% of de-emulsification was achieved. Mixed culture products exhibit high de-emulsifying activity as compared with the most effective pure culture (Nadarajah, Singh, and Owen, 2002).

**Wetting:** It is the spreading and penetrating power a substance that lowers the SFT, when added to a liquid. It reduces attractive forces of similar molecules and increases the attraction towards unlike surfaces. Surfactants act as wetting agents by getting into the pores and fissures rather than bridging



**Figure 1.** Pictorial representation of different functional properties of biosurfactants/bioemulsifiers: (A) Surface tension; (B) Surface and interfacial tension; (C) Emulsification; (D) De-emulsification; (E) Wetting; (F) Foaming; and (G) Adsorption. Note: Based on definitions and information, Figure 1C-G have been constructed.



them with the SFT (Figure 1E). A strong wetting agent is considered by the increase in spreading ability of a liquid over a surface area and the lowering of the contact angle of liquid surfaces to solids. This is extremely important when reconstituting dry powders, dry beads or reagents in solid-phase devices. Wetting properties, emulsification and micellar solubilization of nonionic surfactants such as Rokanol L10, Triton X-100 and BS JBR 425 have been investigated (Pastewski, Hallmann, and Medrzycka, 2006).

**Foaming:** Surfactants get concentrated at a gas-liquid interface leading to the formation of bubbles through the liquid and on the interface resulting in foam formation (Figure 1F). Bubbling techniques help studying the foaming properties of surfactin, sodium dodecyl sulphate (SDS), and bovine serum albumin (BSA). Surfactin exhibit excellent foaming properties as compared with SDS (Dubey, Juwarkar, and Singh, 2005).

**Adsorption:** This functional property helps the surfactant molecules to get adsorb on hydrophobic substrates (Figure 1G). Wei, Mather, and Fotheringham, (2005) recovered rhamnolipid BS JBR215 from Jeneil BS Company, USA by using this technique. More than 95% of BS can be recovered successfully by the adsorption technique. Adsorption property of a surfactant is an important factor to enhance oil recovery. Adsorption facilitates strong interactions of surface active molecules with the rock than with the oil and hence can increase the recovery of oil from rocks (Curbelo et al., 2007). Best example for adsorption property of a surfactant is a pulmonary surfactant. The mixture of lipid and protein surfactant present at air/alveolar interface of the lungs, lower the SFT to very low values, thereby facilitate breathing and prevent alveolar collapse (Schürch, Goerke, and Clements, 1976).

**Dispersion:** A dispersants is a material that reduces the cohesive attraction between similar particles. This property of surfactant keeps insoluble particles in suspension by preventing insoluble particles to from aggregations with each other. This property also leads desorption of hydrophobic molecules from rock surfaces enhancing mobility and recovery and has application in oilfield chemistry.

**Detergency:** Washing and cleansing activity is associated with detergency. BS/BE act in similar way as that of detergents.

**Flocculation:** It is a process, where the emulsion droplets stick together to form a cluster that can be broken up by mechanical action restoring the emulsion to its original form. Microelectrophoresis measurements and optical microscopy testing are used to evaluate flocculation. Interesting work on flocculation of fine fluorite particles by the bacterium *Corynebacterium xerosis* has been reported by Haas et al. (1999). The cells of *C. xerosis* adhere to the fluorite surfaces and promote the aggregation of the particles to achieve high quality flocks.

**Phase separation:** Once the surfactant is added to immiscible liquids, it leads to the formation of emulsion. However, after some period, the emulsion droplet of like molecules begins to assemble and come together leading to

the separation of the two phases. This process is called phase separation.

**Viscosity reduction:** Due to high viscosity of crude oil, it resists to flow and becomes very difficult for transport. Heavy crude oil (high density) contributes significant contents of nitrogen, oxygen, sulphur compounds and heavy metal contaminants. Such viscosity of heavy oils is reduced by using surfactants to increase mobility and ease of transportation.

**Solubilization:** Surfactants enhance solubilization of insoluble material. At high concentration of surfactant, micellar structures are formed. Insoluble molecules are encapsulated into a micellar structure and brought into solution at higher level. This property is important to form water-insoluble substances in aqueous solutions, or water-soluble substances in organic solvents. BS/BE are more effective than the synthetic surfactants to solubilize complex compound mixture to an aqueous solution. Perfumo et al. (2009) reported on the roles of BS and BE in accessing hydrophobic substrates while Wong et al. (2004) also reported similar observation on the effects of synthetic surfactants and BS

**Corrosion inhibition:** Corrosion inhibitors are material that protects against the wearing away of appliance surfaces. Sodium silicate is a corrosion inhibitor which is commonly used in detergents and builds soap for laundry, dishwasher products to prevent under solid deposit corrosion. Li and Mu (2005) investigated a nonionic surfactant Tween-40 and reported a satisfactory level inhibition of corrosion of cold rolled steel in 0.5–7.0 M sulphuric acid as detected by weight loss and potentiodynamic polarization methods.

**Temperature, pH and ionic strength tolerance:** Surfactants are stable at various temperature and pH. Lichenysin obtained from *B. licheniformis* JF-2 is stable up to 50°C, pH of 4.5–9.0 and at NaCl (50 g/L), Ca (25 g/L) concentrations (McInerney, Javaheri, and Nagle, 1990). Similarly, lipopeptide BS from *B. subtilis* LB5a is highly stable at 121°C/20 min and even after 6 months was found to be stable at –18°C. Surface activity of this lipopeptide remains unchanged in pH range of 5–11 and NaCl (20%) (Nitschke and Pastore, 2006).

### Screening methods for detection of biosurfactant and bioemulsifier producers

There are 11 main methods used to screen, detect or evaluate potential BS and BE producing microorganisms, each has its own advantages and disadvantages as discussed below:

**Agar plate overlaid with hydrocarbons:** Pure isolates are streaked on oil coated agar plates and incubated for one week at desired temperature. Colonies surrounded by an emulsified halo are detected as BS producers (Morikawa, Ito, and Imanaka, 1992). This is the efficient method where observation of emulsified halo around the culture is the direct indication of BS producer.

**Aximetric drop shape analysis (ADSA):** This technique determines the CA as well as SFT for the profile of a liquid droplet resting on a solid surface. Cells are suspended in buffer solution or could be in broth cultures. Consequently, droplet of each suspension is placed on fluoroethylenepropylene

surface and the profile of a droplet is determined with counter monitor as a function of time up to 2 h. The SFT of the suspensions are calculated from the droplet profile with ADSA. Only BS producing suspensions shows reduction in SFT, which is dependent on product concentration and or number of BS producing microorganism. It is an excellent technique which requires very small numbers of cells (Van der Vegt et al., 1991).

**Cell surface hydrophobicity technique:** There is a direct correlation between cell surface hydrophobicity and BS production. The cells are harvested by centrifugation (12,000g/30 min/4°C) and washed twice with 50 mM phosphate buffer (pH 7.0) and resuspended using the same buffer to absorption ( $A_{600}$ ) of 0.5. Cell suspensions (3 mL) are added to hydrocarbons (0.5 mL) and vortexed for 3 min and allowed to settle for 10 min for the hydrocarbon phase to rise completely. The aqueous phase is removed and transferred to a 1 mL cuvette to measure  $A_{600}$ . The decrease in  $A_{600}$  of the aqueous phase is taken as a measure of the cell surface hydrophobicity (H%), which is calculated as follows:  $H\% = [(A_0 - A)/A_0] \times 100$ , where  $A_0$  and  $A$  were  $A_{600}$  before and after mixing with hydrocarbon, respectively (Rosenberg, Gutnick, and Rosenberg, 1980; Pan, Li, and Liu, 2006; Maneerat and Dikit, 2007). Depending upon the hydrocarbon uptake behavior, microorganisms may have high and/or low surface hydrophobicity. Generally, those microbes which can take hydrocarbon by direct uptake mode, shows high surface hydrophobicity. Cell bound BS production is also associated with hydrocarbon uptake. This phenomenon is discussed in detail by Franzetti et al. (2008) who worked on *Gordonia*. On the other hand, Bouchez-Naïtali and coworkers (1999) demonstrated that microbes show low surface hydrophobicity when BS/BE are released extracellularly, where hydrocarbon uptake is mediated through the BS. Hydrophobic interaction chromatography (Smyth et al., 1978), salt aggregation test (Lindahl et al., 1981), bacterial adherence (Rosenberg and Gutnick, 1980) and adhesion (Rosenberg, 1984) by replica plating technique helps to identify BS producers.

**Blue agar plate method:** This technique was specially developed for detection of glycolipids such as rhamnolipids by *Pseudomonas* sp. It can be applied for detection of similar type of BS from other Gram negative isolates. Mineral salts agar medium (MSA) (as per Siegmund and Wagner, 1991) supplemented with carbon source (2%) and cetyltrimethylammonium bromide (CTAB: 0.0005%)–methylene blue (MB: 0.0002%). Anionic BS forms insoluble ion pair with the cationic CTAB-MB and formation of dark blue halo around the culture is considered as positive for BS production. It is an excellent technique that has been used generally for detection of glycolipids BS.

**Haemolytic activity:** It is a qualitative screening test for detection of BS producers. Solid media such as Luria agar (LA), nutrient agar (NA), supplemented with 5% fresh whole blood are used (Carrillo et al., 1996; Banat, 1993). Isolates are streaked and incubated at required temperature for 48 h. Visual inspection for haemolysis may be an indication of red blood cell lysis due to cell membrane rupture caused by the

presence of surface active molecules. Blood agar is a complex medium hence; it is very difficult to test the BS productivity of a culture at different culture conditions directly on the agar (Youssef et al., 2004). Haemolytic activity however has been considered an unreliable criterion for the detection of BS activity (Satpute et al., 2008).

**Modified drop collapse method:** Microtitre plates are thinly coated with Pennzoil. A sample of 5  $\mu$ L (culture broth) is added to the centre of the well and observations are carried out for 1 min. If the drop of a sample collapses from the coated oil it is an indication of the presence BS in the culture broth (Jain et al., 1991; Bodour and Miller-Maier, 1998). However if the sample contains negligible amount of surfactant, it may give false negative results (Satpute et al., 2008; Satpute, 2008).

**Oil spread method:** Crude oil of 20  $\mu$ L is added to 50 mL of distilled water (DW) in a Petri plate. Culture broth of 10  $\mu$ L is added on oil coated water surface. Colony surrounded by an emulsified halo is considered positive for BS production (Morikawa, Hirata, and Imanaka, 2000). It is one of the best methods to detect the presence of BS producers.

**Tilted glass slide test:** Isolates are grown for 24 h on agar plates. A sample of colony is mixed with a droplet of 0.9% NaCl at one end of the glass slide. The slide is tilted and droplet observed. BS producers are detected by observation of droplet collapsing down (Persson and Molin, 1987). This technique is effectively a modification of the drop collapse method.

**Direct colony-thin layer chromatographic (TLC) technique:** This method characterizes BS producers. In this technique, a bacterial mass is directly placed on pre-developed (chloroform; methanol; 2:1) TLC plate. After drying the bacterial mass, the plate is run in chloroform; methanol; 5 M ammonia (85:25:4 v/v) and developed with developers. Resulting chromatograph indicates the characteristic lipid compositions of organism (Matsuyama, Sogawa, and Yano, 1987). The method is fast and easy to perform without any special requirement.

**Emulsification assay (EA):** Culture broth centrifuged at 10,000 rpm/15 min/RT. Supernatant (3 mL) is mixed with oil/hydrocarbon (0.5 mL) and vortexed vigorously for 2 min. It is left undisturbed for 1 h to separate aqueous and oil phase (Jagtap et al., 2009). Uninoculated broth is used as a blank. Absorbance of aqueous phase is measured by using a spectrophotometer. An absorbance of 0.01 units at 400 nm multiplied by dilution factor is considered as one unit of emulsification activity per milliliter (EU/mL) (Patil and Chopade, 2001a,b; 2003).

**Emulsification index (EI):** Emulsification activity is measured by calculating EI (Cooper and Goldeberg, 1987). Kerosene is added to culture broth (1:2 v/v), vortexed for 2 min and allowed to stand for 24 h. The height of emulsion is measured by taking the layer formed in between aqueous and kerosene layer. There are some modifications reported by some authors such as using 1 mL of broth, 4 mL of water and 6 mL of kerosene are vortexed to obtain maximum emulsification. EI is calculated by measurement of emulsion height. Ellaiah et al. (2002) and Haba et al. (2000) selected

BS producers from the measurement of EI. The EI stability designates the strength of a surfactant.

**Turbidity assay:** This method was developed by Rosenberg et al. (1979) and later modified by Neu and Poralla (1990). Culture broth is filtered and filtrate is dried and added to a buffer. Further optical density (OD) is measured at 446 nm. To this, hydrocarbon is added and the mixture is vortexed for 2 min. It is allowed to stand for 10 min and again the OD is measured. EA is measured from the difference between initial and final OD.

**Tensiometric measurement of SFT:** Measurement of SFT using a tensiometer is one of the common methods. Wilhelmy plate method, DuNouy ring method, maximum pull force method, pendant drop methods are all known for SFT measurement. SFT measurement is not feasible to apply for large number of isolates at preliminary screening level. Cell free extract is used for SFT measurement.

**Molecular tools to identify biosurfactant producing genes:** Biotechnological applications have been extended for screening methodology. Comprehensive data on molecular biology of BS/BE is given in detail by Satpute et al. (2009). Research team of Hsieh et al. (2004) used *sfp* locus for PCR based detection of BS producing *B. amyloliquefaciens* and *B. circulans*. Such methods would authenticate the conventional screening methods. Similarly, *P. rugulosa* NBRC 10877 was also identified as mannosylerythritol lipid producer on the basis of rDNA sequence (Morita et al., 2006). Direct search for genes involved in BS production is faster and less laborious. Newer inventions such as those of Whiteley, Lee, and Greenberg, (1999) could be used to identify modulators and genes of Quorum sensing signal (QSS) in bacteria. Novel indicator strains and vectors have been engineered successfully.

#### **Recovery or purification of biosurfactants/bioemulsifiers**

Recovery and/or purification of biotechnological products in downstream processing costs usually account for approximately 60% of the total production costs which makes commercial production of BS and BE quite expensive. Methods to reduce costs through the use of inexpensive and renewable substrates are therefore necessary (Desai and Banat, 1997; Banat, Makkar, and Cameotra, 2000; Makkar and Cameotra, 1997). However, a great deal of monetary input is required in the purification processes (Rodrigues et al., 2006). During all these process the risk of contamination with undesired compounds from fermentation procedures always exist. Ionic charge (chromatography), solubility (water/organic solvents) and location (intracellular, extracellular, cell bound) ultimately determines the purification procedure for BS/BE to be extracted.

Generally, purification and precipitation of high molecular weight BS is carried out using ammonium sulphate, followed by dialysis to remove any small molecules. Other methods also involved the use of tri-chloroacetic acid (TCA), acetone precipitation, ethanol and chloroform/methanol. Several conventional methods known for recovery of BS/BE are mentioned as follows.

**Acetone precipitation:** Culture is grown in a minimal medium supplemented with required constituents. Cell free supernatant is mixed with ice-cold acetone to precipitate emulsifiers, which is further suspended in phosphate buffer. Then mixture is incubated at 4°C for 15–20 h to get the precipitate of emulsifier. BE is analyzed for emulsifying activity, polysaccharide and protein fractions. This method has been used by several workers, to purify BS/BE (Rosenberg et al., 1979; Patil and Chopade, 2001a,b; 2003).

**Ethanol precipitation:** Like acetone, ethanol is a popular solvent for obtaining crude extract of BE from the culture supernatant of microbes such as *Acinetobacter*, *Pseudomonas*, *Bacillus*, *Cyanobacterium* and yeast, species. Culture broth is centrifuged (11,000g/20 min/4°C) and BE is precipitated from the supernatant by using cold ethanol. Phetrong, H-Kittikun, and Maneerat, (2008) found that precipitation of emulsifier from *A. calcoaceticus* subsp. *Anitratus* SM7 with ethanol was the most efficient method when compared with other precipitation methods.

**Ammonium sulphate precipitation:** High molecular weight BE such as emulsan, biodispersion (protein rich compounds) are precipitated using  $(\text{NH}_4)_2\text{SO}_4$ . This method was basically introduced by Rosenberg et al. (1979) for precipitation of BE from *Arthrobacter* RAG-1. For this purpose, 30% of  $(\text{NH}_4)_2\text{SO}_4$  was added directly to the fermentation broth without removal of cells and allowed to stand for overnight. Further this precipitate was suspended in 3% saturated  $(\text{NH}_4)_2\text{SO}_4$  and after centrifugation the supernatant was clarified. Additional  $(\text{NH}_4)_2\text{SO}_4$  was added to reach the final concentration of 40%. The resulting precipitate was centrifuged and extracted with ether. Kaplan and Rosenberg, (1982) obtained extracellular emulsifier from *A. calcoaceticus* BD413 by successively increasing concentration of  $(\text{NH}_4)_2\text{SO}_4$  added to cell free extracts. Now laboratories generally proceed with the cooling of cell free broth at 4°C followed by saturating solution by addition of  $(\text{NH}_4)_2\text{SO}_4$ . After overnight refrigeration, pellet is further re-suspended in  $(\text{NH}_4)_2\text{SO}_4$ . The pellet obtained after centrifugation is dissolved in water and extracted with equal volume of hexane for the removal residues. The product is further purified by a dialysis procedure and lyophilized. Depending upon the type of BE, different workers have used different concentrations of  $(\text{NH}_4)_2\text{SO}_4$ . Bach, Berdichevsky, and Gutnick, (2003) added 60%  $(\text{NH}_4)_2\text{SO}_4$  to *A. venetianus* RAG-1 cell free supernatant while Toren et al. (2001, 2002) precipitated alasan with 65% of  $(\text{NH}_4)_2\text{SO}_4$ . Lipopeptide BS has been partially purified from *Bacillus* sp. by precipitating with 40%  $(\text{NH}_4)_2\text{SO}_4$  (Youssef, Duncan, and McInerney, 2005).

**Acid precipitation:** This method is easy inexpensive and readily available to recover crude BS such as surfactin, lipopeptides, glycolipids etc.

**Surfactin:** *Bacillus* sp. produces different types of surface active peptides (Arima, Kakinuma, and Tamura, 1968) which are purified from cell free supernatant. Acid hydrolysis is carried out by using concentrated HCl to bring down the pH 2.0. BS becomes insoluble at lower pH (Mukherjee, Das, and Sen, 2006) and precipitates proteins and lipid containing BS



at 4°C overnight (Cooper et al., 1981). It is followed by centrifugation and pellet is further extracted by using various solvents (Nitschke and Pastore, 2006; Thaniyavarn et al., 2003). Extracted material is filtered for removal of residues and evaporated to dryness using rotary evaporator. Lipopeptide BS from microbes grown under simple or complex growth conditions are also purified by this method (Jennings and Tanner, 2004).

**Rhamnolipid:** Culture supernatant is acid hydrolyzed with HCl to precipitate glycolipid which becomes insoluble in aqueous solution, which is then allowed to stand for overnight at 4°C and collected by centrifugation and solvent extraction. During acidification, BS is present in protonated form, which is less soluble in water. Acidification, centrifugation, and extraction procedure are similar for other glycolipids. However, different solvents viz., chloroform, methanol, ethyl acetate are used generally to purify rhamnolipids. Organic phase is removed separately and mixed with Na<sub>2</sub>SO<sub>4</sub> to remove water and can be concentrated in a rotary evaporator at 40°C to obtain crude product. Residue is further dissolved in NaHCO<sub>3</sub> to purify BS. There are a number of publications reporting on rhamnolipid purification by acid precipitation (Haba et al., 2000; Smyth et al., 2009a). Sophorolipids (Nunez et al., 2001), trehalose lipids, mannosylerythritol lipids (MELs) (Rapp et al., 1979) are extracted similarly to that of rhamnolipids.

**Adsorption-desorption:** Some BS molecules can adsorb and desorb from Amberlite XAD 2 or 16 polystyrene resins and therefore, this interaction is used for purification of BS. The processes is initiated by applying cell-free culture broth directly to the adsorbent column and 0.1 M phosphate buffer (pH 6.1) is used to equilibrate it. Exhaustion of the adsorbent resin is observed by SFT or ultra violet (U.V.) absorption (Reiling et al., 1986). A wash of DW is given to the resin (for removal of pigments and free fatty acids, FAs) (Abalos et al., 2001). Further, elution is carried out with methanol, which can be evaporated to obtain crude BS. Adsorption-desorption techniques have been further extended by Dubey, Juwarkar, and Singh, (2005). Conventional methods such as solvent extraction, precipitation, crystallization, centrifugation and foam fractionation cannot be used when distillery wastewater is used as the nutrient medium for BS production by *P. aeruginosa*. Extraction with those methodologies, impart color to the BS. Therefore, to overcome this difficulty, new downstream techniques have been developed to recover BS. In this newly developed approach, BS are adsorbed on polymer resins and subsequently desorbed with organic solvents. Polystyrene resin is packed in glass columns. The main advantages of this technique are quick, one-step recovery and high quality purified BS is obtained. Adsorption-desorption on wood based activated carbon can be used. Dubey, Juwarkar, and Singh, (2005) suggested that same carbon could be reused for three consecutive cycles for BS adsorption. This process offers good examples of continuous recovery of BS from fermentation broth as well as from concentrated foam, through an *in situ* method that avoids end product inhibition. This ultimately reduces the

use of high cost solvents and results in less degradation of product.

**Ion exchange chromatography:** Charged BS such as rhamnolipids which possess a negative charge at higher pH environments may be attached to ion-exchange resins and can be eluted with buffer containing 10% (v/v) ethanol. The addition of a minimum of 0.6 M NaCl to the buffer leads to the release of rhamnolipid from resin. The adsorption process can be repeated to remove salt from rhamnolipid BS. Ion-exchange resins are reusable. Treatment with buffer (2M NaCl and 20% ethanol) is essential. Thus high quality purified BS can be achieved with ion exchange chromatography. Rhamnolipid BS from *Pseudomonas* sp. has been purified by this method (Matsufuji, Nakata, and Yoshimoto, 1997).

**Centrifugation:** Following acid precipitation, BS containing broth can be centrifuged at 12,000 rpm for 15 min at 4°C to be easily collected as crude product (Nitschke and Pastore, 2006). Once the pellet is obtained, it can be dried under N<sub>2</sub> and extracted with solvents.

**Crystallization:** Once, BS is precipitated/extracted, it is re-dissolved in an organic solvent (glycolipids such as rhamnolipids are concentrated). Reaction is also coupled with a temperature reduction, which crystallizes the BS (rhamnolipid). Therefore, it becomes less soluble in solvents. With the help of this process it is possible to obtain pure crystals of Rha-Rha-C10-C10. Lyophilization process in 0.05 M sodium bicarbonate solution leads to the formation of monorhamnolipids. These pure crystals cannot be achieved by solvents evaporation in the presence of water (Manso Pajarron et al., 1993).

**Filtration and precipitation:** BS produced by *P. aeruginosa* is obtained successfully by using a precipitation method. Precipitation was carried out with ethanol, acetone, ethanol-acetic acid (1%)/5 N HCl in an equal volume of culture liquid. Extraction was performed twice to enhance the yield of BS (Turkovskaya, Dmitrieva, and Muratova, 2001).

**Foam fractionation:** In this method, foam is collected (through fractionation column) and acidified with HCl down to pH 1.0–2.0 to precipitate BS, which can be extracted with solvents (Cooper et al., 1981). High yield of BS can be achieved by increasing the residence time of foam in the fractionation columns. Gravitational forces allow drainage of liquid in the form of lamella which helps to recover BS more efficiently. Abraham, Meyer, and Yakimov. (1998) extracted glucose lipid from the cell wall of *Alcanivorax borkumensis*. For this purpose, wet cells were suspended in solvents such as methanol/dichloromethane (DCM)/phosphate buffer followed by treatment with an ultrasonic probe. Further, sample was centrifuged and lipids were fractionated using column chromatography by sequential elution with DCM, acetone and methanol. Three fractions viz., neutral, glycolipid and phospholipids were successfully eluted with this technique. Neu and Poralla, (1990) purified BS from *Bacillus* sp. by blowing the foam out of the fermentor. It was centrifuged and extracted with acetone precipitation. Noah et al. (2002) conducted a combined experiment of surfactin production from potato process

effluents with direct foam fractionation techniques to enhance the yield of BS.

**Isoelectric focusing:** Zinjarde et al. (1997) reported a rapid and simple procedure for the isolation and purification of BE from marine yeast *Yarrowia lipolytica* NCIM. It is one of the novel approaches used for purification of BS. IEF unit consist of a single column which is filled with electrolyte, density gradient solutions and nonion conducting polymer. In the presence of important factors such as electric influence, pH, density gradient, the ampholyte moves in the column until it reaches a neutral pH. This procedure requires 10–12 h at 400 V and a current of 1.5 A. Crude BE is applied in IEF and pH, emulsification activity needs to be checked. Columns help to separate fractions with the changes in pH. Once total separation occurs, electro-focusing is discontinued and the activity of purified BE is compared with the crude form.

**Solvent extraction:** Hydrophobic moieties of BS are soluble in some solvents which help in extraction and separation of crude product. Different solvents such as chloroform, methanol, ethyl acetate, di-chloromethane, butanol, pentane, hexane, acetic acid, di ethyl ether, isopropanol are commonly used for extraction of BS/BE. However, these solvents are toxic and costly. Therefore, it is very necessary to use inexpensive and less toxic solvents for recovery of BS. Several types of BS such as rhamnolipid, trehalose lipids, sophorolipids, cellobiolipids, liposan produced by different microbial population have been purified by solvent extraction (Desai and Banat, 1997; Smyth et al., 2009 a,b). BS is concentrated from the supernatant by addition of  $ZnCl_2$  and is extracted twice with solvents. The organic phases are evaporated to dryness and analysed by TLC technique (Tuleva, Ivanov, and Christova, 2002).

**Ultrafiltration:** It is used generally to concentrate and purify BS such as surfactin, rhamnolipids. It is a low pressure driven mechanical processes commonly carried out using amicon filter paper or other types of filters of 0.22  $\mu$ , 0.45  $\mu$  pore sizes. BS are recovered from the fermentation broth by ultrafiltration with 30 kDa molecular weight cut-offs (MWCO) at a pressure in the range of  $6.9 \times 10^4$  and  $2.1 \times 10^5$  Pa (Mulligan and Gibbs, 1990). Hollow fiber ultrafiltration cartridges are also used for continuous operation at a pressure of  $1.7 \times 10^5$  Pa. MWCOs ultrafiltration permits passing of small molecules from a fermentation broth such as salts, amino acids, organic acids, alcohols and other metabolites whereas, macromolecules viz., extracellular proteins with nominal molecular diameter higher than that MWCO of the membrane are concentrated. At above CMC level, BS forms micelles that are collected in polymeric membranes. Lin and Jiang (1997) used different MWCO membranes which had affected the percentages of surfactin retention. Surfactin is associated in the form of supramolecules with 10,000 and 30,000 Da, where MWCO membranes retention were 98.8% and 97.9% respectively. The same percentage of surfactin is decreased significantly up to 86% and 53% when 50,000 and 100,000 Da MWCO membranes were used. The behavior of surfactin micelles as macromolecules depends upon the MWCO membranes. Zinjarde et al. (1997) purified BE from yeast *Yarrowia*

*lipolytica* NCIM through ultrafiltration. Lin et al. (1998a) concentrated BS from *B. licheniformis* mutant JF2 by ultrafiltration through MWCO hollow fiber. High MWCO membranes enhance the quality of BS. Sometimes the ultra centrifuged supernatant product resulted in low SFT value without any emulsifying activity. In such cases surfactant can be removed by solvent extraction. When the same medium is extracted without ultrafiltration, it leads to the removal of surfactant without affecting the emulsifying property (Cooper and Goldenberg, 1987). Concentrated lipopeptide from mutated *Bacillus* is achieved by ultra centrifuging supernatant through 30 kDa MWCO (Lin et al., 1998b). BE from a nonfluorescent strain of *P. putida* ML2 is concentrated through ultrafiltration (Bonilla et al., 2005). Ultrafiltration is fast, easy and yields highly pure BS.

**Preparative thin layer chromatography (TLC):** This method results in a pure quality of product. Preparative TLC on a silica-coated glass plate with variable thickness is applied with BS sample and allowed to run in a solvent system. Bands are visualized under UV or some other technique (nondestructive technique) (depending upon type of BS) and are scraped and extracted further with solvents.

**Dialysis and lyophilization:** Seamless cellulose tubing dialysis bags are used for the purification of BE. The collected precipitate samples containing BS/BE can be dissolved in 5–10 mL of sterile DW and dialyzed against double DW for 48 h at 10°C. The storing of this dialysate is carried out at 4°C in an airtight container till it is used further. Kaplan and Rosenberg (1982) reported production of BE from *A. calcoaceticus* BD4 and BD413 which after  $(NH_4)_2SO_4$  precipitation was dissolved in deionized water; dialyzed in cold DW and lyophilized till it is used further. Shah and Prabhune (2007) reported a simple method for resolution of sophorolipids using dialysis tubing instead of a glass column for silica gel chromatography. Silica gel was activated at 11°C for 4 h before packing in dialysis bag. Activated silica gel of 100 g was mixed thoroughly with 10 mL of DW and packed in 4.40 cm dialysis bag which is sealed at one end. Further, 10 mL of DW is mixed to maintain the same moisture level and reproducibility of the  $R_f$  value. BS of 1 g is dissolved in ethyl acetate and mixed with an equal amount of dry activated silica gel. The resultant slurry is packed on the top of the column. Solvent system is allowed to migrate till it reaches to the end of the column. After 2 h, the column was examined under UV visible illumination at 254 nm. Bands are identified, cut out individually with a surgical knife and eluted. This method allows easy, rapid and cost effective purification of BS. Dialysis and ultra filtration techniques are commonly used to enhance the purity of BS. Concentrating these compounds is carried out under reduced pressure at temperatures not more than that of 45°C and products are preserved by lyophilization (Shah and Prabhune, 2007).

#### **Preliminary characterization of biosurfactants by thin layer chromatography**

TLC, one of the most commonly used technique to detect BS/BE. It is based on the principle that the solutes compete with the solvent for the surface sites of the adsorbent. Depending



upon the distribution coefficient, compounds are distributed on surface of the adsorbents. Each sample is separated on fresh layers so that problems involved in carry over, cross contamination of samples and sorbent regeneration procedures are avoided. To detect BS/BE, solvent system depends upon the type of compound of interest. Organic and inorganic solvents which can be dissolved and are nonvolatile are preferred. Solvents which can not be used with HPLC, UV detection due to interference can be used in TLC. Sometimes acetic acid, diethyl ether, ethyl acetate, *n*-hexane, pyridine are needed for the mobility of functional groups of BS. Spot detection of samples from the TLC plates is done by destructive and nondestructive techniques. Nondestructive techniques involve iodine, water methods and UV radiation. Destructive method involves use of H<sub>2</sub>SO<sub>4</sub>. Various developers such as orcinol, resorcinol, iodine, sulphuric acid, ninhydrin are used for detection of carbohydrate, lipid and protein. A summary of various solvents and developers used for detection of different functional groups of BS/BE from diverse microorganisms is given in Table 1. Occasionally single solvent systems are enough for mobilization of different functional groups which can be sequentially identified with different developing reagents as reported of BS obtained from *Bacillus* sp. (Makkar and Cameotra, 1997; Haba et al., 2000).

### Chemical analysis

High molecular weight BS/BE are huge, structurally complex polymers. Therefore, these lipopolysaccharides are analyzed by colorimetric assays (Lowry's method, Bradford assay), mass spectrometry (MS) and sequencing techniques. Fatty acid content and peptide sequence is determined with the help of automated Edman degradation sequencing and MS. Combination of all these different methodologies are important to predict the complete structure of BS/BE.

**Determination of protein content:** High molecular weight BS/BE usually contains protein-lipid complexes containing lipoproteins, proteins, polysaccharides, lipopolysaccharides or a combinations thereof. Proteins from these complex BS/BE are generally quantified with Folin phenol method (Lowry et al., 1951). Emulsan, produced by *A. calcoaceticus* was reported to contain 11.2% of protein (Rubinovitz, Gutnick, and Rosenberg, 1982), while, *A. calcoaceticus* BD413 produces extracellular emulsifying agents containing 71 µg/mL of protein. *Acinetobacter* RAG-1 produces a polymeric extracellular emulsan, which forms hydrocarbon in water emulsions containing 20% of protein. *Y. lipolytica*, IMUFRJ50682 wild strain isolated from Guanabara Bay in Rio de Janeiro produces BE containing 15% protein (Amaral et al., 2006). Both the Folin phenol and the Bradford method (1976) have been used generally for protein estimation by many investigators. Culture of 10 mL is digested with 1 mL of 17.6% NaOH for 10 min at 90°C in water bath. About 100 µL of the supernatant from centrifuged digested biomass suspension is mixed with 5 mL of Bradford reagent. After 10 min absorbance is measured at 595 nm. In Bradford's assay, Coomassie Brilliant Blue binds with protein to change the color and thus proteins from BS/BE are quantified.

**Use of proteases for the digestion of proteins:** Obtaining the full protein structures of some BS/ BE is sometimes necessary. Identifying the amino acid sequence of a whole protein structure is not always possible due to several factors such as an inability to obtain accurate measurements for larger proteins and the fact that Edman degradation and mass spectrometry techniques are more suitable for smaller peptide structures analysis. The need to digest such large protein structure into smaller peptides of 6–20 amino acids is necessary to be able to use such individual peptides in the Edman degradation and MS/MS techniques to provide the information required to identify structure and piece together the full protein structure. Proteases are used for such digestion using trypsin for example to cleaves proteins at lysine and arginine residues in the C-terminal direction. Other proteases can also be used to help determine the order of each peptide sequence in a protein structure through allowing overlapping portions of a sequence to be identified. For detailed description of such digestion procedure see Strader et al., 2006 and Smyth et al., 2009b.

**Edman degradation for amino acid sequence:** The represents the classic technique for sequencing peptides chemically and can be applied to peptides and proteins where the N-terminus has not been modified (Zachara, and Gooley, 2000). The method provides an assignment for each residue in the peptide, unlike amino acid analysis which provides an indication of the ratio of amino acids in the peptide. Edman experiments take place in an oxygen-free environment and involve the modification of the N-terminal residue with phenylisothiocyanate to provide a cleaved phenylthiohydantoin (PTH) amino acid. This process take place on automated sequencers and is followed by a chromatographic step where the retention time of the cleaved PTH amino acid is compared with the retention times of a series of PTH modified amino acid standards to ascertain its identity. This is a relatively slow method taking approximately 45 min for each residue. The amino acid assignment however is called with a high degree of confidence. This method has an advantage over the MS/MS method. The quality of the sequence information obtained by the Edman method depends on the amount of starting material and their purity. Lipopeptides need to be in the open ring form for this type of analysis, which is carried out using mild alkaline hydrolysis.

**Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) for protein separation and determination of molecular mass of bioemulsifiers:** This method involves the use of an electrophoresis unit where sample is applied in a loading buffer containing reducing buffer and SDS. The main role of the reducing buffer is to cleave the disulphide bonds to facilitate protein arrangement in a linear fashion. It is followed by the attachment of SDS molecules to protein (depending upon molecular mass of protein) to create negatively charged protein. Under the influence of electric current, protein molecules (depending upon molecular mass) get separated. Each protein can be eluted from their band can then be removed and the proteins extracted (Toren et al., 2001). This research group treated alasin from *A. radioresistens* KA53 by dissolving

**Table 1.** Summary of different solvents systems and developers used for the characterization of biosurfactants produced by microorganisms using thin layer chromatographic techniques.

Organism	Biosurfactant type	Solvent system	Identification of functional groups	Developer	Reference
<i>Pseudomonas aeruginosa</i>	Methyl rhamnolipids	Chloroform; methanol; water 60:30:5 Chloroform; methanol; acetic acid; water 60:50:10:4 Chloroform; methanol; 7 N NH <sub>4</sub> OH 60:30:5	Rhamnolipids	Not stated	Hirayama and Kato, 1982
<i>Pseudomonas aeruginosa</i>	Rhamnolipid	Chloroform; methanol; acetic acid 65:15:2 n-propanol; ammonium hydroxide; water 6:2:1	Glycolipid Sugars Free fatty acid General lipids	Diphenylamine 4-methoxy-Benzaldehyde and Anthrone Bromocresol-green 2',7'-dichlorofluoresceine	Wu and Ju, 1998
<i>Pseudomonas aeruginosa</i> mutant	Rhamnolipid	Chloroform; methanol; acetic acid 65:15:2	Glycolipid	$\alpha$ -naphthol	Tahzibi et al., 2004
<i>Pseudomonas aeruginosa</i> 47T2 NCIB 40044	Rhamnolipid	Chloroform; methanol; water 65:25:4	Free amino Iodine Sugar	Ninhydrin Lipids $\alpha$ -naphthol; H <sub>2</sub> SO <sub>4</sub>	Haba et al., 2000
<i>Pseudomonas</i> sp.	Glycolipid	chloroform; methanol; water 65:25:5	Glycolipids	Anisaldehyde reagent (1 mL con H <sub>2</sub> SO <sub>4</sub> , 0.5 mL anisaldehyde in 50 mL Acetic acid)	Ellaiah et al., 2002
<i>Pseudomonas aeruginosa</i>	Rhamnolipids	chloroform; methanol; water 65:25:4	Rhamnolipid	Orcinol	Matsufuji, et al., 1997
<i>Pseudomonas fluorescense</i>	Viscosin; a peptidolipid antibiotic	Chloroform; methanol; water 65:25:4	Viscosin	Ninhydrin 4,4'-tetra methyl diamino-diphenylmethane	Neu et al., 1990
<i>Bacillus</i> sp.	Surfactin and Iturin Viscosin	Chloroform; methanol; water 65:25:4	Surfactin and Iturin Viscosin	Ninhydrin 4,4' tetra methyl diamino-diphenylmethane	Neu and Poralla, 1990
<i>Bacillus</i> sp.	Glycolipid	Chloroform; methanol; acetic acid; water 25:15:4:2	Carbohydrate Lipid	Chromosulphuric acid $\alpha$ -naphthol	Tabatabaee et al., 2005
<i>Bacillus subtilis</i> C - 1	Lipopeptide	Butanol; acetic acid; water 4:1:1 Methanol; 6 N HCl; water; pyridine 60:3:19:5:15	Amino acids	2% Ninhydrin in acetone	Vater et al., 2002
<i>Bacillus licheniformis</i> JF - 2	Biosurfactant	Chloroform; methanol; 28% NH <sub>4</sub> OH 65:35:5	Amino group Lipids Surfactin	Ninhydrin (in methanol; water 1:1) Rhodamine B (0.25 g in 100 mL ethanol) H <sub>2</sub> SO <sub>4</sub>	McInerney et al., 1990
<i>Bacillus subtilis</i>	Surfactin	Chloroform; methanol; 25% NH <sub>4</sub> OH 65:25:4	Surfactin Amino groups Lipids	Sulphuric acid; methanol (5:85) Ninhydrin Rhodamine B	Queiroga et al., 2003
Thermophilic <i>Bacillus subtilis</i>	Biosurfactant	Chloroform; methanol; water 65:15:1	Surfactin Surfactin Free Amino groups	Distilled water, 50% H <sub>2</sub> SO <sub>4</sub> , Ninhydrin (0.2%) in acetone	Makkar and Cameotra, 1997
Mesophilic and thermophilic <i>Bacillus subtilis</i>	Biosurfactant	Chloroform; methanol; water 65:15:1	Surfactin	Distilled water	Makkar and Cameotra, 1998
<i>Rhodococcus</i> sp. H13-A	Glycolipid	Chloroform; methanol; 5 M NH <sub>4</sub> OH 65:30:5 Chloroform; methanol; water 65:25:4 Ether; ethyl ether; glacial acetic acid 10:30:1	Glycolipid Free amino groups Neutral lipids	0.2% Orcinol in 75% H <sub>2</sub> SO <sub>4</sub> Ninhydrin 50% H <sub>2</sub> SO <sub>4</sub>	Vogt Singer and Finnerty, 1990
<i>Rhodococcus</i>	Biosurfactant	Chloroform; methanol; water 85:15:2 Chloroform; methanol; water 65:25:4 Chloroform; methanol; n-hexane; chloroform; acetic acid 20:80:0.5 n-hexane; chloroform; acetic acid 20:80:0.5 Saturated hydrocarbon with n-hexane; unsaturated hydrocarbon with n-hexane, dichloromethane 9:1	Glycolipid Phospholipids Fatty alcohols Acylglycerol Fatty acids Lipids	Anthrone % phenol-sulphuric acid Ninhydrin Anisaldehyde hydroxylamine ferric chloride 2,7-Dichlorofluorescein-aluminium chloride-ferric chloride 50% H <sub>2</sub> SO <sub>4</sub>	Kuyukina et al., 2001
<i>Yarrowia lipolytica</i> NCIM 3589	Emulsifier Glycolipid	Butanol; pyridine; water 60:40:10 Butanol; pyridine; water; acetic acid 60:40:30:3	Neutral sugars Hexosamines	0.4% Phthalic acid, 0.3% p-anisidine in ethanol 0.2% Ninhydrin in acetone	Zinjarde et al., 1997
<i>Ustilago maydis</i>	Glycolipids Ustilagic acid Ustilipids	Chloroform; methanol; water 65:25:4	Sugar	Glacial acetic acid: H <sub>2</sub> SO <sub>4</sub> -p-nisaldehyde 50:1:0.5	Hewald et al., 2005; 2006

Table 1. continued on next page

Table 1. Continued.

Organism	Biosurfactant type	Solvent system	Identification of functional groups	Developer	Reference
<i>Rhodococcus</i> sp. H 13A	Glycolipids	Chloroform; methanol; 5 M NH <sub>4</sub> OH 65:30:5 n-propanol ethyl acetate; water 65:10:25	Glycolipids Deacylated glycolipid backbone Amino acids and protein	200mg of orcinol in 100ml of 74% H <sub>2</sub> SO <sub>4</sub> 200mg of orcinol in 100ml of 74% H <sub>2</sub> SO <sub>4</sub> 0.2% Ninhydrin and 2% pyridine in acetone	Bryant, 1990
<i>Capnocyto - phaga</i>	Neutral glycolipid Phospholipids isoprenoids	Chloroform; methanol; 7 N NH <sub>4</sub> OH 60:35:5 Hexane; ether; acetic acid 70:30:4 Chloroform; methanol; acetic acid; water 5:2:1:0.5	Lipids Phospholipids Choline Polyprenols Glycolipids Amino groups	Iodine vapour and Rhodamine 6G Ammonium molybdate Dragendrof reagent Anisaldehyde Diphenylamine Ninhydrin	Holt et al., 1979
<i>Candida bombicola</i>	Alkyl-sophorosides	Methanol; water 90:10	Sophorolipids	$\alpha$ -Naphthol/H <sub>2</sub> SO <sub>4</sub>	Brakemeier et al., 1998a 1998b
Marine <i>Caulobacter</i>	Glycolipids	Chloroform; methanol; 2,6-Dimethyl-4 Heptanone-pyridine-0.5M ammonium Chloride hydrochloride buffer (pH 10.4) 60:35:50:70:12	Carbohydrate	Silver nitrate reagent Rhodamine 6G	De Siervo, 1985
<i>Candida cylindracea</i>	Biosurfactant	Chloroform; methanol; water 60: 10: 1	Monoester	2.5% H <sub>2</sub> SO <sub>4</sub> in ethanol NaIO <sub>4</sub> in acetone Spray with solution of benzidine in acetone	Chopineau et al., 1988

in 2% SDS, 4%  $\beta$ -mercaptoethanol, 8% glycerol, 50 mM Tris-HCl (pH 6.8), and 0.02% bromophenyl blue and then heated to 100°C for 10 min. Up to 100 mg of sample can be loaded. Oil degrading *A. vanetianus* RAG-1 produces emulsan, which stabilizes oil in water emulsions with hydrocarbons. The protein portion (34.5 kDa) of this emulsan product was characterized by this method in a Hoffer apparatus (Bach, Berdichevsky, and Gutnick, 2003).

**Determination of carbohydrate, lipid/fatty acids (FA) content:** Exact composition of BS/BE is carried out by the digestion procedure to separate the carbohydrate and fatty acid moiety. Individual moieties can be observed by gas chromatography mass spectrometry (GC-MS). Estimations of sugars and related substances give an orange yellow color when treated with phenol and concentrated H<sub>2</sub>SO<sub>4</sub>. The reaction is very sensitive and the color of the reaction is stable. It determines the sub micro amounts of sugars (Dubois et al., 1956). This is one of the common methods chosen by the researchers. Reducing sugars are determined by dinitrosalicylic acid (DNSA) (Miller, 1959). A gravimetric method is also used to determine lipid content by weighing the pooled di-ethyl ether extracts of cell-free broth while another method introduced by Reddy et al. (1983) is also commonly used for quantification of lipids using standards such as cholesterol.

**Colorimetric assay for detection and quantification of glycolipids:** Glycolipids are quantified by color development using the anthrone or orcinol test. The anthrone assay detects and quantifies glycolipid present through acidification and heating rhamnose, which forms a color with anthrone. Color intensity is measured as absorbance at 625 nm by a spectrophotometer against a calibration curve with rhamnose or rhamnolipid at concentrations of 0–50 mg/mL (Hodge and Hofreiter, 1962). All types of glycolipid BS and carbohydrate moiety of BS can be quantified with the help of this assay. Several researchers have used the assay as described by Chandrasekaran and BeMiller, (1980).

Rhamnose molecules within rhamnolipids react with H<sub>2</sub>SO<sub>4</sub> and orcinol (1,3-dihydroxy-5-methylbenzene) at high temperatures (30 min/80°C) to give a blue-green color which is similarly measured at 421 nm (Koch et al., 1991). Although these are quick, simple methods their accuracy can vary in samples containing mixtures of different congeners of glycolipids with one or more sugar moieties.

#### Chemical characterization of biosurfactants/bioemulsifiers

Various analytical methods namely, TLC, HPLC, IR, GC-MS, NMR, and FAB-MS are used to characterize BS/BE either individually or in combination and are listed in Table 2.

#### Chromatography

**Thin layer chromatography:** This technique was described earlier in this review and Table 1 gives the summary of various solvents and developers used for detection of different functional groups from BS/BE using this method.

**High pressure liquid chromatography (HPLC):** This consists of a mobile, stationary phase and a detector. The mobile phase carries the sample solution injected through the injector port. Stationary phase is a solid, over which the mobile phase continuously flows the components of sample solution. Components migrate according to the noncovalent interactions of compound with the column. The detector emits a response due to the elution of sample and subsequently signals a peak on the chromatogram. Some of the common detectors used include refractive index (RI), UV, fluorescent, radiochemical, electrochemical, near-infrared, MS, NMR, and light scattering. HPLC is used generally for separation of lipopeptide type BS (Aguilar, 2004), free rhamnose from rhamnolipid (Siegmond and Wagner, 1991). For sample analysis in a HPLC facility, it is treated with trifluoroacetic acid (TFA) and further centrifuged for the removal of solid particles. Glycolipids BS can be separated and identified successively when a HPLC device coupled



**Table 2.** Chemical characterization of biosurfactants produced by different microorganisms using analytical Methods.

Microorganism	Biosurfactant/ Bioemulsifier type	Gravimetric Emulsifier	Gel filtration	Chromatography			Spectroscopy										Reference	
				TLC	HPLC	SDS-PAGE	NMR	GC	CLC	MS	GC-MS	LC-MS	LT-MS	IR	FT-IR	FAB-MS		
<i>Acinetobacter radioresistens</i>	KA53	Arthrobaacter	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Belsky et al., 1979
<i>Arthrobacter sp. Strain</i>	MIS38	Lipopeptide	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	Morikawa et al., 1993
<i>Pseudomonas aeruginosa</i>		Methyl rhamnolipid	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	Hirayama and Kato, 1982
<i>Pseudomonas aeruginosa</i>		Rhamnolipid	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	Wei and Chu, 1998
<i>Pseudomonas aeruginosa mutant</i>		Rhamnolipids	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Tahzibi et al., 2004
<i>Pseudomonas aeruginosa</i>		Rhamnolipid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Edward and Hayashi, 1965
<i>Pseudomonas strain</i>		EPS	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Royan et al., 1999
<i>Pseudomonas aeruginosa</i>	47T2NCIB 40044	Rhamnolipid	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	Haba et al., 2000
<i>Pseudomonas putida</i> ML2		Bioemulsifier	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	Bonilla et al., 2005
<i>Pseudomonas putida</i> 21BN		Rhamnolipid	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	Tuleva et al., 2002
<i>Pseudomonas aeruginosa</i>		Rhamnolipid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Benincasa et al., 2004
<i>Bacillus licheniformis</i>		Lipopeptide	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	Lin et al., 1998a; 1998b
<i>Bacillus sp. IAF 343</i>		Biosurfactant	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	Cooper and Goldenberg, 1987
<i>Bacillus subtilis mutant</i>		Surfactin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Mulligan, Chow, and Gibbs, 1989
<i>Bacillus</i>		EPS	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Yun and Park, 2000
<i>Bacillus licheniformis</i> F2		Biosurfactant	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	Thaniyavarn et al., 2003
<i>Bacillus subtilis</i>		Lipopeptide	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	Lin and Jiang, 1997
<i>Bacillus subtilis</i>		Surfactin	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	Queiroga et al., 2003
<i>Bacillus pumilis</i> A-1		Surfactin	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	Morikawa et al., 1992
<i>Bacillus licheniformis</i>	BASS0	Lipopeptide	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	Yakimov et al., 1995
<i>Thermophilic Bacillus subtilis</i>		Biosurfactant	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	Makkar and Cometra, 1997
<i>Rhodococcus sp. H 13-A</i>		Glycolipid	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	Vogt Singer and Finnerty, 1990
<i>Rhodococcus sp. H13A</i>		Glycolipid	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	Bryant, 1990
<i>Candida (Torulopsis) apicola</i>		Glycolipid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Hommel et al., 1994
<i>Marine bacterium</i> MMI		Marine biosurfactants IV	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	Passeri et al., 1992
<i>Flavobacterium</i> MTN11		Flavolipid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Bodour et al., 2004

+: Test carried out by authors to characterize biosurfactant/bioemulsifier.  
-: Test not done by authors.

with evaporative light scattering detector (ELSD) or mass spectrometry is employed. In this technique separation of various components is based on their polarity and the separated products can be detected and fractions collected for individual peaks to analyze the structure of each moiety. HPLC along with MS facility are important to provide the molecular mass of each fraction.

### Spectroscopy

**Chemical analysis using gas chromatography (GC) and mass spectrometry (MS):** This consists of a GC column and a mass interface. The ionization source is electron impact or chemical. The mass analyzer magnetic sector has quadrupole, ion trap, time of flight and mass detector. It is provided with software MS facility which acts as a gas chromatograph. MS measures the molecular weight of a compound. Separate peaks arise in the GC column and enter in MS. The heat transfer line keeps the compound in the gaseous phase as they enter in the MS. In the ionization chamber, under high voltage, the filament is heated up and provides an electron source. Peaks get transferred to a mass analyzer via focused charged plates and they focus the ions. The mass analyzer analyses only those particular fractions, which can strike it. GC coupled with MS is the most sensitive method for identification and quantification of glycolipids type BS. High amounts of lipid are associated with high molecular weight BS. Analysis of this lipid moiety of BS is crucial for structural elucidation. To analyze the BS sample in a GC or GC-MS device, the sample needs hydrolytic cleavage between the carbohydrate or peptide/protein part of the BS and the lipid portions. Consequently, derivatization of fatty acid chains to fatty acid methyl esters (FAME) and further conversion to trimethylsilyl (TMS) derivatives results analysis by GC or GC-MS (Yakimov et al., 1995). The esterification step by diazomethane is important for detection of compounds using GC-MS. Peng et al. (2007) characterized BS from oil degrading *R. erythropolis* 3C-9. FA from crude extracts was esterified with 2 mol/L HCl in methanol at 100°C (40 min). FA methyl esters were recovered with hexane, and then concentrated to 1 mL under nitrogen blowing for GC-MS analysis. Temperature gradient was maintained between 60°C and 260°C (increasing at 5°C/min). One microlitre of sample was applied to a Shimadzu GC-MS. Pure Carbohydrate sample is prepared by freeze-drying the aqueous phase and extracting with pyridine (to remove all ions). Pyridine was then removed by rotary evaporation (40°C). The saccharide portion was dissolved in DW and used for further analysis. The GC does not give any information regarding the structure of BS. It quantitatively analyses sugar from the converted trimethylsilyl esters.

**Infra red (IR) spectroscopy:** Absorption of different IR frequencies of a sample positioned in the path of an IR beam is measured. It is a form of radiation that can travel through a vacuum while heat is associated with the motion and kinetic energy of molecules. The spectrometer consists of a radiation source, monochromator and detector. The term "infra red" covers the range of the electromagnetic spectrum between 0.78 and 1000 mm. Absorption of IR is

restricted to compounds with small energy differences in the possible vibrational and rotational states. This technique determines the functional groups of gases, liquids and solids samples and gives a structural elucidation of compound. Surfactin, lichenysin and rhamnolipids have been characterized by the IR technique (Das, Mukherjee, and Sen, 2008). The IR Spectrophotometer used is in the range of 4000–400<sup>cm</sup><sup>-1</sup>. For each spectrum 100 scans are used in 0.23 mm KBr liquid cell. Alkyl, carbonyl, ester compounds of BS are detected clearly (Tuleva, Ivanov, and Christova, 2002). Thavasi et al. (2007) used this method for an IR spectrum for Freeze-dried crude BS (10 mg) which was added to potassium bromide (100 mg) and pressed with 7500 kg for 30 sec to obtain translucent pellets. The IR absorption spectrum was recorded on a Thermo Nicolet, AVATAR 330 FTIR systems.

**Nuclear magnetic resonance (NMR):** This is based on transitions in atoms with a magnetic moment when an external magnetic field is applied. It is the absorbance of radio frequency radiation by a nucleus in a strong magnetic field. Radiation absorption causes the nuclear spin to re-align or flip in the higher-energy direction. Once the energy is absorbed, the nuclei will re-emit radiation and return to the lower-energy state. NMR transition energy totally depends on the magnetic-field strength and a proportionality factor for each nucleus called, magnetogyric ratio. NMR provides information regarding the functional groups as well as the position of linkages within the carbohydrate and lipid molecules. Exact location of each functional group can be obtained and information about the structural isomers is also possible with the help of series of NMR experiments. Solvents such as acetic acid, acetone, benzene, chloroform, dimethyl sulfoxide, methanol pyridine and water are used. Samples are hydrolysed (HCl) and then FA extraction is carried out with solvents and detected by NMR.

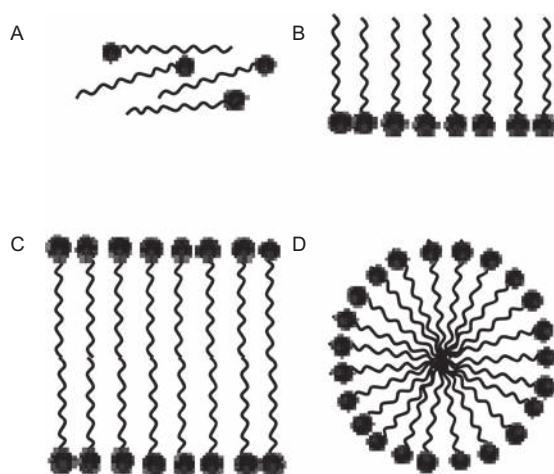
The glycolipids should be dissolved in deuterated chloroform and a series of 1D (1H and 13C) and 2D (such as COSY, ROSY, HMQC, and HMBC) experiments should be carried out by NMR. Detailed analyses of glycolipid BS was carried out with the help of NMR methodology and has recently been reported in the literature (Smyth et al., 2009a).

**Fast atom bombardment- mass spectrometry (FAB-MS):** This is a soft ionization method which requires a direct insertion probe for sample introduction. A high energy beam of xenon atoms/caesium ions is used to stammer the sample and matrix from the probe's surface. The matrix (m-nitrobenzyl alcohol) dissolves the sample and helps for desorption, ionization. BS sample is dissolved in methanol, mixed with matrix and used for analysis. The research group of Manso Pajarron et al. (1993) have identified rhamnolipid mixtures using this method.

It is important to mention here that time requirements to carry out some of the extraction, purification and analysis for some of the procedures described above can be affected by several factors such as sample volume, the presence of residual carbon or other production substrates such as oil mixtures or complex medium compositions.

**Experimental strategies for micelle characterization**

All surfactants belong to a class of molecules with surface active properties due to their amphiphilic structure, which contains both a polar or hydrophilic head and a nonpolar or hydrophobic tail (Israelachvili, 1997). They are normally classified according to the head group type viz. ionic (anionic and cationic), nonionic and amphoteric (zwitterionics). Adsorption of ionic surfactants onto interfaces generates a charge. Cationic surfactants will lead to a positively charged surface and anionic surfactants will give a negatively charged surface. Nonionic surfactant molecules have no charge in aqueous media but normally consist of a highly polar region such as polyoxyethylene groups. Amphoteric surfactants develop negative or positive charges depending on the pH of the solution. At low concentrations, surfactant molecules are unassociated monomers. As the concentration of surfactant is increased, the attractive and repulsive forces between the molecules cause self-assembly to form structures termed micelles (Figure 2). When surfactant is added into the aqueous phase, they are distributed arbitrarily throughout the water. As the surfactant concentration increases the molecules assemble themselves in hollow spheres, rods and disks called micelles (Figure 2). The concentration at which these micelles form is called the CMC. The surface of a micelle represents the layer of polar hydrophilic head group dissolved in the water, while the inner hydrophobic tails are screened from the water by the hydrophilic heads. In general, spherical type of micelle conformations are formed and the process is known as micellization. Other conformations such as ellipsoids, cylinders and bilayers are also known. The characteristics of micelles can be controlled by small changes in the chemical structure of the surfactant molecules or by varying the conditions of the disperse phase. Changes in the pH, ionic strength and temperature are all known to influence the size and shape of surfactant micelles. In a few cases, the micelle size can be affected by the concentration of the surfactant.



**Figure 2.** Assembly of surfactant monomer in different structures. (A) Isolated monomers when surfactant monomer concentration is below CMC. When the concentration is above the CMC, the monomers can aggregate in the form of micelles such as (B) linear chains, (C) planar bilayers, and (D) spherical or cylindrical micelles.

The size or aggregation number ( $N$ ) of micelles is crucial to prove their efficiency for solubilization, CMC and also with the viscosities of their solutions. The aggregation number of a micelle is defined as the number of surfactant molecules per micelle and is often dependent upon the conditions of the disperse phase (Phillie and Yambert, 1996). The aggregation number of a micelle can be determined if the molecular weight of the micelle and the surfactant monomer are known. Determination of absolute molecular weight can be achieved through static light scattering techniques and hydrodynamic characterization (Mattison and Kaszuba, 2003). Surfactant micelles have been studied using various techniques and extensively reported by several authors (Dominguez et al., 1997; Tuzar and Kratochvil, 1993; Munk, 1996; Chu and Zhou, 1996; Webber, 1996; Mortensen, 2000; Zana, 2000; Hamley, 1998).

Microscopic techniques such as transmission electron microscopy (TEM), Cryo-TEM, scanning electron microscopy (SEM), atomic force microscopy (AFM) are regularly used for the characterization of shape and the size of micelles. Egelhaaf, Müller, and Schurtenberger, 1998 determined the size of polymer-like micelles with the help of cryo- transmission electron microscopy (cryo-TEM). Concurred size of the sample micelles were obtained similar with independent light-scattering measurements. Their studies showed that cryo-TEM experiments are crucial in providing direct structural information and in the quantitative resolving of the bulk properties of micelles entities. Fluorescence spectroscopic techniques are useful to provide information on chain dynamics, CMC, hybridization of micelles (Lin et al., 2007). Ultra-centrifugation in combination with electron microscopy are also important for understanding micelle density and the molecular weight of a micelle (Vogel et al., 2003). Warr and Grieser (1986) used fluorescence quenching for determination of micelle size and polydispersity and confirmed that this method was sensitive for determination of the weight-average aggregation number as well as to the shape of the micelle size distribution.

Dynamic light scattering (DLS) techniques can also be used for particle sizing of samples, typically in the sub-micron range. The technique measures the time-dependent fluctuations in the intensity of scattered light from a suspension of particles undergoing random Brownian motion. Analysis of these intensity fluctuations allows for the determination of the diffusion coefficients, which in turn yield the particle size through the Stokes - Einstein equation. Conventional DLS instruments use a detection angle of  $90^\circ$  and this optical configuration may not be sensitive enough for the successful measurement of surfactant micelles. Hydrodynamic radius, ( $R_h$ ) is measured in DLS (Alexandridis and Lindman, 2000; Aivaliotis et al., 2003; Nakahara et al., 2005).

Small-angle neutron scattering (SANS) and small-angle X-ray scattering (SXAS) are useful for the determination of molecular weight, radius of gyration ( $R_g$ ), core radius of micelle ( $R_c$ ), macrolattice structures. The techniques such as shear rate viscosity are used to determine the hydrodynamic radius and intrinsic solution viscosity. Stop flow techniques



measure the kinetics of micelle formation and dissociation. Zhang et al. (2007) investigated the kinetics and mechanism of sphere-to-rod transitions of sodium alkyl sulfate micelles induced by hydrotropic salt, *p*-toluidine hydrochloride (PTHC) with stopped-flow by light scattering. The equilibrium structures of the micelles can be characterized via a combination of <sup>1</sup>H NMR and laser light scattering (LLS) (Wang et al., 2007).

Quite early, Tanford (1974) successfully calculated the micelle size as well as the CMC by self-consistent procedure. The micelle size and other properties of micelle forming systems were determined by separate estimation of the components of the free energy arising from hydrophobic attraction and from head group repulsion and each as a function of micelle size. It was claimed that, this approach was self-consistent and could give exact similar observations with prediction of experimental micelle sizes and CMCs for two distinctly different ionic detergents. Gel filtration chromatographic (GFC) techniques were also used for determination of the size and shape of nonionic surfactants such as polyoxyethylene nonyl phenols. From the retention time, the Stokes radius (*R*) was measured. Further, frictional ratios, the hydration of micelles were calculated (Birdi, 1974, 1997). Nelson, Rutledge, and Hattona, (1997) have studied the equilibrium size and shape distributions of self-assembled micelles using lattice Monte Carlo simulation techniques and concluded that the equilibration of simulated micellar systems was better monitored using the average aggregate size rather than the configurational energy of the system. Nichol and Ogston (1981) also reported a method of ampholyte-micelle equilibria to estimate the micelle size and the association constant.

## Future prospects

Various techniques and solvents have been successively employed to isolate low as well as high molecular weight BS/BE, however future trends will mainly focus on the production of high yields and cost effective downstream processing. 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 use of these pure carbon sources is extremely expensive; as a result other agricultural resources such as sunflower oil and soybean are becoming more suitable for use as more cost effective resources. Indeed recent trends, and even more attractive, would be the use of waste oil sources such as 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 to purchase and thus further drive down 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, should 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.

## Summary

BS/BE possesses outstanding functional properties which form the basic foundation for different industries. Extensive knowledge of different properties has helped to mould these surface active compounds for potential applications. The search for potent BS/BE producers is becoming easier due to the availability of several different screening methods to obtain producing microorganisms and the ability to purify diverse amphiphilic molecules. Techniques such as preparative TLC, dialysis, HPLC, mass spectrometry, column chromatography, ultrafiltration, and lyophilization are essential and have been proven to be efficient in obtaining purified products. More recent approaches such as IEF, dialysis tubing are offering novel methods to purify these compounds. Various analytical methods such as TLC, HPLC, GC-MS, NMR, FAB-MS, protein digestion and amino acids sequencing are facilitating preliminary characterization, prediction and structure determination of novel surface active compounds. Due to the discoveries of new BS/BE it has become possible to enter in the area of chemical synthesis. Single methods or combinations of various analytical methods is essential. Demand of BS and BE is steadily increasing and the need can be satisfied by improving detection, production and purification techniques.

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## Declaration of interest

The author report no conflicts of interest.

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