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# Green surfactant: Production, purification, characterization and application- a review

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***Abstract:*** Microbial surface-active agents (biosurfactant) are amphiphilic compounds with both hydrophilic and hydrophobic moieties produced by diverse group of microorganisms mostly as secondary metabolite. The commercial demand of biosurfactant emerges rapidly due to its wide application in chemistry, ecology, medicine as well as in biological fields. Economically preparations of surfactant by green synthesis using biological entities are gradually increase. With increasing environmental concern about chemical surfactants triggers attention to microbial-derived surface-active compounds essentially due to their low toxicity and biodegradable nature. Being capable of lowering surface- and interfacial-tension (liquid/liquid, gas/liquid or solid/liquid), interest in biosurfactants has increased considerably in recent years, as they are potentially used in many commercial applications in cosmetic industry, food processing, pharmaceuticals and environmental bio-remediation particularly in enhanced oil recovery (EOR) and cleaning of oil spills. These compounds are mainly classified according to their molecular weight, physico-chemical properties and mode of action. Diverse types of biosurfactant are reported and patented are characterized by different physical, chemical and biological means. Here we are focusing on the production, purification, types and applications of biosurfactants in various industries, as environmental compatibility becoming an increasingly important factor in the selection of industrial chemicals and the use of biosurfactants is increasing.

**Key words**: Green synthesis, Biosurfactants, Classification, Rhamnolipid, Critical Micelle Concentration (CMC), Microbial Enhanced Oil Recovery (MEOR)

## *I. INTRODUCTION*

Surfactants the term coined by Antara products in 1950 are the organic amphiphilic compounds which means, that they contain both hydrophilic groups (their "heads") and hydrophobic groups (their "tails"). Therefore, they are soluble in both water and organic solvents. It is surface active compound that reduce the interfacial tension between two liquids, or between liquid and solid. Surface tension is the free surface enthalpy per unit area and is the force acting on the surface of a liquid leading to minimization of the area of that surface. Surfactant or surface-active agents can be classified into two main groups; synthetic surfactant and natural surfactant. Both synthetic and natural surfactants capable of reducing the surface tension of water from 72 mN $m^{-1}$ to around 27 mN $m^{-1}$ (Christofi and Ivshina 2002). Due to a great variety of household and industrial applications worldwide surfactant produce about 17-20 million metric tons annually but most of them are petroleum based and are chemically synthesized again they are environmentally toxic pollutant and are non-biodegradable. Therefore, biosurfactants are the natural choice for such processes as they possess a host of advantages over synthetic surfactants, such as lower toxicity, low irritancy, compatibility with human skin, and effectiveness at a wide range of pH and temperature values as well as biodegradability (Banat et al. 2010; Cameotra et al. 2010).

Biosurfactants are also surface-active compound like others chemical surfactants but unlike the chemical surfactant, biosurfactant are synthesized by diverse group of microbes like bacteria, fungi and yeast, which either adhere to cell surface or are excreted extracellularly in the growth medium. Having both hydrophobic and hydrophilic moieties, biosurfactants are able to reduce surface tension and interfacial tension between two fluids at the surface and interface respectively. These are also able to form microemulsion where hydrocarbons can solubilize in water or where water can solubilize in hydrocarbon. The hydrophilic group of biosurfactant consists of mono-, oligo-, or polysaccharides, peptides or proteins while the hydrophobic moiety usually contains saturated, unsaturated and hydroxylated fatty acids or fatty alcohols (Pacwa-Plociniczak et al. 2011). Biosurfactants comprise the properties of dropping surface tension, stabilizing emulsions, promoting foaming and are usually non-toxic and biodegradable and thus ecofriendly then chemical surfactant. Interest in microbial surfactants has been steadily increasing in recent years due to their diversity, environmentally friendly nature, the possibility of their production through fermentation. The potential applications of biosurfactants in industrial include emulsification and foaming for food processing, crude oil recovery, health care, wetting and phase dispersion for cosmetics and textiles, or solubilization for agrochemicals. In addition, biosurfactants can be used in environmental applications such as bioremediation and dispersion of oil spills. Biosurfactants can be divided into six groups based on their overall structures and functional ability. They are glycolipids, lipopeptides/lipoprotein, phospholipids and fatty acids, surface-active antibiotics, polymeric surfactants and particulate biosurfactants.

Even though interest in biosurfactants is increasing, these compounds do not compete economically with synthetic surfactants. Commercial viability of biosurfactants is still limited by their high production costs, associated with inefficient recovery methods and with the use of expensive raw materials. To reduce production costs, different routes could be investigated such as the increase of yields and product accumulation; the development of economical engineering processes, and the use of cost-free or cost-credit feedstock for microorganism growth and surfactant production. One of the strategies to improve production is to optimize the growth media in order to get maximum production.

Formulation of an optimized production medium involves selection of the right nutrients at their correct levels to provide an ideal microenvironment for supporting growth and metabolite production. The choice of inexpensive raw materials is important to the overall economy of the process because they account for 50% of the final product cost and also reduce the expenses with wastes treatment.

Due high demand, in the last few years, significant work on the medium formulation, fermentative production, genetics, and commercial applications of biosurfactants has been done. In this review we try to give a brief idea about choice of inexpensive method for production and also give an idea about characterization and recent applications of biosurfactant.

## *II. CLASSIFICATION OF BIOSURFACTANT*

Various types of biosurfactant are synthesized by a number of microbes, particularly during their growth on water immiscible substrate. Unlike the chemically synthesized surfactants that are generally categorized on the basis of on the type of the polar group present, biosurfactants are in general classified chiefly by their chemical composition and microbial origin. The majority biosurfactants are either anionic or neutral and the hydrophobic moiety is based on long-chain fatty acids or fatty acid derivatives, whereas the hydrophilic moiety can be a carbohydrate, phosphate, amino acid, or cyclic peptide (Nitschke and Coast, 2007). According to Rosenberg and Ron (1999) biosurfactants are classified in to two major groups one is low molecular weight surface active agent call biosurfactant and high molecular weight substance called bio-emulsifier that is especially used as enhancement of emulsification of hydrocarbon. The main classes of low-mass surfactants are lipopeptides, glycolipids and phospholipids, whereas large-mass surfactants include polymeric and particulate surfactants (Fig 1). In Table 1 there are presented examples of different groups of biosurfactants with their microbial origin.

Table 1: Major biosurfactant classes and microorganisms involved

| Biosurfactant class | **Chemical nature** | **Microbial strain** | **Reference** |
|---|---|---|---|
| Glycolipids | Rhamnolipids | *Nocardioides* sp. | Vasileva-Tonkova and Geshevaa, 2005 |
| | Sophorolipids | *Candida* sp. | Hirata et al., 2009 |
| | Trehalose lipids | *Rhodococcus* sp. | Lang and Philip, 1998 |
| | Cellobiolipid | *Ustilago* sp. | Fiechter, A. 1992 |
| Lipopeptides and lipoproteins | Fengycin | *Bacillus* sp. | Vanittanakom et al., 1986 |
| | Arthrofactin | *Arthrobacter* sp. | Morikawa et al., 1993 |
| | Serrawettin | *Serretia* sp. | Tanikawa et al.,2006 |

| | | | |
|---|---|---|---|
| | Viscosin | *Pseudomonas* sp. | Neu et al.,1990 |
| | Surfactin | *Bacillus* sp. | Carrillo et al.,2003 |
| Phospholipids and fatty acids | Bile salts | *Myroides* sp. | Maneerat et al., 2005 |
| | Fatty acids | *Mycobacterium* sp., *Nocardia* sp., *Candida* sp., *Cladosporium* sp. | Rehm and Reiff, 1981 |
| | Phosphatidyl-ethanolamine | *Rhodococcus* sp. | Kretschmer et al., 1982 |
| Surface-active antibiotics | Gramicidins | *Brevibacterium sp.* | |
| | Polymyxins | *Bacillus sp.* | Falagas et al., 2006 |
| Polymeric biosurfactants | Alasan | *Acinetobacter* sp. | Navon-Venezia et al. 1995 |
| | Bioemulsan | *Gordonia* sp. | Franzettia et al., 2008 |
| | Biodispersan | *Acinetobacter* sp. | Rosenberg, E, 1993 |
| | Liposan | *Candida* sp. | Cirigliano et al., 1984 |
| Particulate biosurfactants | Whole cells | *Yarrowia* sp. | Zinjarde and Pant, 2002 |
| | Vesicles | *Serratia* sp. | Matsuyama et al., 1986 |

## II.1 GLYCOLIPIDS

Glycolipids are carbohydrates in combination with long-chain aliphatic acids or hydroxyaliphatic acids. Most of the biosurfactants are glycolipids and important member of this group are rhamnolipids, trehalolipids and sophorolipids.

Rhamnolipids is a group of biosurfactant that studied extensively. It consists of one or two molecules of rhamnose that are linked to one or two molecules of ß-hydroxydecanoic acid. The production of rhamnose which contains glycolipid was first studied in *Pseudomonas aeruginosa* by Jarvis and Johnson (Jarvis and Johnson, 1949). L-Rhamnosyl-L-rhamnosyl-ß-ydroxydecanoyl-ß-hydroxydecanoate and L-rhamnosyl-ß-hydroxydecanoyl-ß-hydroxydecanoate, referred to as rhamnolipid 1 and 2, respectively, are the principal glycolipids produced by *P. aeruginosa (*Edwar, and Hayashi, 1965).

Trehalolipid is other most important types of glycolipids biosurfactant synthesized by most species of *Mycobacterium, Nocardia, and Corynebacterium*. These bacteria produce disaccharide trehalose linked to a long chain, α-branched and β-hydroxy fatty acids called mycolic acids. Trehalolipids from diverse organisms vary in the size and structure of

mycolic acid, the number of carbon atoms present and the extent of unsaturation (Asselineau and Asselineau, 1978). Many different structural types of trehalolipid biosurfactants have been reported. Trehalose lipids obtained from *Rhodococcus erythropolis* and *Arthrobacter* sp. reduced the surface tension and interfacial tension in culture broth (Kretschmer et al., 1982; Desai and Banat, 1997).

Sophorolipids are synthesized mainly by yeast such as *Torulopsis bombicola* (Cooper and Paddock, 1984; Hommel *et al.,* 1987), *T. petrophilum* and *T. apicola* consists of a dimeric carbohydrate sophorose attached to a long-chain hydroxyl fatty acid by a glycosidic linkage. These biosurfactants are a mixture of at least six to nine different hydrophobic sophorosides. *Candida apicola* and *Candida bombicola* produced extracellular sophorolipids biosurfactant which was a mixture of acidic and lactonic forms (Thaniyavarn et al., 2008). The sophorolipids reduce surface tensions between individual molecules at the surface, although they are effective emulsifying agents (Hirata et al., 2009).

## II.2 LIPOPEPTIDES AND LIPOPROTEINS

Lipopeptide biosurfactants are cyclic compounds mainly consist of hydrophilic peptides, generally they consist 7 and 10 amino acids long, linked to a hydrophobic fatty acid structure and mostly isolated from Bacillus and Pseudomonas type bacteria.

The cyclic lipopeptide surfactin produced by *B. subtilis*, is one of the most powerful biosurfactants. Surfactin is the most commonly studied and it contains 7 amino acid cyclic sequences connected to a C13–C16 fatty acid (Kakinuma A, 1969). It also exhibits excellent temperature, pH, and salt stability. An important feature of the biosurfactant is its ability to lyse mammalian erythrocytes and form spheroplasts. Beside surfectin other cyclic lipopeptides produce by Bacillus are iturin and fengycin families.

## II.3 FATTY ACIDS, PHOSPHOLIPIDS, AND NEUTRAL LIPIDS

Many bacteria and yeast synthesize large amounts of fatty acids and phospholipid surfactants during their growth on n-alkanes (Cirigliano and Carman, 1985). *Arthrobacter* strain and *P. aeruginosa* are reported to accumulate up to 40 to 80% (w/w) of phospholipids, when cultivated on hexadecane and olive oil, respectively (Banat and Desai, 1997). The large quantity of phospholipids has been detected in *Acinetobacter* sp., *Aspergillus* sp. and *Thiobacillus thiooxidans*.

## II.4 POLYMERIC BIOSURFACTANTS

Polymeric biosurfactant are high weight molecular biopolymers which consisting polysaccharides, proteins, lipopolysaccharides, lipoproteins or mixture of theses biopolymers. Examples of polymeric biosurfactants include emulsan, liposan and mannoprotein.

Emulsan synthesizes by *Acinetobacter calcoaceticus* is an extracellular potent polyanionic amphipathics heteropolysaccharide bioemulsifier (Rosenberg *et al.,* 1979). The heteropolysaccharide backbone of emulsan comprises of a repeating trisaccharide of N-acetyl-D-galactosamine, N-acetyl-galactosamine uronic acid and an unidentified N-acetyl amino sugar. Fatty acids are covalently linked to the polysaccharide through O-ester linkages.

Liposan is an extracellular water-soluble emulsifier synthesized by *Candida lipolytica* and is composed of 83% carbohydrate and 17% protein (Cirigliano and Carman, 1984). The carbohydrate portion is a heteropolysaccharide consisting of glucose, galactose, galactosamine, and galacturonic acid (Desai and Banat, 1997).

### II.5 PARTICULATE BIOSURFACTANTS

Particulate biosurfactants are of two types, extracellular vesicles and whole microbial cell. The formation of an extracellular microemulsion plays an important role in alkane uptake by microbial cells. Extracellular membrane vesicles of *Acinetobacter* sp. with 20-50 nm diameters are composed of protein, phospholipid, and lipopolysaccharide (Desai and Banat, 1997). Sometimes the whole bacterial cell itself can work as surfactant. Most hydrocarbon-degrading microorganisms, many nonhydrocarbon degraders, some species of *Cyanobacteria*, and some pathogens have a strong affinity for hydrocarbon-water and air-water interfaces. In such cases, the microbial cell itself is a surfactant (Karanth et al. 1999).

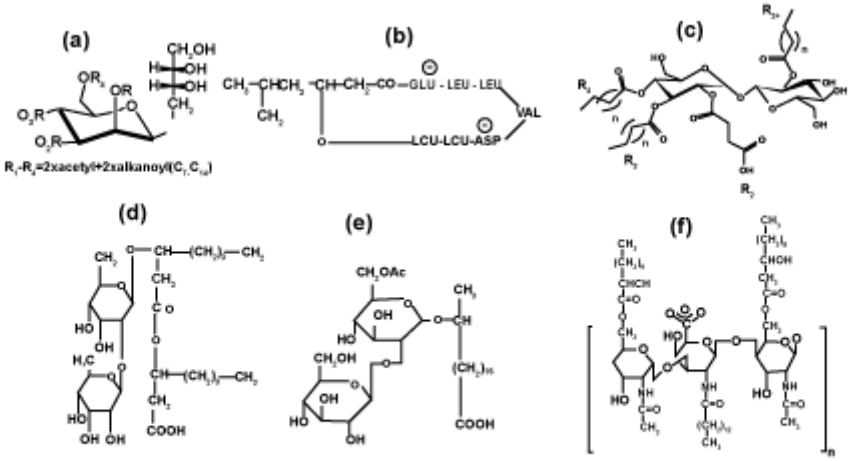


**Figure 1**: Chemical structures of some common biosurfactants (a) Mannosylerythritol lipid (b) Surfactin (c) trehalose lipid (d)Sophorolipid (e) Rhamnolipid (f)Emulsan.

## *III. ISOLATION OF BIOSURFACTANT PRODUCING MICROORGANISM*

In natural environments, microbes are ubiquitous and occur almost always in a mixed population composed of different strains and species. For isolating particular types of microorganism from natural environment a pure culture is required form mixed population. It is very difficult to isolate defined strain directly by diluting and plating, enrichment cultures with hydrophobic substrates are very promising for the isolation of biosurfactant producing microbes.

The principle of enrichment culture is to provide growth conditions that are very favorable for the organisms of interest and as unfavorable as possible for competing organisms. Hence, the microbes of interest are selected and enriched. For the screening of biosurfactant producing microbes, enrichment cultures utilizing hydrophobic compounds as the sole carbon source are applied (Enfors S. et al.1979; Giani C, et. al 1997). The biosurfactant producing bacteria form natural environment can be isolated with polyaromatic hydrocarbons (PAHs)-amended liquid minimal medium (Willumsen and Karlson; 1997), lubricating oil as the sole carbon source (Mercadé et al.1996), mineral media with $C_{14}$ and $C_{15}$ *n*-alkanes (Schulz et al; 1991), crude oil as the sole carbon source (Rahman et al; 2002) and various other means. Thus, the sampling of contaminated sites combined with direct isolation or enrichment culture is an approved strategy for discovering new biosurfactant producing strains.

## *IV. SCREENING OF BIOSURFACTANT PRODUCING ORGANISMS*

Biosurfactants are structurally a very diverse group of biomolecules, e.g., glycolipids, lipopeptides, lipoproteins, lipopolysaccharides or phospholipids. Therefore, most methods for a general screening of biosurfactant producing strains are based on the physical effects of surfactants. Alternatively, the ability of strains to interfere with hydrophobic interfaces can be explored. The isolated colonies were tested for their biosurfactant production by different methods; CTAB Agar Plate; Oil Spreading Technique; Blood Hemolysis Test and Drop collapsing test.

### IV.1 OIL SPREADING ASSAY

In oil spreading assay for oil displacement activity of surfactants was done as per the method described by Morikawa et al. (1993). The principle of this method was based on the ability of the biosurfactant to alter the contact angle at the oil-water interface. In this method 30ml of distilled water was taken in the petri dish (25cm in diameter). 10µl of crude oil (e.g., kerosene) was added to the center of the plates containing distilled water. Now add 10µl of the supernatant of the culture suspension to the center. If the oil displaces and clear zone forms then it shows the presence of biosurfactant. The biosurfactant producing organism can displace the oil and spread in the water. The diameter and area of clear halo visualized under visible light were measured and calculated after 30 seconds. This is also known as oil displacement activity. Measured area is express in BS unit, known as biosurfactant unit. One biosurfactant unit (BS unit) was defined as the amount of surfactant forming 1 $cm^2$ of oil displaced area (Thaniyavam et al. 2003).

### IV.2 HEMOLYTIC ACTIVITY TEST

Biosurfactants can cause lysis of erythrocytes. This principle is used for the hemolysis assay which was developed by Mulligan et al; 1984. Hemolytic activity appears to be a good screening criterion for surfactant-producing strains because biosurfactant producing capacity was found to be associated with hemolytic activity. There are three types of hemolysis α, β, and γ. α hemolysis is said when the agar under the colony become dark and greenish, β is said when it becomes lighten yellow and transparent and when no any change then it is said γ hemolysis. In this method fresh single colonies from the isolated cultures were taken and streaked on blood agar plates. The plates were incubated for 48-72 hours at 37°C. Hemolytic activity was detected as the occurrence of a defined clear zone around a colony (Carrillo et al., 1996). These clear zones indicate the presence of bio-surfactants producing bacteria.

Although the blood agar method is often used for a preliminary screening of microorganisms for the ability to produce biosurfactants on hydrophilic media but the method has some limitations. First, the method is not specific, as lytic enzymes can also lead to clearing zones. Second, hydrophobic substrates cannot be included as sole carbon source in this assay. Third, diffusion restriction of the surfactant can inhibit the formation of clearing zones. In addition, it has been shown that some biosurfactants do not show any hemolytic activity at all. Mulligan *et al.,* 1984 recommend the blood agar method as a preliminary screening method which should be supported by other techniques based on surface activity.

### IV.3 DROP COLLAPSING TEST

Jain et al (1991) developed the drop collapse assay. This assay relies on the destabilization of liquid droplets by surfactants. Therefore, drops of culture supernatant are placed on an oil coated, solid surface. If the liquid does not contain surfactants, the polar water molecules are repelled from the hydrophobic surface and the drops remain

stable. If the liquid contains surfactants, the drops spread or even collapse because the force or interfacial tension between the liquid drop and the hydrophobic surface is reduced. The stability of drops is dependent on surfactant concentration and correlates with surface and interfacial tension. The assay was done in following way: 2µl of mineral oil was added to each well of a 96-well micro-titer plate lid. The lid was equilibrated for 1 hour at room temperature, and then 5 µl of the cultural supernatant was added to the surface of oil. The shape of the drop on the oil surface was inspected after 1 min. Biosurfactant-producing cultures giving flat drops were scored as positive '+'. Those cultures that gave rounded drops were scored as negative '-', indicative of the lack of biosurfactant production (Youssef et al., 2004).

### IV.4 *CTAB AGAR PLATE*

It was developed by Siegmund and Wagner (1991). The CTAB agar plate method is a semi-quantitative assay for the detection of extra cellular glycolipids or other anionic surfactants. Blue agar plates containing cetyltrimethylammonium bromide (CTAB) (0.2 mg $ml^{-1}$) and methylene blue (5 mg $ml^{-1}$) were used to detect extracellular glycolipid or anionic surfactant production. If anionic surfactants are secreted by the microbes growing on the plate, they form a dark blue, insoluble ion pair with cetyltrimethylammonium bromide and methylene blue. Thus, productive colonies are surrounded by dark blue halos. The CTAB agar assay is a comfortable screening method, but it is specific for anionic biosurfactants.

### IV.5 PENETRATION ASSAY

In 2007 Maczek et al developed another assay suitable for high throughput screening, the penetration assay. This assay relies on the contacting of two unsoluble phases which leads to a color change. For this assay, the wells of a 96 microplate are filled with 150µl of a hydrophobic paste consisting of oil and silica gel. The paste is covered with 10µl of oil. 100µl of color supernatant (10µl of a red staining solution plus 90 µl of the supernatant) is applied on the surface of the pest. If biosurfactant is present, the hydrophilic liquid will break through the oil film barrier into the paste. The silica is entering the hydrophilic phase and the upper phase will change from clear red to cloudy white within 15 minutes. Biosurfactant free supernatant will turn cloudy but stay red.

## *V. EXTRACTION OF BIOSURFACTANTS*

For studying the biosurfactant activity, the selected isolates were inoculated in production medium broth containing mixtures of oils (petrol + kerosene + diesel) in 1:1:1 ratio and incubated for appropriate time at optimized temperature (e.g., for bacterial culture 5-10 days at 30°C). Downstream processing in many industrial processes is responsible for up to 60% of the total production cost. Due to economic considerations, most biosurfactant would have to involve either whole-cell spent culture broths or other crude preparations. In addition, biosurfactant activity may be affected by other materials present in these preparations. Biosurfactant recovery depends mainly on its ionic charge, water solubility, and location (intracellular, extracellular or cell bound). The most commonly used biosurfactant recovery techniques are listed in Table 2.

There are different methods for extraction of biosurfactant from culture broth. In one method all the microbial cells were removed by centrifugation (12,000 x g, 4°C, 20 min). The supernatant was taken and the pH of the supernatant was adjusted to 2, using 6N HCl or 1M $H_2SO_4$. The extraction was performed twice with an equal volume of ethyl acetate or chloroform: methanol (2:1) mixture. Pooled solvent extracts were concentrated using an

evaporator under reduced pressure. White colored sediment was obtained as a result i.e., the "Biosurfactants".

In another method the biosurfactant can be extracted in following way. The bacterial cells were recovered by centrifugation, washed twice in demineralized water, and resuspended in 50 mL of phosphate-buffer saline (PBS: 10 mM $KH_2PO_4/K_2HPO_4$ and 150 mM NaCl, pH adjusted to 7.0). The suspensions were maintained at room temperature for up to 2 hours, with gentle stirring to encourage release of biosurfactants. The bacteria were removed from the solution by centrifugation, and the remaining supernatant liquid (containing the biosurfactants) was filtered through a 0.22 $\mu$m pore-size filter (Millipore) for analysis and evaluation.

TABLE 2. Downstream processes for recovery of important biosurfactants

| Process | **Reference(s)** |
|---|---|
| Batch mode | |
| Ammonium sulfate precipitation | |
| Emulsan | Rosenberg, E. et al,1979; Zukerberg, A. et al,1979 |
| Biodispersan | Rosenberg, E. et al 1988 |
| Bioemulsifier | Palejwala, S. et al 1989 |
| Acetone precipitation | |
| Bioemulsifier | Singh, M. et al 1989; Chameotra, S. et al 1990 |
| Acid precipitation | |
| Surfactin | Arima, K. et al 1968; Javaheri, M.et al 1985 |
| Solvent extraction | |
| Trehalolipids | Cooper, D. G. et al 1980; Li, Z. Y. et al 1984 |
| Sophorolipids | Ristau, E.et al 1983 |
| Liposan | Cirigliano, M. C.et al 1985 |
| Crystallization | |
| Cellobiolipids | Spencer, J. F. T.et al 1979; Tulloch, P. et al 1967 |
| Glycolipids | Oberbremer, A.et al 1990 |
| Continuous mode | |
| Centrifugation | |
| Glycolipids | Hauser, G.et al 1958; Kitamoto, D.et al 1993 |

| | |
|---|---|
| Adsorption<br><br>Rhamnolipids<br><br>Lipopetide<br><br>Glycolipids | <br><br>Yamaguchi, M.et al 1976<br><br>Matsuyama, T. et al 1990; Reiling, H. E.et al 1986<br><br>Cooper, D. G. et al 1984; Gobbert, U.et al 1984 |
| Foam separation and precipitation<br><br>Surfactin | <br><br>Cooper, D. G. et al 1981; Mulligan, C. N.et al 1989 |
| Diafiltration and precipitation<br><br>Glycolipids | <br><br>Bryant, F. O. 1990; Chameotra, S. S. et al 1990 |
| Ultrafiltration<br><br>Glycolipids | <br><br>Mulligan, C. N. et al 1990 |

## *VI. SURFACTANT ACTIVITY DETERMINATION*

The surfactant activity is an indicator of a chemical to be performed as surface active agent. The surfactant activity of isolated compound can be determined by different method, some of them are: Emulsification index ($E_{24}$), Surface tension test, Parafilm M test and Tensiometric method.

### VI.1 EMULSIFICATION INDEX (E24)

The emulsifying capacity was evaluated by an emulsification index ($E_{24}$). The $E_{24}$ of culture samples was determined by adding 2 ml of kerosene and 2 ml of the cell-free broth in test tube, vertexing at high speed for 2 min and allowed to stand for 24h. The E index is given as percentage of the height of emulsified layer (cm) divided by the total height of the liquid column (cm). The percentage of emulsification index calculated by using the following equation (Cooper and Goldenberg, 1987):

$$E = \frac{\text{Height of emulsion formed}}{\text{Total height of solution}} \times 100$$

### VI.2 SURFACE TENSION TEST

Surface tension was measured using capillary tube method according to Martin (1993) and applying the formula: $T = f \times r \times g \times h$

Where: T = Surface tension. $f$ = Density of the fluid (1000kg/$m^3$); r = Radius of capillary tube in meter; g = Acceleration gravity (9.81m); h = Highness of fluid in the capillary tube in meter.

### VI.3 PARAFILM M TEST

Youssef et al (2004) demonstrated that the biosurfactant producing microorganism can also be performed by Parafilm M test. In this test 20µl of bacterial supernatants when mixed with 1% xylene-cyanole were added to the hydrophobic surface of parafilm M. The shape of the drop on the surface was inspected after 1 min. The diameters of droplets

were evaluated. The sodium lauryl sulfate and phosphate buffer (pH 7.0) or distill water were used as a positive and negative control, respectively.

### VI.4 SURFACE ACTIVITY DETERMINATION

The surface activity of the biosurfactant was determined by measuring the surface tension of the samples with the ring method. The surface tension of the PBS extract containing the biosurfactants produced by bacteria was measured using a Tensiometer.

## VII. CHARACTERIZATION OF BIOSURFACTANT

### VII.1 THIN LAYER CHROMATOGRAPHY (TLC)

Preliminary characterization of the biosurfactant was done by Thin Layer chromatography (TLC) followed by post chromatographic detection method. A spot of biosurfactant extract was placed on the silica plate (Merck & Co., Mumbai, India) and the biosurfactant was separated on the plate using chloroform: methanol: water (10:10:0.5) or by chloroform/methanol/glacial acetic acid (65:15:2 v/v) as mobile phase. The plate was developed with different color developing reagents. Ninhydrin reagent (0.5g ninhydrin in 100ml anhydrous acetone) was used to detect lipopeptide biosurfactant as red spots and Anthrone reagent (1g Anthrone reagent in 5ml sulfuric acid mixed with 95 ml ethanol) to detect glycolipid biosurfactant as yellow spots (Yin et al., 2008). In another ways the polysaccharides moieties were stained with Syldatk reagents (anisaldehyde: sulfuric acid: glacial acetic acid 0.5:1:50), whereas the fatty acid moieties were stained with ammonium molybdate/cerium sulfate (0.42%, w/v; ammonium molybdate and 0.2%, w/v, cerium (IV) sulfate in 6.2% sulfuric acid).

### VII.2 BIOCHEMICAL TEST

#### VII.2.1 Phenol-$H_2SO_4$ Method

To 1ml of supernatant, 1ml of 5% phenol was added. To this mixture, 2-5 ml of conc. $H_2SO_4$ was added drop by drop, until orange color was developed. The development of orange color indicated the presence of glycolipids containing biosurfactant (Ellaiah P. et. al).

#### VII.2.2 Biuret Test

This test was used to detect the lipopeptide containing bio-surfactant. 2ml of supernatant was heated at $70^0$C, and then was mixed with 1ml of 1M NaOH solution. Drops of 1% $CuSO_4$ were added slowly until violet or pink ring was observed. Formation of violet or pink ring indicates the presence of lipopeptides containing biosurfactant (Feigner C. et al).

#### VII.2.3 Phosphate Test

Up to ten drops of 6M $HNO_3$ was added to 2ml of supernatant, and was heated at $70^0$C. 5 % (w/v) ammonium molybdate was added to this mixture, drop by drop, slowly until the formation of yellow color, and then the yellow precipitate. This indicates the presence of Phospholipids containing biosurfactant (Okpokwasili G.C. et.al).

### VII.3 FOURIER TRANSFORM INFRARED SPECTROSCOPY (FT-IR)

Fourier transform infrared spectroscopy (FTIR) is particularly useful for identifying different types of chemical bonds (functional groups) and can therefore be used to

identify the components of mixtures of unknown composition. Molecular characterization was performed with FTIR spectroscopy of crude biosurfactant extract obtained from the cell free culture supernatant. Infrared spectrophotometer was used to determine the chemical nature of the biosurfactant by the KBr pellet method (Das *et al.,* 2008; Mukherjee et al., 2009).

### VII.4 STABILITY TESTING

To determine the stability 1mg/ml (or cell-free broth obtained after centrifuging of the cultures) of the biosurfactant was taken. The thermostability was maintained at a constant temperature range between 25 – 125°C for 15 min, and cooled at room temperature. Evaluating the effect of pH on biosurfactant activity, the pH was adjusted between 2.0-12. The effect of NaCl addition (0– 50ppt) with different concentration on the biosurfactant activity was investigated. The emulsification value of each treatment was performed as described above.

### VII.5 SURFACE TENSION AND CMC DETERMINATION

The measurement of the surface tension was carried out on the cell-free broth obtained by centrifuging the cultures by the ring method using a Tensiometer at room temperature. The critical micelle concentration (CMC) was determined by measuring the surface tensions of dilutions of isolated biosurfactant in distilled water up to a constant value of surface tension. Each result was the average of 10 determinations after stabilization. The value of CMC was obtained from the plot of surface tension against surfactant concentration.

### VII.6 IONIC PROPERTY OF BIOSURFACTANT

The ionic property of cell bound biosurfactant was determined by using agar well diffusion method (Meylheuc et al. 2001). Briefly, 3 uniformly spaced wells were made on a soft agar (1%) plate; central well was filled with 10 µl of biosurfactant. Either side of wells were filled with anionic compound (Sodium dodecyl sulfate, 20 mM) and cationic compound (Cetyl trimethyl ammonium bromide (CTAB), 20 mM). Plates were incubated at 25°C for 24 h and observed for the precipitation lines.

### VII.7 THERMAL GRAVIMETRIC ANALYSIS (TGA)

Thermal stability of BS is a significant property for its commercial application at elevated ranges of temperature such as microbial enhanced oil recovery and food industries. Thermal degradation of BS was conducted by TG analysis. Approximately (1%) of weight loss was observed with increase in initial temperature from 50 to 200°C possibly due to the loss of solvents and moisture molecules. Briefly, 5–8 mg of lyophilized sample was loaded in a platinum pan and its energy level was scanned in the ranges of 30–480°C and 30–450°C respectively under a nitrogen atmosphere, with a temperature gradient of 10°C $min^{-1}$. Analysis was performed under gradual increase in temperature, plotting the weight percentage and heat flow against temperature respectively.

### VII.8 CYTOTOXICITY ASSESSMENT

There are little publications strictly devoted to toxicity of biosurfactants. Toxicity tests are rather a part of wider research over applicational functions. In spite of this biosurfactants are commonly considered as low- or non-toxic. The cytotoxicity of BS was evaluated on mouse fibroblast (ATCC L929) cell line (Cochis et al. 2012) or other cell line at different concentration and at different time scale.

## *VIII. APPLICATION OF BIOSURFACTANT*

The diverse properties of biosurfactants allow their use and possible replacement of chemically synthesized surfactants in a great number of industrial operations. Much attention has been directed towards biosurfactants due to their broad range of functional properties and diverse synthetic capabilities of microbes and environmental acceptability and because they are readily biodegradable and have low toxicity than synthetic surfactants. A number of applications of biosurfactants have been researched into and published. The huge demand for surfactants is currently met by numerous synthetic, mainly petroleum-based chemical surfactants which are usually toxic to the environment and as well as been non-biodegradable. It has become necessary that tightening environmental regulations and increasing awareness for the need to protect the ecosystem have effectively resulted in an increasing interest in biosurfactants as possible alternates to chemical surfactants (Banat *et al.*, 2000; Benincasa, 2007). Industrial applications of surfactants are classified according to how they are applied. These are surfactants used in detergents and cleaners (54%); as auxiliaries for textiles, leather and paper (13%); in chemical processes (10%); in cosmetics and pharmaceuticals (10%); in the food industry (3%); in agriculture (2%) and in others (8%) (Rahman and Gakpe, 2008).

### VIII.1 ENVIRONMENTAL APPLICATIONS

Owing to industrialization, the environmental contamination caused by industrial activity is due to accidental or deliberate release of organic and/or inorganic compounds into the environment. Such compounds pose problems for remediation, as they become easily bound to soil particles. Biodegradation of hydrocarbons by native microbial populations is the primary mechanism by which hydrocarbons contaminants are removed from the environment. The aims of biosurfactant application are to increasing their bioavailability or mobilizing and removing the contaminants by pseudo-solubilization and emulsification in a washing treatment. Biosurfactant reduced the surface tension by accumulating at the interface of immiscible fluids, increasing the surface area of insoluble compounds which leads to increased bioavailability and subsequent biodegradation of the hydrocarbon. Van Dyke et al. demonstrated a 25 to 70% and 40 to 80% increase in the recovery of hydrocarbons from contaminated sandy-loam and slit-loam soil, respectively, by rhamnolipid from P. aeruginosa. Zhang et al. tested the effect of two rhamnolipid biosurfactant on dissolution and bioavailability of phenanthrene and reported increase in both solubility and degradation rate of phenanthrene (Francy, D.S. et al 1991; Zhang, Y. 1994).

### VIII.2 INDUSTRIAL APPLICATIONS

Since traditional oil recovery technologies can only recover approximately 40– 45% of the oil present in the reservoir, some technologies, collectively defined as enhanced oil recovery (EOR), have been developed (Banat 1995; Dastgheib et al. 2008). One of the most important applications for biosurfactants is in the field of oil recovery and processing. Microbial enhanced oil recovery (MEOR) includes use of microorganisms and the exploitation of their metabolic processes to increase production of oil from marginally producing reservoirs. Microbial surfactants are widely used in oil recovery in recent times. When poor oil recovery from oil wells is due to either low permeability of the rocks forming the reservoir or to the high viscosity of the crude oil, the ability of biosurfactants to reduce the oil/water interfacial tension and to form stable emulsions can improve the process efficiency. The mechanism responsible for oil release is acidification of the solid phase. Certain microorganisms, such as *Bacillus subtilis, Pseudomonas aeruginosa, Torulopsis bombicola* have been reported to utilize crude oil & hydrocarbons

as sole carbon sources & can be used for oil spill clean-ups (Das K, Mukherjee AK; 2007 ).

### VIII.3 BIOMEDICAL APPLICATIONS

The antimicrobial activity of several biosurfactants has been reported in the literature for many different applications (Cameotra S, 2004). Biosurfactant function valuable for medical application due their ability to disrupt membranes leading to cell lysis through increased membrane permeability leading to metabolite leakage. This occurs due to changes in physical membrane structure or through disrupting protein conformations which alters important membrane functions such as transport and energy generation (Van Hamme et al. 2006; Ortiz et al. 2009; Sotirovaetal.2008; Sánchez et al. 2010; Zaragozaetal. 2009). The use and potential commercial applications of biosurfactants in the medical field has increased during the past decade. Biosurfactants are considered relevant molecules for applications in combating many diseases and as therapeutic agents due to their antibacterial, antifungal and antiviral activities. Furthermore, their role as anti-adhesive agents against several pathogens illustrate their utility as suitable anti-adhesive coating agents for medical insertional materials leading to a reduction of a large number of hospital infections without the use of synthetic drugs and chemicals (Rodrigues LR, 2011). Mukherjee *et al.* (2010) elucidated on the wide range of applications of biosurfactants in medicine. Day by day use of antimicrobial agent increase the incidence of drug resistance against existing drugs so attention towards new drug is in increasing. Some biosurfactants have been reported to be suitable alternatives to synthetic medicines and antimicrobial agents and may therefore be used as effective and safe therapeutic agents (Cameotra and Makkar 2004; Singh and Cameotra 2004; Banat et al. 2000). Among the antimicrobial biosurfantant are, Surfactin, produced by *B. subtilis* (Arima et al. 1968), fengycin, iturin, bacillomycins and mycosubtilins produced by *B. subtilis* (Vater et al. 2002), Lichenysin, pumilacidin and polymyxin B (Naruse et al. 1990; Yakimov et al. 1995; Grangemard et al. 2001; Landman et al. 2008) produced by *B. licheniformis*, *Bacillus pumilus* and *Bacillus polymyxa*, respectively. Other reported biosurfactants having antimicrobial activity are daptomycin, a cyclic lipopeptide from *Streptomyces roseosporus* (Baltz et al. 2005), viscosin, a cyclic lipopeptide from *Pseudomonas* (Neu et al. 1990; Saini et al. 2008), rhamnolipids produced by *P. aeruginosa* (Abalos et al. 2001; Benincasa et al. 2004) and sophorolipids produced by *C. bombicola* (Kim et al. 2002; Van Bogaert et al. 2007). Mannosylerythritol lipids (MEL-A and MEL-B) produced by *Candida antarctica* strains have also been reported to exhibit antimicrobial action against Gram-positive bacteria (Kitamoto et al. 1993). Probiotics have long been known for their antimicrobial activity and for the capacity to interfere with the adhesion and formation of biofilms of pathogens to epithelial cells of urogenital and intestinal tracts, catheter materials and voice prostheses, and the mechanisms of this interference have been demonstrated to include, among others, the release of biosurfactants (Gudina EJ et al, 2010; Rodrigues L et al, 2006).

The antiviral activity of biosurfactants, mainly surfactin and its analogues has been reported (Naruse N et al, 1990). The more effective inactivation of enveloped viruses, such as retroviruses and herpes viruses, compared to non-enveloped viruses, suggests that this inhibitory action may be mainly due to physicochemical interactions between the virus envelope and the surfactant (Vollenbroich et al. 1997). Sophorolipids are also claimed to have activity against human immunodeficiency virus (Shah et al. 2005) and rhamnolipid of *Pseudomonas* sp. showed significant antiviral activity against herpes simplex virus types 1 and 2 (Remichkova et al. 2008).

The key microbial activities in the colonization of a surface and therefore can increase the chance of nosocomial infections on different medical devices are due to biofilm

formation and swarming motility of microorganisms (Khardori and Yassien 1995; Vinh and Embil 2005; McCann et al. 2008; Harriott and Noverr 2009). The microbial surfactants or biosurfactants have been found to inhibit the adhesion of pathogenic organisms due to its anti-adhesive property to the surgical instruments or to infection sites thus might constitute a new alternative and effective means of combating colonization of pathogenic microorganisms (Neu 1996; Federle and Bassler 2003; Rasmussen and Givskov 2006). It has been demonstrated that pre-coating vinyl urethral catheters treated with surfactin solution before inoculation with media resulted in decreased amount of biofilm formation by gram negative bacteria like *S. typhimurium, S. enterica, E. coli*, and *P. mirabilis.*

### VIII.4 APPLICATIONS IN FOOD PROCESSING INDUSTRY

Biosurfactants have been used for various food processing industry, due to their useful properties as emulsifiers (Banat et al. 2000), anti-adhesive, and antimicrobial agents (Singh and Cameotra 2004). The basic purpose behind processing food is not only to make it safe to eat, but also to make it look, taste, and smell as enticing and as close to freshly prepared as possible. They used to control the agglomeration of fat globules, stabilize aerated systems, improve texture and shelf-life of starch-containing products, modify rheological properties of wheat dough and improve consistency and texture of fat-based products (Kachholz & Schlingmann, 1987).

The particular combination of characteristics such as emulsifying, anti-adhesive, and antimicrobial activities presented by biosurfactants suggests their application as multipurpose ingredients or additives. They promote the formation and stabilization of emulsion due to their ability to decrease the surface and interfacial tension that is why they are widely used as food formulation ingredient. Considering that biosurfactants exhibit surface-active and emulsifier action, there is a great potential market for effective biosurfactants in the food industry not only due to their surface activity, but also for their environmentally friendly nature (Mohan et al. 2006), low toxicity (Flasz et al. 1998), their unique structures and properties, and the increasing customer demand for natural or organic over synthetic ingredients.

A wide range of biosurfactants has shown antimicrobial activity against bacteria, yeast, fungi, algae, and viruses (Nitschke and Costa 2007). Among biosurfactants, lipopeptides (surfactin, fengycin, iturin, bacillomycins, and mycosubtilins) from *Bacillus* sp. have wide antimicrobial action (Das *et al.* 2008, Fernandes *et al.* 2007).

The involvement of biosurfactants in microbial adhesion and detachment from surfaces has been investigated (Busscher, van der Kuij-Booij, & van der Mei, 1996). This anti-adhesive property of bisurfectant prevents biofilm formation by microbial community on the food surface. The bacterial biofilms present in food industry surfaces are potential sources of contamination, which may lead to food spoilage and disease transmission (Hood & Zottola, 1995).

### VIII.5 APPLICATIONS IN COSMETIC INDUSTRY

In the cosmetic industry, biosurfactants been proposed to replace chemically synthesized surfactants for their multifunctional applications (Kleckner and Kosaric., 1993) due to their exceptional surface properties such as detergency, wetting, emulsifying, solubilizing, dispersing and foaming effects (Rieger, 1997). The most widely used biosurfactant glycolipids in cosmetics are sophorolipids, rhamnolipids and mannosylerythritol lipids. Sophorolipids have good skin compatibility and excellent moisturizing properties, rhamnolipids are natural surfactants and emulsifiers that can replace petrochemical based surfactants used in most of the cosmetic products. These surfactants are used as emulsifiers, foaming agents, solubilizers, wetting agents,

cleansers, antimicrobial agents, mediators of enzyme action, in insect repellents, antacids, bath products, acne pads, anti-dandruff products, contact lens solutions, baby products, mascara, lipsticks, toothpaste, dentine cleansers to mention but a few (Gharaei-Fathabad E; 2011).

### VIII.6 APPLICATIONS IN AGRICULTURE

The biosurfactants synthesized by environmental isolates also has promising role in the agricultural industry. These biosurfactants can be widely exploited in areas related to agriculture for enhancement of biodegradation of pollutants to improve the quality of agriculture soil, for indirect plant growth promotion as these biosurfactants have antimicrobial activity and to increase the plant microbe interaction beneficial for plant. In agriculture, biosurfactants are used for hydrophilization of heavy soils to obtain good wettability and to achieve even distribution of fertilizer in the soil. They also prevent the caking of certain fertilizer during storage and promote spreading and penetration of the toxicants in pesticides (Makkar RS & Rockne KJ; 2003). In agriculture rhamnolipid and fengycins are used as antimicrobial and antifungal respectively in biocontrol of plant diseases (Kachholz T & Schlingmann M, 1987). Lipopeptide biosurfactants produced by several bacteria exhibit insecticidal activity create new tools in the field of biopesticide to prevent emergence of conventional pesticide resistance insect/pest.

## *IX. CONCLUSION*

The microbial biosurfactant is widely distributed in nature and are of diverse chemical composition synthesized by bacteria, yeast and fungi extracellularly or as part of the cell membrane. Day by day use of this versatile chemical create a new filed to develop ecofriendly sustainable environment. In this review we try to represent different types of green surfactant and their commercial as well as household applications. Here we represent the process of isolation of microorganism from natural environment, downstream processing of biosurfactants production and characterization of the isolated surfactant by their physical, chemical and biological property. Finally, we give a keen idea about commercial application of biosurfactant and its utilization in human welfare. Biosurfactants hold promise for reducing dependence over environmentally harsh chemical detergents, and also open up opportunities for novel applications. Biosurfactants have gained importance as emulsifiers, deemulsifiers, foaming agents, wetting agents, spreading agents, functional food ingredients and detergents in various industrial sectors such as, organic chemicals, petroleum and petrochemicals, mining and metallurgy (mainly bioleaching), agrochemicals and fertilizers, foods and beverages, cosmetics and pharmaceuticals, and many others. We hope with increasing public awareness in the environment, biosurfactant would most likely replace the usage of chemical surfactants in the near future.

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