2. Review of literature

2.1. Biosurfactant

Biosurfactants are surface-active substances synthesized by living cells. Biosurfactants (BS) are amphiphilic compounds produced by living surfaces, mostly microbial cell surfaces, or excreted extracellularly and contain hydrophobic and hydrophilic moieties that reduce surface tension (ST) and interfacial tensions between individual molecules at the surface and interface, respectively (Karanth et al. 1999).

Microorganisms utilize a variety of organic compounds as the source of carbon and energy for their growth. When the carbon source is an insoluble substrate like a hydrocarbon (C_xH_y), microorganisms facilitate their diffusion into the cell surface by producing a variety of substances, the biosurfactants. Some bacteria and yeasts excrete ionic surfactants which emulsify the C_xH_y substrate in the growth medium (Mazaheri et al. 2010). Some examples of this group of biosurfactant are rhamnolipids which are produced by different *Pseudomonas sp*. (Neu, 1996), or the sophorolipids which are produced by several *Torulopsis sp*. Some other microorganisms are capable of changing the structure of their cell wall, which they achieve by synthesizing lipopolysaccharides or nonionic surfactants in their cell wall. Although most biosurfactants are considered to be secondary metabolites (Mulligan et al. 2014).

Biosurfactants are amphipathic molecules with both hydrophilic and hydrophobic moieties that partition preferentially at the interface between fluid phases that have different degrees of polarity and hydrogen bonding, such as oil and water or air and water interfaces. This property explains their broad use in environmental and industrial applications.

2.1.1. Biosurfactants: mechanisms of interaction

Biosurfactant**s** are microbial amphiphilic polymers that tend to interact with the phase boundary between two phases in a heterogeneous system, defined as the interface. For all interfacial systems, it is known that organic molecules from the aqueous phase tend to immobilize at the solid interface. There they eventually form a film known as a conditioning film (Figure 2.1), which will change the properties (wettability and surface energy) of the original surface (Neu, 1996). In an analogy to organic conditioning films, biosurfactant**s** may interact with the interfaces and affect the adhesion and detachment of microorganism. In addition, the substratum surface properties determine the composition and orientation of the molecules conditioning the surface during the first hour of exposure.

Figure.2.1 Biosurfactant produced by microbe

Biosurfactants are produced mainly by aerobically growing micro-organisms in aqueous media from a carbon source feedstock, e.g. carbohydrates, hydrocarbons, oils and fats or mixtures of there. The emulsifiers are secreted into the culture medium during the growth of the micro-organism and assist in the transport and translocation of the insoluble substrates across cell membranes (Silva et al., 2014).

In common with all surface-active species, biosurfactants contain one or several lipophilic and hydrophilic moieties the lipophilic moiety can be a protein or a peptide with a high proportion of hydrophobic side chains, but is usually the hydrocarbon chain of a fatty acid with 10–18 carbon atoms, although higher molecular weight fatty acids have been reported. The hydrophilic moiety can be an ester, a hydroxy, a phosphate or carboxylate group or a carbohydrate (Neu et al. 1990).

Bacteria growing on hydrocarbons, the growth rate can be limited by the interfacial surface area between water and oil (Ron et al. 2002; Sekelsky et al. 1999). When the surface area becomes limiting, biomass increases arithmetically rather than exponentially. The evidence that emulsification is a natural process brought about by extracellular agents is indirect, and there are certain conceptual difficulties in understanding how emulsification can provide an (evolutionary) advantage for the microorganism producing the emulsifier. Stated briefly, emulsification is a cell-density-dependent phenomenon: that is, the greater the number of cells, the higher the concentration of extracellular product. The concentration of cells in an open system, such as an oil-polluted body of water, never reaches a high enough value to effectively emulsify oil. Furthermore, any emulsified oil would disperse in the water and not be more available to the emulsifier-producing strain than to competing microorganisms (Ron et al. 2002).

2.1.2. Biosynthesis

As may be expected from the wide variety of biosurfactant structures that have been determined so far, their formation involves an equally diverse range of biosynthetic pathways. For simplicity, three classes of pathways can be distinguished depending on whether the hydrophobic domain, the hydrophilic domain, or both, are synthesized ***de***

nouo. Obviously, this classification does not reflect the many different biosynthetic routes that are involved in the formation of the lipid and hydrophilic domains. Those components that are not synthesized ***de nouo*** are produced by modification of the carbon source, i.e. sugars, alkanes, etc. Often, a variety of different carbon substrates can be incorporated into the biosurfactant, giving rise to a family of related molecules.

The following possibilities exist for the synthesis of different moieties of biosurfactants and their linkage: (i) the hydrophilic and hydrophobic moieties are synthesized de novo by two independent pathways; (ii) the hydrophilic moiety is synthesized de novo while the synthesis of the hydrophobic moiety is induced by substrate; (iii) the hydrophobic moiety is Synthesized de novo, while the synthesis of the hydrophilic moiety is substrate dependent; and (iv) the synthesis of both the hydrophobic and hydrophilic moieties is substrate dependent (Satpute et al., 2010).

The fatty acid components of biosurfactants are synthesized by the rather well characterized pathways of lipid metabolism. The hydrophilic moieties; on the other hand, exhibit a greater degree of structural complexity which is the outcome of a wide variety of biosynthetic mechanisms (Ochsner et al. 1994).

2.2. Types of Biosurfactant

Unlike chemically synthesized surfactants, which are classified according to the nature of their polar grouping, biosurfactants are categorized mainly by their chemical composition and their microbial origin. In general, their structure includes a hydrophilic moiety consisting of amino acids or peptides anions or cations; mono-, di-, or polysaccharides; and a hydrophobic moiety consisting of unsaturated, saturated, or fatty

acids. Accordingly, the major classes of biosurfactants include glycolipids, lipopeptides and lipoproteins, phospholipids and fatty acids, polymeric surfactants, and particulate surfactants (Desai et al. 1997).

Glycolipids: Most known biosurfactants are glycolipids. They are carbohydrates in combination with long-chain aliphatic acids or hydroxyaliphatic acids. Among the glycolipids, the best known are rhamnolipids, trehalolipids, and sophorolipids.

Rhamnolipids: Rhamnolipids, in which one or two molecules of rhamnose are linked to one or two molecules of b-hydroxydecanoic acid, are the best-studied glycolipids. Production of rhamnose-containing glycolipids was first described in *Pseudomonas aeruginosa* by Jarvis and Johnson (Cooper et al. 1984) Rhamnolipids from *Pseudomonas* spp. have been demonstrated to lower the interfacial tension against *n*-hexadecane to 1 mN/m and the surface tension to 25 to 30 mN/m depending on the pH and salt conditions (Hommel et al. 1987; Muthusamy et al. 2008). Rhamnolipids function as a natural surfactant, emulsifier, foaming agent, fungicide, antibiotic and anionic complexation agent. In an aqueous solution pure dry form, rhamnolipids are a white powder. They may range from clear to milky white or tan in color (US20140294925 A1, 2014).

Trehalolipids: Several structural types of microbial trehalolipid biosurfactants have been reported. Disaccharidetrehalose linked at C-6 and C-69 to mycolic acids is associated with most species of *Mycobacterium, Nocardia,* and *Corynebacterium*. Mycolic acids are long-chain, a-branched-b-hydroxy fatty acids.Trehalolipids from different organisms differ in the size and structure of mycolic acid, the number of carbon atoms, and the degree of unsaturation. Trehalose lipids from *R. erythropolis* and *Arthrobacter* sp. lowered the

surface and interfacial tensions in the culture broth to 25 to 40 and 1 to 5 mN/m respectively (Guerra-Santos et al. 1986).

Sophorolipids: Sophorolipids, which are produced mainly by yeasts such as *Torulopsis bombicola*, *T.petrophilum*, and *T.apicola*, Consist of a dimeric carbohydrate sophorose linked to a long-chain hydroxy fatty acid. These biosurfactants are a mixture of at least six to nine different hydrophobic sophorosides (Parra et al., 1989).

Although sophorolipids can lower surface and interfacial tension, they are not effective emulsifying agents. Both lactonic and acidic sophorolipids lowered the interfacial tension between *n*-hexadecane and water from 40 to 5 mN/m and showed remarkable stability toward pH and temperature changes (Kosaric et al. 1993).

Lipopeptides and Lipoproteins: Lipopeptide is a molecule consisting of a lipid connected to a peptide. They are bound by TLR 1, and other, Toll-like receptors. Certain lipopeptides are used as antibiotics. Lipopeptides have been shown in scientific studies, to boost cell's natural productivity levels and are considered to be one of nature's most powerful anti-agers by interacting with cell membranes to boost and revitalize the natural function of the cells, renewing them to maximum growth potential. Unlike water-soluble peptides, lipopeptides are highly biocompatible with skin's natural structure. (Reviews, 2017).

Among the several categories of biosurfactants, lipopeptides are particularly interesting because of their high surface activities and antibiotic potential. Lipopeptides can act as antibiotics, antiviral and antitumor agents, immunomodulators or specific toxins and enzyme inhibitors (Rodrigues et al. 2006).

A large number of cyclic lipopeptides including decapeptide antibiotics (gramicidins) and lipopeptide antibiotics (polymyxins), produced by *Bacillus brevis* and *B. polymyxa*, respectively, possess remarkable surface-active properties. Ornithine- containing lipids Surfactin from *P. rubescens* and *Thiobacillus thiooxidans*, exhibit excellent biosurfactant activity. The cyclic lipopeptide surfactin, produced by *B. subtilis* ATCC 21332, is one of the most powerful biosurfactants. It lowers the surface tension from 72 to 27.9 mN/m (Desai et al. 1997).

Surfactin, one of the most active biosurfactants produced by *B. subtilis* is a cyclic lipopeptide surfactin. The yield of surfactin produced by *B. subtilis* can be improved to around 0.8 g/l by continuously removing the surfactant by foam fractionation and addition of either iron or manganese salts to the growth medium (Karanth et al.1999).

Lipopeptides belonging to the surfactin family are formed by nonribosomal peptide synthetases and / or polyketide synthases. They are amphiphilic cyclic peptides composed of 7 amino acids linked to one ÃŸ-hydroxy fatty acid. Surfactins are not fungitoxic by themselves but have some synergistic effects on the antifungal activity of iturin A (Al-Araji et al. 2007).

With its exceptional surface activity, this compound inhibits fibrin clot formation and lyses erythrocytes and several bacterial spheroplasts and protoplasts. *Bacillus Subtilis* cells secrete surfactins in order to aid with surface motility. In other words, it helps them move across surfaces to colonize new areas.

Fatty Acids, Phospholipids, and Neutral Lipids: Fatty acids are aliphatic monocarboxylic acids derived from, or contained in esterified form in, an animal or

vegetable fat, oil, or wax. Natural fatty acids commonly have a chain of 4 to 28 carbons (usually unbranched and even numbered), which may be saturated or unsaturated. By extension, the term is sometimes used to embrace all acyclic aliphatic carboxylic acids (Chauhan et al. 2009).

Phospholipids are a class of lipids and are a major component of all cell membranes as they can form lipid bilayers. Most phospholipids contain a diglyceride, a phosphate group and a simple organic molecule such as choline; one exception to this rule is sphingomyelin, which is derived from sphingosine instead of glycerol. The 'head' of a phospholipid is hydrophilic (attracted to water), while the hydrophobic 'tails' repel water. The hydrophillic head contains the negatively charged phosphate group, and may contain other polar groups. The hydrophobic tail usually consists of long fatty acid hydrocarbon chains (Thaniyavarn et al., 2003).

Several bacteria and yeasts produce large quantities of fatty acid and phospholipid surfactants during growth on *n*-alkanes (Cirigliano & Carman, 1985). The quantitative production of phospholipids has also been detected in some *Aspergillus* spp. and *Thiobacillus thiooxidans*. *Arthrobacter* strain AK-19 (Sen, 2010) and *P. aeruginosa* 44T1 accumulate up to 40 to 80% (wt/wt) of such lipids when cultivated on hexadecane and olive oil, respectively (Sen, 2010).

Polymeric Biosurfactants: The best-studied polymeric biosurfactants are emulsion, liposan, mannoprotein, and other polysaccharide-protein complexes. *Acinetobacter calcoaceticus* RAG-1 produces a potentpolyanionic amphipathic heteropolysaccharide

bioemulsifier called emulsan. Fatty acids are covalently linked to the polysaccharide through *o*-ester linkages (Rosenberg et al. 1979).

Emulsan is a very effective emulsifying agent for hydrocarbons in water even at a concentration as low as to 0.01%. It is one of the most powerful emulsion stabilizers known today and resists inversion even at a water-to-oil ratio of 1:4. Liposan is an extracellular water-soluble emulsifier synthesized by *Candida lipolytica* and is composed of 83% carbohydrate and 17% protein (Muthusamy et al., 2008). The carbohydrate portion is a heteropolysaccharide consisting of glucose, galactose, galactosamine, and galacturonic acid (Kosaric, 1987)

Particulate Biosurfactants: Extracellular membrane vesicles partition hydrocarbons to form a microemulsion which plays an important role in alkane uptake by microbial cells. Vesicles of *Acinetobacter* sp. Strain HO1-N with a diameter of 20 to 50 nm and a buoyant density of 1.158 g/cm^3 are composed of protein, phospholipid, and lipopolysaccharide. The membrane vesicles contain about 5 times as much phospholipid and about 350 times as much polysaccharide as does the outer membrane of the same organism (Käppeli et al. 1979).

Surfactant activity in most hydrocarbon-degrading and pathogenic bacteria is attributed to several cell surface components, which include structures such as M protein and lipoteichoic acid in the case of group A streptococci, protein A in *Staphylococcus aureus*, layer A in *Aeromonas salmonicida*, prodigiosin in *Serratia* spp., gramicidins in *Bacillus brevis* spores, and thin fimbriae in *A. calcoaceticus* RAG-1 (Fattom et al. 1985;

Sen, 2010). Table 2.1 Shows list of different types of biosurfactant and their respective producing microorganisms.

Table 2.1. Type and microbial origin of biosurfactants (Lin, 1996; Shekhar et al. 2015)**.**

Type of Biosurfactant	**Microorganism**
Trehalose lipids	*Arthrobacter paraffineus, Corynebacterium spp., Mycobacterium spp., Rhodococus erythropolis, Nocardia sp.*
Rhamnolipids	*Pseudomonas aeruginosa, Pseudomomas sp., Serratia rubidea*
Sophorose lipids	*Candida apicola, Candida bombicola, Candida lipolytica, Candida bogoriensis*
Glycolipids	*Alcanivorax borkumensis, Arthrobacter sp., Corynebacterium sp., R. erythropolis, Serratia marcescens, Tsukamurella sp.*
Cellobiose lipids	*Ustilago maydis*
Polyol lipids	*Rhodotorula glutinus, Rhodotorula graminus*
Diglycosyl diglycerides	*Lactobacillus fermentii*
Lipopolysaccharides	*Acinetobacter calcoaceticus (RAG1), Pseudomonas sp., Candida lipolytica Arthrofactin Arthrobacter sp. Lichenysin A, Lichenysin B, Bacillus licheniformis*
Surfactin	*Bacillus subtilis, Bacillus pumilus*
Viscosin	*Pseudomonas fluorescens*
Ornithine, lysine peptides	*Thiobacillus thiooxidans, Streptomyces sioyaensis, Gluconobacter cerinus*

Phospholipids	*Acinetobacter sp.*
Fatty acids	*Capnocytophaga sp., Penicillium spiculisporum, Corynebacterium lepus, Arthrobacter paraffineus, Talaramyces trachyspermus, Nocardia erythropolis*
Alasan	*Acinetobacter radioresistens*
Streptofactin	*Streptomyces tendae*
Particulate surfactant (PM)	*Pseudomonas marginalis*

2.3. Applications of Biosurfactants -

2.3.1. Microbial enhanced oil recovery

An area of considerable potential for Biosurfactant application is microbial enhanced oil recovery (MEOR). In MEOR, microorganisms in reservoir are stimulated to produce polymers and surfactants which aid MEOR by lowering interfacial tension at the oil–rock interface. To be useful for MEOR *in situ*, bacteria must be able to grow under extreme conditions encountered in oil reservoirs such as high temperature, pressure, and salinity. Several aerobic and anaerobic thermophiles tolerant of pressure and moderate salinity have been isolated which are able to mobilize crude oil in the laboratory. It was estimated that about 27% of oil reservoirs in USA are amenable to microbial growth and MEOR. The effectiveness of MEOR has been reported in field studies carried out in US, Czechoslovakia, Romania, USSR, Hungary, Poland, and The Netherlands. Significant increase in oil recovery was noted in some cases (Shibulal et al., 2014).

In situ removal of oil is due to multiple effects of the microorganisms on both environment and oil. These effects include gas and acid production, reduction in oil viscosity, plugging by biomass accumulation, reduction in interfacial tension by biosurfactants and degradation of large organic molecules. These are all factors responsible for decreasing the oil viscosity and making its recovery easier (Jack, 1985).

MEOR has several advantages compared to other Enhanced Oil Recovery (EOR) processes in that it does not consume large amounts of energy, as do thermal processes, nor does it depend on the price of crude oil, as do many chemical processes. MEOR can also be cost-effective, since microbial products can be produced from inexpensive, renewable resources, and several MEOR processes have been shown to produce incremental oil for about \$19 per m^3 (\$3 per barrel) (Brown et al. 2002; McInerney et al. 2005).

Hydrocarbon degradation: Hydrocarbons, which include petroleum products, oil products and halogenated compounds, form an important class of pollutants on a global scale. The presence of these hydrocarbons in the environment is of considerable public health and ecological concern, because of their persistence, toxicity and ability to accumulate.

One of the problems associated with the biodegradation of hydrophobic compounds, which include petroleum hydrocarbons, is that they bind to soil particles and have limited solubility in water, resulting in limited availability to soil microorganisms, which in turn can retard and/or stop the degradation process. It is generally assumed that, if you can increase the availability of the hydrophobic compounds to the microorganisms, you can increase the rate of bioremediation.

One method which has been investigated to address the problem is the use of surfactants and emulsifiers. These chemicals increase the solubility and dispersion of hydrophobic compounds. Biosurfactants, as they are produced naturally, may be more ecologically acceptable and, unlike many synthetic surfactants, they are biodegradable (Brooijmans et al. 2009; Das et al. 2011).

Oil storage tank cleaning Sludges and heavy oil factions that settle at the bottom of oil storage tanks are highly viscous or even solid deposits that cannot be lifted by conventional pumps. Their removal usually requires solvent washing or manual cleaning, both being hazardous, time consuming and expensive processes (Mansur et al. 2015).

Surfactants have been studied for use in reducing the viscosity of heavy oils, thereby facilitating recovery, transportation and pipelining. Two tons of biosurfactant-containing whole-cell culture was used to mobilize and clean 850 m^3 oil sludge. Approximately 91% (774 m^3) of this sludge was recovered as re-sellable crude oil and 76 m^3 non-hydrocarbon materials remained as impurities to be manually cleaned. The value of the recovered crude covered the cost of the cleaning operation (US $100,000±150,000 per tank). Such a clean-up process is therefore economically rewarding and less hazardous to persons involved in the process compared to conventional processes. It is also an environmentally sound technology leading to less disposal of oily sludge in the natural environment (Banat et al. 2000).

2.3.2. Biomedical and therapeutic applications of biosurfactants

Some biosurfactants are a suitable alternative to synthetic medicines and antimicrobial agents and may be used as safe and effective therapeutic agents. There has been increasing interest in the effect of biosurfactants on human and animal cells and cell lines.

The mannosylerythritol lipid MELs produced by *Candida antartica*, rhamnolipids produced by *P. aeruginosa* and lipopeptides produced by *B. subtilis* and *B. licheniformis* have been shown to have antimicrobial activities. Yakimov *et al*. demonstrated the antibacterial activity of lichenysin A, a biosurfactant produced by *B. licheniformis* more recently; Grangemard *et al*. reported the chelating properties of lichenysin, which might explain the membrane-disrupting effect of lipopeptides. In another study, Carrillo *et al*. noted a molecular mechanism of membrane permeabilization by surfactin, which may explain surfactin-induced pore formation underlying the antibiotic and hemolytic action of these lipopeptides. This study also suggested that the membrane barrier properties are likely to be damaged in the areas where surfactin oligomers interact with the phospholipids, at concentrations much below the onset for solubilization. Such properties can cause structural fluctuations that may well be the primary mode of the antibiotic action of this lipopeptide (Kitamoto et al., 1993).

Lipopeptide surfactin was also reported to have an antitumour activity against Ehrlich's ascite carcinoma cells and an antifungal activity as well as various pharmacological applications such as inhibiting fibrin clot formation and haemolysis and formation of membrane ion channels. In addition, surfactin and surfactin analogues have been reported as antiviral agents: a significant inhibitory effect of pumilacidin on HSV-1

was demonstrated as well as an inhibitory activity against H^+, K^+-ATPase and protection against gastric ulcers *in vivo*. The potential of surfactin against human immunodeficiency virus 1 (HIV-1) was reported by Itokawa *et al*. The antiviral action of surfactin was suggested to be due to physicochemical interactions between the membrane-active surfactant and the virus lipid membrane (Itokawa et al., 1994; Naruse et al., 1990).

The deficiency of pulmonary surfactant described earlier which is responsible for respiration failure in premature infants may be corrected through the isolation of genes for protein molecules of this surfactant and cloning in bacteria for possible fermentative production and use in medical application. Respiratory Distress Syndrome – Breathing problems in newborn babies. Surfactant deficiency cause pulmonary haemorrhage, pulmonary oedema, and Pneumonia. Use of surfactant therapy in treating respiratory disease in the neonate (Rodrigues et al., 2006). Table 2.2 Shows the biosurfactant produced from their respective microorganism and their activity/applications in medical field.

Table 2.2. Biosurfactant application in medical field (Rodrigues et al., 2006)

Microorganism	**Biosurfactant type**	**Activity/application in medical field**
Pseudomonas sp.	rhamnolipid	*antimicrobial activity against Mycobacterium tuberculosis * anti-adhesive activity against several bacterial and yeast strains isolated from voice prostheses

Bacillus subtilis	surfactin	* antimicrobial and antifungal activities * inhibition of fibrin clot formation * haemolysis and formation of ion channels in lipid membranes * antitumour activity against Ehrlich's ascite carcinoma cells *antiviral activity against human immunodeficiency virus 1 (HIV-1)
Bacillus pumilus	pumilacidin	*antiviral activity against herpes simplex virus 1 (HSV-1) * inhibitory activity against H+, K+-ATPase and protection against gastric ulcers in vivo
Bacillus subtilis	iturin	*antimicrobial activity and antifungal activity against profound mycosis * effect on the morphology and membrane structure of yeast cells * increase in the electrical conductance of biomolecular lipid membranes *non-toxic and non-pyrogenic immunological adjuvant

Bacillus licheniformis	lichenysin	* antibacterial activity * chelating properties that might explain the membrane-disrupting effect of lipopeptides Mannosylerythritol
Candida antartica	Lipids	*antimicrobial, immunological and neurological properties * induction of cell differentiation in the human promyelocytic leukemia cell line HL60 * induction of neuronal differentiation in PC12 cells
Rhodococcus erythropolis	treahalose lipid	*antiviral activity against HSV and influenza virus
Streptococcus thermophilus	glycolipid	* anti-adhesive activity against several bacterial and yeast strains isolated from voice prostheses
Streptococcus mitis	not identified	* anti-adhesive activity against *S*treptococcus Mutans.
Lactobacillus sp.	surfactin	* anti-adhesive activity against several Pathogens including enteric bacteria.

2.3.3. Heavy Metal Removal by Biosurfactants

The addition of biosurfactant may promote desorption of heavy metals from soils in two ways. The first is through complexation of the free form of the metal residing in solution which decreases the solution-phase activity of the metal and therefore promotes desorption. The second occurs under conditions of reduced interfacial tension; the biosurfactants accumulate at the solid solution interface, which may allow direct contact between the biosurfactant and the sorbed metal (Banat et al., 2000).

Due to the anionic nature of rhamnolipids, they are able to remove metals from soil and ions such as cadmium, copper, lanthanum, lead and zinc due to their complexation ability (46). Cations of lowest to highest affinity for rhamnolipid were $K^{+} < Mg^{2+} < Mn^{2+} < Ni^{2+} < Co^{2+} < Ca^{2+} < Hg^{2+} < Fe^{3+} < Zn^{2+} < Cd^{2+} < Pb^{2+} < Cu^{2+} < Al^{3+}$. The affinities were approximately the same or higher than those that organic acids (acetic, citric, fulvic and oxalic acids) have for metals, thus indicating the potential of the rhamnolipid for metal remediation. Rhamnolipids were then applied to a soil in the presence of oil contamination and from sediments to remove heavy metals (shown in Figure 2.2). Biosurfactant could also be added as a soil washing process for excavated soil. Due to the foaming property of the biosurfactant, metal–biosurfactant complexes can be removed by addition of air to cause foaming and then the biosurfactant can be recycled through precipitation by reducing the pH to 2. Improvements in the fermentation and purification process will continue to decrease costs Also increases in applications will enable more full-scale production (Ochoa et al. 1998; Tan et al. 1994).

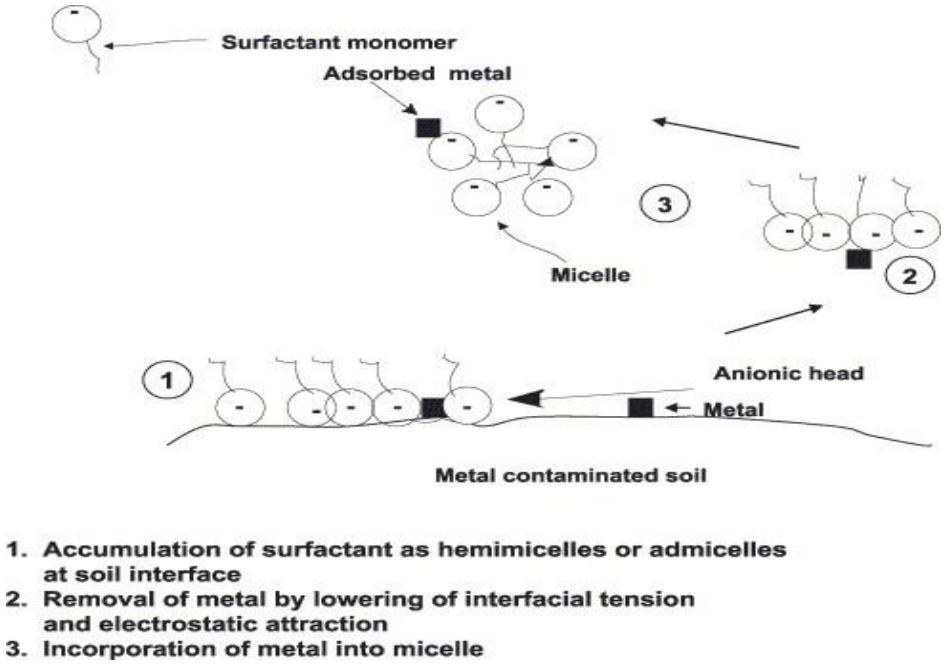

Figure 2.2. Removal of heavy metal from soil (Ochoa Loza, 1998)**.**

2.3.4. Biosurfactants as Pesticide

The biosurfactant has zoosporicidal activity against species of Pythium, Phytophora, and Plasmopara at concentrations ranging over 5 to 30 µg/ml. The proposed mechanism for the biosurfactant action is that the biosurfactant intercalates with and disrupts the plasma membrane, although this evaluated the biological control potential of rhamnolipid-producing strains and concluded that biosurfactants have potential for the biological control of zoosporic plant pathogens as shown in Figure 2.3 (Stanghellini et al. 1994).

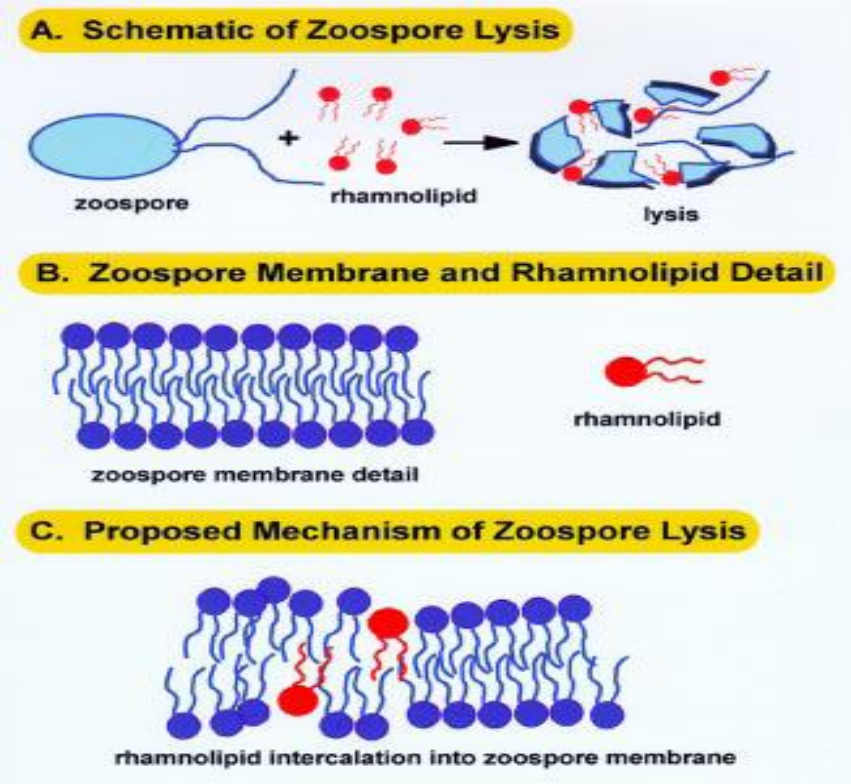

Figure 2.3. Zoosporicidal activity of biosurfactant

Surface-active agents are needed for the hydrophilization of heavy soils to obtain good wettability and also to achieve equal distribution of fertilizers and pesticides in the soils. Biosurfactants have also been used in formulating poorly soluble organophosphorus pesticides.

Two Bacillus strains producing an emulsifier, possibly a glycolipopeptide, were able to form a stable emulsion in the presence of the pesticide fenthion .The compound had some activity against other liquid-immiscible organophosphorus pesticides, but not solid organophosphorus or organochlorine pesticides, or hydrocarbons. A biosurfactant produced by *P. aeruginosa* has been reported to solubilize toxic organic chemicals and increase the

solubility and recovery of hexachlorobiphenyl from soil slurries by 31% (Banat et al., 2000; Berg et al. 1990). It was found that the addition of a biosurfactant produced by B. subtilis MTCC 2423 enhanced the rate of biodegradation of the chlorinated pesticide α- and β-endosulfan by 30±40%. Rhamnolipid biosurfactant was initially registered (licensed for sale) on March 23, 2004. As of that date, EPA had registered one pesticide product, ZONIX™ Biofungicide, Jeneil Biosurfactant Company.

2.3.5. Use in cosmetic industry

All cosmetics are composed of one or more surfactants. The developed biosurfactants present superb skin moisturizing characteristics equivalent to those of natural ceramides, and it can be used in functional cosmetics and for other skin care products. The biosurfactants consist of only sugar and fatty acids, and are thus highly environmentally friendly. They can also be used for high-performance washing detergents and advanced nanomaterials, because they easily form a variety of liquid crystals in aqueous solutions. The cosmetic industry demands surfactants with a minimum shelf life of 3 years. Therefore, saturated acyl groups are preferred over the unsaturated compounds. Sophorolipid is commercially used for cosmetic makeup brands. Mode of action of biosurfactant in skin was shown in Figure 2.4.

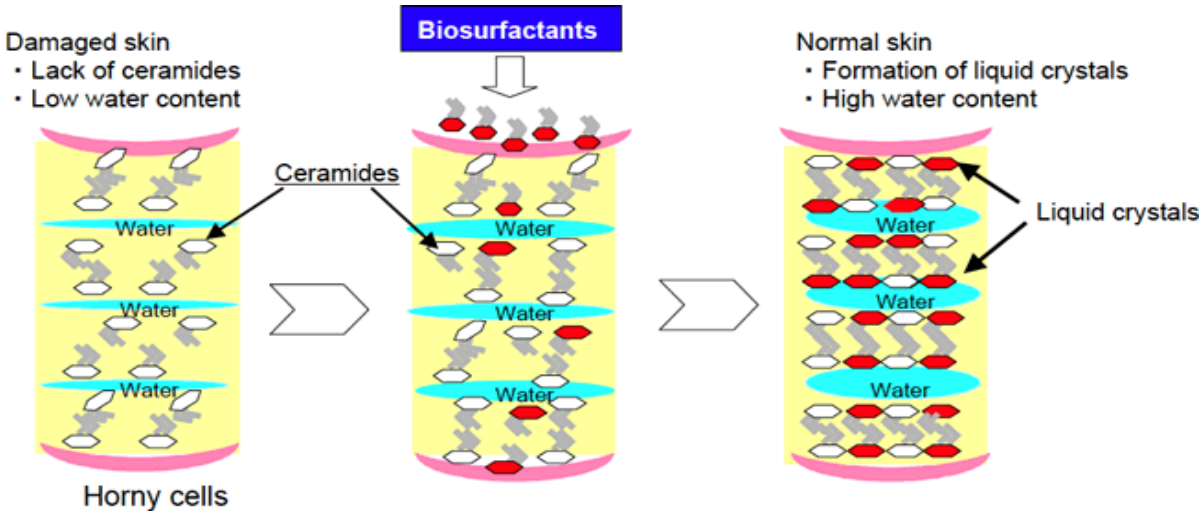

Figure 2.4. Action of Biosurfactant on Skin (Fattom & Shilo, 1985)

Monoglyceride, one of the widely used surfactants in the cosmetic industry, has been reported to be produced from glycerol-tallow (1.5:2) with a 90% yield by using *P. fluorescens* lipase treatment (McNeill et al. 1990; McNeill et al. 1991). Biosurfactants also exhibit a spontaneous formation of capsules of nanometer size (1 nanometer = 1 x 10^{-9} meters), so-called liposomes, which considerably improve the stability of cosmetic ingredients and their permeability to the skin. Natural ceramides have been praised for its moisturizing properties, and together with hyaluronic acids, they have become a crucial material for skin care applications (Sil et al. 2015).

2.3.6. Use In food industry

Apart from their obvious role as agents that decrease surface and interfacial tension, thus promoting the formation and stabilization of emulsions, surfactants can have several other functions in food. For example 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 et al. 1987).

In bakery and ice cream formulations biosurfactants act controlling consistency, retarding staling and solubilizing flavor oils; they are also utilized as fat stabilizer and antispattering agent during cooking of oil and fats. An improvement of dough stability, texture, volume and conservation of bakery products was obtained by the addition of rhamnolipid surfactants (Kosaric, 2001; Van Haesendonck et al. 2006).

2.4. Recovery Methods

Downstream processing of biosurfactant is an important factor to determining the feasibility of a bioprocess on a commercial scale and economic recovery. In many biotechnological processes, the downstream processing operations constitute upto 60%-70% of the total production cost. Biosurfactant recovery depends mainly on its ionic charge, water solubility, and intracellular or extracellular yield with respect to the cell surface. Several conventional and most widely used methods for recovery of biosurfactants are acid precipitation, solvent extraction, crystallization, ammonium sulfate precipitation and centrifugation, they have been widely reported in literature. The most widely used techniques are acid precipitation and solvent extraction, most common solvents used are: chloroform-methanol, dichloromethane-methanol, butanol, ethyl acetate, pentane, hexane, acetic acid, ether, etc. solvent extraction method is advantageous because of low cost, less toxic and highly available solvents may be used to significantly cut off the recovery expenses. More recently, other unconventional and interesting recovery methods for biosurfactants recovery have been reported, Such as foam fractionation, ultrafiltration and

adsorption-desorption on polystyrene resins and ion exchange chromatography (Mukherjee et al. 2006; Santos et al. 2016).

To make more cost effective biosurfactant recovery single downstream processing technique is not enough. In such cases, a multi-step recovery strategy is very useful to obtain required degree of biosurfactant purity. Further, physicochemical and structural characterizations of crude biosurfactant were elucidated by IR, MS and NMR studies. Table 2.3 shows the common biosurfactant recovery techniques with their working principles and advantages (Karadi et al. 2014).

Crude or impure biosurfactants obtained at the initial stages of recovery process will be available at lower costs and can be used for environmental applications, oil recovery as well as in paint and textile industry but highly pure biosurfactants are required for pharmaceutical, food and cosmetic industries.

Table 2.3. Biosurfactant recovery methods and their relative advantages (Desai et al. 1997; Mukherjee et.al., 2006)

Downstream recovery procedure	**Biosurfactant property responsible for separation**	**Instrument/ setup required**	**Advantages**
Acid precipitation	Biosurfactants become insoluble at low pH values	No set-up required	Low cost, efficient in crude biosurfactant recovery
Organic solvent extraction	Biosurfactants are soluble in organic	No set-up required	Efficient in crude biosurfactant

	solvents due to the hydrophobic end		recovery and partial purification, reusable nature
Ammonium sulfate precipitation	Salting-out of polymeric or protein rich biosurfactants	No set-up required	Effective in isolation of certain type of polymeric biosurfactants
Centrifugation	Insoluble biosurfactants are precipitated due to centrifugal force	Centrifuge required	Reusable, effective in crude biosurfactant recovery
Ion-exchange chromatography	Charged biosurfactants are attached to ion-exchange resins and can be eluted with buffer	Ion exchange resins packed in columns	High purity, reusability,fast recovery
Ultrafiltration	Biosurfactants form micelles above their critical micelle concentration (CMC), which are trapped by polymeric membranes	Ultrafiltration units having porous polymer membrane	Fast, one step recovery, high level of purity
Foam fractionation	Biosurfactants due to surface activity forms and partition into foam	Specially designed bioreactors which facilitate foam recovery during fermentation	Useful in continuous recovery procedures, high purity of product
Adsorption to	Biosurfactants are	Polystyrene resin	Fast, one step

polystyrene resines	adsorbed to polystyrene resins and subsequently desorbed using organic solvents	packed in glass columns	recovery, high level of purity, reusability
Adsorption to wood-activated carbon	Biosurfactants are adsorbed to activated carbon and can be desorbed using organic solvents	No setup required, can be added to culture broth, can also be packed in glass columns	Highly pure biosurfactants, cheaper, reusability, recovery from continuous culture

2.5. Estimation methods

Due to their unique properties and vast array of application, identification of new biosurfactant producing microbes is in great demand. There are different screening methods that have been reported as criteria to screen biosurfactant producing microbes such as hemolytic assay, bacterial adhesion to hydrocarbons (BATH) assay, drop collapse assay, oil spreading assay, emulsification assay, surface tension measurement, titled glass slide test, blue agar plate and hydrocarbon overlay agar assay. It was found that the drop collapse and oil spreading assays directly correlated to each other and easy to perform without any specialized equipment's. However, surface tension measurement results also had a direct correlation with drop collapse and oil spreading assays, but it needs a surface tensiometer to perform the assay (Thavasi et al. 2011).

Drop collapse method: Drop collapse test was performed by following the procedure described by Jain et al. Furthermore, Bodour and Miller-Maier showed that for pure surfactant, this assay can even be quantitative by measuring the drop size with a

micrometer (Bodour et al. 1998; Jain et al. 1991). This method is sensitive rapid and easy to carry out, requires no specialized equipment and just a small volume of sample. Drop of culture will be added on the oil surface. The shape of the drop will be inspected after 1 minute, if the culture will give flat drop or drop collapse; this shows that biosurfactant will produced. The stability of drops was dependent on biosurfactant concentration and it correlated with surface tension but not with emulsifying activity. Microbial colonies grown in the presence of carbohydrates or hydrocarbons could be readily screened for surfactant production by this method (Jain et al., 1991). But it displays a relative low sensitivity since a significant concentration of surface active compounds must be present in order to cause a collapse of the aqueous drops on the oil or glass surfaces.

Oil spreading method: The oil spreading assay was developed by Morikawa et. al., in drop collapse method oil spreading method is also sensitive rapid and easy to carry out, requires no specialized equipment and just a small volume of sample and it is also known as oil displacement activity. It can be applied when the activity and quantity of biosurfactant is low. The principle of this method was based on the ability of the biosurfactant to alter the contact angle at the oil-water interface. The surface pressure of the biosurfactant displaced the oil. In this method the oil added on the surface of water then cultured supernatant gently placed on the center of oil layer. If the oil displaces and clear zone forms then it shows the presence of biosurfactant (Morikawa et al. 2000).

Hemolytic activity assay: This is also known as blood agar method, biosurfactants can cause lysis of erythrocytes. This principle is used for the hemolysis assay which was developed by Mulligan *et. al.*, Biosurfactant can causes hemolysis of red blood cell because

of either permeabilization or disruption of membrane (Zaragoza et al., 2010). Hemolysis activity depends on biosurfactant concentration, nature of compound and its physicochemical Properties. When Cultures are inoculated on blood agar plates and incubated. Positive strains will cause lysis of the blood cells and exhibit a colorless, transparent clear zone. The diameter of the clear zone is a qualitative method used as an indicator of biosurfactant production. But the method has some limitations. Such as, the method is not specific, as lytic enzymes can also lead to clearing zones, hydrophobic substrates cannot be included as sole carbon source in this assay, and diffusion restriction of the surfactant can inhibit the formation of clearing zones. Mulligan *et. al.*, recommend the blood agar method as a preliminary screening method which should be supported by other techniques based on surface activity measurements (Mulligan et al. 1984).

Surface tension measurement: The direct measurement of the interfacial or surface activity of the culture supernatant is the most straightforward screening method and very appropriate for a preliminary screening of biosurfactant producing microbes. 24 This gives a strong indication on biosurfactant production. The interfacial or surface tension of a liquid can be measured by a variety of methods. The surface tension decreases with increasing surfactant concentration until the critical micelle concentration (CMC) is reached. Critical micelle concentration (CMC) is the minimum biosurfactant concentration needed to reduce the surface tension to the maximum extent. Furthermore, the measurements are strongly affected by factors such as pH, temperature and ionic strength. Du-Nouy-Ring method is quite easy and most frequently used method for measuring the surface tension. The Du-Nouy-Ring method is based on measuring the force required to detach a ring or loop of wire from an interface or surface. The detachment force is

proportional to the interfacial tension (Rufino et al. 2014; Tadros, 2005). This can be measured by the use of Tensiometer to measure the surface tension (dyn/ cm).

High Performance Liquid chromatography (HPLC): High Performance Liquid chromatography (HPLC) is a powerful and reliable tool for the Identification and quantification of biosurfactant and has been described in various reports in the literature. Although methods like ion exchange chromatography, thin layer chromatography, gel permeation chromatography, and ultrafiltration have been used for the purification of lipopeptide biosurfactants, these techniques have a serious limitation as they do not separate individual isoforms present in the crude lipopeptide mixture. In this regard, reverse-phase high-performance liquid chromatography has been extensively used and found to be efficient in separation and purification of isoforms (Lin et al. 1998; Sivapathasekaran et al. 2009; Thaniyavarn et al., 2003). HPLC analysis, it can be done by comparative study of the biosurfactant sample produced by microorganism chromatogram showed similar retention peaks to those observed with standard commercial biosurfactant (Mata-Sandoval et al. 1999).

Fourier Transform Infrared Spectroscopy (FTIR): Fourier transform infrared spectroscopy (FTIR) spectroscopy is the one of best method to characterize functional groups, bonding types and nature of compounds, therefore can be used to elucidate some components of an unknown mixture. The technique helps to explore the functional groups and the chemical bonds present in the crude biosurfactant extract (Varadavenkatesan & Murty, 2013). This technique is very useful to determine the primary chemical structure and composition of an unknown compound. The crude active fraction of biosurfactant samples (0.3-0.5 mg) were ground in about 80 mg of spectral-grade potassium bromide and

pressed into pellets under about 5–6 tons/cm^2 pressure. The spectra, measured in the absorbance mode, were acquired with the use of 4 /cm resolution yielding infrared traces over the range of 400–4000 /cm (Pereira et al., 2013).

Emulsification Index Assay: One of the most popular assay based on the emulsification capacity of biosurfactants was developed by Cooper and Goldenberg (Cooper et al. 1987). Emulsification Index is calculated by measurement of emulsion height. Ellaiah *et al.*, 2002 and Haba *et al.*, 2000, selected biosurfactant producers from the measurement of emulsification index. The emulsification index stability designates the strength of a surfactant (Ellaiah et al., 2002; Haba, et al. 2000). Emulsification assay is an indirect method used to screen biosurfactant production. If the cell free culture broth used in this assay contains biosurfactant then it will emulsify the hydrocarbons present in the test solution. In this biosurfactant and equal volume of oil mixed (vortex) for 1minute and leave it for 24 Hrs. After 24 hours, the height of the stable emulsion layer is measured. The emulsion index E_{24} is calculated as the ratio of the height of the emulsion layer and the total height of liquid (Cooper et al. 1987).

$$\mathbf{E_{24}} = \frac{\text{Height of emulsified layer (cm)}}{\text{Height of total liquid (cm)}} \times 100$$

Emulsification index assay is a simple and most popular for a first screening of biosurfactant producing microbes. It is applied in many screenings by many researchers (Walter et al. 2013). The stability of biosurfactant over wide range of temperature, pH, and salinity was evaluated by estimating their emulsification capacity by calculating Emulsification index over such a stress conditions (Lima et al. 2009).

It was reported that drop collapse and oil spreading methods are reliable methods to screen large number of samples for biosurfactant production because these methods require very small amount of sample (5-10 µl of biosurfactant) solution and have direct correlation with each other. Drop collapse and oil spreading methods are considered as semi-quantitative methods and after screening the strains for biosurfactant production using oil spreading and drop collapse assay, for confirmation surface tension method can be included in the screening criteria (Thavasi et al., 2011). Emulsifications assay also a popular and most widely used for primarily screen of biosurfactant production it is considered as a reliable assay.

Further, structural characterization of crude biosurfactant can be done by Infrared (IR) spectroscopy, Mass spectroscopy (MS) and NMR analysis. These techniques are very much helpful for determining the class of biosurfactant and structural determination of novel biosurfactant.