Chapter 2

Literature Survey

An overview of the agro based residues that can be effectively used for biosurfactant production and information about different types of biosurfactants, different microorganisms, substrates used for biosurfactant production, factors affecting biosurfactant production with emphasis to surfactin, various techniques used for recovery, concentration, identification and purification of biosurfactants is discussed herewith.

2.1 Overview of agro based residues with potential for biosurfactant production

Agriculture is an important sector of the Indian economy accounting for 14% of its GDP and about 13.08% of its exports. Almost half of the population still relies on agriculture as its principal source of income. India's strength in agriculture sector is predominant and therefore proper utilization of the waste of its agricultural produce is essential.

The agro waste generated in huge amount during the processing of grains, cereals, pulses sugarcane, other food processing industries and dairy industry finds its application mainly as cattle feed. However, these agricultural wastes owing to their high carbohydrate and protein content can be utilized for the production of high value added products as well as biofuels. A study undertaken by the Central Institute of Post Harvesting Engineering and Technology (CIPHET) has calculated wastage in various food and dairy produce as listed in Table 2.1

Table 2.1: Wastage in food and dairy produce (Source: CIPHET, Ludhiana).

Crop	Crop Cumulative Wastage (percent)
Cereals	3.9-6
Pulses	4.3-6.1
Oil Seeds	6.0
Fruits & Vegetables	5.8-18
Milk	0.8
Fisheries	2.9
Meat	2.3
Poultry	3.7

There is therefore, considerable choice available in the selection of agricultural feedstock that are rich in carbohydrate content for commercial production of biosurfactants in agricultural economies. Much of the material which is discarded as waste that leads to environmental degradation due to enhancement of BOD in waste water can be effectively processed to yield biosurfactants that have considerable commercial value. The pattern of consumption and waste generation from some key agricultural raw materials / feedstocks that are geographically abundantly available in India, South East Asia and other tropical countries are detailed below. However, we are fully aware that there are a number of other sources as well that can be harnessed for the production of biosurfactants.

2.1.1 Potato

Potato is the third most important agricultural crop in the world after rice and wheat. China and India contribute to 1/3 rd of the global potato productivity. Figure 2.1 shows the potato production scenario reported by national horticulture board potato production for the year 2012-13 (NHB, Govt. of India- 2012-13). 50-60% of potato sales are the processors for French fries, chips and other potato produce. Potato processing market is reported to be growing at 7-8% annually (Pandey et al., 2009).

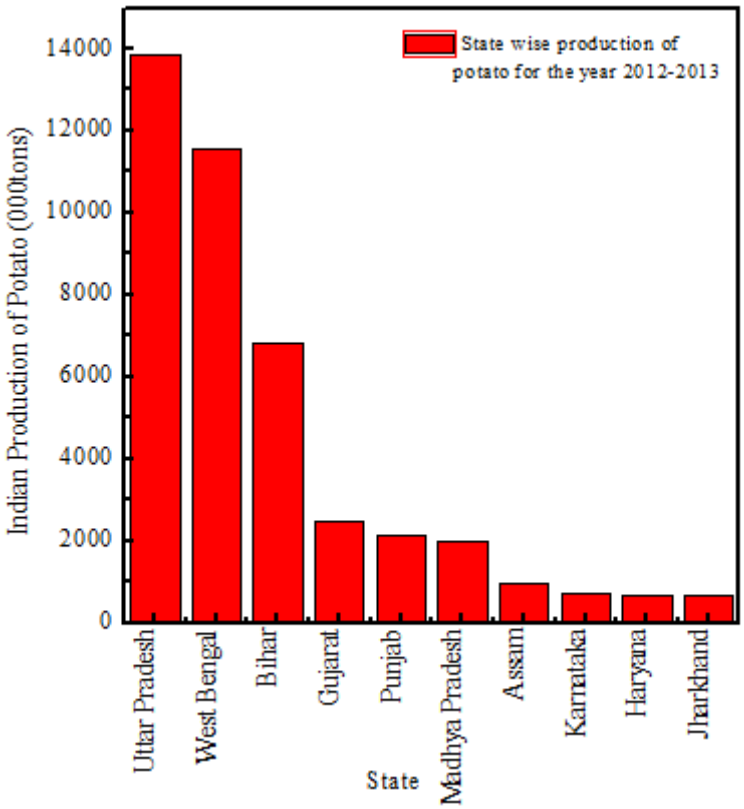

Fig. 2.1: Indian potato production for the year 2012-13 (National Horticulture Board – Government of India)

Potato chips are estimated to constitute ~ 85% of India's salty snack market and account for over 60% of the total potato processing capacity of the industry (Pandey et al., 2009). The processing of potato chips involves essentially slicing of peeled potatoes, washing them in cold water, rinsing, partial drying and frying. Considerable amount of starch is released during slicing and washing process. Water used during potato processing strongly depends on the type of product being processed and equipment used. According to Mishra et al. (2004) for potato chip processing industry ~ 5Tonnes of water is required per ton of potato processed. This process water contains high concentration of starch, protein, in addition to a high concentration of COD, TSS and PKN. The analysis of effluent from a major potato chips producing industry is shown in Table 2.2 (Mishra et al., 2004)

As per the data available from Jabsons food processing industry, Bharuch, the potato processing industry effluent consists of 2-3% starch (w/v). Based on these statistics and the amount of water required during chips manufacture, the total starch available from potato process effluent from the potato processing industry all over the country would be substantial. The predicted future growth of potato processing industry represents a corresponding increase in waste water. Potato processing industry waste waters have been successfully utilized for the production of biosurfactants (Fox and Bala, 2000; Pandey et al., 2000; Thompson et al., 2000).

Table 2.2: Analysis of effluent from potato chips processing industry (Mishra et al., 2004)

Parameters	Concentration
TSS	0.64 g/l
Carbohydrate	19.47 g/l
Reducing Sugar	0.04 g/l
Total Protein	2.88 g/l
N	0.46 g/l
P	0.22 g/l
K	0.15 g/l
BOD	1950 mg/l
COD	8122 mg/l
pH	7.5

2.1.2 Molasses

Worldwide around forty-five countries produce sugarcane. Sugarcane is an importantt agricultural produce of India. Sugarcane contains about 11-15% sucrose out of which only 8-11 % crystallizes, remaining sugar goes as byproduct like reducing sugar and molasses. Main molasses producers worldwide are Brazil, India and Thailand. Nearly 90% of molasses produced is consumed by industrial alcohol manufacturers and remaining 10% for various other uses like potable liquor. There are legal impediments on the use and storage of molasses and one has to get legal permission for the same. In Brazil 45% of the total sugarcane goes to sugar production while the remaining 55% to ethanol production from sugarcane juice, which is primarily used as a biofuel. Figure 2.2 and 2.3 show the fluctuating trends of sugar and molasses production in India.

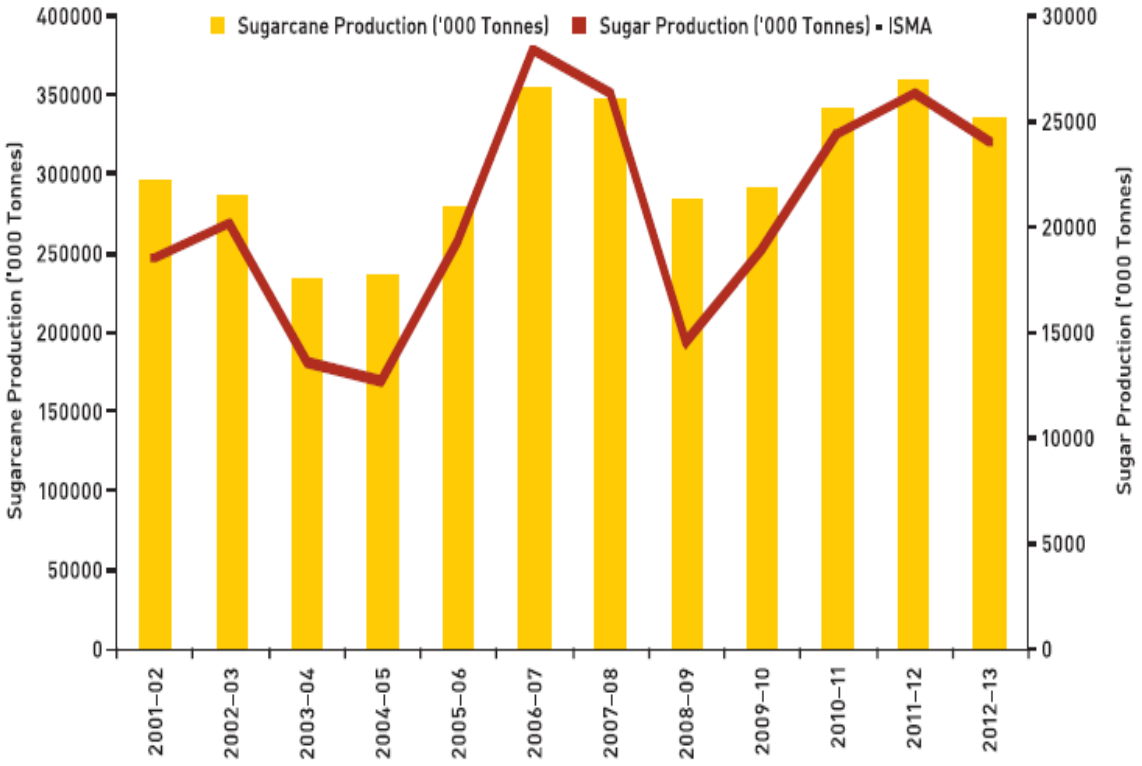

Fig 2.2: Trends in sugar and sugarcane production in India
Source – Indian Sugar Mills Association (ISMA)

India produces ethanol from molasses of sugarcane to blend with petrol. Use of sugarcane directly to produce biofuel is not likely in India given the need for producing food from scarce land and water resources. In a bid to renew its focus and strongly implement the Ethanol Blending Program (EBP), the Cabinet Committee of Economic Affairs (CCEA) on November 22, 2012 recommended 5 percent mandatory blending of ethanol with gasoline.

The government's current target of 5 percent blending of ethanol in gasoline has been partially successful in years of surplus sugar production and remains unfulfilled when sugar production declines. In the calendar year 2013 the ethanol blending target of 2.9 percent could be met.

Cane molasses a product of tropical agriculture is widely known as black strap molasses. 100 T of sugarcane gives 10-11 T of sugar and 3-4 T of molasses. Sugar in particular, sucrose is the major component of molasses, molasses is actually solution of sucrose, some glucose, fructose and other organic and inorganic matter in water. Molasses being a rich source of carbohydrate can be used as a source for biosurfactant production although quite some restrictions exist that in a way inhibit the free use of this fine raw material.

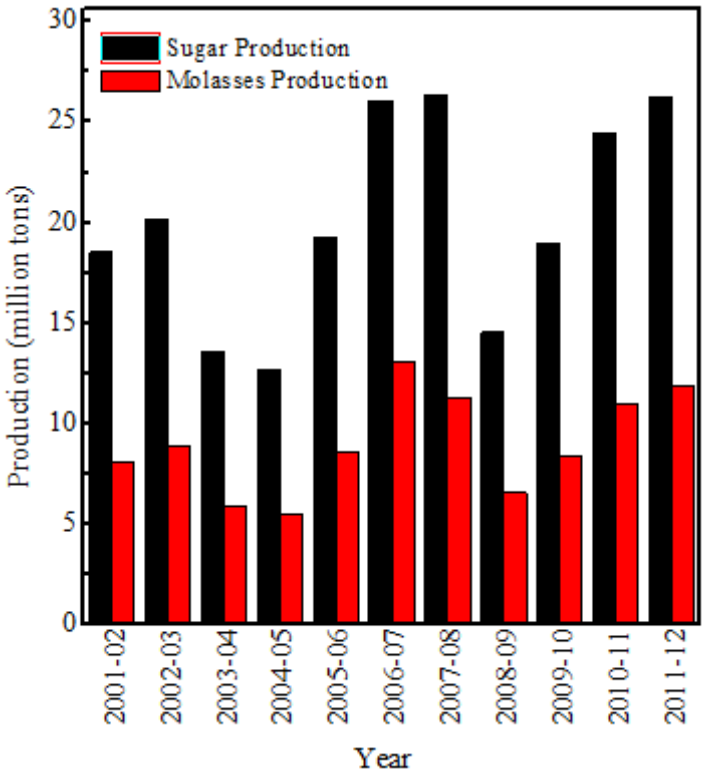

Fig 2.3: Trends in sugar and molasses production in India (Source-Cooperative Sugar 45(1), September 2013)

2.1.3 Rice

India is the second largest producer of rice in the world next to China producing ~100 MMT per year. The crop occupies 37% of the total cropped area and 44% production of food grains in India. Rice grain has a hard outer coating (husk or hull) surrounding the rice endosperm. Between the grain and husk is a composite dark brown colored layer commonly called as bran. Rice bran contains 20% oil holding and more than 65% of the

nutrients present in bran. Unfortunately, this invaluable product is mostly treated as a byproduct by the rice industry.

India produces 9 million tons (MT) of rice bran of which 5MT is processed for oil and the rest is used as cattle feed. India is the major producer of rice bran oil globally. Almost 900,000 MT is produced annually while global production is ~1.2Million Tons. Of 900,000T only 300,000T is consumed as edible oil rest is used by the vanaspati industry or blended with other oils. Domestic rice bran oil consumption is increasing at 25% CAGR (Roy, 2014). Rice bran oil is extensively used in Japan, Korea, China, Taiwan and Thailand as premium edible oil. Japan and Thailand produce 70,000T and 60,000T respectively while China produces 50,000T.

A survey conducted by us in various local rice mills of Gujarat revealed that around 10-12 kg of rice bran is produced from 100 kg of raw rice during the polishing process in the mill. This waste account to 10-12% of the raw rice produced by farmers, and looking at the total rice production in India, the quantity of rice bran available would be huge. Rice bran therefore could be utilized as cheap source of carbon for the microbial growth and subsequent biosurfactant production.

2.2 Biosurfactants overview

Biosurfactants are biomolecules that consist of a hydrophobic and a hydrophilic part. The hydrophobic role is based upon long-chain fatty acids, hydroxy fatty acids or α-alkyl-α-hydroxy fatty acids. The hydrophilic part can be based on carbohydrate, amino acid, cyclic peptides, phosphate, carboxylic acid or alcohol (Mulligan and Gibbs, 2004).

Microbial surfactants have recently become an important product of biotechnology due to their varied applications in cosmetic, medicinal, enhanced oil recovery and many other fields. The reason behind their popularity, as high value biotech products, is primarily in their specific action of large surface tension lowering ability, high foaming, high selectivity, retaining specific activity at higher temperatures, pH and salinity, lower toxicity, higher biodegradability, better environmental compatibility, relative ease of preparation and widespread applicability (Desai and Banat, 1997).

2.2.1 Classification of biosurfactants

Biosurfactants are biosynthesized by utilizing bacteria or yeast with different substrate sources including sugars, oils and other agricultural as well as petroleum wastes (Lin,

1996). The critical micelle concentration (CMC) of most of the biosurfactant lies in the range of 1 to 200 mg/L and molecular mass from 500 to 1500 Daltons (Lang and Wagner, 1987). Most microbial surfactants are complex molecules, comprising of different structures that include lipopeptides, glycolipids, polysaccharide protein complexes, fatty acids and phospholipids (Nitscke and Pastore, 2006). Biosurfactants of different types and their characteristics are discussed below.

- ***Sophorolipids***

Sophorolipids (SLs) are classified as glycolipids produced by fermentation process with yeast such as *Candida bombicola*, *Candida apicola*, and *Wickerhamiella domercqiae* and are composed of a dimeric sugar called sophorose linked with a glycosidic bond to a hydroxyl fatty acid. Sophorolipids have generated interest in the pharmaceutical arena because of their wide array of therapeutic benefits (Shah et al., 2007). Natural sophorolipids and their first-generation chemical derivatives are efficient microbicidal spermicides, having activities similar to that of nonoxynol-9 (Shah et al., 2005) and are effective septic shock antagonists (Bluth et al., 2006). They are known to possess antifungal, antiviral and spermicidal properties.

Sophorolipids have been demonstrated to be effective anticancer agents against cancerous cell lines (Chen et al., 2006). These lipids are produced on a commercial scale when the organism is cultured on substrates containing glucose and a source of alkyl moieties, such as alkanes or seed oils, which influence the nature of the fatty acid constituent. Yield of sophorolipids can be as high as 300g/L from organisms during the stationary phase. The chemical structure of sophorolipid as produced by *C.bombicola* is shown in Figure 2.4 (Mulligan and Gibbs, 2004).

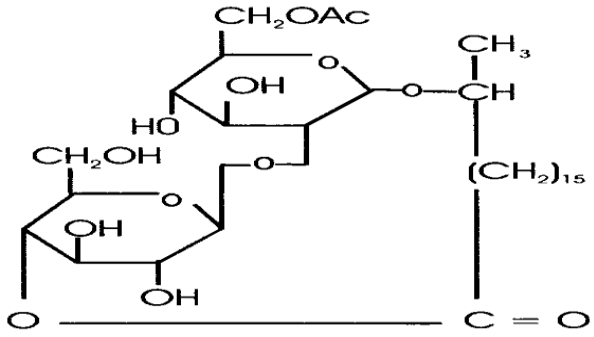

Fig. 2.4: Structure of sophorolipid produced by *C. Bombicola* (Mulligan and Gibbs, 2004)

- ***Mannosylerythritol lipid***

Mannosylerythritol is classified as a glycolipid type of biosurfactant. The microorganism *C.Antartica* is reported to produce mannosylerythritol type of biosurfactant (Krishnaswamy et al., 2008). Soybean oil has been utilized for production of this type of biosurfactant with *Candida sp* SY16 with maximum yield of 95g/L (Kim et al., 2006). The structure of mannosylerythritol lipid is illustrated in Figure 2.5

- ***Rhamnolipids***

Rhamnolipids, as produced by *Pseudomonas aeroginosa* is a group of biosurfactant that has been studied extensively and possesses good surface tension lowering ability (Hitsatsuka et al., 1971)

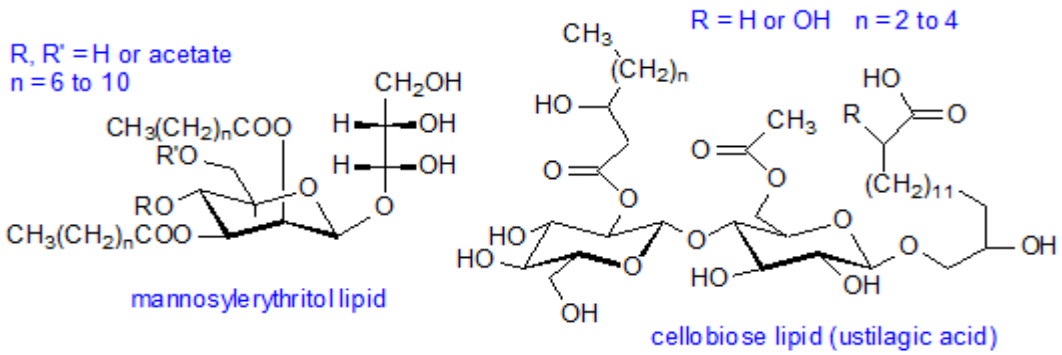

Fig. 2.5: Structure of Mannosylerythritol lipid (Mulligan and Gibbs, 2004)

Surface tensions of 29mN/m are characteristic of these compounds. Upto seven homologues have now been identified (Abalos et al., 2001). The two types of rhamnolipids contain either two rhamnoses attached to α-hydroxy decanoic acid or single rhamnose linked to the identical fatty acid (Mulligan and Gibbs, 2004). The structure of one rhamnose linked to α-hydroxy decanoic acid produced by *P.aeruginosa* is illustrated in Figure 2.6.

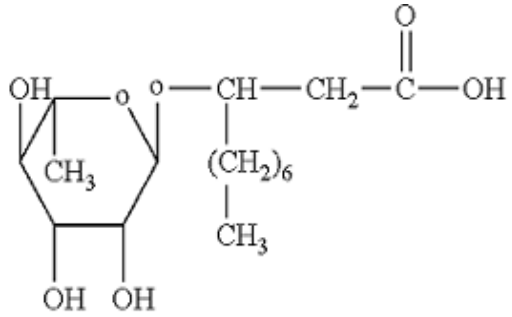

Fig. 2.6: Structure of Rhamnolipid as produced by *P.aeruginosa* (adapted from Mulligan and Gibbs, 2002)

Rhamnolipids have found to be useful for *in situ* soil remediation provided they do not adsorb strongly to the soil. They exhibit excellent property as non-toxic oil dispersing agents (Mulligan et al., 2001).

- ***Lipopeptides and lipoproteins***

A number of bacterial species produce lipopeptides or peptidolipids, most of which have important biological functions. For example, many have surfactant, antibacterial or antifungal properties and hence have attracted interest from industry. They can consist of short linear chains or cyclic structures of amino acids, linked to a fatty acid via ester or amide bonds or both.

A large number of cyclic lipopetides, including decapeptide antibiotics (gramicidins) and lipopeptide antibiotics (polymyxins) are produced. These consist of a lipid attached to a polypeptide chain. *Bacillus licheniformis* produces several biosurfacants which act synergistically and exhibit excellent temperature, pH and salt stability. These are also similar in structural and physico-chemical properties to surfactin. The surfactants produced by *B. licheniformis* are capable of lowering the surface tension of water to 27 mN/m and the interfacial tension between water and *n* hexadecane to 0.36 mN/m (Muthusamy et al., 2008).

Lipopeptides are produced by several *Bacillus* species. Surfactin, a cyclic lipopeptide produced by *B.subtilis* is the best studied lipopeptide type of biosurfactant. It is known to be one of the most powerful biosurfactants because the presence of surfactin in concentrations as low as 0.005% reduces the surface tension to 27 mN/m (Mulligan and Gibbs, 2004). Surfactin consists of seven amino acids bonded to the carboxyl and hydroxyl groups of a 14-carbon acid. Figure 2.7 shows the structure of surfactin (Kakinuma et al., 1969).

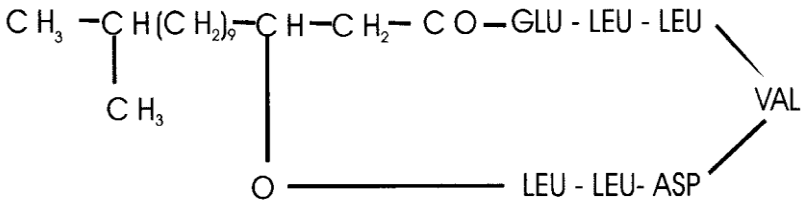

Fig. 2.7: Structure of Surfactin (Kakinuma et al., 1969)

Mass spectrometry and High-energy tandem mass spectrometry revealed that the length of the acyl chain in surfactin structure can vary from 12 to 15 carbons (Hue et al., 2001). The solubility and surface-active properties of the surfactin are dependent on the orientation of the residues. However, yields are typically low for surfactin (0.02g/g glucose). Iron and manganese salt addition could enhance yield of surfactin up to 0.7g/L (de Roubin et al., 1989).

Cyclic surfactins adopt a conformation such that the fatty acid chain remains folded on to the peptide. The folding is governed by the increased intramolecular hydrophobic interactions with lipopeptide chain length. These observations therefore provide an insight towards the interaction of surfactin with the lipid membranes (Eeman et al., 2006; Bouffioux et al., 2007; Magnet-Dana et al., 1995). Surfactin exhibits 'detergent like' action on cell membranes due to its unspecific mechanism of membrane permeabilization at concentrations much below the CMC values (Heerklotz and Seelig, 2001). A host of very interesting features of surfactin has opened various avenues for its applications such as antimicrobial, antiviral agent, anti-adhesive and anti-inflammatory uses. (Shaligram and Singhal., 2010). Surfactin has also been found to be an active agent in biodegradation of pesticides (Singh et al., 2007).

- ***Polymeric biosurfactants***

High molecular weight biopolymers generally exhibit useful properties, such as high viscosity, tensile strength, and resistance to shear. It is, therefore not surprising that polymeric biosurfactants have found a variety of industrial uses. The best-understood polymeric biosurfactants are emulsan, liposan, alasan, lipomanan and other polysaccharide–protein complexes.

(a) Emulsan: Emulsan is a polyanionic amphipathic heteropolysaccharide. Emulsan does not appreciably reduce interfacial tension, but it is a very effective emulsifying agent for hydrocarbons in water emulsions even at a concentration as low as 0.001%-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.

(b) Biodispersan: *A. calcoaceticus* A2 has been reported to produce an extracellular, nondialyzable dispersing agent called biodispersan (Kosaric and Fazilet, 1993). The active component of biodispersan is an anionic heteropolysaccharide, with an average molecular weight of 51,400 and four reducing sugars namely,

glucosamine, 6-methyl aminohexose, galactosamine uronic acid, and an unidentified amino sugar.

(c) Liposan: Extracellular water-soluble emulsifier, designated as liposan, is be synthesized by *C. lipolytica*. It is composed of 83% carbohydrate and 17% protein (Krishnaswamy and Muthusamy, 2008). The carbohydrate portion is a heteropolysaccharide consisting of glucose, galactose, galactosamine, and galacturonic acid (Kosaric and Fazilet., 1993).

Other polysaccharide protein complexes: Kosaric and Fazilet (1993) observed that the polysaccharide alone does not exhibit emulsification activity, but polysaccharide released with protein during the growth of a parent strain on ethanol had potent emulsification activity. The purified mannoprotein emulsifier contains 44% carbohydrate and 17% protein.

- ***Fatty acids, phospholipids, and neutral lipids***

Several bacteria and yeast produce large quantities of fatty acids and phospholipid surfactants during growth on *n*-alkanes (Cirigliano and Carman, 1985). The hydrophilic and lipophilic balance (HLB) is directly related to the length of the hydrocarbon chain in their structures. In *Acinetobacter* sp. strain HO1-N, phosphatidylethanolamine-rich vesicles are produced (Kappeli and Finnerty, 1979), which form optically clear microemulsions of alkanes in water. Phosphatidylethanolamine produced by *R. erythropolis* grown on *n*-alkane causes a lowering of interfacial tension between water and hexadecane to less than 1 mN/m and has a critical micelle concentration (CMC) of 30 mg/L (Muthusamy et al., 2008)

2.2.2 Biosurfactants in comparison with chemically synthesized surfactants

The exceptional properties of biosurfactants allow their utilization and possible replacement of chemically synthesized surfactants in many different industrial operations. Surfactants are used by almost all types of modern industrial operations. The potential application of biosurfactants in industries is also a reality and there are commercial scale products incorporating biosurfactants (Kosaric et al., 1987)**.** There are many advantages of biosurfactants as compared to their chemically synthesized counterparts. Some of those are:

a) Biodegradability.
b) Generally low toxicity.

c) Biocompatability and digestibility finding application in cosmetics, pharmaceuticals and as functional food additives.
d) Availability of raw materials: Biosurfactants can be produced from relatively cheap raw materials which are available in large quantities. The carbon source may come from hydrocarbons, carbohydrates and/or lipids, which may be used separately or in combination with each other.
e) Acceptable production economics: Depending, on the application, biosurfactants can also be produced from industrial wastes and byproducts and this is of particular interest for bulk production (e.g. for use in petroleum related technologies).
f) Use in environmental control: Biosurfactants can be efficiently used in handling industrial emulsions, control of oil spills, biodegradation and detoxification of industrial effluents and in bioremediation of contaminated soil.
g) Specificity: Biosurfactants, being complex organic molecules with specific functional groups, are often specific in their action. This would be of particular interest in detoxification of specific pollutants, de-emulsification of industrial emulsions, specific cosmetic, pharmaceutical and food applications. (Kosaric et al., 1992)

Pertaining to disadvantages, the bottleneck is to bring down the production cost for commercialization and large scale production of biosurfactants. Large quantities are particularly required in many industrial operations, which, due to the bulk use, may be expensive. To overcome this problem, processes should be coupled with the utilization of waste substrates and simultaneously reducing their polluting effect, which balances the overall cost (Kosairic, 1992)

2.3 Microbial origin of biosurfactants:

Microorganisms produce various types of microbial surface active agents (biosurfactants) that have very different chemical structures and surface properties. Due to their diverse structure, they play different roles and can be utilized in different applications. Table 2.3 lists various microorganisms that could be utilized for production of specific type of biosurfactant. The genetics of the organisms used for biosurfactant production is an important factor affecting the overall yield of all biotechnological products. In addition to the natural biosurfactant producer strains, a few varieties of

mutant and recombinant strains have also been reported in literature that have enhanced biosurfactant production characteristics.

Table 2.4 lists such mutant hyper producing strains. These mutant varieties can be produced using various agents for example, transposons, chemical mutagens such as N-methyl-N'-nitro-N-nitrosoguanidine or by radiation. Some of the recombinant strains producing biosurfactants also provide enhanced yields (Mukherjee et al., 2006).

Table 2.3: Types and microbial origin of biosurfactants (Mulligan and Gibbs., 1993)

Surfactant class	Microorganism
Trehalose lipids	*Arthrobacter paraffineus, Corynebacterium spp., Mycobacterium spp., Rhodococus erythropolis*
Rhamnolipids	*Pseudomonas aeruginosa, Pseudomonas sp.*
Sophorose lipids	*Candida apicola, Candida bombicola, Candida lipolytica, Candida bogoriensis*
Glucose-, fructose-, saccharose lipids	*Arthrobacter sp., Corynebacterium sp., R. erythropolis*
Cellobiose lipids	*Ustilago maydis*
Polyol lipids	*Rhodotorula glutinus, Rhodotorula graminus*
Diglycosyl diglycerides	*Lactobacillus fermentii*
Lipopolysaccharides	*Acinetobacter calcoaceticus (RAG1), Pseudomonas sp., Candida lipolytica*
Lipopeptide	*Arthrobacter sp., Bacillus pumilis, Bacillus licheniformis*
Surfactin	*Bacillus subtilis*
Viscosin	*Pseudomonas fluorescens*
Ornithine, lysine peptides	*Thiobacillus thiooxidans, Streptomyces sioyaensis Gluconobacter cerinus*
Phospolipids	*Acinetobacter sp.*
Sulfonylipids	*T.thiooxidans, Corynebacterium alkanolyticum*
Fatty acids (corynomycolic acids, etc.)	*Capnocytophaga sp., Penicillium spiculisporum, Corynebacterium lepus, Arthrobacter paraffineus, Talaramyces trachyspermus, Nocardia erythropolis*

Table 2.4: Recombinant and mutant strains of microorganisms resulting in enhanced yield of biosurfactants. (Mukherjee et al., 2006)

Mutant and/or recombinant strains	Characteristic feature	Enhanced yield
Pseudomonas aeruginosa 59C7	Transposon Tn5-GM induced mutant of *Pseudomonas aeruginosa* PG201	2 times more production
Pseudomonas aeruginosa	Random mutagenesis with *N-methyl-N-nitro-N-nitrosoguanidine*	10 times more production
Bacillus licheniformis	Random mutagenesis with *N-methyl-N-nitro-N-nitrosoguanidine*	12 times more production
Bacillus subtilis ATCC 55033	Random mutagenesis with *N-methyl-N'-nitro-N-nitrosoguanidine*	4-6 times more production
Bacillus subtilis Suf-1	Ultraviolet mutant of *Bacillus subtilis* ATCC 21332	3-4 times more production
Recombinant *Bacillus subtilis* MI 113	Incorporation of a plasmid containing *Ipa-14* gene	8 times more surfactin production
Acinetobacter calcoaceticus RAG-1 mutants	Mutant selection on basis of resistance to cationic detergent CTAB	2-3 times more production
Recombinant *Gordonia amarae*	Stable maintenance and expression of *Vitreoscilla hemoglobin gene (vgb)*	4 times more production of trehalose lipid biosurfactant
Recombinant *Bacillus subtilis*	Produced by whole enzyme module swapping	Production of lichenysin
B. subtilis SD901	Random mutagenesis with *N-methyl-N'-nitro-N-nitrosoguanidine*	4-25 times more surfactin production

2.4 Metabolic pathways for the synthesis of biosurfactant

The natural products such as lipopeptide generated by microorganisms possess structural diversity apparently as a result of modifications and combinations of reactions from primary metabolic pathways. For example, the formation of lipopeptide is probably due to variations in the pathways responsible for the combination of fatty acid biosynthetic machinery with acetyl-CoA biosynthetic pathways (Burja et al., 2001).

Very rarely completely novel reactions are found in secondary metabolisms that are not precedent in primary metabolism. Various biochemical and genetic analysis of the biosynthesis of secondary metabolites revealed that majority of them are synthesized via limited number of core biosynthetic pathways. Figure 2.8 illustrates the metabolic pathways to microbial secondary metabolites (Burja et al., 2001).

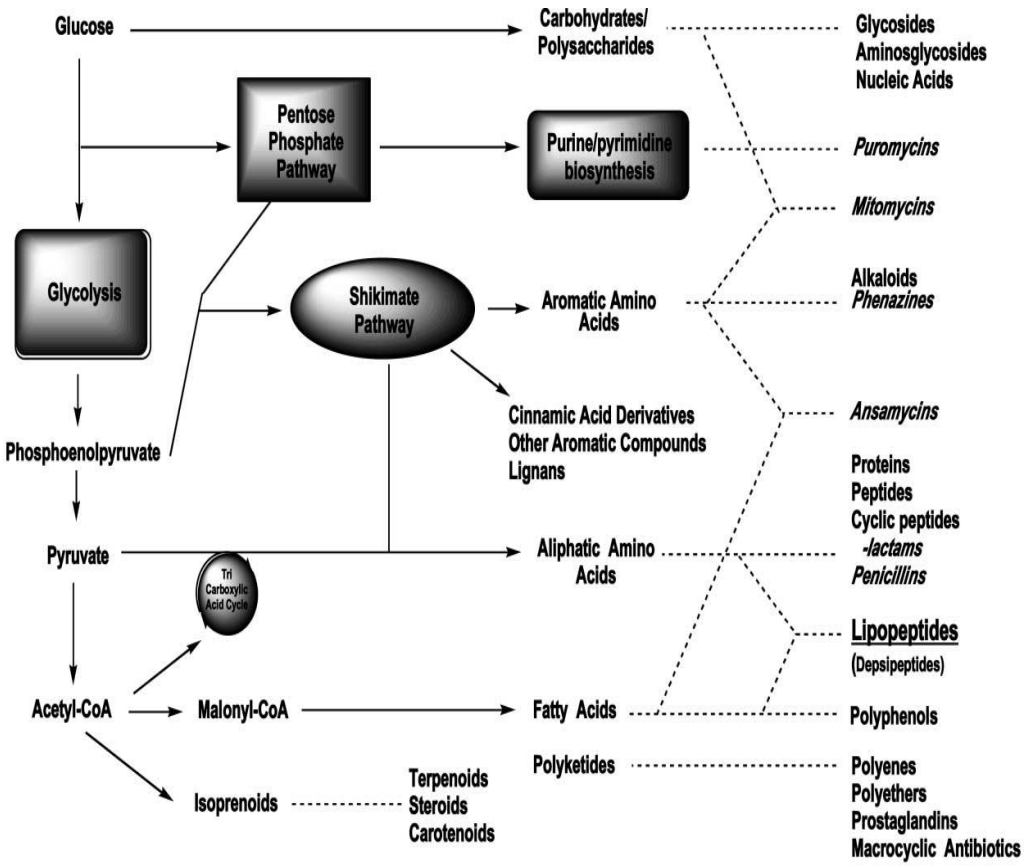

Fig.2.8: Core pathways to microbial secondary metabolites including the postulated pathway for lipopeptide production (Burja et al., 2001)

2.5 Parameters affecting the growth of microorganisms and subsequent biosurfactant production

Microorganisms particularly bacteria and yeast contribute to the synthesis of biosurfactants. Microorganisms such as *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Bacillus licheniformis* , and *Candida bombicola* species have been explored well for the production of biosurfactant.

Apart from providing proper nutrients for the growth of bacteria, it is essential to know the physical environment that will be suitable for the best growth of bacteria. Bacteria exhibit diverse responses to physical conditions such as temperature, pH, nutrients provided and gaseous conditions. Overview about the growth requirements of microorganisms include:

2.5.1 Effect of nutrients provided

(a) Almost every organism requires source of energy which may be obtained from chemicals by chemotrophs and from radiant energy by phototrophs. Both forms exist among bacteria.

(b) A source of electrons is required for the metabolism. Some organisms utilize inorganic compounds in reduced form as electron donors and are termed as lithotrophs. Other organism use organic compounds as electron donors and are termed as organotrophs.

(c) Carbon is a primary requirement for all organisms in some form for use in synthesizing cell components. CO_2 is required by most of the organism in small amount. Autotrophs utilize CO_2 as their major or even sole source of carbon while heterotrophs utilize organic compounds as their carbon source (Pelczar et al., 2004)Makkar and Cameotra (2002) found that sucrose (2%) served as good carbon source for the production of biosurfactant by *Bacillus subtilis* MTCC 2423.

(d) Majority of organisms require nitrogen in some form for cell components. Bacteria are highly versatile in utilizing either atmospheric nitrogen or inorganic nitrogen from nitrates, nitrites and even from organic compounds such as amino acids (Pelczar et al., 2004).

Abouseoud et al. (2007) studied the effect of carbon and nitrogen source on production of biosurfactant by *Pseudomonas fluorescens* Migula 1895-DSMZ. The biosurfactant productivity of the culture was examined by variations in carbon and nitrogen sources as well as C: N ratio. The best results for biosurfactant production were obtained when using olive oil and ammonium nitrate as carbon and nitrogen sources respectively with a C: N ratio of 10.

Investigations carried out by Makkar and Cameotra (2002) revealed that potassium nitrate (0.3%) was the best nitrogen source for biosurfactant production with *Bacillus subtilis* MTCC 2423. Tabatabaee et al (2005) and Haghighat et al (2008) reported that *Bacillus* isolates produced more biosurfactants in the presence of nitrogen sources such as ammonium chloride rather than sodium nitrate. The work reported by Robert et al (1989); Desai and Banat (1997); Cameotra and Makkar (1998) showed that ammonium chloride and urea were the best nitrogen sources among the inorganic salts tested for biosurfactant production by *Anthrobacter paraffineus.*

(e) All organisms depend on availability of oxygen, sulfur and phosphorus for their cell components. Oxygen is provided by means of water while sulphur is utilized from the organic sulfur compounds, inorganic sulfur compounds, and some can even

utilize elemental sulfur. Sulfur is needed for the synthesis of certain amino acids such as methionine, cystine and cysteine. Phosphorus is an essential component of nucleotides, nucleic acids, phospholipids and other compounds which is supplied in the form of phosphates.

(f) All living organisms require metal ions, such as K^+, Ca^+, Mg^{2+}, and Fe^{2+} for normal growth. Other metal ions are needed as well, but in very low concentrations, such as Zn^{2+}, Cu^{2+}, Mn^{2+}, Ni^{2+} which are usually present as trace elements and occur as contaminants in culture media which are sufficient for the bacterial growth. All the biological functions of metal ions are not known, however the ions such as Fe^{2+}, Mg^{2+}, Zn^{2+}, Mo^{6+}, Mn^{2+}, and Cu^{2+} are known to be cofactors for various enzymes.

(g) Most of the living organisms require vitamins and vitamin like compounds which function as coenzymes for several enzymes or as building blocks for coenzymes. Some bacteria are able to synthesize while other require additional vitamin supplement.

(h) All organisms require water. All the nutrients must be in aqueous solution before they can enter the cells. Water being highly polar compound, has ability to dissolve or disperse cellular components and provide suitable milieu for the various metabolic reactions of a cell. (Pelczar et al., 2004)

2.5.2 Effect of temperature

All microbial growth processes depend on chemical reactions and the rate of reaction is influenced by temperature, bacterial growth can be extensively influenced by change in temperature. The optimum growth temperature is that temperature that allows rapid growth during a short period of time. (It must be noted here that the optimum temperature for growth may not always be the same for other cellular activities and synthesis of certain metabolites).

On the basis of growth and temperature relationships, bacteria are divided into three main groups:

(a) *Psychrophiles:* They are the group of bacteria that are able to grow at 0°C or lower; however they grow best at optimum temperature of 15°C or lower and a maximum temperature of about 20°C

(b) *Mesophiles:* Mesophiles grow best in the temperature range of approximately 25 to 40°C. Most of the pathogenic bacteria for humans are mesophiles and grow best at about the body temperature of 37°C.

(c) *Thermophiles:* This group of bacteria grows best at the temperatures above 45°C. The growth range of some thermophiles extends into that of mesophiles while others are strictly thermophiles and cannot grow in mesophilic region.

The effect of temperature on surfactin (biosurfactant) production was explored by Sen and Swaminathan (1997). They found that at appropriate pH and agitation rate, surfactin production was maximal at 37.4°C.

2.5.3 Effect of agitation and gas supply

Gases such as oxygen and carbon dioxide are the main ones that can affect the bacterial growth. On the basis of utilization of oxygen, the organisms are divided into four groups

(a) Aerobic bacteria: They require oxygen for growth and can grow when incubated in an air atmosphere

(b) Anaerobic bacteria: These bacteria do not use oxygen to obtain energy; moreover, oxygen can be toxic for them. Some can tolerate low levels of oxygen while others cannot tolerate even low levels and may die when exposed to air.

(c) Facultative anaerobic bacteria: They do not require oxygen for growth, however they utilize it for energy production if it is available. They may grow in the presence of oxygen and are not inhibited by its presence

(d) Microaerophilic bacteria: They cannot tolerate levels of oxygen present in an air atmosphere and requires low levels of oxygen.

The effect of agitation rate on surfactin production as studied by Yeh et al. (2005) revealed that the increase in the dissolved oxygen at higher agitation rates had positive impact on surfactin production. However, very high agitation rates above 250 rpm led to higher foam production and decreased oxygen transfer as well as surfactin yield.

Yeh et al. (2007) optimized the fermentation parameters for the surfactin production in a 5-litre jar fermentor. The effect of different aeration and agitation rates on oxygen transfer and mass transfer rate was studied. As per the study carried out with *B. subtilis* ATCC 21332 along with a solid carrier in an iron enriched fermentation medium, it was found that there exists strong relationship between oxygen consumption, pH and surfactin yield.

Oxygen supply and efficient mass transfer played important role on the kinetics of surfactin production. Very high agitation rates above 300 rpm and aeration above 2.0 (vvm) resulted in excessive foaming thereby, decreasing the cell recycling and surfactin production. The best surfactin yield was found to be at mass transfer coefficient (K_La) of 0.012 s^{-1}.

2.5.4 Effect of alkalinity or acidity (pH*)*

Optimum pH for the growth of bacteria lies between 6.5 and 7.5, and limits generally lie somewhere from 5 to 9. Few bacteria prefer extreme pH conditions. It is very obvious that the pH of the medium originally adjusted to 7.0 is likely to change as a result of the chemical activities of the organism. A carbohydrate present in the medium initially gets fermented or oxidized to organic acids, thus decreasing the pH of the medium. The oxidation of organic salts such as sodium malate may result into increase in the pH of the medium. Such changes in pH may be so great that further growth of the organism is eventually inhibited (Pelczar et al., 2004).

Radical shifts in pH of the medium can be prevented by the addition of a buffer into the medium. Some large capacity fermentors are equipped with continuous control for the maintenance of constant pH. Sen and Swaminathan (1997) studied the effect of pH along with temperature, rates of agitation and aeration as parameters for the production of surfactin by response surface methodology using *B. subtilis* DSM 3256. Their results revealed that surfactin production was maximal at 37.4 °C and pH=6.75, agitation at 140 rpm and aeration at 0.75 gas volume flow per unit liquid volume per minute (vvm.).

2.6 Low cost agro based substrates for the production of biosurfactants

Economy is often the bottleneck of biotechnological processes, especially in the case of biosurfactant production. The four key factors that influence biosurfactant production costs as suggested by Kosaric et al. (1984) are

- The microbes (selected, adapted or engineered for higher yields of products),
- The process (selected and engineered for low capital and operating costs),
- The microbial growth substrate or process feed stock (adapted for low cost),
- The process byproducts (minimal or managed as saleable products rather than as wastes).

Agro-industrial wastes are often not utilized to develop high end products. However, due to presence of high content of carbon and other nutrients, they could be utilized for

microbial growth and subsequent biosurfactant production. The success of biosurfactant production relies largely on the processes based on low cost raw materials, which account for 10–30% of the overall cost (Cameotra and Makkar, 1998).Salient aspects of various agro- wastes that qualify to be used as resources for production of biosurfactants are listed below:-

2.6.1 Starchy substrate

Fox and Bala (2000) highlighted the potential environmental threat and economic liability of starch-rich wastes from potato-processing industries. Potatoes are composed of 80% water, 17% carbohydrates, 2% protein, 0.1% fat and 0.9% vitamins, inorganic minerals and trace elements. They are a rich source of carbon (in the form of starch and sugars), nitrogen and sulfur (from protein), inorganic minerals, trace elements and vitamins (Makkar and Cameotra, 2002). Cassava wastewater, another carbohydrate-rich residue, which is generated in large amounts during the preparation of cassava flour, is also an attractive substrate and has been used for surfactin production by *B. subtilis* (Nitschke and Pastore, 2006).

Several other starchy waste substrates, such as rice water (effluent from rice processing industry and domestic cooking), corn steep liquor and wastewater from the processing of cereals, pulses have tremendous potential to support microbial growth and biosurfactant production (Krishnaswamy et al., 2008).

Agricultural wastes such as barley bran, trimming vine shoots, corn cobs, and eucalyptus globulus chips has been utilized for the simultaneous production of biosurfactant and lactic acid (Moldes et al., 2007). Ramnani et al. (2005) exploited cornstarch, potato starch and rice starch along with soluble starch and lactose as the source of carbon for the production of biosurfactant utilizing *Bacillus Licheniformis* RG1.

2.6.2 Molasses, a byproduct of sugar industry

Molasses is a byproduct of the sugar industry and is a major raw material for the production of alcohol, baker's yeast, citric acid, feed yeasts, acetone, organic acids and amino acids. Molasses is comparatively low in price compared to other sources of sugar and is rich in various nutrients besides sucrose. Average values for the constituents of cane molasses (75% dry matter) are: total sugars, 48–56%; nonsugar organic matter, 9–12%; protein, 2.5%; potassium, 1.5–5.0%; calcium 0.4–0.8%; magnesium, 0.06%;

phosphorus, 0.06–2.0%; biotin, 1.0–3.0 mg/kg; pantothenic acid, 15–55 mg/kg; inositol, 2,500–6,000 mg/kg; and thiamine 1.8 mg/kg (Makkar and Cameotra, 2002).

Molasses and cornsteep liquor were utilized as the sole source of carbon and nitrogen for the production of rhamnolipid biosurfactant from *P. aeruginosa* GS3. The biosurfactant production reached a maximum when 7% (v/v) of molasses and 0.5% (v/v) of cornsteep liquor were utilized. Maximal surfactant production occurred after 96 hours of incubation, when cells reached the stationary phase of growth. Rhamnose concentration of 0.25 g/l and reduction of interfacial tension between surfactant and crude oil of 0.47 mN m^{-1} was observed (Patel and Desai, 1997).

Biosurfactant production was explored by *Bacillus licheniformis K51, B. subtilis 20B, B. subtilis R1* and *Bacillus strain HS3* utilizing molasses as a sole source of nutrition at 45 °C (Joshi et al., 2008). The production of surfactin by *Bacillus pumilus* UFPEDA 448 in solid-state fermentation (SSF), using a medium based on okara with the addition of sugarcane bagasse as a bulking agent was explored. The optimum proportion of sugarcane bagasse and okara was 50 % (wt) each (Slivinski et al., 2012).

2.6.3 Soapstock, a by-product from oilseed processing industry

Soapstock a gummy, amber-coloured by-product of oilseed processing industry is produced during extraction and refinement of edible oil from the seeds with hexane and other chemicals. Shabtai (1990) reported the production of two extracellular capsular heteropolysaccharides, emulsan and biodispersan by *A. calcoaceticus* RAG-1 and *A. calcoaceticus* A2 respectively, utilizing soapstock as a major carbon source. Emulsan could be utilized to form stable oil-water emulsion (Kim et al., 2000), whereas biodispersan facilitates dispersion of the large solid limestone granules, forming micron-sized water suspension (Rosenberg et al., 1998).

2.6.4 Olive oil industry effluent

Olive oil extraction involves an intensive consumption of water and produces large amounts of olive oil mill wastewater, thus causing deleterious environmental effects. Mercade et al. (1993) found that *Pseudomonas* sp. could reduce the surface tension in culture medium comprising olive oil mill effluent (OOME; 100 g/l) and $NaNO_3$ (2.5 g/L). Surface-active compounds produced from *Pseudomonas* sp. cultured in OOME

medium included rhamnolipids biosurfactant with a total yield of 14 grams of rhamnolipids per kg of OOME after 150 hours of cultivation time.

2.6.5 Animal fat

Animal fat and tallow can be obtained in large quantities from meat-processing industries and have been used as a cooking medium for food. Animal fat was utilized for the production of sophorolipid biosurfactant by yeast, *C. bombicola.* On using only fat as a sole carbon source, the growth was poor however; a mixture of 10% glucose and 10% fat resulted in the highest level of growth. Sophorolipid was produced at levels of 97 g/L and 12 g/L without and with pH control respectively (Deshpande and Daniels, 1995).

2.6.6 Lactic whey and distillery wastes

The effluent from the dairy industry, known as dairy wastewater, supports good microbial growth and has been used as a cheap raw material for biosurfactant production (Dubey and Juwarkar, 2004). Whey waste was utilized for cultivation of *P. aeruginosa* BS2; within 48 hours of incubation the yield of biosurfactant obtained was 0.92 g/l (Dubey and Juwarkar, 2001). The isolated biosurfactant possessed the potent surface active properties, as it effectively reduced the surface tension of water from 72 to *27* mN/m and formed 100% stable emulsions of a variety of water insoluble compounds. Cultures grown on distillery and whey waste gave biosurfactant yields of 0.91 and 0.92 g/l, respectively (Sudhakar et al., 1996). Curd whey was utilized as a medium for the cost-effective production of biosurfactant by *Pseudomonas aeruginosa* strain – PP2 and *Kocuria turfanesis* strain-J and the biosurfactant produced was effective even under extreme pH and temperature conditions (Dubey et al., 2012)

2.6.7 Vegetable oil and oil wastes

Plant-derived oils such as, rapeseed oil (Trummler et al., 2003), babassu oil and corn oil (Vance-Harrop et al., 2003; Pekin et al., 2005) have shown to be useful as cheap raw materials for biosurfactant production. Identically, vegetable oils such as sunflower and soybean oil have been used for the production of rhamnolipid, sophorolipid and mannosylerythritol lipid biosurfactants using various microorganisms (Rahman et al., 2002; Kim et al., 2006). Apart from various vegetable oils, oil wastes from vegetable-oil refineries or soap industries and the food industry are also reported as good substrates for biosurfactant production (Haba et al., 2000; Bednarski et al., 2004; Nitschke et al., 2005).

Peanut oil cake a byproduct produced in large quantity during the production of peanut oil consists of high carbohydrate, protein and lipid content and is relatively cheap. Thavasi et al., (2011) utilized peanut oil cake as the sole source of carbon for the production of biosurfactant using bacteria *Lactobacillus delbrueckii*. The biodegradation of crude oil was promoted to a large extent by biosurfactant alone produced utilizing peanut oil cake as substrate without the use of fertilizer. Zhang et al. (2012) utilized waste frying oil for the growth of three strains of *Pseudomonas aeruginosa* to produce rhamnolipids as biosurfactants. The production of biosurfactant by *Bacillus subtilis* LSFM-05 was carried out with raw glycerol as a sole source of carbon, obtained from a vegetable oil biodiesel plant in Brazil (Fonseca de Faira et al., 2011).

2.7 Upstream processing techniques for biosurfactant production with emphasis on surfactin - a lipopeptide type of biosurfactant.

Commercialization of any biotechnological product is dependent upon its bioprocess economics. Even though a large number of biosurfactant producer microorganisms are reported in the literature but production and product enhancement has been limited to a few organisms such as *Bacillius*, *Pseudomonas* and *Candida* (Mukherjee et al., 2006)

Submerged fermentation (SMF) and solid- state fermentation (SSF) have been tried for surfactin production. Several approaches have been tried for increasing the surfactin yield at shake flask level as well as fermentor level by making variations in process parameters or in the design of reactor. Productivity in different types of media using SMF and SSF is listed in Table 2.5.

Product concentration in diluted solutions, low yields and the product inhibition are some of the major obstacles observed in the production of biosurfactants. Different techniques and reactor types have been employed to enhance surfactin production. Drouin and Cooper (1992) explored the production of surfactin in aqueous two-phase fermentor. An aqueous two-phase system was prepared using polyethylene glycol (PEG-8) and dextran (D-40) to partition the surfactant and surfactant-producing organism in a 2-litre cyclone fermentor. Noah et al. (2002) utilized potato process effluent for the continuous production of surfactin by *B.subtilis*. Airlift reactor (3L) was utilized for batch culture with potato starch, where surfactin was stripped out into the foam and there was better transfer of O_2 into the liquid phase thereby enabling *B. subtilis* to utilize the nutrients more effectively than the indigenous bacteria.

Table 2.5: Productivity of surfactin in various media and type of fermentation

Strain	Medium	Type of fermentation	Reference	Yield of surfactin (mg/L)
B. subtilis ATCC 21332	Semisynthetic	SMF	Arima et al., (1968)	100
B. subtilis ATCC 21332	Synthetic	SMF	Cooper et al., (1981)	250
B. subtilis ATCC 21332	Synthetic	SMF	Cooper et al.,(1981)	800
B. subtilis ATCC 21332	Meat hydrolysate	SMF	Sheppard and Mulligan., (1987)	160
B. subtilis ATCC 21332	Synthetic	SMF, aqueous two phase	Drouin and Cooper., (1992)	350
B. subtilis RB14	Semisynthetic	SMF	Ohno et al., (1992)	250
B. subtilis RB14	Okara	SSF	Ohno et al.,(1992)	200-250 (mg/kg wet mass)
B. subtilis MI113 (pC112)	Semisynthetic	SMF	Ohno et al., (1992)	350
B. subtilis MI113 (pC12)	Okara	SSF	Ohno et al., (1992)	2000
B. subtilis ATCC 55033	Semisynthetic	SMF	Carrera et al., (1992)	3500-4300 (mg/kg wet mass)
Suf-1, a mutant of	Synthetic	SMF	Mulligan et al., (1989)	550

Ohno et al. (1995) used recombinant *B. subtilis* MI113 and the original strain of *B.subtilis* RB14 for the production of surfactin using both SSF and SMF fermentation techniques. A maximum of 1.8–2.0 g/kg of surfactin wet mass was achieved by *B. subtilis* MI113 at 37 °C in 48 hours of fermentation time. High yield of surfactin was obtained in SSF rather than in SMF. In SMF the homogenous distribution of the nutrients and oxygen in the liquid medium favor the cell growth rather than the production of surfactin while the lower growth rate of cell biomass in SSF indicates that nutritional limitations could have triggered the synthesis of surfactin. The effect of temperature was studied on the dual production of iturin A and surfactin by *B.subtilis* RB14 in SSF using *okara* as a substrate. *Bacillus polyfermenticus* KJS-2 (BP-KJS-2) was

utilized for the production of surfactin in SSF using soybeans. It exhibited antimicrobial activity against bacteria at a concentration of 0.05 mg/mL (Kim et al., 2009)

2.8 Downstream processing techniques for recovery and purification of biosurfactant

Downstream processing costs accounts for more than 60% of the total production costs during the synthesis of biotechnological products (Mukherjee et al., 2006). Utilization of appropriate recovery techniques is therefore an important aspect of biosurfactant production process and dictates the overall economy of the process.

Various conventional techniques such as acid precipitation, solvent extraction, crystallization, ammonium sulfate precipitation and centrifugation have been employed in the recovery of biosurfactants (Desai and Banat, 1997; Kuyukina et al., 2001; Philip et al., 2002; Sen and Swaminathan, 2004; Dubey et al., 2005; Nitachke and Pastore, 2006). Table 2.6 lists different techniques used in the recovery process.

Apart from the conventional techniques, some of the interesting and unconventional recovery methods include foam fractionation (Davis et al., 2001; Noah et al., 2002), ultrafiltration (Sen and Swaminathan, 2005), adsorption-desorption on polystyrene resins and ion exchange (Reiling et al., 1986). These unconventional techniques are based upon the properties of biosurfactant such as surface activity or their ability to form micelles and are particularly useful in large scale continuous extraction of extracellular biosurfactants with high level of purity. Some of the unconventional techniques utilized in the recovery of biosurfactants are discussed in greater detail.

2.8.1 Ultrafiltration

Ultrafiltration (UF) a membrane separation technique, based on transmembrane pressure for recovery of dissolved and suspended materials is based on size and molecular scale. Sen and Swaminathan (2005) used UF as the downstream technique to recover surfactin. The operating pressure used in UF was between 100kPa to 500 kPa. At higher initial concentration beyond 0.5 g/L of surfactin, formation of a gel layer of surfactin was observed on membrane surface. Under these conditions, concentration polarization became an important aspect of filtration process. Therefore a tangential flow ultrafiltration was prescribed by them for better recovery of surfactin on a large scale.

Table 2.6: Downstream processing techniques used for recovery of biosurfactants (Desai and Banat, 1997; Krishnaswamy et al., 2008)

Downstream recovery technique	Biosurfactant property responsible for separation	Biosurfactant separated	Advantages
Acid precipitation	Biosurfactants become insoluble at low pH values	Surfactin	Low cost, efficient in crude biosurfactant recovery
Organic solvent extraction	Biosurfactants are soluble in organic solvents due to the presence of hydrophobic end	Sophorolipids, trehalose lipids	Efficient in crude biosurfactant recovery and partial purification, reusable nature
Ammonium sulphate precipitation	Salting-out of the polymeric or protein-rich biosurfactants	Bioemulsifiers, emulsan	Effective in isolation of certain type of polymeric biosurfactants
Centrifugation	Insoluble biosurfactants are precipitated because of centrifugal force	Glycolipids	Reusable, effective in crude biosurfactant recovery
Foam fractionation	Biosurfactants, due to surface activity, form and partition into foam	Surfactin	Useful in continuous recovery procedures, high purity of product
Membrane ultrafiltration	Biosurfactants form micelles above their critical micelle concentration, which are trapped by polymeric membranes	Glycolipids, surfactin	Fast, one step recovery, high level of purity, reusability
Adsorption on polysterene resins	Biosurfactants are adsorbed on polymer resins and subsequently desorbed with organic solvent	Glycolipids, lipopeptides, rhamnolipids	Fast, one step recovery, high level of purity,
Adsorption on wood-activated carbon	Biosurfactants are adsorbed on activated carbon and can be desorbed using organic solvent	Glycolipids, lipopeptides, rhamnolipids	Highly pure biosurfactants, cheaper, reusability, recovery from continuous culture
Ion-exchange chromatography	Charged biosurfactants are attached to ion-exchange resins and can be eluted with proper buffer	Surfactin	High purity, reusability, fast recovery
Solvent extraction (using methyl tertiary-butyl ether)	Biosurfactants dissolve in organic solvents owing to the hydrophobic ends in the molecule	Sophorolipids, trehalose lipids	Less toxic than conventional solvents, reusability, cheap

The advantage of ultrafiltration technique is the purity of the product obtained in just one step during downstream recovery which is 70% comparatively higher than the chemically purified surfactin 32.1%. Ultrafiltration technique has been effectively utilized in downstream processing for concomitant purification of protease and biosurfactant with 95% recovery of both (Ramnani et al., 2005).

2.8.2 Foam Fractionation

Foam fractionation allows continuous extraction of surface active compounds directly from the fermentation broth (Cooper et al., 1981). In particular, foaming has potential application in specific conditions such as for dilute solutions, cheap operations (Uraizee et al., 1990), and for crude mixtures of compounds (Mohan et al., 1994). Foam fractionation has been efficiently utilized for the recovery of the surface active compounds such as surfactants (Hines, 1996) and proteins (Brown et al., 1999a; Brown et al., 1999b). Moreover, it is possible further to integrate the production and continuous recovery of surface active compounds. Recoveries of over 60% can be typically obtained for biosurfactant such as surfactin by pooling foam fractions (Davis et al., 2001).

In certain fermentation processes, foaming creates problems including the undesirable stripping of product, nutrients and cells into the foam and further containment becomes difficult. Addition of anti foaming agents is sometime essential to avoid excess foaming. Chemical antifoams are costly, can reduce the oxygen transfer rate and may exert adverse effect on cell's physiology (Koch et al., 1995). Addition of antifoams can be avoided by recovering the foam produced during microbial fermentation.

2.8.3 Adsorption-desorption on polystyrene resins

Dubey and Juwarkar (2005) reported a novel downstream technique for biosurfactant recovery comprising of adsorption-desorption processes with wood-based activated carbon (WAC). WAC served as the best adsorbent among the ones tested (i.e. silica gel, activated alumina and zeolite). The WAC (1% w v^{-1}), equilibrium time (90 min), pH range of 5-10 and temperature of 40 °C were optimum to achieve 99.5% adsorption efficiency. Biosurfactant adsorption was of the chemisorption type. The WAC could be regenerated utilizing acetone and the reuse of WAC could be allowed up to 3 cycles. Acetone facilitated a recovery of 89% from WAC. This process reduces the use of high cost solvent, avoids end product inhibition and minimizes product degradation (Dubey et al., 2005).The adsorption-desorption process was utilized for continuous recovery of biosurfactant from fermented distillery wastewater and concentrated foam.

2.8.4 Ion Exchange Chromatography

Ion exchange chromatography technique was utilized for the recovery of rhamnolipids (Reiling et al., 1986). This technique was used in combination with adsorption

chromatography and subsequent anion exchange chromatography for further enrichment of the biosurfactant. Utilization of anion exchange chromatography as secondary enrichment step followed by adsorption chromatography yielded a high product purity of over 90%. However, the disadvantages associated with ion-exchange chromatography concern the cost of exchanger and relatively low flow rates.

2.9 Applications of biosurfactants

The interest in utilization of biosurfactants has increased in recent years owing to its unique environmental acceptability. They are readily biodegradable and have low toxicity compared to synthetic surfactants. Some of the potential applications are in *Microbial Enhanced Oil Recovery (MEOR)*, pollution control and environmental hazard elimination such as hydrocarbon degradation in soil environment, heavy metal removal from contaminated soil and hexa-chloro cyclohexane degradation.

Biosurfactants such as rhamnolipids, sophorolipids and surfactin have found promising applications in various industries such as food, environmental, specialty chemicals, cosmetics and pharmaceutical industries. Table 2.7 lists some other noteworthy applications in food, cosmetics, health care, therapeutics and biomedical fields. Rhamnolipids have been successfully utilized in bioremediation of soil. Scheibenbogen et al. (1994) found that the rhamnolipids from *P.aeruginosa* UG2 were able to effectively remove a hydrocarbon mixture from a sandy loam soil and that the degree of removal was dependent on the type of hydrocarbon removed and the concentration of the surfactant used. Pentachlorophenol (PCP) from soil was also removed by them utilizing rhamnolipids in the form of foam.

Metals and ions such as cadmium, lead, copper, lanthanum and zinc could be removed utilizing rhamnolipids due to their complexation ability (Tan et.al., 1994, Harman et al., 1995 and Ochoa-Loza et al., 2001)**.** L-Rhamnose has considerable potential as a precursor for flavoring. It is already used industrially as a precursor of high-quality flavor component like furaneol. Another development is the feasibility of rhamnolipid in dispersion of oil spills (Muthuswamy et al., 2008).

Sophorolipids have generated interest in the pharmaceutical arena because of their wide array of therapeutic benefits. Shah et al. (2005) first reported that natural sophorolipids

Table 2.7: Industrial applications of biosurfactants (Muthusamy et al., 2008; Mulligan and Gibbs, 1993)

Industry	Application	Role of biosurfactant
Petroleum	Enhanced oil recovery	Improving oil drainage into well bore, lowering of interfacial tension, dissolving oil, stimulating release of oil entrapped by capillaries, wetting of solid surfaces, reduction of oil viscosity and oil pour point
	De-emulsification	De-emulsification of oil emulsions, oil solubilization, viscosity reduction, wetting agent
Environmental	Bioremediation	Emulsification of hydrocarbons, lowering of interfacial tension, metal sequestration
	Soil remediation and flushing	Emulsification through adherence to hydrocarbons, dispersion, foaming agent, detergent, soil flushing
Food	Emulsification and de-emulsification	Emulsifier, solubilizer, demulsifier, suspension, wetting, foaming, defoaming, thickener, lubricating agent
	Functional Ingredient	Interaction with lipids, proteins and carbohydrates, protecting agent
Biological	Microbiological	Physiological behaviour such as cell mobility, cell communication, nutrient accession, cell-cell competition, plant and animal pathogenesiss
	Pharmaceutical and therapeutics	Antibacterial, antifungal, antiviral agents, adhesive agents, immunomodulatory molecules, vaccines, gene therapy
Cosmetic	Health and beauty products	Emulsifiers, foaming agents, solubilizers, wetting agents, cleansers, antimicrobial agents, mediators of enzyme action
Agricultural	Biocontrol	Facilitation of biocontrol mechanisms of microbes such as parasitism, antibiosis, competition, induced systemic resistance and hypovirulence
Bioprocessing	Downstream processing	Biocatalysis in aqueous two-phase systems and microemulsions, biotransformations, recovery of intracellular products, enhanced production of extracellular enzymes and fermentation products

and their first-generation chemical derivatives are efficient microbicidal spermicides, having activities similar to that of nonoxynol-9. Bluth et al. (2006) showed that natural sophorolipids are effective septic shock antagonists. Preliminary investigations by Scholz et al. (1998) concerning its anticancer activity were performed with sophorolipids and its derivatives prepared by alcoholysis and enzyme-catalyzed acetylations.

Sophorolipid has been derivatized and also used as a protective substance in cosmetic industries (Inoue, 1988). Sophorolipids in combination with propylene glycol is ideal as a skin moisturizer (Yamane, 1987). There are few applications reported so far concerning its potential to enhance the remediation of hydrocarbon-contaminated soils (Mulligan et al., 1999a; 2001).

Surfactin has very high surface tension lowering ability even at very low concentration and hence can be utilized for replacing chemical surfactants. Surfactin exhibits remarkable property to inhibit blood coagulation, protein denaturation and to accelerate fibrinolysis. It has been found that surfactin possesses antimycoplasmic properties (Vollenbroich et al., 1997).

Surfactin is a stabilizing agent that is renewable, low in toxicity and biodegradable and is thus an environmentally friendly additive. Therefore, surfactin has been found to be useful in the field of nanotechnology in development of nanoparticles. Reddy et al. **(**2008) showed that synthesis of silver nanoparticles could be stabilized by surfactin. Availability of two negative charges on the structure of surfactin, one on aspartate and another on glutamate among the amino acid chain in the cyclic lipopeptide helps surfactin bind with metals. Therefore, it has been utilized for the removal of heavy metals from contaminated soil and sediments (Thimon et al., 1992).

2.10 Isolation and identification of biosurfactants with emphasis to surfactin

Identification or the characterization of the bio surfactant molecule is an involved task, it primarily comprises of separating the targeted molecule from other similar molecules which could be isomers or polymorphs using suitable techniques, identification of

chemical moieties and the molecular structure and environment, determination of molecular mass and ultimately the surface activity

.2.10.1 Thin Layer Chromatography (TLC)

Thin layer chromatography (TLC) technique has been used for the separation of two or more different types of biosurfactants present in the same liquid solution. Pepoux et al. (1991) utilized TLC to separate two different types of surfactin isomers into different spots on silica gel 60 with butanol/acetic acid/water (32:48:8, by vol.) solution as the mobile phase. Chloroform/methanol/acetic acid (92:5:3, by vol.) followed by toluene/pyridine/chloroethanol/0.8M ammonia (upper phase 50:15:30:30, by vol.) were utilized in TLC for the determination of C-terminal amino acid as dinitrophenyl derivative. The derivative after solvent extraction was subsequently quantified by spectrophotometry (Pepoux et al., 1991).

The lipopeptide nature of the biosurfactant using the TLC technique was observed by Mukherjee et al., (2008). These investigators showed that on chromatographic development of the TLC plate the biosurfactant spot was visible under UV lamp (254nm) and it stained yellow when developed with iodine vapor confirming the presence of lipid in the molecule.

2.10.2 Fourier Transform Infrared Spectroscopy (FTIR)

Fourier transform infrared spectroscopy (FTIR) is one of the most useful techniques for the identification of chemical bonds (functional groups) present and therefore can be used to ascertain components in an unknown mixture (Rodrigues et al., 2006). Surfactin, lichenisyn and rhamnolipids have been characterized by the IR technique (Das et al., 2008). Identification of functional groups with FTIR technique confirmed the presence of aliphatic and peptides like moieties in the biosurfactant (Makkar and Cameotra., 1999). The lipopeptide biosurfactant produced by *B.licheniformis* KGL11 was characterized using similar technique and bands for peptides as well as for aliphatic chains were seen at specific wavenumbers (Lin et al., 1998).

2.10.3 Nuclear Magnetic Resonance (NMR)

NMR is based on magnetic moment under which transition in atoms occurs in the presence of an external magnetic field. The absorbance of radio frequency radiation by a nucleus in a strong magnetic field causes the nuclear spin to realign or flip in to higher-

energy direction. Upon absorption of energy, the nuclei will re-emit radiation and return to the lower-energy state. NMR reveals information regarding the functional groups as well as the position of linkages within the carbohydrate and lipid molecules.
Solvents such as acetic acid, acetone, benzene, chloroform, dimethyl sulfoxide, methanol pyridine and water are used. Samples are hydrolysed and extracted in the solvent and detected further by NMR (Satpute et al., 2010).

Nuclear magnetic resonance (NMR) spectroscopy technique has been utilized for identification of the molecular structure based on chemical environment of the magnetic nuclei like 1H, 13C, 31P etc. Yakimov et al., (1995) utilized 2D NMR to characterize a lipopeptide surfactant produced by *Bacillus licheniformis* BAS50. NMR spectroscopy revealed the amino acid composition and sequence of lipopeptide. The amino acid spin systems were identified from a 2D ^{1}H phase-sensitive TOCSY spectrum starting from the backbone amide protons in the region from 10 to 7 ppm and confirmed by correlations of the H_{α}s in the region from 4.4 to 4.0 ppm. The β-hydroxy fatty acid signals were also observed in 2D TOCSY spectrum of the lipopetide. NMR technique utilized for the characterization of biosurfactant produced by *Bacillus subtilis* under thermophilic conditions revealed a lipopeptide nature with the presence of lipid and peptide moiety (Makkar and Cameotra, 1999).

Tang et al., (2007) determined the molecular structure of surfactin isomer isolated from the mangrove bacterial strain '*Bacillus sp'* utilizing combined mass spectrometry, 1D and 2D NMR experiments including DEPT, ^{1}H–^{1}H COSY, HSQC, HMBC, TOCSY, ROESY and HSQC-TOCSY. Assignment of full spectral data for ^{1}H and ^{13}C NMR were also determined.

2.10.4 High Pressure Liquid Chromatography (HPLC)

The separation of various components using HPLC is based on their polarity and the separated products are further detected and fractions collected for analysis of the structure of each moiety. HPLC device can be coupled with evaporative light scattering detector (ELSD) or mass spectrometry (MS) for separation and successive identification of glycolipid type of biosurfactant (Satpute et al., 2010).

Siegmund and Wagner, (1991) used HPLC for separation of free rhamnose from rhamnolipid product mixture, whereas Lin et al., (1998) used HPLC for the

characterization of lichenisyn produced by *B.licheniformis mutant*. It was also used for separation of lipopeptide type biosurfactant (Aguilar, 2004).

2.10.5 Electrospray Ion Mass Spectrometry (ESI-MS) and Secondary Ion Mass Spectrometry (SIMS)

ESIMS and SIMS have been utilized for the characterization of biosurfactant i.e. to determine the molecular weight per charge (m/z) value. The molecular weight of the various components of biosurfactant such as lichenysin, a lipopeptide was determined by the positive and negative-ion SIMS and ESIMS analyses (Yakimov et al., 1995). A rapid method for online detection of surfactin isomers was developed based on HPLC-ESIMS analyses (Tang et al., 2010). Romano et al. (2011) reported the isolation and structural characterization of the three new cyclic lipopeptides from organic extract of *Bacillus amyloliquefaciens* strain. The stereostructures of the same were elucidated using NMR and ESI-MS.

2.10.6 Surface activity of biosurfactants

Surface tension (SFT) and interfacial tension (IFT) constitute two important functional properties of surfactant. The water molecules strongly remain together by intermolecular cohesive forces. These forces develop tension on the surface called as SFT. SFT of the distilled water is 72 mN/m and is further reduced by the addition of surfactant. Interfacial tension exists at the interface of the two phases (Satpute et al., 2010). The quantity of surfactants constituting 1 cm^3 of oil displaced area is defined as one BS unit (Thaniyavarn et al., 2003).

Surface activity of biosurfactant depends on the selection of microorganisms, carbon sources and various process parameters (Zajic et al., 1977; Syldak & Wagner, 1987; Desai et al., 1994; Hommel, 1994) Surfactin produced by *Bacillus sp.* is one of the most efficient BS in reducing surface tension of water from 72 to 27 dynes/cm (Cooper and Goldenberg, 1987; Banat et al., 1993).

Critical micelle concentration (CMC) is one of the important parameter in defining the surface activity of biosurfactant. CMC is the concentration of biosurfactant in the solution at which the formation of micelles is initiated. Above this concentration, no further reduction in surface tension is expected. Therefore it is highly desirable to establish CMC of biosurfactants for their varied applications (Rodrigues et al., 2006).

Emulsification index, wetting and foaming ability of biosurfactants are other important functional properties for defining the surface activity of biosurfactants (Satpute et al., 2010)

2.11 Conclusions:

Biosurfactants are an important class of chemicals derived from biological origin and are obtained from the fermentation of complex sugars. They find wide applications in the food, cosmetics and pharmaceutical industries for environmental applications and are also used as specialty chemicals. Surfactin, an important class of biosurfactant has specific applications in enhanced oil recovery. Surfactin has primarily been obtained by the fermentation of starch however other sources that have been used include byproducts from the sugar and oil industry, wastes from distilleries, oil and the meat processing industry.

Though there are pointers on using molasses a byproduct from the sugar industry as substrate for surfactin production, there is no literature available for synthesizing surfactin using rice bran, a byproduct from the rice polishing industry which is rich in vitamins, minerals and useful chemicals

This work primarily is oriented towards the synthesis of biosurfactant surfactin from molasses and rice bran as agro based substrate materials rich in sugars and nutrients and often treated as waste, although both are valuable and there are legislations on the use of molasses. In case surfactin is obtained in moderate amounts and it would be an economically viable process, then it would open up new vistas of utilizing these wastes as substrates and eventually one would generate wealth from waste. The growing demand and commercial value of biosurfactant could be in a way be the driving force for transformation of agrarian economics into industrial ones.