Chapter 1

Introduction

Biosurfactants are microbially produced compounds which exibit surface activity. At present, biosurfactant synthesis has been studied extensively (Fiechter, 1992; Banat *et al*., 2000). These biosurfactants are amphipathic molecules consisting of hydrophobic and hydrophilic domains. Due to their amphipathic nature, biosurfactant can partition at the interfaces between different fluid phases such as oil/water or water/air interfaces.

Biosurfactants have applications in the environmental protection, crude oil recovery, agriculture, mining, health care and food-processing industries (Fiechter, 1992; Desai and Banat, 1997). Biosurfactants have special advantage over the chemical surfactants, such as lower toxicity, higher biodegradability, better environmental compatibility, higher foaming, high selectivity and specific activity at extreme temperature, pH and salinity (Desai and Banat, 1997; Makkar and Cameotra, 1997a). As environmental compatability becoming an increasingly important factor in the selection of industrial chemicals, the use of biosurfactants in environmental application, such as biodegradation and dispersion of oil spill is increasing (Banat, 1995). In addition, biosurfactants have other use in petroleum industry, such as enhancing oil recovery process and the transportation of crude oil (Kim *et al.*, 1997). Other possible application fields are in the food, cosmetic and pharmaceutical industries. In these industries, most biosurfactants are used as emulsifiers (Khire *et al.*, 1994; Banat, 1993). H-Kittikun *et al*. (1993) has been studied *Bacillus* MUV4 could produce antibiotic that was macrolactin A compound against the growth of *Bacillus anthracis*, *Salmonella* sp. and *Aeromonas hydrophila*. During antibiotic production, foam formation was observed and decreased so it was observed that this strain could produce biosurfactant .

The aim of this research is to optimize conditions for biosurfactant production by *Bacillus* MUV4. In addition, some properties of the biosurfactant and its application will be investigated.

Literature Review

1. Surfactants

Surfactants are amphipathic molecules consisting of hydrophobic and hydrophilic moieties in one molecule.

1.1 Hydrophilic moieties (water soluble) or polar group appear in variations such as amino acid or peptide, carbohydrate, phosphate, alcohol and carboxylic acid.

1.2 Hydrophobic moieties (oil soluble) or non-polar, is frequently a hydrocarbon chain such as unsaturated fatty acid and saturated fatty acid.

Both hydrophilic and hydrophobic moieties partition preferentially at the interface between fluid phases with different degrees of polarity and hydrogen binding such as oil/water or air/water interface. These properties make surfactants capable of reducing surface and interfacial tension and foaming microemulsion where hydrocarbons can solubilize in water or where water can solubilize in hydrocarbon. Such characteristics confer excellent detergency, emulsifying, foaming and dispersing traits (Desai and Banat, 1997; Finnerty, 1994).

The surfactant character of molecules is due to their mixed hydrophilic/hydrophobic nature. They are able to form rod-shaped micelles, bilayers and vesicles (Figure 1).

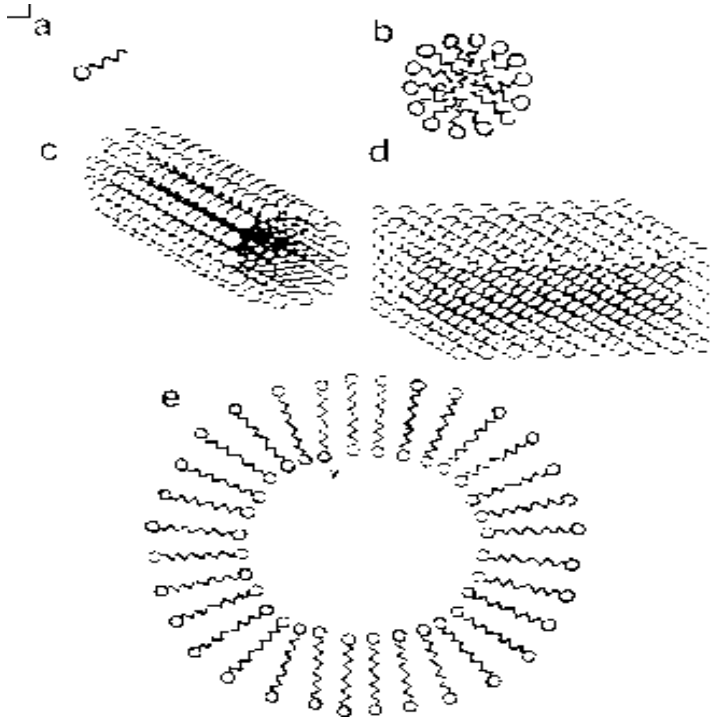

Figure 1 Surfactants are characterized by an amphipathic structure.
Hydrophobic and hydrophilic properties depend on the charge of the polar groups (anionic, cationic, neutral or amphoteric types).
a) surfactant monomer
b) circular micelle
c) rod-shaped micelle
d) micellar layer
e) vesicle representation

Source : Fiechter (1992)

2. Nature of surfactants

Cell membranes are composed mainly of ampliphiles, which are self-assembled into a bilayer structure with the molecules oriented so that the hydrophilic groups are on the out side of the membrane and the hydrophobic groups avoid contact with the aqueous regions by being packed closely together inside the membrane. The complete membrane contains other materials such as

proteins, polysaccharides and cholesterol but the primary structure owes its form to surfactant aggregation. In mammalian cells, the primary ampliphiles are phospholipids, which are derivatives of glycerol esters with long-chain fatty acid.

Fats, which are important sources of the fatty acid used by the body to store chemical energy, are triglycerides that are not really thought of as amphiphiles since the head group is not sufficiently hydrophilic. In milk, the fat is mainly in the form of triglycerides but a small amount is present as phospholipids and diglycerides, which are surfactant that help to stabilize the emulsion in the water. Pancreatic lipase hydrolyses the triglyceride at the oil/water interface of this emulsion to produce free fatty acids and monoglycerides. Both of the materials are strongly surface active and form mixed micelles with bile salts to produce essencially solubilized fat, which can pass through the walls of the intestine. Bile salts are surfactants produced in the liver and stored in the gall-bladder. In the blood stream, the fatty acids leave these surfactant complexes to combine with proteins such as serum albumin to form lipoproteins, which self-organize into species suitable for transport to various destinations around the body.

A more complex state of aggregation is shown by the lipoprotein in myelin, which is a more specialized membrane that surrounds nerve fibers. The basic structural unit is the back-to-back bilayer of the cell membranes, however, in myelin these are built into multilayers, which resemble the lamellar structure shown by lamellar liquid crystals. At an even higher level, complex glycolipids form intricate structure in brain tissue, which are the basis for the rapid neural processes controlling all body functions (Clint, 1992).

3. Synthetic surfactants

Many operations and processes in the both domestic and industrial situations rely on surfactants and, in most cases, these surfactants are synthetic rather than naturally occurring. Synthetic surfactants may be produced from

petroleum derived feedstocks (*e.g.* alcohol, alkylbenzenes, alkylphenols) or natural raw materials (vegetable-and animal-derived oils and fats, fatty acids and alcohols, carbohydrates, *etc*.) by one more chemical conversion processes.

The best example is detergency. A detergent is a cleaning product formulated with several components of which surfactants are major and important ingredients. Soap was at first home made detergent responsible for the introduction of commercial manufacturing in the thirteenth century (Cosson, 1987 cited by Clint, 1992). Although soap is still a valuable and indeed preferred ingredient for certain applications, it was the introduction of synthetic detergents in the 1940s that saw the start of a revolution in both domestic and industrial cleaning products.

In addition to these domestic uses of surfactants, there are large industrial uses other than for detergency. These include dyestuffs, fibers, mineral processing, oil field chemicals, paints, pesticides, pharmaceuticals and plastics. Each of these is a major industry and the total usage of surfactants represents a significant factor in the economy.

4. Types of surfactants (Clint, 1992)

Types of surfactant are concerned mainly with aqueous solutions of surfactants, the two parts of the amphiphilic molecules are hydrophilic and hydrophobic. An enomerous variety of chemical groups have been used but the following represents some of the major classes of commercial importance.

4.1 Hydrophilic groups

The hydrophilic group of a surfactant is usually referred to as the 'head group' and is either strongly polar or charged. If the charged group is anionic, the counterion is usually Na^+ and for cationic surfactants the counterion is usually Cl^-

4.1.1 Anionics. These include the traditional soaps ($-CO_2^-$) and the early synthetic detergents, the sulphonates ($-SO_3^-$) and the sulfates ($-O-)SO_4$). All of these compounds are used extensively in cleaning formulations. The major

advantage of the sulphonates and sulfates over the carboxylates is their greater tolerance of divalent metal ions in hard water.

4.1.2 Cationics. These are usually quaternary ammonium, imidazolinium or alkyl pyridinium compounds. The positive charge on the head group gives the surfactant a strong substantivity on charged fibers, such as cotton and hair, and they are therefore used as fabric and hair conditioners.

4.1.3 Zwitterionic surfactants (often referred to as amphoteric). These are used in the form of betaines ($-N^+(CH_3)_2CH_2CO_2^-$) or sulphobetaines ($-N^+(CH_3)_2CH_2SO_3^-$). These compounds are milder on the skin than the anionics and have especially low eye-sting effects, which leads to their use in toilets and baby shampoos. Among the naturally occurring surfactants in this class are the lecithins or phosphatidyl cholines, which have the head group: $-O-PO_3^- -CH_2\ CH_2-N^+(CH_3)_3$.

4.1.4 Non-ionic surfactants. These are dominated by the ethoxylates, $-(OCH_2\ CH_2)_nOH$. They are used extensively in low-temperature detergency and as emulsifiers. This class of surfactants also includes several so-called semi-polar compounds such as the amine oxides, sulphoxides and phosphine oxides, although only the amine oxide is commercially important. More recently of non-ionic head groups have been extended to include, for example, pyrolidones (Rosen *et al*., 1988 cited by Clint, 1992) and even sugars. Also to be included in the non-ionic classes are the alkanolanides and their ethoxylated derivatives.

4.1.5 Combinations. The most common are those that have both non-ionic and anionic groups such as the alkyl ethoxysulfates, $-(OCH_2CH_2)_nOSO_3^-$. Surfactants of this type are mild on the skin and are therefore used in formulations where skin contact is not usually avoided, for example dishwashing liquids and shampoos.

4.2 Hydrophobic groups

This part of the surfactant is usually called the 'tail' and is most commonly a simple hydrocarbon group. When soaps were the only detergents the tail would

be a mixture of those alkyl groups occurring in the fatty acids derived from hydrolysis of natural fats and oils. These would be straight alkyl chains of length around C_{12} to C_{20} with a certain fraction of unsaturation depending on the source of the fat.

Early soapless detergents were based on alkyl benzene sulphonates, where the alkyl group was often branched chain material derived from a readily available tetrapropylene feedstock. Such branched alkyl chains are not easily biodegraded, they therefore persist in water after sewage treatment, causing foaming in the rivers and streams. However, these have been superseded by linear alkyl benzene sulphonates, which are biodegradable and where the alkyl group is often approximately n-C_{12} H_{25}. Also, on safety and environmental grounds, the trend is now towards tail groups with no aromatic component.

5. Biosurfactants

Biosurfactants are a structurally diverse group of surface-active molecules synthesized by microorganisms. These molecules reduce surface and interfacial tensions in both aqueous solutions and hydrocarbon mixtures.

Biosurfactants activities can be determined by measuring the charge in surface and interfacial tensions, stabilization or destabilization of emulsion. The surface tension at the air/water and oil/water interfaces can easily be measured with a tensiometer. The surface tension of distilled water is 72 mN/m, and addition of surfactant lowers this value to 30 mN/m (Desai and Banat, 1997).

An emulsion is formed when one liquid phase is dispersed as microscopic droplets in another liquid continuous phase. Biosurfactant may stabilize (emulsifiers) or destabilizer (deemulsifiers) emulsion. The emulsification activity is assayed by the ability of the surfactant to generate turbidity due to suspended hydrocarbons such as a hexadecane-2-methylnaphthalene mixture (Rosenberg *et al*., 1979) or kerosene (Cooper and Goldenberg, 1987) in an aqueous assay system.

5.1 Biosurfactant classification

The major classes of biosurfactants include glycolipids, lipopeptides and lipoproteins, fatty acids, phospholipid and neutral lipids, and polymeric surfactants.

5.1.1 Glycolipids. Most known biosurfactants are glycolipids. They are carbohydrates in combination with long-chain aliphatic acid or hydroxyaliphatic acid. Among glycolipids, the best known are rhamnolipids, trehalolipids and sophorolipids (Figure 2).

5.1.2 Rhamnolipids. Rhamnolipids are composed of rhamnose and linked to one or two molecules of β-hydroxydecanoic acid. Production of rhamnose-containing glycolipids was first described in *Pseudomonas aeruginosa* by Javis and Johnson (1949 cited by Desai and Banat, 1997). They demonstrated a glycosidic linkage of β-hydroxydecanoyl-β-hydroxydecanoate with two rhamnose molecules after cultivation of *Pseudomonas aeruginosa* on 3% glycerol.

5.1.3 Trehalolipids. Structure of trehalolipid biosurfactants are disaccharide trehalose linked at C-6 and C'-6 to mycolic acids. Mycolic acids are long-chain, α-branched-β-hydroxy fatty acid. Trehalolipids from different organisms differ in the size and structure of mycolic acid, the number of carbon atoms, and degree of unsaturation. Trehalose dimycolate produced by *Rhodococcus erythropolis* has been extensively studied (Kretschmer *et al*., 1982). Trehalose lipid from *Arthrobacter* sp. lowered the surface and interfacial tensions in the culture broth to 1-5 mN/m (Li *et al*., 1984).

5.1.4 Sophorolipids. Sophorolipids, which are produced mainly by yeast such as *Torulopsis bombicola* (Cooper and Paddock, 1984; Gobbert *et al.*, 1984) and *Torulopsis petrophillum* (Cooper and Paddock, 1983), consist of a dimeric carbohydrate sophorose linked to a long-chain hydroxy fatty acid. These biosurfactants are a mixture of at least 6 to 9 different hydrophobic sophorosides.

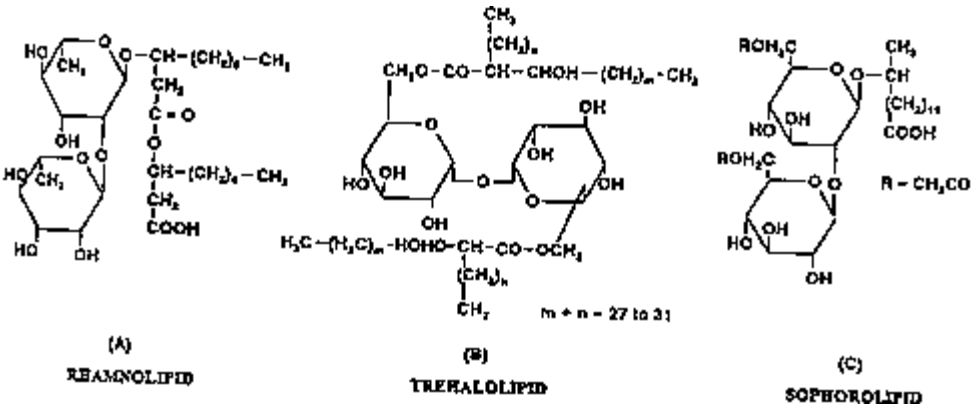

Figure 2 Structure of glycolipid biosurfactants.

(A) Rhamnolipid from *Pseudomonas aeruginosa.*

(B) Trehalolipid from *Rhodococcus erythropolis.*

(C) Sophorolipid from *Torulopsis bombicola.*

Source : Desai and Banat (1997)

5.2 Lipopeptides and lipoproteins. A large number of cyclic lipopeptides including decapeptide antibiotic (gramicidins) and lipopeptide antibiotics (polymyxins) produced by *Bacillus brevis* and *B. polymyxa*, respectively. The cyclic lipopeptide surfactin (Figure 3) produced by *B. subtilis* ATCC 21332, is one of the most powerful biosurfactants. It lowers the surface tension of water from 72.0 to 27.9 mN/m at concentration as low as 0.005% (Arima *et al*., 1968 cited by Desai and Banat, 1997). *Bacillus licheniformis* produces several biosurfactants which act synergistically and exhibit excellent temperature, pH and salt stability (McInerney *et al*., 1990; Yakimov *et al*., 1995). The biosurfactant BL-86, produced by *B. licheniformis* 86 is capable of lowering the surface tension of water to 27 mN/m and the interfacial tension between water and hexadecane to 0.36 mN/m (Horowitz *et al*., 1990). Structural analysis revealed that it is a mixture of lipopeptide with major components ranging in size from 979 to 1,091 Da. Each molecule contains seven amino acids and a lipid portion which is composed of 8

to 9 methylene groups and a mixture of linear and branched tails (Horowitz and Griffin, 1991).

Yakimov *et al.* (1995) have shown production of a new lipopeptide surfactant, lichenysin A, by *B. licheniformis* BAS50 reduces the surface tension of water from 72 to 28 mN/m with critical micelle concentration (CMC) of as little as 12 μM, comparing favorably with surfactin (24 μM). The detailed characterization of lichenysin A showed that isoleucine was the C-terminal. Amino acid instead of leucine and an asparagine residue was present instead of aspartic acid as in the surfactin peptide. Addition of branched chain α-amino acid to the medium caused similar changes in lipophilic moieties of lichenysin A and lowering of surface tension activity.

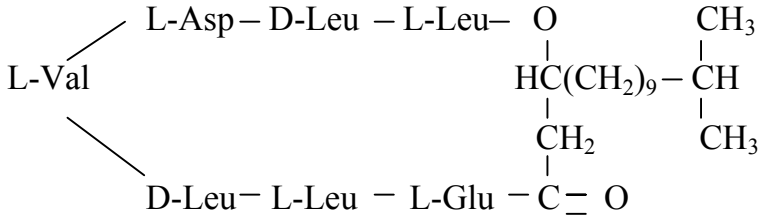

Figure 3 Structure of cyclic lipopeptide surfactin produced by *Bacillus subtilis*
Source : Desai and Banat (1997)

5.3 Fatty acids, phospholipids and neutral lipids. Several bacteria and yeast produce large quantities of fatty acid and phospholipid surfactants during growth on n-alkanes (Cirigliano and Carman, 1985; Robert *et al*., 1989). In *Acinetobacter* sp. strain H01-N phosphatidylethanolamine rich vesicles are produced (Kappeli and Finnerty, 1979). The potent surfactant properties of these vesicles are evident from the observation that they are able to generate optically clear microemulsions of alkanes in water. Phosphatidylethanolamine (Figure 4) produced by *R. erythropolis* grown on n-alkane caused a lowing of interfacial

tension between water and hexadecane to less than 1 mN/m and a critical micelle concentration (CMC) of 30 mg/l (Kretschmer *et al*., 1982).

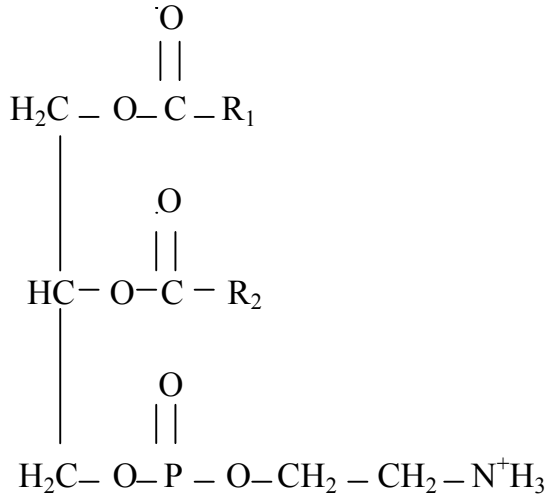

Figure 4 Structure of phosphatidylethanolamine, a potent biosurfactant produced by *Acinetobacter* sp. R_1 and R_2 are hydrocarbon chains of fatty acids.
Source : Desai and Banat (1997)

5.4 Polymeric surfactants. The best-studied polymeric biosurfactants are emulsan, liposan, mannoprotein and other polysaccharide protein complexes. Novonvenezia *et al.* (1995) described the isolation of alasan, an anionic alanine-containing heteropolysaccharide protein biosurfactant with a molecular approximately 1 MDa produced by *Acinetobacter radioresistens* KA-53. It was found to be 2.5 to 3 times more active after being heated at 100 ^{o}C under neutral or alkaline condition. Alasan produced by a strain of *Acinetobacter radioresistens*, is a complex of an anionic polysaccharide and protein. The polysaccharide component of alasan is unusual in that it contains covalently bound alanine. The protein component of alasan appears to play an important role in both the structure and activity of the complex (Navon-Venezia *et al*., 1998).

Liposan is an extracellular water-soluble emulsifier produced by *Candida lipolytica* and is composed of 83% carbohydrate and 17% protein. The carbohydrate portion is a heteropolysaccharide consisting of glucose, galactose, galactosamine and galacturonic acid (Cirigliano and Carman, 1984). Kitamoto *et al.* (1993) demonstrated the production of two kind of manosylerythritol lipid in *Candida antarctica* T-34.

The biosurfactant-producing microbes are destributed among a wide variety of genera. The major types of biosurfactants, their properties and microbial species of origin are listed in Table 1.

Table 1 Types of microbial surfactants

Surfactants	Microbial sources
Glycolipids	

Rhamnolipids	*Pseudomonas sp*.
	P. aeruginosa
Trehalolipids	*Rhodococcus erythropolis*
	Nocardia erythropolis
	Mycobacterium sp.
Sophorolipids	*Torulopsis apicola*
	T. bombicola
	T. petrophilium
Lipopeptides and lipoproteins	
Peptide-lipid	*Bacillus licheniformis*
Serrawettin	*Serratia marcescens*
Viscosin	*P. fluorescens*
Surfactin	*B. subtilis*
Subtilisin	*B. subtilis*
Gramicidins	*B. brevis*
Polymyxins	*B. polymyxa*
Fatty acids, neutral lipids and phospholipids	
Fatty acids	*Candida lepus*
Neutral lipids	*Nocardia erythropolis*
Phospholipids	*Thiobacillus thiooxidans*
Polymeric surfactants	
Emulsan	*Acinetobacter calcoaceticus*
Biodispersan	*A. calcoaceticus*
Mannan-lipid-protein	*Candida tropicalis*
Liposan	*C. lipolytica*
Carbohydrate-protein-lipid	*P. fluorescens*
Protein PA	*P. aeruginosa*

Source : Desai and Banat (1997)

6. Medium components affecting on growth and biosurfactant production by microorganisms

6.1 Carbon source. The carbon source used in bacterial culture was very important in biosurfactant production. The carbon source generally used in

biosurfactant production could be devided into three categories, carbohydrates, hydrocarbons and vegetable oils. Water-soluble carbon sources such as glycerol, glucose, mannitol and ethanol were all used for rhamnolipid production by *Pseudomonas* spp. However, biosurfactant production was interrior to that obtained with water-immiscible substrate such as n-alkane and olive oil (Robert *et al*., 1989). Javaheri *et al*. (1985) showed the presence of large amounts of biosurfactant bound to *Bacillus licheniformis* cells when grown on mineral salts medium containing glucose and 0.1% yeast extract. The surface tension of medium was reduced from 70 to 74 mN/m to as low as 28 mN/m due to the production of an anionic biosurfactant.

Davila *et al.* (1992) demonstrated a high yield of sophorose lipids by overcoming product inhibition in *Candida bombicola* CBS6009 through the addition of ethyl esters of rapseed oil fatty acids in D-glucose medium. Lee and Kim (1993) reported that in batch culture, 37% of the carbon input produced 80 g of sophorolipid per liter by *Torulopsis bombicola*. However, in fed-batch cultures about 60% of the carbon input was incorporated into biosurtactant with the yield of 120 g/l.

Banat (1993) isolated biosurfactant-producing bacterial from 35 bacterial strains only two strains AB-2 and Y12-B had a capability to reduce surface tension in their culture broth to values below 35 mN/m. Strain AB-2 grew on glucose and other organic media in addition to hydrocarbons. The culture grew rapidly on glucose (5 g/l) and hydrocarbon (oleic acid 2% v/v) added after 8 h fermentation. Emulsification Index (E-24) value also increased rapidly once the hydrocarbon was added to the culture broth and reached 95% after 14 h fermentation.

Kim *et al*. (1997) studied effect of carbon source on the biosurfactant production by *B. subtilis* C9. Glucose, n-hexadecane and soybean oil were used as carbon sources. *B. subtilis* C9 reduced the surface tension of the culture broth

from 72.8 to 28.2 dyne/cm and highly emulsified crude oil, soybean oil and hydrocarbon when cultured in medium containing a glucose.

Makkar and Cameotra (1997a) showed growth and biosurfactant production on different carbon source by *Bacillus subtilis* MTCC 2423 found that the surface tension reduction of culture broth was greater when glucose, sucrose, tri-sodium citrate, sodium pyruvate, yeast extract and beef extract were used as carbon source. Sodium acetate inhibited growth and was not used by *B. subtilis* MTCC 2423 for biosurfactant production. The organism was able to grow on n-hexadecane but did not produce biosurfactant on n-hexadecane. None of the other water-insoluble carbon source (hydrocarbon) was used by *B. subtilis* MTCC 2423 for biomass and biosurfactant production.

Makkar and Cameotra (1997b) studied the utilization of molasses for biosurfactant production by two strains of *Bacillus subtilis* (MTCC 2423 and MTCC 1427). Two strains grew and produced biosurfactant when cultured on molasses as substrate. Biosurfactant production started after 24 h and continued till 96 h of fermentation. As a result of biosurfactant accumulation, the surface tension of the medium was lowered to 23 and 31 dyne/cm, respectively. *Bacillus subtilis* MTCC 2423 produced biosurfactant more than *Bacillus subtilis* MTCC1427. Maximal biosurfactant production in both strains was achieved in the late stationary phase.

Yakimov *et al*. (1995) found that sucrose and glucose supported the best biosurfactant production (lichenysin A) of *Bacillus licheniformis* BAS50. The amount of lichenysin A isolated from stationary-phase cells was approximately 160 mg/l by cultivation in glucose- or sucrose-containing medium. Increasing glucose or sucrose concentration above 2% (w/v) did not affect surfactant production.

6.2 Nitrogen source. Medium constituents other than carbon source also affect the production of biosurfactants. The structure of surfactin is influenced by the L-glutamic acid concentration in the medium to produce either Val-7 or Leu-7

surfactin (Peypoux and Michel, 1992). Similarly, lichenysin A production is enhanced two- and four-folds in *B. licheniformis* BAS50 (Yakimov *et al*., 1996) by addition L-glutamic acid and L-asparagine, respectively, to the medium.

Robert *et al*. (1989) and Abu-Ruwaida *et al.* (1991b) observed nitrate to be the best source of nitrogen for biosurfactant production by *Pseudomonas* strain 44T1 and *Rhodocuccus* strain ST-5 growing on olive oil and paraffin, respectively. The production started after 30 h of growth when the culture reached nitrogen limitation, and continued to increase up to 58 h of fermentation. In *P. aeruginosa,* a simultaneous increase biosurfactant production and glutamine synthetase activity was observed when growth slowed as the culture became nitrogen limiting (Milligan and Gibbs, 1989). Similarly, nitrogen limitation caused increased biosurfactant production in *C. tropicalis* ITP-4 (Singh *et al.,* 1990).

Guerra-Santos *et al.* (1984) showed maximum rhamnolipid production after nitrogen limitation at a C:N ratio of 16:1 to 18:1 and no surfactant production below a C:N ratio 11:1, whereas the culture was not nitrogen limited. Sophorose lipid production increased with increasing initial ammonium sulfate concentration. Both growth and product formation were strongly reduced at 73.5 mM ammonium. With 58.9 mM ammonium, a microcrystalline sophorose lipid was formed. The ratio of the two isomers of the sophorose lipid, harbouring either ω- or ω -1 hydroxy fatty acid was influenced by the initial concentration of ammonium (Hommel *et al.,* 1994).

Kim *et al*. (1997) showed that the best yield of biosurfactant was obtained using NH_4HCO_3 as the main nitrogen source. The final culture pH measured after 3 days culture indicated that the high yield of biosurfactant resulted from the prevention of a decrease in the pH by NH_4HCO_3. The maximum biosurfactant concentration was attained when 13.5 g/l of NH_4HCO_3 was used.

Makkar and Cameotra (1997a) have studied the effect of nitrogen source on biosurfactant production of *Bacillus subtilis* found that in nitrogen-free medium, the organism grew and produced the biosurfactant at least, whereas

sodium nitrate, potassium nitrate and urea were the best sources of nitrogen of those tested. Ammonium sulfate was not good for growth and biosurfactant production but the organism was able to utilize ammonium nitrate for growth.

6.3 Salt and minerals

Salt concentration also affected biosurfactant production depending on its effect on cellular activity. Some biosurfactant products, however, were not affected by salt concentrations up to 10% (wt/vol), although slight reductions in the critical micelle concentration (CMC) were detected (Abu-Ruwaida *et al*., 1991a).

Sodium salt is a major component of seawater, and calcium salt, included in industrial water, frequently break the emulsion between oil and water in the practical process (Jung *et al*., 1995). Kim *et al*. (1997) studied the resistance of biosurfactant to NaCl and $CaCl_2$. Biosurfactant, C9-BS retained its surface tension reducing activity up to a concentration of 1,000 mM NaCl and 10 mM $CaCl_2$ with 70% and 87% emulsification activity of the initial activity, respectively.

Sutthivanitchakul *et al*. (1999) showed that addition of 5% NaCl in the culture broth improved the surface activity of the biosurfactant from *Bacillus licheniformis* F2.2 but the activity to reduce surface tension will drop when NaCl concentration was presented at more than 10%.

Yakimov *et al*. (1995) isolated *Bacillus lichenifomis* BAS50 from a petroleum reservoir at a depth of 1,500 m. *B. licheniformis* BAS50 grew and produced a lipopeptide surfactant when cultured on a variety of substrates at salinities of up 13% NaCl. The biosurfactant production occurred both aerobically and anaerobically and was optimal at 5% NaCl and temperature between 35 and 45 ^{o}C. For *Arthrobacter protophormiae*-an antarctic strain could grow and produce biosurfactant in the presence of high NaCl concentrations (10.0 to 100.0 g/l). The biosurfactant reduced the surface tension of the medium from 68.0

mN/m to 30.6 mN/m and exhibits good emulsifying activity (Pruthi and Cameotra, 1997b).

Makkar and Cameotra (1997a) reported that the *Bacillus subtilis* grew and produced biosurfactant at different NaCl concentration (0.01-4%). High NaCl concentration in the medium did not affect the capability of the organism to grow and synthesis biosurfactant, but biosynthesis of biosurfactant was reduced at 4% NaCl.

Iron concentration has a dramatic effect on rhamnolipid production by *P. aeruginosa*, resulting in a 3 fold increase in production when cells were shifted from medium containing 36 μM iron to medium contain 18 μM iron. Phosphate, iron, magnesium and sodium were all important elements for a biosurfactant-producing *Rhodococcus* sp. much more than either potassium or calcium (Abu-Ruwaida *et al*., 1991b). Cooper *et al.* (1981) showed that a biosurfactant-producing *Bacillus subtilis* required large amount of iron (1.3×10^{-3} M), as $FeSO_4$ for higher growth and biosurfactant production. However, higher iron concentration did not improve its biosurfactant yield.

6.4 Environmental factors

Environmental factors and growth conditions such as pH, temperature, agitation and oxygen availability also affect biosurfactant production through their effects on cellular growth or activity.

6.4.1 pH

Rhamnolipid production in *Pseudomonas* spp. was at its maximum at pH range from 6.0 to 6.5 and decreased sharply above pH 7.0 (Guerra-Santos *et al*., 1984). In contrast, the pH range 7.0-9.0 were appropriated value for growth and biosurfactant production by *Bacillus licheniformis* F2.2. The bacterium was unable to reduce the surface tension of culture medium when pH was 4.0 and 4.5 (Sutthivanitchakul *et al*., 1999).

Cooper and Goldenberg (1987), who found that increasing the pH from 6.5 to 7.0 in the cultivation medium affected neither biosurfactant synthesis nor yield by a *Bacillus* sp., whereas lowering the pH to below 5.5 decreased both growth and biosurfactant production. However, growth of *P. aeruginosa* was less influenced by cultivation pH, whereas biosurfactant production dropped when the cultivation pH was changed from the optimum 6.2 to 6.4 (Guerra-Santos *et al*., 1986).

The surface tension reducing activity of biosurfactant C9-BS was stable to pH over the range of pH of 5.0 to 9.5. At the lower end of the pH scale (<4.0), surface tension reducing activity was reduced due to precipitation of biosurfactants. The emulsification activity of C9-BS was stable in regard to pH in the range tested (pH 4.0-10.3), while the level of emulsification of hydrocarbons by chemically synthesized surfactants such as sodium lauryl sulfate (sodium dodecyl sulfate, SDS) and dodecylbenzene sulfonate (linear alkylbenzene sulfonate, LAS) decreased at pH 4.0 and 10.3, respectively (Kim *et al*., 1997). Budsabun (1996) found that the pH of the medium is an important factor in the reduction of the surface tension of culture broth by *B. subtilis* 3/38.

The effect of initial pH on growth and biosurfactant production of *Rhodococcus* showed that highest growth was obtained at pH values of 6.5 and 6.8. Biosurfactant production, as determined by surface tension, critical micelle dilution (CMD), interfacial tension and emulsification index measurements was less influenced by the cultivation pH, since the biosurfactant production or activity was highest with in a wide pH range (6.5-7.2) suggest that the culture, although its highest growth occurred within a narrow pH range, can still produce biosurfactant effectively in a wider pH range (Abu-Ruwaida *et al*., 1991b).

6.4.2 Temperature

A thermophilic *Bacillus* sp. grew and produced biosurfactant at temperature above 40^{o}C (Banat, 1993). Temperature dependency of the production of iturin A and surfactin by a dual producer, *Bacillus subtilis* RB14, in solid-state fermentation was investigated. The optimal temperature for iturin A was 25 ^{o}C while that for surfactin was 37 ^{o}C (Ohno *et al*., 1995). Gurjar *et al*. (1995) reported the effect of temperature on growth and production of emulsifier of *Bacillus stearothermophilus* VR-8 showed that maximum growth of the organism was in the temperature range 45 to 70 ^{o}C, the highest emulsifier production (0.6 g/l) occurred at 50 ^{o}C. The emulsification activity was stable over a broad range of temperature (50 to 80 ^{o}C).

Abu-Ruwaida *et al*. (1991b) demonstrated that optimum growth and biosurfactant production of *Rhodococcus* sp. were obtained at 37 ^{o}C. At this temperature, the highest biomass yield of 2.3 g/l, emusification activity of 40% and biosurfactant concentration as determined by CMD measurements (31 mN/m) were obtained. Lower or higher temperatures generally had a depressing effect on growth and biosurfactant production, while the yield obtained at 35 ^{o}C and 30 ^{o}C were about 1.0 and 0.75 g/l, respectively. The biosurfactant properties were not affected within wide range of temperatures (30 to 41 ^{o}C).

Two new strains of fermenting bacteria were isolated from oily sludge under conditions of enhanced salt concentration (approx. 8.0% w/v) and temperature (50 ^{o}C). They produced emulsifier or biosurfactant, that enrichment medium was supplemented with hexadecane and oil. Both strains could grow at 37, 50 and 55 ^{o}C, but not at 30 and 60 ^{o}C (Denger and Schink, 1995).

An incubation temperature of 30 ^{o}C promoted cell growth an glycolipid production of *Tsukamurella* sp., while a higher (34 ^{o}C) and lower (20, 25 and 27 ^{o}C) temperature the culture broth became inhomogeneous and cell aggregation occurred with a decrease in the glycolipid yield (Vollbrecth *et al*., 1998). *Arthrobacter protophormiae*-an antarctic strain could grow and produce

biosurfactant under psychophilic condition (10 ^{o}C). It exhibited stable activity over a wide range of temperature (30 to 100 ^{o}C) (Pruthi and Cameotra, 1997b).

6.4.3 Agitation and aeration

An increase agitation speed results in the reduction of biosurfactant yield due to the effect of shear in *Nocardia erythropolis* (Margaritis *et al*., 1979 cited by Desai and Banat, 1997). While studying the machanism of biosurfactant production in *A. calcoaceticus* RAG-1, Wang and Wang (1990) revealed that the cell-bound polymer/dry-cell ratio decreases as the shear stress increases. An increase of agitation speed from 250 to 500 rpm caused a decrease in biosurfactant by *Nocardia erythropolis* due to a shear rate effect on the growth kinetics of the microorganism (Syldalk and Wanger, 1987).

Sheppard and Cooper (1990) have concluded that oxygen transfer was one of the key parameters for the process optimization and scale-up of surfactin production in *B. subtilis*. Increasing the aeration rate to K_La 169.9 h^{-1} caused an increase in biosurfactant production by *Pseudomonas aeruginosa* LBI (Benincasa *et al*., 2001).

Yakimov *et al*. (1995) studied biosurfactant production by *B. licheniformis* BAS50 when cultured in aerobic and anaerobic condition. In comparison to anaerobic cultivation, aerobic cultivation of *B. licheniformis* BAS50 was characterized by a shorter lag phase of growth and higher biomass concentration. The surface tension of aerobic medium decreased during early exponential growth phase, reaching a minimum of 28.3 mN/m. During the different growth phases, the surface tension of anaerobic cultures was similar to that of aerobic cultures, but its minimum values was approximately 35 mN/m.

7. Recovery of biosurfactant

Downstream processing in many biotechnological process is responsible for up to 60% of the total production cost. Due to economic considerations, most biosurfactant would have to involve either whole-cell spent culture broth or other

crude preparations. In addition, biosurfactant activity may be affected by other material present in these preparations. Biosurfactant recovery depends mainly on its ionic charge, water solubility and location (intracellular, extracellular or cell bound).

The most commonly, used biosurfactant recovery techniques are shown in Table 2. The most widely used techniques are extractions with chloroform-methanol, dichloromethane-methanol, butanol, ethyl acetate, pentane, hexane, acetic acid, ether, *etc*. Trehalose lipids of *Arthrobacter paraffineous* (Li *et al*., 1984), trehalose corynemycolates and tetraesters of *Rhodococcus erythropolis* (Kretschmer *et al.*, 1982 ; McDonald *et al*., 1981), liposan from *Candida lipolytica* (Cirigliano and Carman, 1985) are some of the well-known examples of biosurfactant recovery by solvent extraction.

Glycolipids produced by *Torulopsis bombicola* (Cooper and Paddock, 1984) was extracted by chilled ethyl acetate after adsorption on charcoal. Biosurfactant from *P. aeruginosa* had also been recovered in similar way, except that extraction was carried out in acetone (Neu *et al*., 1990). Manosylerythritol lipid produced by *Candida* sp. was sedimented as heavy oils upon centrifugation and then extracted in either ethanol or methanol (Kim *et al*., 1999). Bryant (1990) improved the method for isolating the biosurfactant glycolipids by using diafiltration and isopropanol precipitation. This method removed the protein, which coisolated with the glycolipids by using organic extraction and salicic acid chromatography.

Table 2 Downstream processes for recovery of important biosurfactants

Process	References
Batch mode	
Ammonium sulfate precipitation	
Biodispersan	Rosenberg *et al*., 1979.
Bioemulsifier	Rosenberg *et al*., 1988.
Acetone precipitation	
Bioemulsifier	Cameotra and Singh, 1990.
Acid precipitation	
Surfactin	Javaheri *et al*., 1985.
Solvent extraction	
Trehalolipids	Li *et al*., 1984.
Sophorolipids	Ristao and Wagner, 1990.
Liposan	Cirigliano and Carman, 1985.
Continuous mode	
Centrifugation	
Glycolipids	Kitamoto *et al*., 1993.
Adsorption	
Lipopeptides	Matsuyama *et al*., 1991.
Glycolipids	Cooper and Paddock, 1981.
Foam separation and precipitation	
Surfactin	Gobbert and Wagner, 1984.
Tangential flow filtration	
Mixed biosurfactant	Cooper *et al*., 1980.
Diafiltration and precipitation	
Glycolipids	Mattei *et al*., 1980. Bryant, 1990.
Ultrafiltration	
Glycolipids	Cameotra and Singh, 1990. Mulligan and Gibbs, 1990.

Source : Desai and Banat (1997)

Surfactin and surfactin-like biosurfactants produced by *Bacillus subtilis* (Yakimov *et al*., 1995) and *B. licheniformis* (Javaheri *et al*., 1985) were recovered by acid precipitation, whereas biosurfactant from *Pseudomonas* spp. (Cameotra and Singh, 1990; Goswami and Singh, 1991) was best recovered by acetone precipitation.

One of the successful technique for recovery was a continuous in situ removal of surfactin from fermentation broth by foam fractionation. In this technique, foam was collected and acidified to pH 2.0 with concentrated HCl and the precipitated surfactin was extracted in dichloromethane (Cooper *et al*., 1981; Mulligan *et al*., 1989). Neu and Poralla (1990), in contrast, recovered a *Bacillus* sp. biosurfactant by blowing the foam out of the fermentor to be collected, centrifuged and extracted by acetone precipitation.

8. Potential application of biosurfactants

Current worldwide surfactant markets are around $9.4 billion per annum and their demand is expected to increase at a rate of 35% toward the end of the century (Desai and Banat, 1997). The total sales volume of specially surfactants in the USA in 1992 was estimated to be growing at a rate of 3-5% annually (Makkar and Cameotra, 1998). Almost all surfactants currently in use are chemically synthesized. Nevertheless, in recent years, much attention has been directed toward biosurfactants due to their broad-range functional properties and the diverse synthetic capabilities of microbes. The structural analysis of biosurfactant has opened possibilities for their chemical synthesis. Most important is their environmental acceptability, because they are readily biodegradable and have lower toxicity than synthetic surfactants (Desai and Banat, 1997). Most of biosurfactant applications are in oil, food and cosmetics industries as well as in therapeutic agent.

8.1 Oil industry

Biosurfactants are used to enhance oil recovery. Due to the potential use in the oil industry with minimum purity specification so that whole-cell broth could be used. Compared with chemical surfactants, they are very selective and required in small quantities and are effective under broad ranges of oil and reservoir conditions.

In a pilot field investigation, Banat *et al.* (1991) tested the ability of biosurfactant produced by a bacterial strain (Pet 1006) to clean oil storage tank and to recover hydrocarbon from the emulsified sludge. Two ton of biosurfactant whole-cell cultures were used to mobilized and clean 850 cm^3 oil sludge. Approximately 91% (744 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.

B. licheniformis JF-2, was isolated from oilfield injection water. In addition to producing most effective biosurfactants (CMC, 10 mg/l; interfacial tension of saline against decane lowered to 10^{-3} dynes/cm), *B. licheniformis* JF-2 had other properties such as being anaerobic, halotolerant and thermotolerant (Desai and Banat, 1997).

Makkar and Cameotra (1997a) observed good sand-pack oil recovery using strains of *Bacillus subtilis* at 45 ^oC. Biosurfactant produced by two strains of *Bacillus subtilis* (MTCC1427 and MTCC2423) accounted for 56% and 62% oil recovery from oil-saturated sand columns. The added advantage of being thermotolerant and stable over a wide range of pH values (4.5-10.5) make them suitable candidates for in situ MEOR (Microbial Enhance Oil Recovery). The two strains grew at 45 ^oC and utilized mollasses, cheap source of nutrient additive.

8.2 Biodegradation of hydrocarbon contaminants

Biodegradation of hydrocarbons by native microbial populations is the primary machanism by which hydrocarbons contaminants are removed from the

environment. Surfactant help degradation by solubilization or emulsification to release hydrocarbon sorbed to soil organic matter and increase the aqueous concentrations of hydrophobic compounds, resulting in higher mass transfer rates (Banat *et al*., 2000). Biosurfactant reduced the surface tension by accumulating at the interface of immiscible fluids, increasing the surface area of insoluble compounds which leads to increased bioavailability and subsequent biodegradation of the hydrocarbon (Barathi and Vasudevan, 2001).

Rhamnolipid biosurfactant from *Pseudomonas* enhanced octadecane dispersion and biodegradation and showed that 20% of octadecane was mineralized in 84 h in the presense of rhamnolipid per liter (Zhang and Miller, 1992). Van Dyke *et al*. (1993) demonstrated a 25 to 70% and 40 to 80% increase in the recovery of hydrocarbons from contaminated sandy-loam and slit-loam soil, respectively, by rhamnolipid from *P. aeruginosa.*

In an investigation of capability of polyaromatic hydrocarbon-utilizing bacteria to produce biosurfactants using naphthalene and phenanthrene. Daziel *et al*. (1996) detected biosurfactant production that was responsible for an increase in the aqueous solubility of naphthalene. It was observed when concentration of glycolipids exceeded the critical micelle concentration, which indicated by the lowered surface tension. This indicated a potential role for biosurfactant in increasing the solubility of such compounds. Zhang *et al*. (1997) tested the effect of two rhamnolipid biosurfactant on dissolution and bioavailability of phenanthrene and reported increase in both solubility and degradation rate of phenanthrene.

8.3 Food industry

Biosurfactants also have several applications in the food industry as food additives. Lecithin and it derivatives, fatty acid esters containing glycerol, sorbitol or ethylene glycol and ethyloxylated derivatives of monoglycerides including a recently synthesized oligopeptide are in use as emulsifiers in the food industry

worldwide (Besson and Michel, 1992). Other applications of biosurfactants are in bakery and meat products where they influence the rheological characteristics of flour or the emulsification of partially broken fat tissue (Fiechter, 1992).

Busscher *et al.* (1996) found that a biosurfactant produced by thermophilic dairy *Streptococcus* spp. could be used for fouling control of heat exchanger plates in pasteurizers, as they retard the colonization of *S. thermophilus* responsible for fouling.

8.4 Cosmetic industry

A broad potential application area is the cosmetic industry where surface–active substances are found in shampoo and many skin-care products (Fiechter, 1992). Biosurfactant have found a niche in the personal care market because of their moisterizing properties and skin compatability (Brown, 1991cited by Desai and Banat, 1997). Production of sophorolipids by microbial fermentation with yield up to 90 to 150 g/l has been documented (Lee and Kim, 1993). Monoglyceride, one of the widely used surfactants in 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 and Yamane, 1991).

8.5 Biosurfactant as therapeutic agents

Biosurfactants have some therapeutic applications. Rhamnolipid produced by *Pseudomonas aeruginosa*, lipopeptides produced by *B. subtilis* (Sandrin *et al.*, 1990), and *B. licheniformis* (Jenny *et al.,* 1991; Fiechter, 1992; Yakimov *et al.,* 1995) and mannosylerythritol lipids from *Candida antarctica* (Kitamoto *et al*., 1993) have been shown antimicrobial activities. Surfactin, one of the earliest known biosurfactants, has various pharmacological applications such as inhibiting fibrin clot formation and formation of ion channels in lipid membranes (Sheppard *et al.,* 1991). Thimon *et al.* (1995) described another anti-fungal biosurfactant,

Iturin, a lipopeptide produced by *B. subtilis*, which affects the morphology and membrane structure of yeast cells.

Objectives

1. To study factors affecting on growth and biosurfactant production by *Bacillus* MUV4
2. To extract and purify the biosurfactant from *Bacillus* MUV4
3. To study properties of biosurfactant from *Bacillus* MUV4
4. To apply biosurfactant from *Bacillus* MUV4 in oil recovery and antimicrobial activity