LITERATURE REVIEW

2.1 Biosurfactants in biotechnology

Chemically synthesized surfactants are widely used in industries. During the last few decades, there has been an increase in the use of biosurfactants mainly due to their biodegradability, production on renewable resources and functionality under extreme conditions particularly pertaining to tertiary crude oil recovery (Banat, 1995). Biosurfactant from microorganisms have a future because there are many different applications, each requiring a slightly different mix of properties, for example, in applications involving crude oil, where there is a broad range of types of oils and resevoir conditions and each requires a slightly different surfactant property (Cooper, 1986).

Biosufactants are classified as microbial compounds which exhibit surface activity (Lin *et al.*, 1994a). They include a variety of surface and interfacially active compounds which include glycolipids, lipopeptides, polysaccharide-protein complexes, phospholipids, fatty acids and neutral lipids (Lin *et al.*, 1994a).

New developments in biological sciences show great potential for the application of biosurfactants in industries (Kosaric *et al.*, 1987). The potential application of biosurfactants lies in their broadly functional properties which include emulsification, phase separation, wetting, foaming, solubilization, deemulsification and viscosity reduction associated with heavy crude oils (Fiechter, 1992).

Surface-wetting and solid dispersal are important properties for froth-flotation separation of ores or preparation of coal slurries for pipelining. Surfactants are also used for colloid preparations for paints and related products whereas the penetration rates of inks and dyes are determined by the surfactants for the pulp, paper and textile industries. Surfactants are used for foam stabilization in fire extinguishers (Cooper, 1986).

A very important aspect of microbially produced surfactant lies in their capacity to be modified by genetic engineering, biological or biochemical techniques which may allow the products to meet specific requirements including biodegradability (Fiechter, 1992).

2.2 Biosurfactants

A surfactant molecule is characterized by 2 functional parts: a hydrophilic (water soluble) or polar part, and a lipophilic (oil soluble) or non-polar part (Margaritis *et al.*, 1979). Biosurfactants are extracellular amphiphilic metabolites produced by bacteria, yeast and fungi (Fiechter, 1992). The hydrophilic part of the molecule may be a mono, oligo or polysaccharide, an amino acid, cyclic peptide, protein or phosphoester whereas the lipophilic portion usually consists of one or more long chained fatty acids, hydroxy fatty acid or alkyl-hydroxy fatty acids (Wagner, 1988; West & Harwell, 1992). The solubility of a surfactant in water depends on the number of -C-C bonds present in the lipophilic tail of the surfactant molecule. For example, if the chain length is less than 12 -C-C- bonds, the surfactant is water soluble because of the polar hydrophilic group carries with it the entire surfactant molecule. Whereas if the non-polar lipophilic tail has more than 16 -C-C- bonds, the surfactant tends to be insoluble in water (Margaritis *et al.*, 1979).

In aqueous solution, surfactants tend to aggregate into larger groups called micelles. At very low concentrations, the individual single molecules are present as their ions. As the surfactant concentration increases, a point is reached called Critical Micelle Concentration (CMC). At this point, there is an abrupt change in the solution properties, such as, surface tension, osmotic pressure, viscosity, density and electrical conductivity. Beyond the CMC, the surfactant molecules form aggregate called micelles. In the micelles, the surfactant molecules are oriented to form spherical aggregates with their lipophilic tails clustered together while the hydrophilic ends extends outward (Margaritis *et al.*, 1979).

Industrially, surfactants are known as surface active compounds capable of forming micelles. Micelles are explained by the "Oil Drop Model" (West & Harwell, 1992). They are generally pictured as 3-4 nanometer diameter droplets of oil with a polar or ionic coating. Micelle formation only occurs above a critical concentration of surfactant monomers which is referred to as the Critical Micelle Concentration (CMC) (West & Harwell, 1992). The efficiency of a biosurfactant is gauged by its Critical Micelle Concentration (CMC) which is in effect the solubility of a surfactant within an aqueous phase (Fiechter, 1992).

Amphiphilic molecules associate readily to form supramolecular structures such as micelles, bilayers and vesicles at concentrations above the Critical Micelle level (Fig. 1). These are held together by forces that include hydrophobic, van der Waals, electrostatic and hydrogen bonding (Georgiou *et al.*, 1992).

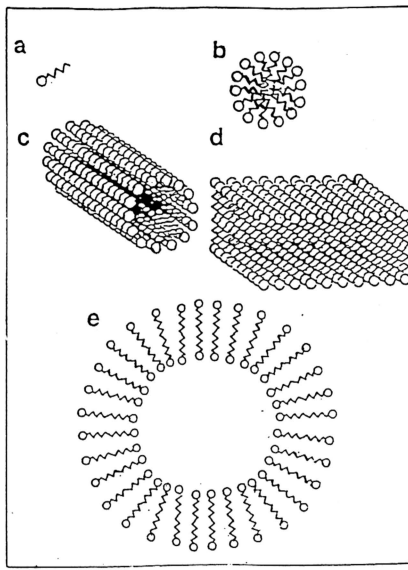

Fig. 1: Surfactants are characterized by an amphipathic structure. Hydrophobic and hydrophilic properties depend on the charge of the polar group (anionic, cationic, neutral or amphoteric). They tend to associate at interfaces or in micelles, favouring a minimal free energy charge of the system. The minimal surface-tension value attained due to the activity of the surfactant, and the critical micelle concentration (cmc) required, give a measure of the efficiency of the surface-active compound. (a) Surfactant monomer, denoted by a cycle representing the hydrophilic head attached to a hydrocarbon tail; (b) Circular micelle; (c) Rod-shaped micelle; (d) Micellar layer and (e) Vesicle representation (Fiechter, 1992).

2.3 Classification of biosurfactants

Biosurfactants can be classified into 6 major groups; the glycolipids, lipopeptides or lipoproteins, phospholipids, neutral lipids, substituted fatty acids and lipopolysaccharides (Jenny *et al.*, 1991). The majority of surfactants are either negatively charged or neutral. The negative charge is attributed to the presence of carboxylate, phosphate or sulphate groups whereas the cationic surfactants usually contain amine functions (Cooper, 1986).

A large number of hydrocarbon utilizing microorganisms produce surfactants belonging to the class of glycolipids (Lang & Wagner, 1987). These include sophorose lipids from *Torulopsis bombicola* (Cooper & Paddock, 1984), Rhamnolipid from *Pseudomonas aeruginosa* (Reiling *et al.*, 1986), Trehalosetetraesters from *Rhodococcus erythropolis* (Ristau & Wagner, 1983), Mannosylerythritol lipids from *Candida antartica* (Kitamoto *et al.*, 1992) and *Shizonella* sp., and cellobiose lipids from *Ustilago zeae* (Fiechter, 1992). Figures 2 and 3 show the structure of sophorose lipid surfactants in 2 different forms produced by *T. bombicola*.

The amino acid containing lipid biosurfactants include lipopeptides and ornithine lipids (Cooper & Zajic, 1980). The most potent and best studied surfactant in this class is surfactin (Fig. 4), an acidic surfactant produced by *Bacillus subtilis*. Structural analysis indicates that surfactin consists of 3-hydroxyl-13-methyl-tetradecanoic acid amidated to the N-terminal amine of a heptapeptide. The ionizable side chains of glutamic and aspartic acids probably contribute significantly to the excellent surface active properties of the molecule (Georgiou *et al.*, 1992).

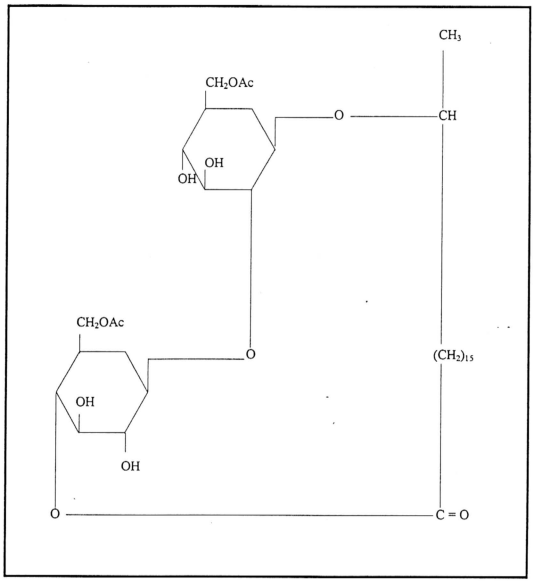

Fig. 2: Lactonic sophorose lipid (Lang & Wagner, 1987)

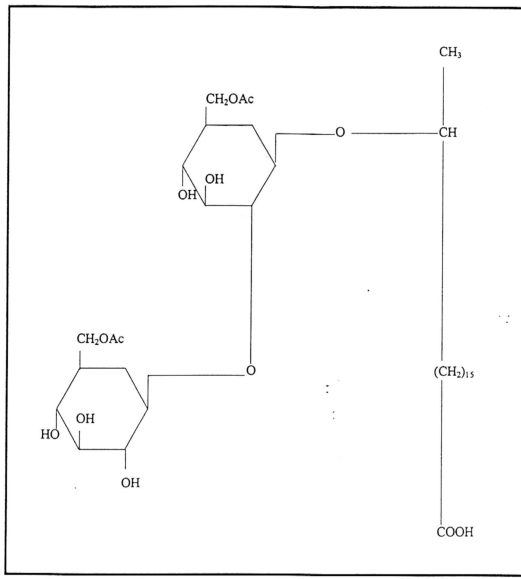

Fig. 3: Acidic sophorose lipid (Lang & Wagner, 1987)

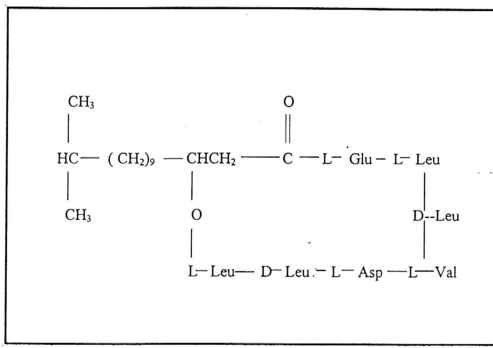

Fig. 4: Structure of surfactin from *Bacillus subtilis* (Cooper, 1986)

Ornithine lipids include lipopetides synthesized by *Thiobacillus thiooxidans* (Fig. 5), (Lang & Wagner, 1987), Orthinine-taurine lipid or cerilipin by *Glucanobacter cerinus* and lysine containing lipid of *Agrobacterium tumefaciens* (Fiechter, 1992). Cerilipin from *G. cerinus*, a zwitterionic surfactant is unique as it contains a sulphate group (Cooper, 1986). The lipid from *P. rubescens* is zwitterionic, having both a free carboxyl group and a free amine group whereas *Thiobacillus thiooxidans* produces a similar ornithine lipid but with different carboxylic acids (Cooper & Zajic, 1980).

Certain microorganisms have the ability to change the structure of their cell wall by building lipopolysaccharides or non-ionic surfactants into their cell walls (Syldatk & Wagner, 1987). *Acinetobacter calcoaceticus* RAG-1 is the source of Emulsan, an extracellular lipoheteropolysaccharide polyanionic bioemulsifier (Pines & Gutnick, 1986). The majority of polysaccharides are located predominantly in the outer membrane of Gram negative bacteria which exert remarkable surfactant activity. The cell walls of yeast such as the alkane-assimilating *Candida tropicalis* contain a polysaccharide-lipid complex related to alkane transfer through the cellular membrane (Fiechter, 1992).

The remaining surface active compounds are polymeric which are often referred to as bioemulsifiers. These are polysaccharides which often contain protein or carboxylic acids (Cooper, 1986). Protein-like substances are known to possess biosurfactant activity. Serraphobin, a 70 kDa protein, isolated from culture broth, cell surface and culture supernatants of *Serraphobin marcescens* binds to hexadecane droplets (Bar-Ness & Rosenberg, 1989).

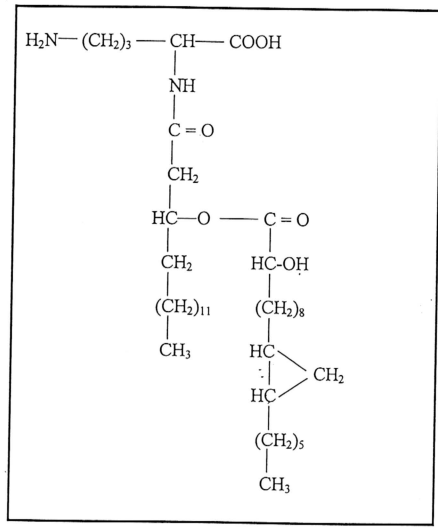

Fig. 5: The structure of ornithine containing lipid from *Thiobacillus thiooxidans* (Lang & Wagner, 1987)

2.4 *Bacillus licheniformis* JF-2.

Bacillus licheniformis JF-2 was isolated from water injection brine obtained from an oil well in Carter County, Oklaholma (Javaheri *et al.*, 1985; Banat, 1995). This bacterium is a Gram positive, spore forming motile rod, 1.5 - 2.0 μm in length and 0.7 - 1.0 μm in width (Plate 1). The spore is oval and subterminally positioned (McInerney *et al.*, 1985).

A lipopetide biosurfactant is produced when *B. licheniformis* JF-2 is grown under aerobic or anaerobic conditions (Lin *et al.*, 1993). This lipopeptide consists of a heterogenous C_{15} fatty acid tail linked to a peptide moiety (Fig. 6) similar to surfactin (Fig. 4) which has a molecular weight of 1035. This biosurfactant exhibited a Critical Micelle Concentration (CMC) of 10 mg/L and reduced the interfacial tension of the fermentation broth to 0.006 dyne/cm (Lin *et al.*, 1994a).

Chemical analysis, immunological reactivity and NMR studies conducted by Lin *et al.* (1994a) showed that the chemical structure of the peptide moiety of the biosurfactant from *B. licheniformis* JF-2 (Fig. 6) is similar to that of surfactin from *B. subtilis* (Fig. 4). The presence of a family of lipopeptides with the same peptide domains but different chain lengths of lipid tails, which has been observed for lipopeptides produced by other microorganisms was not seen for the biosurfactant produced by *B. licheniformis* JF-2. The lipid tail of the JF-2 biosurfactant was found to be in 3 different forms; n-, iso- and anteisoforms by ^{13}C NMR analysis. The anteisoform of fatty acids has not been observed in surfactin or other lipopeptide surfactants. This difference in the lipid moieties between surfactin and the JF-2 biosurfactant does not affect the interfacial activities (Lin *et al.*, 1994a).

Plate 1: Photomicrograph of *Bacillus licheniformis* JF-2 (magnified 1000x)

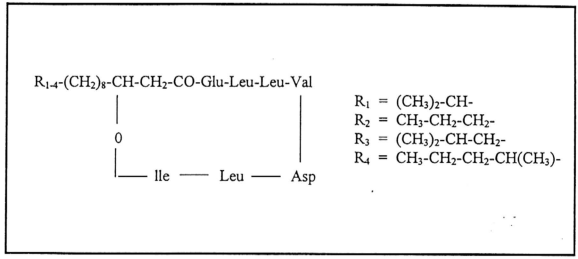

Fig. 6: Structure of the surface-active lipopeptide of *Bacillus licheniformis* JF-2 (Fiechter, 1992)

2.5 The industrial use of *Bacillus licheniformis*.

The fermentation industry has for many years used microorganisms isolated from nature to produce antibiotics, enzymes, amino acids and other useful products (de Boer *et al.*, 1994). *Bacillus licheniformis* is widely distributed in the environment which includes common soil, nutrient poor soils such as moorland and desert (de Boer *et al.*, 1994). The genus *Bacillus*, comprises of five physiologically similar species. These are *B. amyloliquefaciens, B. atrophaeus, B. licheniformis, B. pumilis* and *B. subtilis*. Phenotypically, these bacteria are very similar. However, DNA homology studies indicate that *B. licheniformis* shares an average of 9-15% homology with *B. amyloliquefaciens* and 8% with *B. pumilus* (Seki & Oshima, 1989).

Bacillus licheniformis is listed in the third edition of the Food Chemical Codex as a source of protease and carbohydrase used in food processing (de Boer *et al.*, 1994). In the biotechnology industry, *B. licheniformis* produces a variety of extracellular enzymes (Bulthuis *et al.*, 1991). Industrially, the α-amylase has been used for the liquefaction of starch at temperatures above 90°C (Dobreva *et al.*, 1994). Carbohydrase and protease enzymes produced by *B. licheniformis* have been affirmed as Generally Recognised As Safe (GRAS) by the Food and Drug Administration (FDA) of the United States (de Boer *et al.*, 1994).

An α-amylase gene from *B. licheniformis* CIB-25 has been cloned and expressed in *Pischia pastoris*. The cloning of the α-amylase gene with its own signal sequence was under the control of the alcohol oxidase 1 (AOX 1) promoter from *P. pastoris* using integrative construction. This was integrated

with high frequency at the AOX 1 locus by gene replacement homologous recombination which led to increased α-amylase amounting to 0.9 g/L in the transformed yeast (Margolles *et al.*, 1992). Studies have shown that *B. licheniformis* is used as an alternative host for the expression of cloned gene products on an industrial scale (de Boer *et al.*, 1994).

Bacillus spp. generally produce a variety of antibiotics of which bacitracin, gramicidin S and tyrocidine are the best studied (Lebbadi *et al.*, 1994). Various strains of *B. licheniformis* have been used in the production of the peptide antibiotic bacitracin (Herzog-Velikonja *et al.*, 1994). Bacitracin A, an antibiotic polypeptide is synthesized by three functional enzymes; bacitracin synthetase (BA) 1, 2 and 3 (Herzog-Velikonja *et al.*, 1994). Other antibiotics produced by various strains of *B. licheniformis* include the phosphorus containing triene, proticin, bacilysin and the licheniformins (Lebbadi *et al.*, 1994).

Bacillus licheniformis occurs in agricultural products such as cereals, and the spores are found in processed or dried food such as cocoa and herbs (Priest, 1989). A study by Sanni & Ogbonna (1991) on Owoh, a Nigerian seasoning from the fermentation of cotton seeds was found to contain *B. licheniformis, B. pumilus* and *Staphylococcus* spp. which caused an increase in the content of total soluble amino acids and total sugar. Studies conducted by Ho *et al.* (1984) on soysauce fermentation in Malaysia showed that *B. licheniformis* JF-2, *Pediococcus halophilus, Saccharomyces rouxii, Torulopsis* spp. and *B. subtilis* occur in the brined fermentation stage producing the specific flavours of soy sauce. The fermentation is dependent on selection of desirable organisms from the environment by specific physical and chemical

parameters present in the various phases of fermentation process. Okpiye, a food condiment, prepared by 96 hours of natural fermentation of *Prosopis africana* seeds in the laboratory was characterized by the growth of microorganisms to 10^6 - 10^8 CFU/g substrate. *Bacillus licheniformis* was among the species of bacteria found which included other *Bacillus* species (Achi, 1992).

The detergent industry produced detergents at 1300 - 1500 tonnes per year which was worth US $300 million. The detergent enzymes used were protease subtilisin Novo by *B. amyloliquefaciens* and *B. licheniformis* (Hubner *et al.*, 1993). *Bacillus licheniformis* is non-pathogenic to humans and the industrial use of recombinant *B. licheniformis* has the approval of authorities in the United States, Europe and Japan for production with and products from recombinant *B. licheniformis* (de Boer *et al.*, 1994).

2.6 Biosynthesis of biosurfactants

The biosynthesis of biosurfactants involves a range of pathways. This is due to the wide variety of biosurfactants that may be produced. Three classes of pathways are distinguished depending on whether the hydrophobic moiety, hydrophilic moiety or both, are synthesized *de novo*. Components that are not synthesized *de novo* are produced by the modification of the carbon source or a variety of different carbon substrates may be incorporated into the surfactant giving rise to a family of related structures (Georgiou *et al.*, 1992).

Trehalose lipids synthesized by *Rhodococcus erythropolis* are typical of compounds in which the fatty acid moiety depends on the chain length of the alkane feed whereas the hydrophilic moiety is not affected by the carbon

substrate (Kretschmer & Wagner, 1983). In the enzymatic reaction which is probably rate limiting, the fatty acid (corynomycolic acid) is esterified to trehalose-6-phosphate, which is subsequently subject to dephosphorylation and further modification (Georgiou *et al.*, 1992). Fructose lipids are produced when *Arthrobacter paraffineus* is grown on fructose as the sole carbon source whereas glucose and sucrose lipids predominate in sucrose grown cultures. The surfactant produced by *A. paraffineus* represents the class of compounds in which the hydrophilic moiety is influenced by the carbon source (Suzuki *et al.*, 1974; Itoh & Suzuki, 1974).

2.7 The physiology of microorganisms affecting the yield of biosurfactants

It is well established that the physiology of a microorganism is not exclusively determined by its genetic information but is also a function of the environmental conditions (Harder & Dijkhuizen, 1983; Meyer *et al.*, 1985). To increase commercial interest, the yield of biologically produced surfactants must be improved. Methods which are widely used to enhance production are strain selection, manipulation of environmental and nutritional factors which bring about marginal increase in production (Mulligan *et al.*, 1989).

2.7.1 Culture conditions affecting the yield of the lipopeptide biosurfactants

The lipopeptide biosurfactant produced by *B. licheniformis* JF-2 is quite similar to surfactin produced by *B. subtilis*. Both surfactants are ionic and contain an amino group and a lipid moiety (Javaheri *et al.*, 1985). The production of the biosurfactant depends on the age and density of the inoculum used (Lin *et al.*, 1994b).

Serial transfer of *B. licheniformis* JF-2, between three to four times, increased the proportion of cells that produced round colonies rather than volcano shaped colonies. Liquid cultures inoculated with cells from round colonies had higher surface tension (approximately 45 mN/m) than those inoculated from volcano shaped colonies (< 30 mN/m). The round colonies are variants of JF-2 and not contaminants (Javaheri *et al.*, 1985). Further studies by Javaheri *et al.* (1985) indicated that serial transfer of cultures above 18 generations under both aerobic and anaerobic conditions produced a biosurfactant which reduced the surface tension of water to 27 mN/m, to a new minimum value of 44 mN/m. This increase in surface tension may be due to the selection of variants which produced lower levels of the biosurfactants (Javaheri *et al.*, 1985).

2.7.2 Nutritional factors affecting the yield of the biosurfactant

Growth of microorganisms under nutrient limitation other than a carbon source may lead to a significant accumulation of intracellular reserve material, extracellular polymers or a variety of low molecular weight metabolites (Guerra-Santos *et al.*, 1986).

The production of surfactin, which is similar to the biosurfactant produced by *B. licheniformis* JF-2 was necessary for the development of DNA transfer competence in *B. subtilis* (Nakano *et al.*, 1991). The biosurfactant from *B. licheniformis* JF-2 may be transported in the cytoplasm following its uptake by cells and this may serve as a signal for development (Lin *et al.*, 1993).

Lipopeptide synthesis and deactivation via internalization are two competing processes which determine the concentration of the biosurfactant in the growth medium (Lin *et al.*, 1993). The addition of phosphate at a concentration of 25 mM was found to inhibit the disappearance of the biosurfactant from the cultivation medium with more than 40% of the maximum amount of biosurfactant present after 24 hours of fermentation compared to the cultivation medium containing higher concentrations of phosphate of up to 100 mM which showed a complete loss of the biosurfactant within 24 hours after inoculation (Lin *et al.*, 1993). Further studies by Lin *et al.* (1993) indicated an increase in the biosurfactant yield from 90 mg/L to 110 mg/L in a mineral salts medium containing 50 mM phosphate compared to 100 mM of phosphate. However, lower concentrations resulted in a decrease in production although the cell growth was not affected.

The optimal concentration of ammonium sulphate for maximum production was 7.5 mM (Lin *et al.*, 1993). The addition of magnesium sulphate from 0.25 mM to 5 mM in mineral salts medium did not affect cell growth but increased the biosurfactant yield from 70 to 97 mg/L (Lin *et al.*, 1993). Higher concentrations of $MgSO_4$ inhibited the removal of biosurfactant from the fermentation broth. When magnesium sulphate was replaced with magnesium chloride, similar findings were observed suggesting that the inhibition of biosurfactant removal from the cultivation medium by *B. licheniformis* JF-2 was caused by magnesium and not by other divalent cation or the counter ion (Lin *et al.*, 1993).

Studies by McInerney *et al.* (1985) showed that lichenysin, the crude lipopeptide biosurfactant from *B. licheniformis* JF-2 was not substantially

inhibited by NaCl concentrations of up to 15g/100mL. This relative insensitivity to NaCl should be particularly effective as a surfactant in the briny millieu permeating most oil formations (averaging about 5% NaCl) and lichenysin appeared to be maximally effective as a surfactant at about 5% NaCl concentration.

2.7.3 Carbon source for biosurfactant production

The structure and yield of microbial surfactants depend largely on the choice of the carbon source. There are microorganisms which produce biosurfactants only when grown on hydrocarbons while and others require simple water soluble substrates such as carbohydrates and amino acids (Robert *et al.*, 1989). In the case of *P. aeruginosa*, the surface active agents are produced when grown with water - soluble or water - insoluble substrates, although in the latter case, production is higher (Rapp *et al.*, 1979).

Biosynthesis of biosurfactants are often associated with microbial growth on hydrocarbons or other lipophilic substrates. The physiological role of biosurfactants in hydrocarbon metabolism is connected with the mechanism for the interaction of the lipophilic substrate and the microbial cell (Wagner, 1988). Other carbon substrates that can be utilized for microbial synthesis of biosurfactants include fats, oils, glycerol or carbohydrates (Haferburg *et al.*, 1986). Oil degrading *Arthrobacter* sp. RAG-1 produced an extracellular non-dialyzable emulsifying agent when grown on hexadecane, ethanol or acetate. Emulsifier production was parallel to growth on either hydrocarbon or non-hydrocarbon substrates during the exponential phase. However, production continued after growth had ceased (Rosenberg *et al.*, 1979). Davila and

coworkers (1992) managed to produce 320 g/L of sophorose lipids with a weight of 65% with respect to the carbon source by growing *Candida bombicola* CBS 600 using glucose and ethyl esters of rapeseed fatty acids. When *Corynebacterium lepus* was grown in fed batch fermentation with kerosene as the sole carbon source, 3 g/L of lipopeptide was produced which contained corynomycolic acids and small amounts of phospholipids and neutral lipids (Cooper *et al.*, 1979).

Lin *et al.* (1993) investigated the production of biosurfactant from *B. licheniformis* JF-2 and found that glucose was the preferred carbon source compared to glycerol where no biosurfactant was produced in the latter even though good cell growth was achieved. Javaheri *et al.* (1985) showed that *B. lichenifromis* JF-2 produced a biosurfactant which decreased the surface tension of a mineral salts medium containing glucose-yeast extract with sodium nitrate ($NaNO_3$), when grown anaerobically. While glucose and sucrose supported the growth of *B. licheniformis* JF-2 oil did not (Jenneman *et al.*, 1983).

Bacillus licheniformis BAS 50 isolated by Yakimov *et al.* (1995) used a variety of carbon sources for growth. These included arabinose, fructose, galactose, glucose, mannitol, methyl-D-glucoside, salicin, sorbitol, starch and xylose. The lipopeptide biosurfactant termed lichenysin A from *B. licheniformis* BAS 50, obtained from stationary phase cells was approximately 160 mg/L in glucose or sucrose medium and, 70 mg/L in Cooper's medium supplemented with other sugars (Yakimov *et al.*, 1995). Molasses of up to 4% (w/v) in Cooper's medium or injection water with a NaCl concentration of 130 g/L supplemented with 0.05% (w/v) yeast extract and 0.1% sodium

nitrate ($NaNO_3$) supported the growth and biosurfactant yield of *B. licheniformis* BAS 50 upto 50°C (Yakimov *et al.*, 1995).

Lichenysin A showed a lowering surface tension at high NaCl concentrations of up to 30% (w/v) and has a CMC of 12 mg/L. The CMC of lichenysin A (12 mg/L) is 2 times lower than that of surfactin (25 mg/L) and lichenysin B (20 mg/L) (Yakimov *et al.*, 1995).

The use of safflower oil and glucose in the cultivation medium of *Torulopsis bombicola* produced 70 g/Lof sophorose lipid which corresponded to 35% conversion of the substrate on a weight loss basis (Cooper & Paddock, 1984). *Torulopsis bombicola* also produced this sophorose lipid in similar yields when grown on sucrose (Klekner *et al.*, 1991). The maximum emulsifier obtained from *P. fluorescens* when grown in a medium containing gasoline was 233 μg/mL as compared to only 164 μg/mL when glucose was used as a growth substrate (Desai *et al.*, 1988). *Nocardia corynebacterium* SM1 when grown on n-alkanes, under nitrogen limiting conditions synthesized a pentasaccharide lipid with remarkable surface and interfacial properties (Powalla *et al.*, 1989).

Nocardia corynebacterium ATCC 4277 grown in mineral salts medium at 4% hexadecane produced a mixture of biosurfactants with most of the activity is due to neutral lipids. The residual surface activity after pentane extraction are due to a mixture of polar lipids and pentane insoluble neutral lipids (Macdonald *et al.*, 1981). *Torulopsis apicola* IMET 43747 cultivated on mineral salts medium, containing n-alkanes, or hydrocarbons, produced a mixture of water-soluble biosurfactants which gave surface tension values of around 30 mN/m. The concentration of the hydrophobic carbon source

determines the conversion of available carbon into biosurfactants. However, the supplementation of additional hydrocarbon to stationary cells initiated no further increase in biosurfactant production. The addition of hydrocarbons or vegetable oils to stationary cells grown on glucose did, however, result in an increase in the production of surfactants (Hommel *et al.*, 1987).

Torulopsis bombicola (ATCC 22214) produced sophorose lipids up to 80 g/L in batch culture containing 11% glucose and 10% soybean oil as carbon and energy sources. According to the carbon mass balance analysis, 13% and 37% of input carbon were channeled to cells and to products, respectively and 50% of the total input carbon was channeled to CO_2 in batch culture. In fed batch culture with intermittent oil feeding, the carbon fractions incorporated into sophorose lipid and cells were 60% and 12%, respectively, and the carbon fraction evolved as CO_2 was 30%. The yield of sophorose lipids based on total input carbon increased from 0.37 g/g-substrate in batch culture to 0.6 g/g-substrate by employing a fed-batch culture (Lee & Kim, 1993).

2.8 Surfactants in the petroleum industry

The demand for petroleum as a source of energy and primary raw material in the chemical industry has resulted in an increase in the world production of over 2,400 million metric tonnes per year (Gutnick & Rosenberg, 1977). Due to the increase in the production of crude oil, numerous related problems have arisen largely with respect to the transportation, accidental spills and deliberate discharge of ballast and wash waters from oil tankers (Gutnick & Rosenberg, 1977). Chronic or accidental pollution of oceans due to spillage of hydrocarbons accounts for 6.3 x

10^9 kg/year. Biological techniques such as seeding has been used to accelerate the biodegradation of naturally occurring hydrocarbons (Dumenil *et al.*, 1988).

Crude oil (5-100 μg/L) contains mutagenic, carcinogenic and growth inhibiting chemicals. Certain petroleum fractions destroy microalgae and other forms of microorganisms. Thus, oil pollution in the ocean and coastal waters presents serious problems to marine life, recreational resources and public health (Gutnick & Rosenberg, 1977). A wide variety of petroleum degrading microorganisms which include bacteria, yeasts, and fungi have been identified and characterized (Chakrabarty, 1985). They are generally emulsifiers which are biological in nature and are mediated either by the cells themselves or by the production of extracellular products.

The production of emulsifying agents and subsequent oil droplet formation enhances pseudo-solubilization of hydrocarbons (Gutnick & Rosenberg, 1977). These have similar physical characteristics to synthetic surfactants and are proposed for the removal of adsorbed compounds from soils and the mobilization of separate phases and immiscible petroleum hydrocarbons (Falatko & Novak, 1992).

Emulsifying agents or surfactants are generally used for the clean up of oil drums, the dispersion of oil slicks (Chakrabarty, 1985), enhanced oil recovery (Javaheri *et al.*, 1985), petroleum deasphalting and viscosity control (Kosaric *et al.*, 1987). Biosurfactants may be used in conjunction with *in-situ* degradation of petroleum hydrocarbons during aquifer and oil remediation (Falatko & Novak, 1992).

The effectiveness of a biosurfactant can be determined by measuring the reduction in surface tension and interfacial tension at the oil-water interface (Chakrabarty, 1985). Several biosurfactants have the ability to reduce the interfacial tension between oil and brine to 0.01 mN/m. This is useful in enhanced oil recovery (Javaheri *et al.*, 1985). They are also used as rock wetting agents in deemulsification and viscosity reduction of heavy crude oil (Guerra-Santos *et al.*, 1984).

The extraction of residual oil from natural oil fields by injection of pressurized water down a new well. The water which displaces the oil and pushes it to the surface through one of these processes can be maximized by increasing the viscosity and decreasing the surface tension of the water used (Ventosa & Nieto, 1995). Biosurfactants produced by many microorganisms reduce the interfacial tension of brine and oil to less than 0.01 mN/m making them potential candidates for Enhanced Oil Recovery (EOR). A majority of these biosurfactants are produced by aerobic microorganisms which would not be suitable for *in-situ* microbially enhanced oil recovery processes since most oil reservoir are anaerobic (Javaheri *et al.*, 1985).

Bacillus licheniformis JF-2 isolated from oil field injection water (Jenneman *et al.*, 1983), is suitable for *in-situ* microbially enhanced oil recovery processes due to its ability to produce the biosurfactant under anaerobic conditions at temperatures and salinities found at many reservoirs. It has a low CMC and generates low interfacial tension (Javaheri *et al.*, 1985).

Bacillus licheniformis JF-2 is suitable for EOR as it is able to grow in NaCl of up to 10% (w/v) over a pH range of 4.6 to 9.0 and is not inhibited by the presence of crude oil (Jenneman *et al.*, 1983). Anaerobically grown cells

produce the biosurfactant during the exponential phase of growth (Javaheri *et al.*, 1985).

Bacillus licheniformis BAS 50 isolated from a petroleum reservoir at a depth of 1500 m by Yakimov *et al.* (1995) grew in Cooper's medium with a range of 0 to 13% (w/v) NaCl, between 25°C to 55°C and at pH between 5.4 and 8.5. Growth under anaerobic conditions resulted in lower biomass and biosurfactant yields, and was influenced by the temperature and salt concentration with optimal growth and surfactant production in Cooper's medium with 5% NaCl at 4° to 45°C (Yakimov *et al.*, 1995). The lowering of surface tension by the purified lipopeptide termed Lichenysin A produced by *B. licheniformis* BAS 50 was not inhibited by NaCl concentrations of upto 10% (w/v) but was inhibited at 30% (w/v) NaCl (Yakimov *et al.*, 1995).