CHAPTER II

THEORETICAL BACKGROUND AND LITERATURE REVIEWS

2.1 Surfactant (surface-active agent)

Surfactant was substance which lowers the surface tension of the medium in which it was dissolved (IUPAC Compendium of Chemical Terminology). In the generally, surfactants were organic molecules with different end portions (moieties). One end of the molecule was distinctly hydrophilic, while the other one was hydrophobic. The hydrophilic moiety allows the surfactant to be soluble in water, while the hydrophobic moiety was more soluble in a nonpolar environment. The most energetically favorable position for such a molecule is at the interface between the aqueous phase and a nonaqueous phase (Figure 2.1).

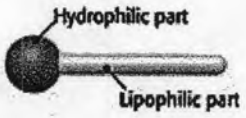

Figure 2.1 Structure of surfactant which composes of head (hydrophilic) and tail (lipophilic) part

A surface-active agent could be classified by the presence of synthetic. Biosurfactants were natural products derived from bacteria, yeasts, or fungi. The complex chemical structures and physical properties of biosurfactants generally resulted in properties equal to, or exceeding, many synthetic surfactants. Synthetic surfactant can be separated chemical surfactant and natural surfactant (Figure 2.2). For example of chemical surfactant such as soaps or detergents, were amphiphilic molecules that had both water-like and oil-like regions to their molecule. Natural

surfactant was extracted from environmental such as moisturizer that extracted from strawberry (www.the-body-shop.com).

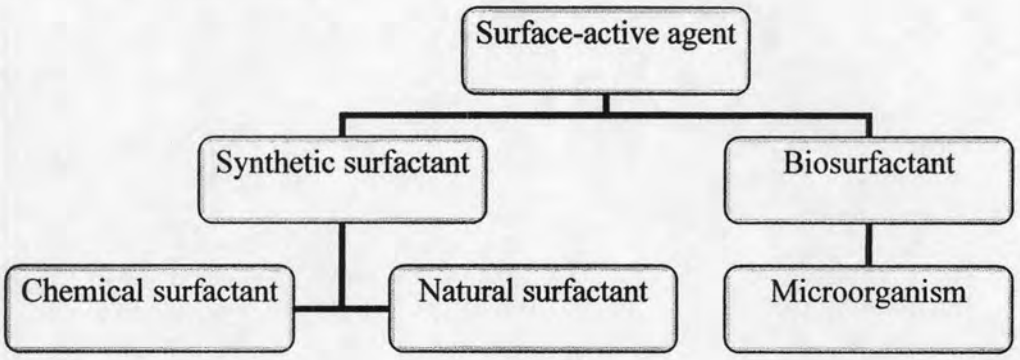

Figure 2.2 Classification of surfactants (surface-active agent)

Surface-active agent preparations were now widely used and have been credited with recent improvements in overall infant mortality (Horbar *et al.*, 1993; Schwartz *et al.*, 1994) which was indispensable components of daily life. A wide variety of surface-active agent preparations have been developed and tested. These include synthetic surfactants and surfactants derived from animal or plant sources (Tooley *et al.*, 1987). They were widely used in the pharmaceutical, cosmetic, petroleum, and food industries. Many different types of surfactants were already being used in industry, but it was important to develop even more new compounds to broaden the spectrum of specific properties and applications (Cameotra and Makkar, 1998). The surfactant industry now exceeds US$ 9 billion per year (Desai and Banat, 1997). Most of these compounds were chemically synthesized and were of petroleum origin.

2.2 Synthetic surfactant

Synthetic surfactants were amphiphilic molecules consisting of a hydrophilic and a hydrophobic domain. The non-polar, hydrophobic part was frequently a hydrocarbon chain. The polar component appears in many variations (Georgiou *et al.*,

1992). The most common non-ionic surfactants were ethoxylates, ethylene and propylene oxide co-polymers and sorbitan esters. Examples of commercially available ionic surfactants included fatty acids, ester sulphonates or sulphates (anionic) and quartenary ammonium salts (cationic). In addition to, synthetic surfactant could reduced surface and interfacial tensions by accumulating at the interface of immiscible fluids and increase the solubility and mobility of hydrophobic or insoluble organic compounds (Prince *et al.*, 2002; Ron and Rosenberg, 2002; Mulligan, 2005).

Synthetic surfactants could be classified according to their processes for production and sources that affected their physicochemical properties (Tiehm, 1994).

2.2.1 Chemical surfactant

Petrochemical surfactants were derived from crude oil and were also known as "chemical" surfactants. The surfactant industry currently used roughly equal amounts of "natural" oleochemicals and "synthetic" petrochemicals (Stalmans and Cavalli, 1993).

Chemical surfactants could mimic the latter effects of biosurfactants and have been exploited, for example, as antimicrobial agents in disease control and to improve degradation of chemical contaminants. Both chemical- and biosurfactants were potentially toxic to specific microbes and may be exploited as antimicrobial agents against plant, animal and human microbial pathogens (Boyette *et al.*, 2001; Cameotra and Makkar, 2004).

2.2.2 Natural surfactant

Oleochemical surfactants, also referred to as "natural," are derived from plant oils such as palm, palm kernel or coconut oil or from animal fats such as tallow, lard or fish oil. Fish oil was no longer used as an oleochemical feedstock, and

animal fats had lost ground in recent years. In contrast, vegetable oils have been gaining ground.

2.3 Biosurfactant

Biosurfactants (microbial surface-active agents) were biological compounds that exhibited high surface active properties (Georgiou *et al.*, 1992). Yeast or bacteria from various substrates including sugars, oils, alkanes and wastes (Lin *et al.*, 1998), biologically produced some surfactants, known as biosurfactants. Microorganisms, plants and animals, including humans, produced them. Biosurfactants had both hydrophilic and hydrophobic (nonpolar) portions in the molecule. They were amphipathic molecules enabling the formation of specialized structures vital to their action. They function by residing at the oil–water interface. The hydrophilic (polar) part of a biosurfactant was usually referred to as the "head", whereas the nonpolar hydrophobic portion was known as the "tail". The latter was a hydrocarbon chain of varying length in different surfactants.

2.3.1 Physicochemical properties

Effective physicochemical properties such as critical micelle concentration (CMC) and temperature stability were characteristics of these compounds. The CMC of biosurfactants had range from 1 to 2000 $mg.l^{-1}$. Surface tension of good biosurfactants was less than 30 $mN.m^{-1}$ (Mulligan and Gibbs, 1993). Other distinct advantages of biosurfactants over synthetic surfactants include higher specificity, lower toxicity, higher biodegradability, better environmental compatibility (Mulligan *et al.*, 2001), and the ability to be synthesized from renewable feedstock.

2.3.1.1 Critical micelle concentration (CMC)

When the aqueous surfactant concentration exceeds a certain level, surfactant molecules self-aggregate into spherical structures known as micelles, which contain fifty or more surfactant molecules. Micelles form when the surfactant concentration exceeded the CMC. Micelle formation was unique to surfactant molecules and differentiates them from alcohols, which done not form such aggregates. Biosurfactant micelles increased the aqueous concentration of low-solubility organic compounds by providing a hydrophobic region into which organic compounds could partition. The micelle concentration increased with increasing biosurfactant concentrations above the CMC. The apparent solubility of the contaminant correspondingly increases. Biosurfactant concentrations well above the CMC (e.g., 10 to 20 times the CMC or more) were used to maximize contaminant solubility and extraction efficiency. The CMC of the biosurfactants generally range from 1 to 200 $mg.l^{-1}$ and their molecular mass was from 500 to 1500 Da (Li *et al.*, 1984). They could be potentially as effective with some distinct advantages over the highly used synthetic surfactants including high specificity, biodegradability and biocompatibility (Cooper and Goldenberg, 1987). For example, glycolipids from *Rhodococcus* species 413A were 50% less toxic than Tween 80 in naphthalene solubilization tests (Kanga *et al.*, 1997).

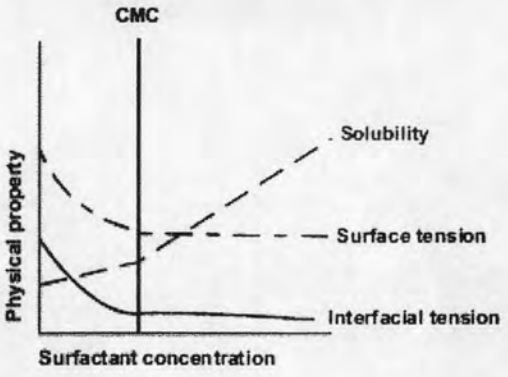

Figure 2.3 Surface tension, interfacial tension and solubilization as a function of surfactant concentration (CMC represents critical micelle concentration) (Mulligan, 2005).

2.3.1.2 Biosurfactants activity on temperature and pH

Some strains of *Bacillus licheniformis* were reported in the literature for production of biosurfactant under aerobic and anaerobic conditions at very wide range of temperatures and in the presence of high salt concentrations of salts but we could not find any report on their ability to degrade hydrocarbons (Jenneman *et al.*, 1983; Jenny *et al.*, 1991; Lin *et al.*, 1994; Thaniyavarn *et al.*, 2003). Jenneman *et al.* (1983) isolated *Bacillus licheniformis* JF-2 from oil field injections which produced a very effective extracellular biosurfactant under aerobic and anaerobic conditions at a very wide range of temperatures and in the presence of high salt concentrations of salts (Javaheri *et al.*, 1985). The biosurfactant isolated from strain JF-2 was further characterized by Lin *et al.* (1994) and found it to be lipopeptide in nature. Yakimov *et al.* (1995) isolated another strain of *Bacillus licheniformis* BAS50 from a petroleum reservoir which produced lipopeptide surfactant on a variety of substrates in the presence of high salt concentrations (13% wv^{-1} NaCl) and temperatures 35 and 45°C. They proposed its suitability for *in-situ* applications in microbially enhanced oil recovery. Thaniyavaran *et al.* (2003) isolated *Bacillus licheniformis* F2.2 from

fermented food with the ability to produce biosurfactant and grew at high salinity and temperature. This strain was reported to produce a new non-lipopeptide type biosurfactant together with lipopeptides, pilpastatin, and surfactin. Maugeri *et al.* (2002) isolated a halophilic thermotolerant *Bacillus licheniformis* B 3-15, which could utilize kerosene and gasoline as sole source of carbon and produced an exopolysaccharide.

Stability of emulsion in presence of salt and pH has been reported as one of the properties of the biosurfactant produced by *Bacillus licheniformis* JF-2 (McInerney *et al.*, 1990) and *Aeromonas* sp. (Ilori *et al.*, 2005). Emulsification index was more or less the same between pH of 5.5 and 8.5 however, emulsification index decreasing was observed at highly acidic and alkaline pH values. Ilori *et al.* (2005) reported biosurfactant that retained its 77% activity after 120 min of exposure to heat at a temperature of 100°C.

2.3.2 Biosurfactants classification

Generally, the typical amphiphilic structure of biosurfactants had a hydrophobic moiety, which was either a long chain fatty acid, alkyl or hydroxyl fatty acid. In addition, it was a hydrophilic moiety, which could be a carbohydrate in glycolipids, an amino acid in lipoproteins, a phosphate in phospholipids, a carboxylic acid, an alcohol, etc (Sreekala and Shreve, 1994).

Biosurfactant molecules were also secreted into the surrounding media. Biosurfactant molecules had the potential to promote cellular attraction to hydrophobic surfaces, to affect the distribution of cells between oil and water phases, to emulsify water-insoluble substances, and to mediate transport of hydrophobic substrate into the cell. Most biosurfactants were either neutral or negatively charged.

The anionic character was due to the presence of carboxylic groups. A small number of cationic biosurfactants contained an amine functional group.

Recently, a considerable number of studies have been performed on biosurfactants produced by a wide variety of microorganisms such as bacteria, yeast and filamentous fungi. Different types of microorganisms produced a variety of biosurfactants (Table 2.1). The most commonly isolated and widely studied group of surfactants produced by microorganisms a glycolipids which contained one or more monosaccharide residues linked by a glycosyl linkage to a lipid part and include trehalose lipids, rhamnolipid, and sophorolipids. Among them, rhamnolipids were the most widely research.

Table 2.1 Type and microbial origin of biosurfactants (Mulligan and Gibbs, 1993)

Type of biosurfactants	**Microorganism**
Trehalose lipids	*Arthrobacter paraffineus,* *Corynebacterium* sp., *Mycobacterium* sp., *Rhodococus erythropolis*, *Nocardia* sp.
Rhamnolipids	*Pseudomonas aeruginosa,* *Pseudomomas* sp., *Serratia rubidea*
Sophorose lipids	*Candida apicola, Candida bombicola,* *Candida lipolytica,* *Candida bogoriensis*
Glycolipids	*Alcanivorax borkumensis,* *Arthrobacter* sp., *Corynebacterium* sp., *R. erythropolis, Serratia marcescens,* *Tsukamurella* sp.

Most of the biosurfactants were high molecular-weight lipid complexes, which are normally produced under aerobic conditions. This was achievable in their *ex-situ* production in aerated bioreactors. The biosurfactant sources, classes and properties have been reviewed. In general, biosurfactants could be classified as:

- Glycolipids,
- Lipopolysaccharide,
- Lipoprotein-lipopeptides,
- Phospholipids,
- Fatty acids and neutral lipids

According to Zajic and Seffens (Zajic and Seffens, 1984), biosurfactants may be classified into five groups:

(1) Glycolipids

These were compounds of a carbohydrate and a lipid. Them the linkage was by way of either ether or an ester group for example trehalose, sophorose, rhamnose lipids and mannosylerythritol lipids. They were involved in the uptake of low polarity hydrocarbons by microorganisms. Glycolipids were low molecular weight compounds containing a mono- or disaccharide unit linked to a fatty acid moiety. The structural diversity of the glycolipids was high (Haferburg *et al.*, 1986) (Figure 2.4). Still many new glycolipids were being discovered (Arino *et al.*, 1998). The largest and best-known group was the glycolipid group, which included a form known as rhamnolipids (Kim *et al.*, 2000; Hong *et al.*, 2002).

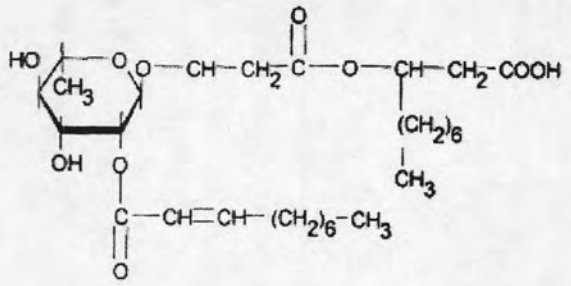

Figure 2.4 A glycolipid produced by a *Pseudomonas* strain (Kosaric, 1987)

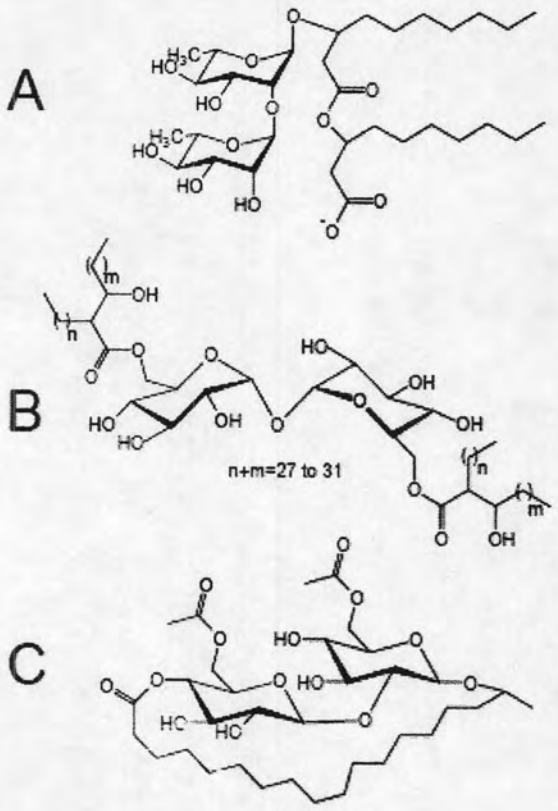

Figure 2.5 Structures of several glycolipid biosurfactants. (A) Rhamnolipid produced by *Pseudomonas aeruginosa*; (B) Trehaloselipid produced by *R. eryrthopolis* (Rapp *et al.*, 1979); (C) Sophorolipid (in lactonc form) produced by the yeast *Torulopsis bombicola* (Rosenberg, 1986).

(2) Liposaccharides were the high molecular weight, water soluble extracellular emulsifiers produced by hydrocarbon degrading bacteria like *Acinetobacter calcoaceticus* (emulsans). The polymeric biosurfactants included (hetero) polysaccharides (e.g. emulsan), polysaccharide-protein complexes, lipopolysaccharides, or proteins with emulsifying properties (Rosenberg, 1986). Only

recently, Kim *et al.* (2000) showed that the biological modification of the fatty acid group (C8-C20) in emulsan, caused by supplementation of fatty acids under conditions inhibiting native fatty acid biosynthesis, influenced the emulsifying activity. Among the emulsans produced from even-numbered fatty acids, the emulsan produced from myristic acid (C14) contained the greatest amount of the same-numbered fatty acids.

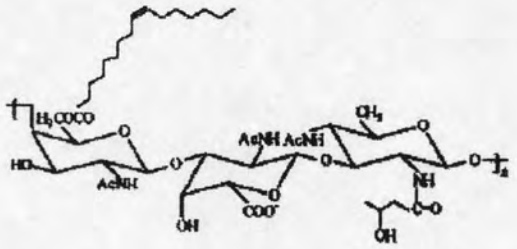

Figure 2.6 The structure of emulsan-like polymer (Zhang *et al.*, 1997).

(3) Lipopeptides

For example ornithine lipids and the subtilysin produced by *B. subtilis*, claimed to be the most effective biosurfactant reported to date. In lipopeptides such as herbicolin A and surfactin, both the lipid and the peptide domains are directly synthesized from carbohydrates. Addition of amino acids or fatty acids in the grown medium could affect the yield but not the structure of the product. The best-known lipopeptides were the surface-active antibiotics (bactericidal, fungicidal) produced by *Bacillus*. These compounds were often cyclic and were composed of 7-16 amino acids and a fatty acid side chain (Fiechter, 1992).

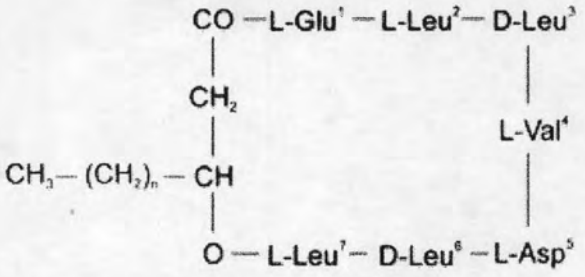

Figure 2.7 Primary structure of surfactin (n = 9-11) (Morikawa *et al.*, 1993).

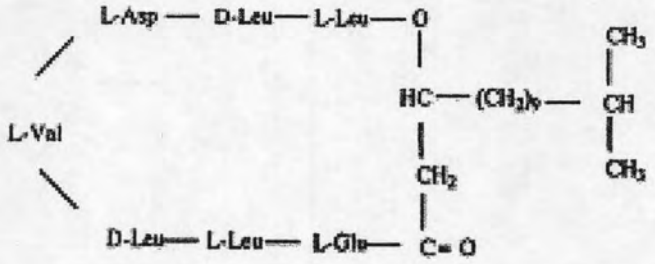

Figure 2.8 Cyclic Lipopeptide produced by a *B. subtilis* (Desai and Banat, 1997).

(4) Phospholipids were the esters formed between the alcohol groups on a lipid and a phosphate such as lecithin, phospholipids. Although they were present in every microorganism, there were very few examples of extracellular production, the most notable one being the biosurfactants produced by *Corynebacterium lepus*. L-a-Phosphatidylcholine (L-a-Lecithin) derived from soybean, Type II-S, with a purity of about 19% was supplied by Sigma Chemical Co., USA. Lecithin chemical structure is shown in Figure 2.9.

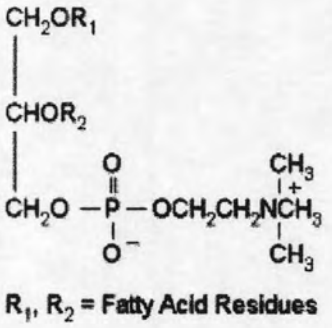

Figure 2.9 Chemical structure of lecithin (Urum *et al.*, 2004).

(5) Fatty acids and neutral lipids

For example, ustilagic acid, the corynomycolic acids, the lipotheichoic acids (sometimes classified as glycolipids) and the hydrophobic proteins produced by varies microorganism. In the case of ustilagic acid, the sugar moiety of ustilagic acid was the disaccharide cellobiose, which was O-glycosidically linked to the ω-hydroxyl group of the unusual long-chain fatty acid 15,16-dihydroxyhexadecanoic acid or 2,15,16-trihydroxyhexadecanoic acid (Figure 2.10).

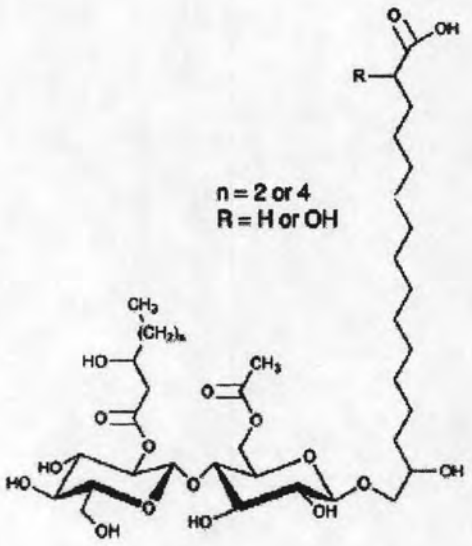

Figure 2.10 Chemical structures of ustilagic acids that produced from *Ustilago maydis* (Hewald *et al.*, 2005).

2.4 Advantage and disadvantage of biosurfactant and synthetic surfactant

Microbial surface-active agents (biosurfactants) were important biotechnological products, with a wide range of applications in many industries. Most of the applications today involve the use of chemically synthesized surfactants. Production of surfactants in the United States and worldwide was estimated at 3.4 x 10^9 kg and 7 x 10^9 kg in 1989, respectively. The US surfactant industry shipments

were $3.65 billion in 1989 (Kosaric, 2001). There were many advantages of biosurfactants if compared to their chemically synthesized counterparts.

Table 2.2 Compared properties between biosurfactants and synthetic surfactants

Properties	Biosurfactants	Synthetic surfactants
1. Biodegradability and solubilization	The application of biosurfactants has been largely directed to the biodegradation of crude oil (Muller-Hurtig *et al.*, 1993) and to enhance oil recovery systems (Jack, 1991; Finnerty and Singer, 1992).	Not easily biodegradable for example Researchers (Vipulanandan and Ren, 2000) have compared the solubilization of the PAH, naphthalene, by rhamnolipid, sodium dodecyl sulfate (SDS), an anionic surfactant and Triton X-100, a non-ionic surfactant. The biosurfactant increased the solubility of naphthalene by 30 times. However, biodegradation of naphthalene (30 $mg.l^{-1}$) took 40 days in the presence of biosurfactant (10 $g.l^{-1}$) compared to 100 hours for Triton X-100 (10 $g.l^{-1}$). It appeared that the biosurfactant was used as a carbon source instead of the naphthalene, which did not occur in the case of Triton X-100. Naphthalene in the presence of SDS was not biodegraded.
2. Toxicity	Biosurfactants have found a niche in the personal care market too because of their low toxicity, excellent moisturizing properties and skin compatibility (Brown, 1991).	There were also indications of higher levels of toxicity by SDS compared to the biosurfactant as the concentration of the surfactants increased above 100 $mg.kg^{-1}$ (Deschenes *et al.*, 1996).
3. Biocompatibility	Sophorolipids are commercially used by Kao Co. Ltd. as humectants in cosmetic make-up brands such as Sofina. The product containing sophorolipid and propylene glycol had specific compatibility to the skin and had found commercial utility as a skin moisturizer (Yamane, 1987).	Increased environmental awareness and strict legislation has made environmental compatibility of surfactants an important factor in their application for various uses (Maier and Soberon-Chavez, 2000).

(Continued)

Properties	Biosurfactants	Synthetic surfactants
4. Availability of raw materials	Could be produced from cheap raw materials which were available in large quantities such as the carbon source may come from hydrocarbons, carbohydrates and/or lipids, which may be used separately or in combination with each other (Makkar and Cameotra, 2002).	Biosurfactants had to advantage with surfactants of petrochemical origin which derived from crude oil and were also known as "chemical" surfactants (Mulligan, 2005).
5. Specificity application	There were widely used in the pharmaceutical, cosmetic, petroleum, and food industries for example being complex organic molecules with specific functional groups, were 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, 2001).	Synthetic surfactants could be cationic, anionic, nonionic or amphoteric, although anionic and nonionic surfactants have been used as oil dispersants (Uysal and Turkman, 2005). Moreover, many different types of surfactants were already being used in industry, but it was important to develop even more new compounds to broaden the spectrum of specific properties and applications (Cameotra and Makkar, 1998).
6. Ecological acceptability	Biosurfactants could be able to grown under the severe environmental conditions encountered in oil reservoirs, such as high temperature, pressure, salinity and low oxygen (Cameotra and Makkar, 1998).	Synthetic surfactants have been shown to inhibit microbial activity when added to the environment in high concentrations (Sreekala and Shreve, 1994).
7. Use in environmental control	Biosurfactants could be efficiently used in handling industrial emulsions, control of oil spills, biodegradation and detoxification of industrial effluents and in bioremediation of contaminated soil (Kosaric, 2001).	Biosurfactants had low toxicity profiles to freshwater, marine, and terrestrial ecosystems, and were potential candidates for a variety of environmental applications when compared synthetic surfactant (Finnerty, 1994).

(Continued)

Properties	Biosurfactants	Synthetic surfactants
8. Acceptable production economics	Makkar and Cameotra (2002) reported in depth on the available information on the used of various non-conventional substrates and the economics of production of biosurfactants on water miscible substrates and hydrophobic wastes.	The manufacture of surfactants was on such a large scale that their production had always been considered to lie within the realms of organic chemistry and chemical engineering (Cameotra and Makkar, 1998).
9. Cost	The economics of producing the biosurfactants had limited commercial applications. Costs could be reduced by improving yields, rates and recovery, and using cheap or waste substrates which disadvantaged of biosurfactant (Mulligan and Gibbs, 1993).	In general, surfactants were used to save energy and consequently energy costs (such as the energy required for pumping in pump and treat techniques) which cheaper than biosurfactant. Thus, synthetic surfactants were cheaper than biosurfactant (Finnerty, 1994).

2.5 Microbial production of biosurfactants

2.5.1 Biosurfactant-producing microorganism

This report showed to produce by bacteria, yeasts and fungi in Table 2.3, and particularly in bacteria which were in state of growth on water-immiscible substrate that was source of food for example crude oil spillage treated with selected microorganisms. By evolution, the bacteria have adapted themselves to feeding on water-immiscible materials by manufacturing and using surface-active product that helped the bacteria that were in the aqueous phase to adsorb, emulsify, wet, and disperse or solubilize the water-immiscible material.

These biotechnological compounds were primarily synthesized by bacteria, yeast or fungi cultured in media with a hydrophobic carbon source such as petroleum fractions, animal fat or vegetable oil (Hommel and Ratledge, 1990). The efficiency of glycolipids synthesis by *Candida antarctica* and *Candida apicola* ranged from 6.5 to 10.4 $g.l^{-1}$ and was considerably higher than the efficiency in cultures without post refinery fatty acids (Bednarski *et al.*, 2004). In addition to mannosylerythritol lipids production, *Ustilago maydis* secreted a second class of glycolipids, the cellobiose lipid ustilagic acid that was first described in 1950 by Haskins, who observed in submerged culture of *U. maydis* the formation of an insoluble compound with antibiotic activity (Haskins, 1950). Production of both types of glycolipids in *U. maydis* occurs readily under conditions of nitrogen starvation and could reach large yields (up to 23 $g.l^{-1}$) (Hewald *et al.*, 2005). Most work on biosurfactant production by microorganisms such as *Pseudomonas* sp., *Bacillus* sp., *Acinetobacter* sp., *Rhodococcus* sp. and *Arthobacter* sp. have been well studied. This work reported the biosurfactant production and its activity of bacterial isolated from the screening.

Table 2.3 Types of biosurfactants produced by microorganisms (Banat *et al.*, 2000).

Microorganism	Biosurfactant type	Reference
Yeast		
Candida antarctica	Mannosylerythritol lipids	Kitamoto *et al.* 1993
Candida bombicola	Sophorose lipid	Brakemeier *et al.* 1995
Candida apicola IMET 43747	Sophorose lipid	Hommel and Ratledge 1990
Candida sp. SY16	Mannosylerythritol lipid	Kim *et al.* 1999
Lactobacillus sp.	Surfactin	Velraeds-Martine *et al.* 1996b
Norcardia SFC-D	Trehalose lipid	Kosaric *et al.* 1990
Saccharomyces cerevisae	Rhamnolipid	Vasileva-Tonkova *et al.* 2001
Fungi		
Botrytis cinerea	Rhamnolipid	Abalos *et al.* 2001
Rhizotecnia solani	Rhamnolipid	Abalos *et al.* 2001
Ustilago maydis	Cellobiose lipids (glycolipid)	Hewald *et al.* 2005
Bacteria		
Alcanivorax borkumensis	Glycolipid	Abraham *et al.* 1998
Acinetobacter radioresistens	Alasan	Navon-Venezia *et al.* 1995
Arthrobacter sp. EK1	Trehalose tetraester	Schulz *et al.* 1991
Arthrobacter sp. MIS 38	Arthrofactin	Morikawa *et al.* 1993
Bacillus pumilus A1	Surfactin	Thaniyavarn *et al.* 2003
Bacillus subtilis	Surfactin	Makkar and Cameotra 1997
Bacillus subtilis C9	Surfactin	Kim *et al.* 1997
Bacillus licheniformis	Lichenysin A	Yakimov *et al.* 1995
Bacillus licheniformis JF-2	Lichenysin B	Lin *et al.* 1994
Pseudomonas aeruginosa GL-1	Rhamnolipid	Arino *et al.* 1996

(Continuted)

Microorganism	**Biosurfactant type**	**Reference**
Pseudomonas aeruginosa GL-1	Rhamnolipid	Patel and Desai 1997
Pseudomonas aeruginosa UW-1	Rhamnolipid	Sim *et al.* 1997
Pseudomonas fluorescens	Viscosin	Koch *et al.* 1991
Pseudomonas marginalis PD 14B	Particulate-surfactant (PM factor)	Burd and Ward 1996
Pseudomonas maltophilla CSV 89	Biosur Pm	Poirier *et al.* 1995
Rhodococcus sp. H13 A	Trehalose lipid	Singer *et al.* 1990
Rhodococcus sp. ST 5	Trehalose lipid	Abu Ruwaida *et al.* 1991
Serratia rubidea	Glycolipid	Matsuyama *et al.* 1986
Serratia marcescens	Glycolipid	Pruthi and Cameotra 1997
Streptomyces tendae TU901/8c	Streptofactin	Richter *et al.* 1998
Tsukamurella sp.	Glycolipid	Vollbrecht *et al.* 1998

In order to introduce biosurfactants into contaminated soil, the *ex-situ* production of biosurfactants was a necessary first stage of technology. Effective production and behavior of biosurfactants played an important role in achieving an efficient treatment technology. In this section, attention was paid to reviewing production of glycolipid biosurfactants, which were used in this research. Production methods of glycolipids from *Pseudomonas* sp. were concluded in the below list:

2.5.2 Growth-associated biosurfactant production

There exists a parallel relationship between growth, substrate utilization, and biosurfactant production. Different carbon sources such as glycerol, glucose, and ethanol could be used for glycolipid production by *Pseudomonas* sp.

(Robert *et al.*, 1989). Reena and Desai (1997) dealt with the glycolipids produced from *Pseudomonas aeruginosa* GS3. When glucose was the substrate, maximum production (0.44 $g.l^{-1}$) was observed during the stationary phase of growth. In addition, *Pseudomonas aeruginosa* GS3 could produce glycolipid biosurfactants during growth on carbohydrates, higher chain length n-alkanes and 1-alkenes, petroleum crude oil and vegetable oils. Pilot-plant studies have shown that the production of glycolipids with a concentration of approximately 2.25 $g.l^{-1}$ was achieved (Haskins, 1950; Guerra-Santos *et al.*, 1986).

2.5.3 Growing cells under growth-limitation conditions

Due to the limitation of nitrogen, iron, and low phosphate concentration, the culture at the stationary phase of growth may increase the production of biosurfactants. Numerous investigators have demonstrated an overproduction of biosurfactants by *Pseudomonas* sp. when the culture reaches the stationary phase of growth due to the limitation of nitrogen and iron {Guerra-Santos (Guerra-Santos *et al.*, 1986; Mulligan and Gibbs, 1989; Rarnana and Karanth, 1989). When bacterial cells were shifted from medium containing 36 μM iron to a medium containing 18 μM iron, glycolipid production dramatically increased three-fold (Guerra-Santos *et al.*, 1986).

2.5.4 Production by resting cells

In this method, although the bacterial cells continue to utilize the carbon source for the synthesis of biosurfactants, there was no ce11 multiplication. *Pseudomonas* sp. could produce the biosurfactants by resting cells. Syldatk *et al.* (1985) reported that the highest yield of glycolipids from alkanes produced by the

resting free cells of *Pseudomonas* sp. DSM 2874 occurred at pH 6.6 and a temperature 37°C. However, the biosurfactant production rate with resting cells was much lower than with growing cells. One of the advantages of using resting cells to produce biosurfactant was that it reduced the cost of product recovery (Guerra-Santos *et al.*, 1986).

There were several nutritional and environmental factors, which affect biosurfactant production and should be considered in the development of the technology, including carbon, nitrogen, phosphate substrate, pH, temperature, oxygen concentration and salinity. Water-soluble carbon sources such as glycerol, glucose, mannitol, and vegetable oils were all used for glycolipid production by *Pseudomonas* sp. Ammonium salts and urea were selected as nitrogen sources for biosurfactant production. However, biosurfactant production in certain *Pseudomonas* species could be enhanced when cells were grown under low nitrogen conditions (Guerra-Santos *et al.*, 1986). The research on glycolipid synthesis had shown that there were non-limiting concentrations of phosphate for the synthesis of glycolipids (Mulligan and Gibbs, 1989).

2.5.5 Optimum of biosurfactant production

In the study of Makkar and Cameotra (1998), growth and biosurfactant production by *Bacillus subtilis* was studied in minimal medium supplemented with sucrose and starch as carbon sources at a concentration of 2% wv^{-1}. Another report of Ilori *et al.* (2005) found *Aeromonas* sp. was isolated from tropical estuarine water that produced of biosurfactant at highest in medium with glucose and lowest in the medium with diesel + acetate. Soybean was the best nitrogen source for biosurfactant production. The activity of the biosurfactant was enhanced optimally at NaCl

concentration of 5% wv^{-1}, pH of 8.0 and temperature of 40°C. In this study the ability of biosurfactant-producing bacteria to grown and produced biosurfactant on different carbon and nitrogen sources under temperature as 37°C was studied. Moreover, determination of the effect of NaCl, temperature and pH on produced of the biosurfactant.

2.6 Brief biosurfactant-producing bacteria

In this experiment, two biosurfactant-producing bacteria were identified as *Enterobacter* sp. and *Burkholderia cepacia*, respectively. Therefore, information of previous studied of two strains in biosurfactant was useful.

2.6.1 *Enterobacter*

Species of *Enterobacter* had been attracting in both environmental and biotechnological applications such as H_2 and ethanol were produced from glycerol containing wastes with *Enterobacter aerogenes* HU-101 (Takeshi Ito *et al.*, 2005), an alkanol degrading by bacterium *Enterobacter* sp. VKGH12 (Grit Neumann *et al.*, 2005) and biosurfactant properties were studied. Nevertheless, *Enterobacter* strains had a few studies to involve biosurfactant properties. For example, emulsification activity of a marine bacterial exopolysaccharide produced by *Enterobacter cloaceae* was superior or comparable with that of other gums (Anita Iyer *et al.*, 2006).

2.6.2 *Burkholderia cepacia*

Pseudomonas cepacia (*Burkholderia cepacia*) produced biosurfactant and degrades the water-insoluble herbicide 2,4,5-trichlorophenoxyacetic acid (Benerjee *et al.*, 1983). This biosurfactant system also formed stable emulsions with herbicides other than chlorophenols, and may be beneficial in the biodegradation of

other toxic chemicals. Moreover, *B. cepacia* hemolysin displayed strong surfactant properties and could be considered a powerful biosurfactant. The critical micellar concentration of the toxin and the γ_{CMC} were determined graphically, by the method of Sheppard and Mulligan (1987), to be 12.6 $mg.ml^{-1}$ and 42.2 $mN.m^{-1}$, respectively. Furthermore, compared with other polyolbased surfactants, i.e., glycerol or sorbitan esters, they were not used as carbon source by *B. cepacia* LB400 (Ferrer *et al.*, 2003).

2.7 Applications of biosurfactant

2.7.1 Usage

Biosurfactants had attained the special status of a chemical in demand in recent years, had many potential applications including enhanced oil recovery, crude oil drilling lubricants, surfactant-aided bioremediation of water-insoluble pollutants, and in the health care and food processing industries as shown in Table 2.4 (Banat *et al.*, 2000). Other developing areas of biosurfactant using were in cosmetic and soap formulations, and foods. Various reviews in the past decade had summarized the possible roles of biosurfactants (e.g. Cameotra and Makkar., 1998; Banat *et al.*, 2000). In recent years, there were reports of applications of biosurfactants other than traditional used such as oil clean up or bioremediation of hydrocarbons. Biosurfactants could be commercially produced at levels of up to 100 $g.l^{-1}$, as reported for rhamnolipids from *Pseudomonas* (Maier and Soberon-Chavez, 2000). At this production level, and combined with the used of cheap renewable substrates and organic wastes, the cost of biosurfactants became competitive with the cost of synthetic surfactant production. Some of the latest applications of biosurfactants will be reviewed in the following sections.

Table 2.4 Biosurfactants used and effected (Kosaric, 2001).

Use	Effect of surfactants
Metals	
Concentration of ores	Wetting and foaming, collectors and furthers
Cutting and forming	Wetting, emulsification, lubrication and corrosion inhibition in rolling oils, cutting oils, lubricants, etc.
Casting	
Rust and scale removal	Mold release additives
Plating	In pickling and electrolytic cleaning
	Wetting and foaming in electrolytic plating
Paper	
Pulp treatment	Deresinification, washing
Paper machine	Defoaming, color leveling and dispersing
Calender	Wetting and leveling, coating and coloring
Paint and protective coatings	
Pigment preparation	Dispersing and wetting of pigment during grinding
Latex paints	Emulsification, dispersion of pigment, stabilize latex, retard sedimentation and pigment separation, rheology
Waxes and polishes	Emulsify waxes, stabilize emulsions, antistat
Petroleum production /products	
Drilling fluids	Emulsify oil, disperse solids, modify rheological properties of drilling fluids for oil and gas wells
Worker of producing wells	Emulsify and disperse sludge and sediment in cleanout of wells
Producing wells	De-emulsify crude petroleum, inhibit corrosion of equipment In
Secondary recovery,	flooding operations, preferential wetting
Refined products	Detergent sludge dispersant and corrosion inhibitor in fuel oils crank-case oils and turbine oils
Textiles	
Preparation of fibers	Detergent and emulsifier in raw wool scoring; dispersant in
Dyeing and printing	viscose rayon spin bath; lubricant and antistat in spinning of
Finishing of textiles	hydrophobic filaments
	Wetting, penetration, solubilization, emulsification, dye leveling, detergency and dispersion
	Wetting and emulsification in finishing formulations, softening, lubricating and antistatic additives to finishes

(Continued)

Use	Effect of surfactants
Agriculture	
Phosphate fertilizers	Prevent caking during storage
Spray application	Wetting, dispersing, suspending of powdered pesticides and emulsification of pesticide solutions; promote wetting, spreading and penetration of toxicant
Building and construction	
Paving	Improve bond of asphalt to gravel and sand
Concrete	Promote air entertainment
Food and beverages	
Food processing plants	For cleaning sanitizing
Fruits and vegetables	Improve removal of pesticides, and in wax coating
Bakery and ice cream	Solubilize flavor oils, control consistency, retard staling
Crystallization of sugar	Improve washing, reduce processing time
Cooking fat and oils	Prevent spattering due to super heat and water
Industrial cleaning	
Janitorial supplies	Detergents and sanitizers
Descaling	Wetting agents and corrosion inhibitors in acid cleaning of boiler tubes and heat exchangers.
Soft goods	Detergents for laundry and dry cleaning
Leather	
Skins	Detergent and emulsifier in degreasing
Tanning	Promote wetting and penetration
Hides	Emulsifiers in fat liquoring
Dyeing	Promote wetting and penetration

2.7.2 Biosurfactants as specialty products

Biosurfactants were being investigated as an alternative to high value synthetic chemicals whose used may had toxic environmental impacts. Ishigami *et al.* (1996) reported the synthesis of a pyrenacylester of rhamnolipids for used in monitoring the polarity and fluidity of solid surfaces and the attendant influenced of coatings on these surface propertied. Rhamnolipids had been source of stereospecific L-rhamnose, which was used in the production of high quality flavor compounds and as starting material for synthesis of some organic compounds (Linhardt *et al.*, 1989). Other sources of rhamnose include polysaccharides from plants or microbes. Isolation of rhamnose from these sources was a technically difficult process. Rhamnolipid from *P. aeruginosa* was a better source of rhamnose as it was excreted in late log and stationary phase allowing easy separation of cells. Rhamnose could then be produced by hydrolysis of the rhamnolipids.

2.7.3 Biosurfactants as therapeutic and health care agents

Biosurfactants had some therapeutic applications. These included possible applications of rhamnolipids produced by *P. aeruginosa*, and lipopeptides produced by *B. subtilis*, and *B. licheniformis* as biocidic agents. Respiration failure in premature infants was caused by deficiency of pulmonary surfactant (Tayler *et al.*, 1985). With the cloning of a gene encoding the protein molecule of the surfactant in bacteria, the fermentative production of this product for medical applications was now possible (Lang *et al.*, 1989). Kosaric (1993) described possible applications as emulsifying aids for drug transport to the infection site, for supplementing pulmonary surfactant and as adjutants for vaccines.

2.7.4 Additional applications of biosurfactants

Biosurfactants may be used for dispersion of inorganic minerals in mining and manufacturing processes. Rosenberg *et al.* (1988) described the production by *A. calcoaceticus* A2 of an anionic polysaccharide called biodispersan, which prevented flocculation and dispersed a 10% limestone in water mixture. Sutton (1992) investigated the used of biosurfactant produced by *Nocardia amarae* for the removal and recovery of non-ionic organics from aqueous solutions. Polman *et al.* (1994) applied biosurfactants for solubilization of coal and achieved partial solubilization of North Dakota Beulah Zap lignite coal using a crude preparation of biosurfactants from *Candida bombicola.* Mulligan *et al.* (2001) used surfactin, rhamnolipids and sophorolipids in batch washing experiments to remove heavy metals from sediments

2.7.5 Environmental applications of biosurfactants

2.7.5.1 Effect of glycolipids on contaminant biodegradation

a) Petroleum hydrocarbons

Various studies had examined the effect of glycolipids on biodegradation of organic contaminants with mixed results. There had been particular focus on various hydrocarbons of low solubility. A recent review by Maier and Soberon-Chavez (2000) indicated that glycolipid addition could enhanced biodegradation of hexadecane, octadecane, n-paraffin, and phenanthrene in liquid systems, in addition to hexadecane, tetradecane, pristine, creosote and hydrocarbon mixtures in soils. Two mechanisms for enhanced biodegradation were possible, enhanced solubility of the substrate for the microbial cells, and interaction with the cell surface, which increased the hydrophobicity of the surface allowing hydrophobic

substrates to associate more easily (Zhang and Miller, 1992; Shreve *et al.*, 1995). Zhang and Miller (1992) demonstrated that concentration of 300 $mg.l^{-1}$ of glycolipids increased the mineralization of octadecane to 20% from 5% for the controls. Beal and Betts (2000) showed that cell surface hydrophobicity increased by the biosurfactant strain more than a non-biosurfactant producing strain during growth on hexadecane. The glycolipid also increased the solubility of the hexadecane from 1.8 to 22.8 $mg.l^{-1}$. There had been indications that inhibition could also occurred. Other studies by Churchill *et al.* (1995) showed that glycolipids with a fertilizer enhanced biodegradation of aromatic and aliphatic compounds in aqueous phase and soil reactors.

b) Polycyclic aromatic hydrocarbons (PAH)

Researchers (Vipulanandan and Ren, 2000) had compared the solubilization of the PAH, naphthalene, by a glycolipid, sodium dodecyl sulfate (SDS), an anionic surfactant and TritonX-100, non-ionic surfactant. The biosurfactant increased the solubility of naphthalene by 30 times. However, biodegradation of naphthalene (30 $mg.l^{-1}$) took 40 days in the presence of biosurfactant (10 $g.l^{-1}$) compared to 100 hours for TritonX-100 (10 $g.l^{-1}$). It appeared that the biosurfactant was used as carbon source instead of the naphthalene, which did not occur in the case of TritonX-100. Naphthalene in the presence of SDS was not biodegraded.

c) Chlorinated hydrocarbons

Pesticides were another group of contaminants that had been studied. Mata-Sandoval *et al.* (2000) compared the ability of the glycolipid mixture to solubilize the pesticides, trifluralin, coumaphos and atrazine, with the synthetic surfactant TritonX-100. The synthetic surfactant was able to solubilize approximately twice as much of all pesticides as the glycolipid. The biosurfactant shown to bind

trifluralin tightly in the micelle and releases the pesticide slowly to the aqueous phase, which could have implications for microbial uptake. This approach of utilizing micellar solubilization capacities and aqueous–micelle solubilization rate coefficients and micellar–aqueous transfer rate coefficients could be useful for future studies on microbial uptake. Addition of glycolipid in the presence of cadmium enabled biodegradation of the hydrocarbon naphthalene to occurre as if no cadmium was present (Maslin and Maier, 2000).

2.7.5.2 Biosurfactant production in halophilic environments

Some microorganisms could survive and grown over a wide range of salt concentration. In aquatic environments the conditions range from fresh waters (containing less than 0.05% wv^{-1} dissolved salts), through sea water with total salinities of 3.2% ± 3.8% (wv^{-1}) to saturate salt solutions up to 30% wv^{-1} and above (Kushner, 1978; Brown, 1983). There were very few reports on hydrocarbon biodegradation in hypersaline environments. Ward and Brock (1978) had shown an inverse relationship between hydrocarbon biodegradation and salinity. Bertrand *et al.* (1990) reported the isolation of halophilic hydrocarbonoclastic bacteria, showing that hydrocarbon metabolism might occur in hypersaline conditions. Halophiles had a unique lipid composition (phytanylglycerol), may had an important role to play as surface-active agents. The biopolymers secreted by halophiles were intrinsically highly stabled and may had applications as mobility controllers and emulsifying agents in the oil industry (Anton *et al.*, 1988; Austin, 1989). The archae-bacterial ether-linked phytanyl membrane lipid of the extremely halophilic bacteria had been shown to have surfactant properties (Post and Al-Harjan, 1988). Jenneman *et al.* (1983) reported the production of biosurfactant by a halotolerant *Bacillus* species and

its potential in enhanced oil recovery. *Bacillus licheniformis* strain BAS 50 was able to grown and produced a lipopeptide surfactant when cultured on variety of substrates at salinities up to 13% wv^{-1} NaCl (Yakimov *et al.*, 1995). The production of bioemulsifiers from *Methanobacterium thermoautotrophicum* had been reported (Trebbau de Acevedo and McInerney, 1996). These bioemulsifiers were active over a wide range of pH (5 ± 10) and at very high salt concentrations (up to $200l^{-1}$).