CHAPTER II
THEORETICAL BACKGROUND AND LITERATURE REVIEW

2.1 Biodegradation of Contaminated Petroleum Hydrocarbon in Environment.

Petroleum hydrocarbons continue to be used as the principle source of energy and hence become an important global environmental pollutant. Apart from accidental contamination affecting directly the ecosystem, the vast amounts of oil sludge generated in refineries from water oil separation systems and accumulation of waste oily materials in crude oil storage tank bottoms pose great problems because of the expensive disposal. In addition, the risk of oil spillage, appearing in many activities of oil refineries, poses a serious environmental problem, due to the possibility of air, water and soil contamination. The degree of association of organic and inorganic pollutants is governed by the complex physico-chemical interactions at interfaces. This association involves sorption onto soil constituents and partitioning in a nonaqueous phase liquid (NAPL) which represents organic substances that are relatively insoluble in water providing a long-term source of pollution. As a result the bioavailability of contaminants to biodegradation may be reduced (Rahman *et al.*, 2003).

An interest in industrial biotechnology has increased recently and many industries whose activities were traditionally remote from any biological system have now recognized the potential of living cells (Benincasa *et al.*, 2002). Despite decades of research, successful bioremediation of petroleum hydrocarbon contaminated soil remains a challenge. The enhancement of natural biological degradation processes can be a preferred cost-effective method for removing contaminants from soil-contaminated and other contaminated environments.

Biodegradation or microbial degradation of hydrophobic compounds is a function of their structure, availability to microorganisms and the physical and chemical interaction affecting the metabolic capability of the microorganisms. This process can break down organic molecules into other substances, such as fatty acids and carbon dioxide. Using surfactants can enhanced the desorption of pollutants from particulates and increase hydrocarbon degradation (Christofi and Ivshina, 2002).

2.2 Background on Surfactants and Biosurfactants

2.2.1 Surfactants

Today, the demand for surfactants world-wide are increased every year. More than one million tons of surfactants are produced each year in Japan for her domestic market. In addition, surfactants are used in various industries like polymers, plastics, textiles, papers, cosmetics, pharmaceuticals, food and machinery manufacture. Surfactants are potentially useful in every industry dealing with multiple phase system (Kitamoto *et al.*, 2002).

Surfactants (surface-active compounds) are amphiphilic compounds that contain a hydrophobic portion with little affinity for the bulk aqueous medium and a hydrophilic portion that is attracted to the bulk aqueous medium. Figure 2.1 below shows the basis structure of a surface-active molecule.

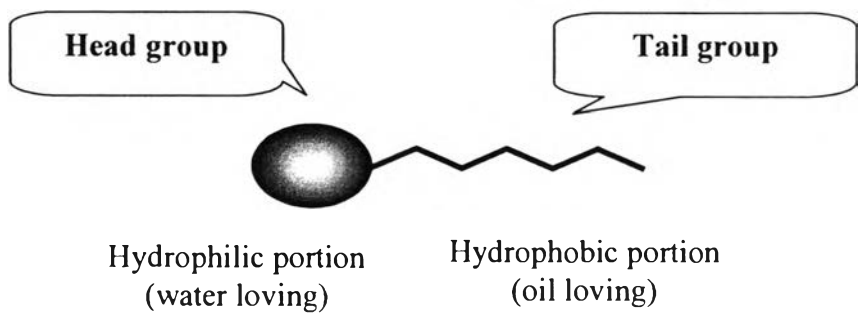


Figure 2.1 Structure of surfactant monomer.

The presence of surfactants reduce the free energy of the aqueous system by replacing the bulk molecules of higher energy at an interface (Mulligan, 2005) that have different degree of polarity and hydrogen bonding, such as oil/water, or air/water and water/solid interfaces (Singh *et al.*, 2004). The surface and interfacial tensions at air/water and oil/water interfaces, respectively are comparatively easily measured quantitatively, most commonly by instruments such as a Du Nouy tensiometer, and such measurements are the basis of most initial evaluations. Surface tension at water/solid interfaces is less conveniently quantifiable (Parkinson, 1985).

The effectiveness of a surfactant is determined by its ability to lower the surface tension which is a measure of the surface free energy per unit area required to bring a molecule from the bulk phase to the surface (Rosen, 1978). The surface tension correlates to the concentration of surfactant until the critical micelle concentration (CMC) is reached, as shown in Figure 2.2 (Mulligan *et al.*, 2001).

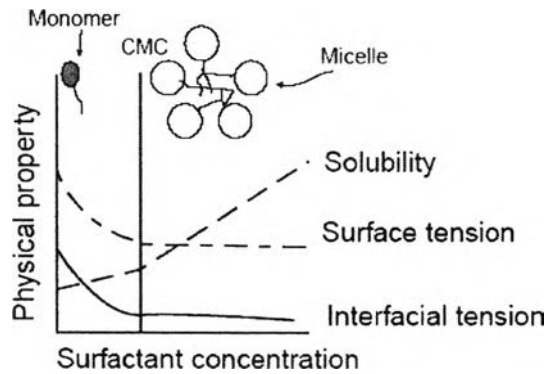


Figure 2.2 Schematic diagram of the variation of surface tension, interfacial and contaminant solubility with surfactant concentration (Mulligan *et al.*, 2001).

With increasing surfactant concentration, the reduction of surface tension is observed up to a critical level, above which surfactant monomers associate readily to form the structures like circular micelles, rod-shaped micelles, micellar layer or vesicle micelles, as shown in Figure 2.3. The CMC is defined as the minimum surfactant concentration necessary to initiate micelle formation. A surfactant has a low value of the critical micelle concentration, indicating that a lower amount of the surfactant is required to obtain the minimum surface tension.

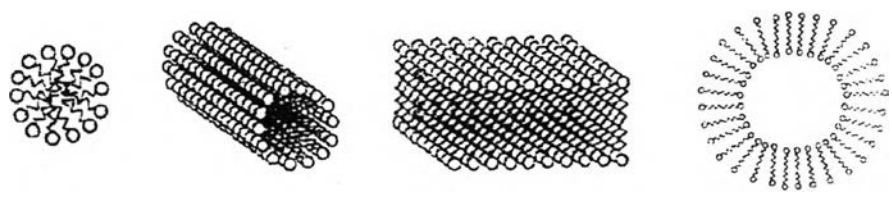


Figure 2.3 The shape of micelle (a) circular micelles; (b) rod-shaped micelles; (c) micellar layer and (e) vesicle micelles (Fiechter, 1992).

An emulsion is defined as a "heterogeneous system", consisting of at least one immiscible liquid dispersed in another in the form of droplets, whose di-

ameters, in general, exceeds 0.1 mm. The term of hydrophilic-lipophilic balance (HLB) is used to classify which type of emulsion will favor (e.g. water-in-oil (w/o) or oil-in-water (o/w) emulsions) (Kosaric, 1993).

The HLB value can be estimated emulsifying properties which assigns values of 1 to oleic acid and of 20 to sodium oleate. Any intermediate value is based proportionately on mixtures of the two. New surfactants are assessed by comparison with surfactants of known HLB. Generally, surfactants with HLB less than 6 will favor the formation stabilization of water-in-oil emulsions; they tend to have a predominance of lipophilic portions and to be more soluble in the oil phase. Besides this value, surfactants with HLB values between 10-18 have the opposite characteristics and favor the formation of oil-in-water emulsions (Parkinson, 1985).

Surfactants can be classified into four types:

1. Anionic surfactants e.g. LAS (linear alkylbenzene sulfonate)
2. Cationic surfactants e.g. CTAB (cetyl trimetylammonium bromide)
3. Nonionic surfactants e.g. AE (alcohol ethoxylate)
4. Amphoteric surfactants e.g. dodecyl dimethyl sulfobetaine and dodecyl dimethyl amine oxide (Rosen, 2004).

Surfactants can either be chemically synthesized (synthetic surfactants) or microbially produced (biosurfactants). Synthetic surfactants are of petrochemical origin whereas biogenic surfactants (biosurfactants) are produced by bacteria, yeast, and fungi (Edwards *et al.*, 2003). Most of the surfactants are chemically synthesized, These compounds are usually toxic to the environment and some of them are non-biodegradable. Moreover, their production processes and by-products can be environmentally hazardous. In recent years, biosurfactants have been considered as possible alternatives to chemical surfactants (Zouboulis *et al.*, 2003).

Both synthetic and natural (biological) surfactants have been used in several applications. The use of biosurfactants in the control of the bioavailability of toxicants in soils is an attractive option because of their biodegradability. These surface-active compounds can be diverse with novel chemical structures and characteristics; they can be produced from cheap raw materials and the organisms producing them can be modified genetically to overproduce or produce new compounds. Addi-

tionally, they are significantly less toxic than synthetic petroleum-based surfactants (Banat *et al.*, 1995).

2.2.2 Biosurfactants

Biosurfactants (microbial surface active agents) are biomolecules containing both a lipophilic and hydrophilic moieties like chemical surfactants. The lipophilic portion is the hydrocarbon chain of a fatty acid or sterol ring whereas the hydrophilic or polar portion is the carboxyl group of fatty acids or amino acids, the phosphoryl group of phospholipids, hydroxyl group of saccharides, and peptides (Morikawa *et al.*, 2000). They produced by a wide variety of microorganisms (e.g. bacteria, yeasts, and filamentous fungi) which are able to grow on water-insoluble substrates like *n*-alkanes or vegetable oils, as carbon sources. These microorganisms have been adapted to any particular substrate to produce biosurfactants, which help them to adsorb, emulsify, wetting, solubilizing or dispersing the water-immiscible material (Zouboulis *et al.*, 2003).

2.2.2.1 Types of biosurfactants

Biosurfactants are mainly classified into four categories based on their biochemical nature and the microbial species (Healy *et al.*, 1996). All of these are:

2.2.2.1.1 Glycolipids

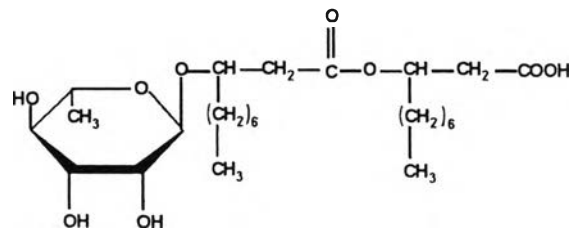
Glycolipids (the most commonly studied biosurfactants) are carbohydrates in combination with long-chain aliphatic acids or hydroxyl aliphatic acids. The main glycolipids which are studied from the point of view of surfactant characterization and properties are (A) rhamnolipid, (B) trehalose lipids, and (C) sophorolipids.

(A) Rhamnolipids

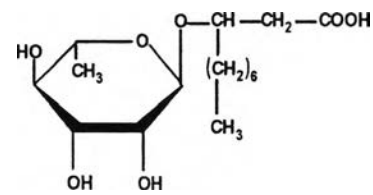
Rhamnolipids are the one of best-studied glycolipids, produced by several species of *Pseudomonas*. They produces mainly two types of rhamnolipid, which consists of one or two molecules of rhamnose linked to one or two molecules of β-hydroxydecanoic acid that called monorhamnolipid and dirhamnoli-

pid, respectively (Edwards *et al.*, 2002). The amounts of different types in the culture liquid are about 90% rhamnolipid 3 and 10% rhamnolipid 1 whereas rhamnolipid 2 and rhamnolipid 4 occur only in trace amounts. The structures of different rhamnolipids are shown in Figure 2.4.

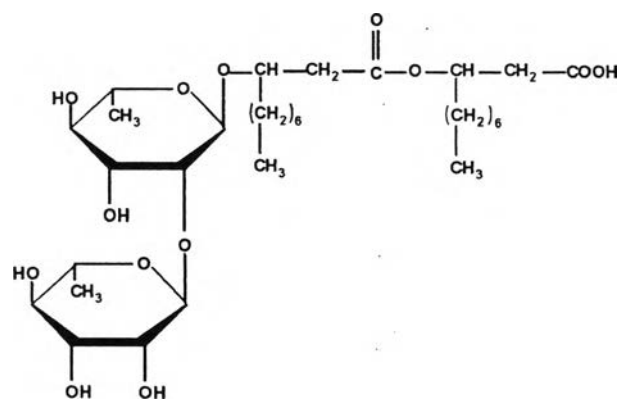
a) Rhamnolipid 1 (one rhamnose subunits are linked to two β-hydroxydecanoic acids in a side chain)



b) Rhamnolipid 2



c) Rhamnolipid 3



d) Rhamnolipid 4

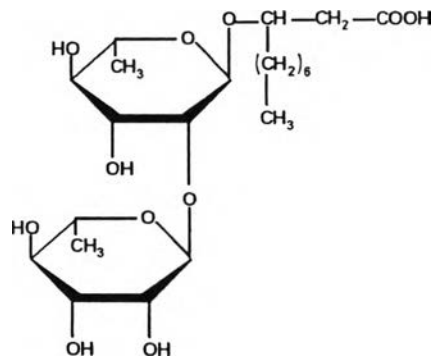


Figure 2.4 Schematic representation of four different rhamnolipids produced by *P.aerugonosa* (Kosaric, 1993).

(B) Trehalose Lipids

The structures of trehalose lipids are found to be widely distributed. Their disaccharide trehalose link at C6 and C6′ to mycolic acids which are associated with most species of *Mycobacterium, Nocardia, Corynebacterium Arthrobacter* and *Brevibacterium.* Mycolic acids are long-chain, a-branched-hydroxy fatty acids. Trehalolipids from different organisms differ in the size and structure of mycolic acid, the number of carbon atoms, and the degree of unsaturation. Desai and Banat (1997) reported the structure of trehalose dimycolate produced by *Rhodococcus erythropolis* as shown in Figure 2.5.

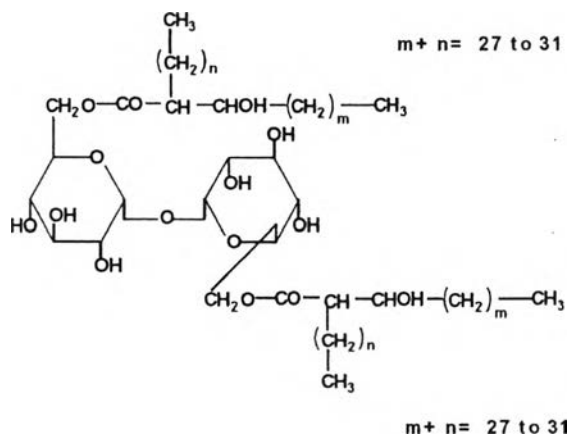


Figure 2.5 Trehalose dimycolate produced by *Rhodococcus erythropolis*, in which disaccharide trehalose is linked to two long-chain α-branched β-hydroxy fatty acids (Desai and Banat, 1997).

(C) Sophorolipids

Sophorolipid biosurfactants, mostly produced by yeasts such as *Torulopsis bombicola*, *Torulopsis petrophilum*, and *Torulopsis apicola*, consist of a dimeric carbohydrate sophorose linked to a long-chain hydroxy fatty acid, as shown in Figure 2.6.

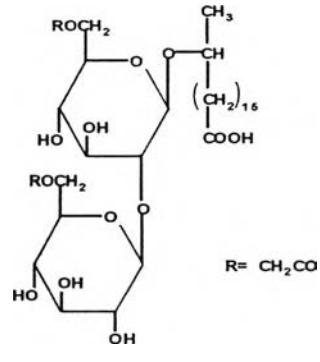


Figure 2.6 Sophorolipid from *Torulopsis bombicola* in which dimeric sophorose is linked to a long-chain (C18) hydroxy fatty acid (Desai and Banat, 1997).

Sophorolipids produced by *T. petrophilum* which grows on water-insoluble substrates such as alkanes and vegetable oils whereas some sophorolipids, which were chemically identical to those produced by *T. bombicola*, do not emulsify alkanes or vegetable oils. Although sophorolipids can lower surface and interfacial tension, they are not effective emulsifying agents (Desai and Banat, 1997).

2.2.2.1.2 Lipoproteins or lipopeptids

These consist of a lipid attached to a polypeptide chain. One of the most effective cyclic lipopeptide biosurfactants is surfactin (SF) produced by *Bacilus subtilis* ATCC 21332. It is capable of lowering the surface tension from 72 to 27.9 mN/m at a concentration as low as 0.005%. The structure of surfactin as shown in Figure 2.7.

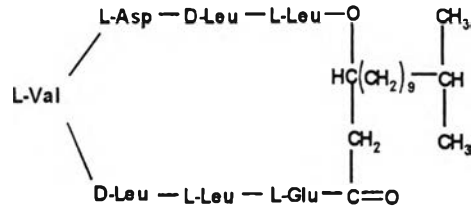


Figure 2.7 Structure of cyclic lipopeptide surfactin produced by *Bacillus subtilis* (Desai and Banat, 1997).

2.2.2.1.3 Phospholipids and Fatty acids

Phospholipids and Fatty acids are produced by bacteria and yeasts when grown on *n*-alkanes. Their structures are ester form between the alcohol group on a lipid and a phosphate. The HLB value of phospholipids is directly related to the length of the hydrocarbon chain in their structures. *R.erythropolis* produced Phosphatidylethanolamine on *n*-alkane causes the reduction of interfacial tension (Desai and Banat, 1997). In Figure 2.8, shows the structure of Phosphatidylethanolamine.

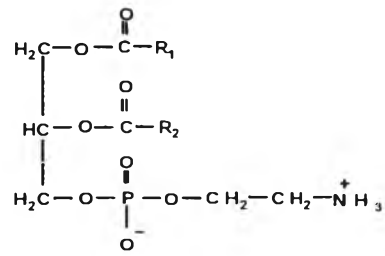


Figure 2.8 Structure of phosphatidylethanolamine, a potent biosurfactant produced by *Acinetobacter* sp. R_1 and R_2 are hydrocarbon chains of fatty acids (Desai and Banat, 1997).

2.2.2.1.4 Polymeric biosurfactants

These biosurfactants are high molecular weight biopolymers and generally demonstrate useful properties such as, high viscosity, tensile strength, and resistance to shear. Accordingly, polymeric biosurfactants have found a variety of industrial uses. Emulsan, liposan, mannoprotein, and other polysaccharide-protein complexes are among of these biosurfactants.

(A) Emulsan

Emulsan has been characterized as a polyanionic amphiphatic heteropolysaccharide as shown in Figure 2.9.

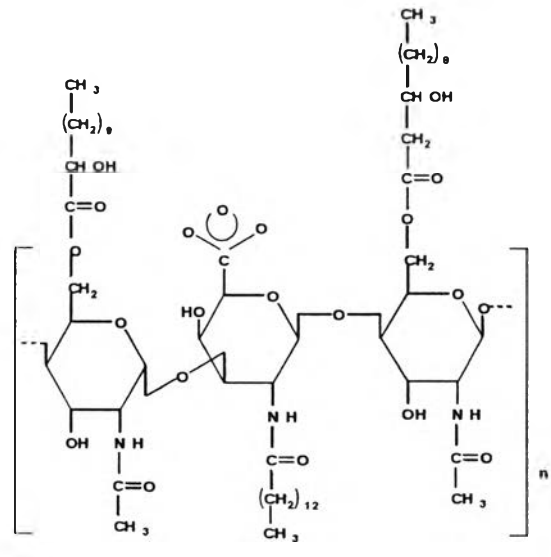


Figure 2.9 Structure of emulsan, produced by *Acinetobacter calcoaceticus*, in which fatty acids are linked to a heteropolysaccharide backbone (Desai and Banat, 1997).

(B) Biodispersan

Biodispersan is an extracellular, nondialyzable dispersing agent produced by *A.calcoaceticus* A2. The active component of biodispersan is an anionic heteropolysaccharide, with an average molecular weight of 51,400 and four reducing sugars (glucosamine, 6-methylaminohexose, galactosamine uronic acid, and an unidentified amino sugar).

(C) Liposan

Liposan is an extracellular water-soluble emulsifier synthesized by *Candida lipolytica* which composed of 83% carbohydrate (heteropolysaccharide containing glucose, galactose, galactosamine, and galacturonic acid) and 17% protein. Desai and Banat, (1997) showed the major types of biosurfactants, with their properties and microbial species of origin, as summarized in Table 2.1.

Table 2.1 Microbial source and properties of important types of Biosurfactants (Desai and Banat, 1997)

Biosurfactant	Organisms	Surface tension (mN/m)	CMC (mg/l)	Interfacial tension (mN/m)
Glycolipids				
Rhamnolipids	P. aeruginosa	29		0.25
	Pseudomonas sp.	25–30	0.1–10	1
Trehalolipids	R. erythropolis	32–36	4	14-17
	N. erythropolis	30	20	3.5
	Mycobacterium sp.	38	0.3	15
Sophorolipids	T. bombicola	33		1.8
	T. apicola	30		0.9
	T. petrophilum			
Cellobiolipids	U. zeae, U. maydis			
Lipopeptides and lipoproteins				
Peptide-lipid	B. licheniformis	27	12–20	0.1-0.3
Serrawettin	S. marcescens	28-33		
Viscosin	P. fluorescens	26.5	150	
Surfactin	B. subtilis	27-32	23-160	1
Subtilisin	B. subtilis			
Gramicidins	B. brevis			
Polymyxins	B. polymyxa			
Fatty acids, neutral lipids, and phospholipids				
Fatty acids	C. lepus	30	150	2
Neutral lipids	N. erythropolis	32		3
Phospholipids	T. thiooxidans			
Polymeric surfactants				
Emulsan	A. calcoaceticus			
Biodispersan	A. calcoaceticus			
Mannan-lipid-protein	C. tropicalis			
Liposan	C. lipolytica			
Carbohydrate-protein-lipid	P. fluorescens	27	10	
	D. polymorphis			
Protein PA	P. aeruginosa			
Particulate biosurfactants				
Vesicles and fimbriae	A. calcoaceticus			
Whole cells	Variety of bacteria			

2.3 Factors Affecting Biosurfactants Production

2.3.1 Carbon Sources

Carbon sources, the raw materials used to produce rhamnolipid biosurfactants, can be divided into water-soluble carbon sources (e.g. glycerol, glucose, mannitol, and ethanol) and water-immiscible substrates (e.g. n-alkanes and vegetable oil) (Rashedi *et al.*, 2006). They influence biosurfactant synthesis by either induction or repression (Makkar and Cameotra, 2002). The different carbon sources in the medium also affect the composition of biosurfactant production. Substrates with different chain lengths exhibit no effect on the chain lengths of fatty acid moieties in glycolipids.

Mata-Sandoval *et al.* (1999) demonstrated that rhamnolipid mixture ($RhC_{10}C_{10}$, $Rh_2C_{10}C_{10}$, $Rh_2C_{10}C_{12}$, $Rh_2C_{10}C_{12}H_2$) was produced by *Pseudomonas aeruginosa* UG2 growing on corn oil as a sole carbon.

Benincasa *et al.* (2002) studied the effect of the carbon source on rhamnolipid production by *Pseudomonas aeruginosa* LBI. The different oily substrates gaved different amounts of rhamnolipid biosurfactants. The most suitable substrates were the residues containing soapstock (the waste from sunflower oil process), with crude oleic acid, soy bean oil and sunflower oil, and olive oil, 4.5, 4.8, 4.9, 5.4 g/l of rhamnolipids were produced, respectively.

Wei *et al.* (2005) evaluated a variety of carbon substrates, including hydrophilic substrates (glucose, glycerol), vegetable oils (sunflower oil, grape seed oil, and olive oil), and mineral oils (diesel and kerosene) for their effectiveness on rhamnolipid production from *Pseudomonas aeruginosa* J4. The results showed a general trend that rhamnolipid production initially increased with increasing carbon substrate concentration, until it reached a maximum value and then leveled off. However, only glycerol behaved differently, as the rhamnolipid level decreased sharply when glycerol concentration was over 2% resulting in negligible rhamnolipid production in the culture. Olive oil was an excellent carbon source for rhamnolipid production with a maximum rhamnolipid concentration of nearly 3.6 g/L occurred at

an olive concentration of 10%. Sunflower oil and grape seed oil (both at a concentration of 6%) achieved a maximum concentration of 2.0–2.1 g/L.

Rashedi *et al.* (2006) showed rhamnolipid production by *P. aeruginosa* MM1011 using sugar beet molasses as a carbon and energy source. With a medium containing 24.2 g/l of glucose, a biosurfactant mass concentration (expressed as rhamnolipids) of up to 1.1 g/l was obtained in the cell-free culture liquid. The rhamnolipid mass concentration was 7.5 mg/ml.

Thaniyavarn *et al.* (2006) concluded the biosurfactant production by *Pseudomonas aeruginosa* A41 that types of carbon sources were found to affect biosurfactant yield. The yields of rhamnolipid biosurfactant were 6.58 g/L, 2.93 g/L and 2.91 g/L determined as rhamnose content when olive oil, coconut oil, and palm oil respectively, were used as a carbon source. These yield of biosurfactant steadily increased even after a stationary phase. Among them, biosurfactant obtained from palm oil was the best in lowering surface tension of the medium. Increases in biosurfactant activities in terms of oil displacement test and rhamnose content were observed to be higher with shorter chain fatty acids than that of the longer chains (C12>C14>C16). In addition, highly unsaturated fatty acid of C18:2, showed higher oil displacement activity and rhamnose content than that of C18:1.

Wu *et al.* (2007) examined the effect of carbon substrates, nitrogen sources and carbon-to-nitrogen (C/N) ratio on rhamnolipid production with an isolated strain *Pseudomonas aeruginosa* EM1 originating from an oil-contaminated site. The carbon sources tested included carbohydrates (glucose and sucrose), glycerol, vegetable oils (olive oil and soybean oil), fatty acid (oleic acid), and hydrocarbon (hexane) whereas, nitrogen sources included inorganic (NH_4Cl and $NaNO_3$) and organic (urea and yeast extract). The results showed that glucose and glycerol were effective for rhamnolipid production. After cultivation for 7 d, the culture with glucose, glycerol, olive oil, soybean oil, oleic acid, hexane and sucrose produced 7.50, and 4.93 g/L, 3.70, 2.63 g/L, 0.55 g/L, 0.12 g/L and 0.07 g/L of rhamnolipid, respectively. *P. aeruginosa* EM1 strain showed a different trend. Vegetable oils were more efficient substrates in rhamnolipid production from *P. aeruginosa* strains as compared with glucose, glycerol, and hydrocarbons. This suggests that the carbon source preference for rhamnolipid production seems to be strain dependent.

2.3.2 Nitrogen Sources

The nitrogen source can be an important key to the regulation of biosurfactant synthesis. Nitrogen limitation not only causes overproduction of biosurfactant but also changes the composition of the biosurfactant produced. Among the inorganic salts tested, ammonium salts and urea were formed to be preferable preferred nitrogen sources for biosurfactant production by *Arthrobacter paraffineus* whereas nitrate supported maximum surfactant production in *Pseudomonas aeruginosa* and *Rhodococcus* spp.

Guerra-Santos *et al.* (1984) studied the influence of nitrogen source ($NaNO_3$, $(NH_4)_2S0_4$) on *Pseudomonas aeruginosa* growth and biosurfactant production. The medium 2 M with glucose concentration of 18.2 g/l served as the basic medium for the optimization experiments. Nitrate as a nitrogen source was found to able to be lower surface and interfacial tension values of the culture broth than ammonium. The influence of C:N ratio on biosurfactant production showed maximum rhamnolipid production after nitrogen limitation at a C:N ratio of 18:1. A decrease or increase in the concentration of nitrate was expressed in a lower rhamnose concentration. At a C:N ratio below 11:1, rhamnose was no longer detected or no biosurfactant production.

Robert *et al.* (1989) observed nitrate to be the best source of nitrogen for biosurfactant production by *Pseudomonas* strain 44T1. Olive oil as a carbon source (2%) supported the highest amount of growth (5 g/l) and surfactant production (CMC $^{-1}$ = 20). Rhamnolipid production started soon after incubation (14 h), when nitrogen limiting conditions were reached, but increased dramatically, for 58 h.

Abu-Ruwalda *et al.* (1991) studied the effect of different nitrogen sources on the growth of *Rhodococcus* sp. ST-5. The result showed that nitrate was the best source of nitrogen for biosurfactant production by using 2% (v/v) n-paraffin as a carbon source.

Wu *et al.*, 2007 reported that nitrate ($NaNO_3$) was a better nitrogen source than ammonium ion (NH_4Cl) for *P. aeruginosa* EM1 to produce rhamnolipid. To obtain a high rhamnolipid yield of 8.63 g/L, the optimal C/N ratios of 26 and 52 were obtained for glucose- and glycerol-based culture, respectively. The effect of

C/N ratio on rhamnolipid production was slightly different when the carbon source was different. Moreover, this work also showed that poor rhamnolipid production performance was obtained when the C/N ratio was too high.

2.3.3 Mineral Sources

Guerra-Santos *et al.* (1986) showed minerals affecting biosurfactant production on *Pseudomonas aeruginosa* DSM 2659. The empirical adjustment of the mineral medium formulation was found to affect the yields of the active compounds rhamnolipids.

2.3.4 Environmental Factors

The pH, temperature, agitation, and oxygen as an environmental factors and growth conditions also affect biosurfactants production because of their effects on the cellular growth as well as the activity.

2.3.4.1 The pH

Gobbert *et al.* (1984) reported that the pH medium played an important role in sophorolipid production by *T. bombicola.* The high production of sophorolipid was found at pH of 3.5 whereas Guerra-Santos *et al.*(1984) showed that the rhamnolipid production in *Pseudomonas* spp. was maximized at a pH range from 6 to 6.5. Any change to both lower or higher pH values caused an appreciable drop in the productivity of biosurfactant. However, above pH 7, the rhamnose concentration decreased rapidly.

2.3.4.2 Temperature

Banat (1993) showed that thermophilic *Bacillus* sp. grew and produced biosurfactant at temperatures of 45°C. Heat treatment of some biosurfactants caused no appreciable change in biosurfactant properties such as the lowering of sur-

face tension and interfacial tension and the emulsification efficiency, all of which remained stable after autoclaving at 120°C for 15 min (Abu-Ruwaida *et al.*, 1991).

Wei *et a.l.* (2005) reported that rhamnolipid production increased with temperature from 25 to 30°C, remained nearly constant for 30 and 37°C, and decreased slightly when temperature was further increased to 42°C. *P. aeruginosa* J4 was unable to grow at 47°C. These results suggest that the optimal temperature for rhamnolipid production with the J4 strain was in the range of 30–37°C.

2.3.4.3 Agitation and Aeration

Sheppard and Cooper (1990) studied the effects of biosurfactant on oxygen transfer in a cyclone column reactor and concluded that oxygen transfer is one of the key parameters for the process optimization and scale-up of surfactin production in *B. subtilis* .

Wei *et a.l.* (2005) showed agitation rate affecting the mass transfer efficiency of both oxygen and medium components which were considered to be crucial to the cell growth and biosurfactant production of the strictly aerobic bacterium *P. aeruginosa* J4, especially when it was grown in a shaking flask. The results from batch fermentation under different agitation rates (50–250 rpm) showed that as the agitation rate increased, the rhamnolipid production increased nearly 80% and the dissolved oxygen (DO) level in the batch culture also increased from approximately 0.12–0.55 mg/L.

Gautam and Tyagi (2006) described an increase in agitation speed causing the reduction of biosurfactant yield of *Nocardia erythropolis* due to the effect of shear. On the other hand, the biosurfactant production by yeast increaseed when the agitation and aeration rate increased.

2.4 Advantages and Disadvantages of Biosurfacants

2.4.1 Advantages

The unique properties of biosurfactants allow their use and possible replacement of chemically synthesized surfactants in a great number of industrial op-

erations because they are many advantages as compared to chemically synthesized counterparts (Kosaric, 2001). The unique properties of biosurfactants can be summarized as follows:

- Biodegradability and low toxicity
- Biocompatibility and digestibility - which allow their application in cosmetics, pharmaceuticals and food additives.
- Availability of raw materials - biosurfactants can be produced from cheap raw materials which are available in large quantities. The carbon source may come from hydrocarbons, carbohydrates and/or lipids, which may be used separately or in combination with each other.
- Acceptable production economics - depending upon application, biosurfactants can also be produced from industrial wastes and by-products and this is of particular interest for bulk production.
- Use in environmental control - biosurfactants can be efficiently used in handling industrial emulsions, control of oil spills, biodegradation and detoxification of industrial effluents and in bioremediation of contaminated soil.
- Specificity - biosurfactants, being complex organic molecules with specific functional groups, are often specific in their action: de-emulsification of industrial emulsions, specific cosmetic, pharmaceutical, and food applications.

2.4.2 Disadvantages

- Low yield production of biosurfactants but large quantities are particularly needed in petroleum and environmental applications.
- Pure substances which is of particular importance in pharmaceutical, food and cosmetic applications which most biosurfactants produced still contains several impurities.

2.5 Sequencing Batch Reactors on Biosurfactants Production

The use of batch processes for treating wastewaters is not a recent development. Sequencing batch reactors (SBR) have been widely used since they provide

high treatment efficiency and high process stability without sedimentation tanks. A part from domestic wastewater, municipalities, resorts, casinos, a number of industrial wastewaters, including dairy, pulp and paper, tanneries and textiles, have been treated successfully by using SBRs. The biological and physical unit processes involved in the SBR and conventional activated sludge systems are essentially the same. Both steps of aeration and sedimentation/clarification are performed in aeration tank without a separate sedimentation tank. This makes the SBRs extremely flexible to adapt to regulatory changes for effluent parameters such as nutrient removal. The SBRs are also very cost effective as compared with all conventional treatment processes (Lahlou *et al.*, 2003).

2.5.1 The cyclic process of SBR

Sequencing batch reactor (SBR) is a typical cyclic process consisting of one or more tanks, each capable of waste stabilization and solids separation. The number of tanks may be varied, depending on the sophistication of the control system. Each tank in the SBR system is filled during a discrete period of time and then operated as a batch reactor. The aeration is stopped to allow the mixed liquor is to settle and the clarified supernatant is then drawn from the tank. The cycle for each tank in a typical SBR is divided into four discrete time periods: Fill, React, Settle, and Draw (Al-Rekabi *et al.*, 2007).

2.5.1.1 Fill

The influent to the tank may be either a raw wastewater or primary effluent. It may be either pumped in or allowed to flow in by gravity. The feed volume is determined based on a number of factors including desired loading and detention time and expected settling characteristics of the microorganisms. The time of Fill depends upon the volume of each tank, the number of parallel tanks in operation, and the extent of diurnal variations in the wastewater flow rate.

2.5.1.2 React

Biological reactions, which were initiated during Fill, are completed during React. As in Fill, alternating conditions of low dissolved oxygen concentrations (e.g., Mixed React) and high dissolved oxygen concentrations (e.g. Aerated React) may be required. The liquid level remains at the maximum throughout react, sludge wasting can take place during this period as a simple means for controlling the sludge age. By wasting during React, sludge is removed from the reactor as a means of maintaining or decreasing the volume of sludge in the reactor and decreases the solids volume. Time dedicated to react can be as high as 50% or more of total cycle time.

2.5.1.3 Settle

In the SBR, solids separation takes place under quiescent conditions (i.e., without inflow or outflow and no aeration) in a tank, which may have a volume more than ten times that of the secondary clarifier used for conventional continuous-flow activated sludge plant. This major advantage in the clarification process results from the fact that the entire aeration tank serves as the clarifier during the period when no both flows air and wastewater enter the tank. Because all of the biomass remains in the tank until some fraction must be wasted, there is no need for underflow hardware normally found in conventional clarifiers. By way of contrast, the mixed liquor is continuously removed from a continuous flow activated-sludge aeration tank and passed through the clarifiers only to have a major portion of the sludge returned to the aeration tank.

2.5.1.4 Draw (Decant)

The withdrawal mechanism may take one of several forms, including a pipe fixed at some predetermined level with the flow regulated by an automatic valve or a pump, or an adjustable or floating weir at or just beneath the liquid surface. In any case, the withdrawal mechanism should be designed and operated in a manner that prevents floating matter from being discharged. The time dedicated to Draw can range from 5 to more than 30% of the total cycle time. The time in Draw,

however, should not be overly extended because of possible problems with rising sludge.

Cassidy *et al.* (2000) compared the performance of a continuous-flow stirred tank reactor (CSTR) and a soil slurry-sequencing batch reactor (SS-SBR) treating the same diesel fuel-contaminated soil. The result showed that the SS-SBR provided markedly enhanced contaminant degradation relative to the CSTR. Diesel fuel removal efficiency was 96% in the SS-SBR, compared with 75% in the CSTR and biosurfactant production was greater in the SS-SBR. Microbial growth was approximately 25% greater in the SS-SBR than the CSTR. However, signifcant biosurfactant production and foaming occurred in the SS-SBR, whereas none was observed in the CSTR. Converting the CSTR to an SS-SBR resulted in surfactant production and enhanced diesel fuel degradation. These results indicate that the fill-and-draw operation is favorable for microbes with a greater ability to produce surfactants and degrade diesel fuel than the CSTR operation.

Cassidy and Hudak (2001) continued a comparison of the CSTR and SS-SBR for biosurfactant production. They found that the SS-SBR operation favored the growth of biosurfactant-producing microorganisms relative to the CSTR. Biosurfactant-producing species comprised 88% of the total microbial concentration in SS-SBR, and 23% in CSTR. However, the numbers of all five species (*C.tropicalis, B.casei, F.aquatile, P.aeruginosa*, and *P. fluorescens*) were significantly different with SS-SBR and CSTR operation except *P.aeruginosa*. *C tropicalis* concentrations achieved with SS-SBR operation were more than 3 orders of magnitude greater than with the CSTR operation. Biosurfactants were produced in the SS-SBR to levels of nearly 70 times the critical micelle concentration (CMC) early in the cycle, but were completely degraded by the end of each cycle. The result also showed that biosurfactant production was not observed in the CSTR. The biodegradation rate of diesel was over 40% greater in the SS-SBR than the CSTR. However, considerable foaming occurred in the SS-SBR, whereas none was observed in the CSTR.

Ong *et al.* (2003) evaluated the effects of the powered activated carbon (PAC) and activated rice husk (ARH) in reducing the toxic effect of copper on the activated sludge microorganisms. The SBR reactor was operated with FILL,

REACT, SETTLE, DRAW and IDLE modes in a time ratio of 0.5: 3.5: 1: 0.75: 0.25 for a cycle time of 6 h. The result showed that the efficiency of copper and COD removal was 90 and 85% respectively.

Andrea *et al.* (2004) studied the total polycyclic aromatic hydrocarbons (PAHSs) removal efficiency and the addition of lactose in the reactor as biosurfactant stimulator to enhance PAH removal efficiency in the sequencing batch reactor (SBR). The results showed that the total PAHs efficiency removal close to 55% was achieved for long (98 days), middle (70 days) and short (35 days) HRT of the SBR. The addition of lactose (external carbon source) in the SBR has not increased the biological activity.

Sarioglu (2005) investigated various pure cultures for the biological phosphorus removal using a Sequencing Batch Reactor (SBR). Pure cultures of *Acinetobacter lwoffii*, *A. lwoffii–Pseudomonas aeruginosa* mixture and *P. aeruginosa* were added into the first, second and third reactors, respectively. The results demonstrated that the use of *A. lwoffii* resulted in 100% PO_4–P removal within one month. On the other hand, the mixed culture of *A. lwoffii* and *P. aeruginosa* and *P. aeruginosa* gave PO_4–P removal efficiencies of 25% and 20%, respectively. The COD removal efficiency of 90% was found in all reactors. The decrease in PO_4–P removal in the two SBRs can be explained by the lower different growth rate of *P. aeruginosa* as compared to that of Acinetobacter lwoffii. The phosphorus removal data implied that population dynamics has a significant effect on phosphorus removal in all three SBRs.

2.5.2 Advantages and disadvantages of SBRs

Some advantages and disadvantages of SBRs are described by Irvine *et al.* (2004).

2.5.2.1 Advantages

- Equalization, primary clarification (in most cases), biological treatment, and secondary clarification can be achieved in a single reactor vessel.

- Operating flexibility and ability to handle shock loads. Organisms in the SBR are exposed to severe organic concentration variations during each Fill cycle, which encourages SBR organisms to excel at accommodating unplanned organic spikes in the feed.

- Complete quiescent settling for improved total suspended solids (TSS) removal.

- Potential capital cost savings by eliminating clarifiers and other equipment.

2.5.2.2 Disadvantages

- A higher level of sophistication is required (compared to conventional systems), especially for larger systems, of timing units and controls.

- Higher level of maintenance (compared to conventional systems) associated with more sophisticated controls, automated switches, and automated valves.

- Potential of discharging floating or settled sludge during the DRAW or decant phase with some SBR configurations.

- Potential plugging of aeration devices during selected operating cycles, depending on the aeration system used by the manufacturer.

- Potential requirement for equalization after the SBR, depending on the downstream processes.

2.6 The potential application of biosurfactants in industries.

Biosurfactants in many cases have been proved to be more effective than chemical surfactants and have the added benefit of being their broad range of novel structural characteristics, physical properties and their production on renewable substrates. Many chemical surfactants cause environmental problems due to their resistance to biodegradation and their toxicity when allowed to accumulate in natural ecosystems. The increasing interest in the potential applications of biosurfactants is based on their broad range of functional properties that includes emulsification, phase separation, wetting, foaming, solubilization and de-emulsification.

2.6.1 Microbially-enhanced oil recovery (MEOR)

Enhanced oil recovery (EOR) processes rely upon the use of chemical or thermal energy to recover crude oil that is trapped in pores of reservoir rock after primary and secondary (water flood) crude oil production has ceased. The residual crude oil in reservoirs makes up about 67% of the total petroleum reserves, indicating the relative inefficiency of primary and secondary production. Chemicals used for EOR include surfactants to reduce the interfacial tension between oil and water, and oil and rock interfaces. The main factor is the low permeability of some reservoirs or the high viscosity of the oil which results in poor mobility. High interfacial tensions between the water and oil may also result in high capillary forces, retaining the oil in the reservoir rock. The use of chemical surfactants for cleaning-up oil reservoirs is an unfavorable practice that is hazardous, costly and will leave undesirable residues which are difficult to dispose of without adversely affecting the environment. Many microorganisms can produce biosurfactants with high activity by using fermentation from low cost raw materials such as molasses.

Bryant (1987) described the microorganisms can also be beneficial in terms of oil recovery. There are three ways to enhance oil recovery (EOR):(a) microorganisms can produce biosurfactants and biopolymers in a separate unit; (b) microorganisms grow in reservoir rock pore throats to produce gases, surfactants, and other chemicals to recover the trapped oil in reservoirs; and (c) microorganisms can selectively plug high-permeability channels in reservoir rock so that the sweep efficiency of the recovery process can be increased.

2.6.2 Biosurfactants Oil Storage Tank Clean-Up

Bognolo (1999) described biosurfactants as emulsifying agents for hydrocarbons in oil storage tanks. Sludges and heavy oil fractions that settle at the bottle of oil storage tanks are highly viscous or even solid deposits that cannot be lifted by conventional pumps. Their removal usually requires solvent washing or manual cleaning: both being hazardous, time consuming and expensive processes. Several attempts have been made to develop an alternative cleaning process by form-

ing concentrated oil-in-water emulsions through the use of surface-active agents. pumping out the mobilized sludge and recovering valuable crude oil after emulsion breaking.

Biosurfactants produced from microorganisms were used to mobilize and clean-up sludges from a crude oil storage tank in Kuwait. A quantity 1.5 tons of biosurfactant was added to about 850 m^3 of sludge, along with 750 m^3 of crude oil and 2000 m^3 of brackish water. Then, circulation in the tank was initiated by suction at the water–oil interface and reinjection through the tank bottom and continued uninterrupted for 5 d. After that, the sludge was found to be dispersed in small droplets. Next, the circulation was stopped an emulsion breaker was added to obtain two separate phase with an upper oil layer whilst the inorganic contaminants collected at the bottom. Using this cleaning technique, 91% of the sludge was recovered as crude oil, and remaining about 75 m^3 of impurities (consisted mainly of non-hydrocarbon materials) at the tank bottom (Banat 1995).

2.6.3 Oil Spill Dispersants

Wei *et al.*, (2005) used rhamnolipids biosurfactant produced by strain JBR215 to clean used oil sorbents. Due to oil is explored, transported, stored and used there will be the risk of a spillage caused major problem on the environment. The process have been developed to remove oil from contaminated areas by using oil sorbents. Of all the sorbents which can be utilized, polypropylene (PP) nonwoven sorbents have high oil sorption capacity and low water uptake, and so PP nonwoven sorbents are ideal materials for oil recovery from the water surface. Most sorbents, however, end up in landfills or in incineration after a single use. These options either produce another source of pollution or increase the oil recovery cost. Biosurfactants have been increasingly used in soil washing and oil removal from contaminated areas. In this results showed that with biosurfactant washing more than 95% removal of the oil from sorbents was achieved, depending on the washing conditions. Biosurfactants were found to have considerable potential for recycling the used sorbents.

2.6.4 Heavy metal removal from sediments by biosurfactants

Mulligan *et al.* (2001) used biosurfactants for the removal of heavy metals from sediments. According to heavy metals to be allowed to pass through the municipal waste treatment facility, they return to the environment where they are persistent, cannot be biodegraded. These metals can adsorb onto the soil. runoff into rivers or lakes or leach in the groundwater, an important source of drinking water. Therefore, rhamnolipids from *Pseudomonas aeruginosa* surfactin from *Bacillus subtilis*, and sophorolipid from *Torulopsis bombicola* were evaluated their performance is heaving metal removal using a metal-contaminated sediment (containing 110 mg/kg copper and 3300 mg/kg zinc). The results sowed that a single washing with 0.5% rhamnolipid removed 65% of copper and 18% of zinc, whereas 4% sophorolipid removed 25% of copper and 60% of zinc. Surfactin was less effective, removing 15% of the copper and 6% of the zinc.

Mulligan (2005) described that rhamnolipids (anionic in nature), they are able to remove metals from soil and ions due to they form biosurfactant–metal complexes. Cations of lowest to highest affinity for rhamnolipid were $K^{+} < Mg^{2+} < Mn^{2+} < Ni^{2+} < Co^{2+} < Ca^{2+} < Hg^{2+} < Fe^{3+} < Zn^{2+} < Cd^{2+} < Pb^{2+} < Cu^{2+} < Al^{3+}$. Thus, the results indicate the potential of the rhamnolipid for metal remediation. The metal–biosurfactant complexes can be removed by addition of air to cause foaming and then the biosurfactant can be recycled through precipitation by reducing the pH to 2. The technique of ultrafiltration and zeta potential measurements were used to determine the mechanism of metal removal by the surfactants. It was then postulated that metal removal by the biosurfactants occurs through the sorption of the surfactant on to the soil surface and complexation with the metal, detachment of the metal from the soil into the soil solution and hence association with surfactant micelles. Sequential extraction of the sediments after washing with the various surfactants indicated that the biosurfactants, rhamnolipid and surfactin could remove the organically-bound copper and that the sophorolipid could remove the carbonate and oxide-bound zinc.