CHAPTER II
LITERATURE REVIEW

2.1 Rhamnolipids: Preparation, Characterization, and Potential Applications

Certain species of *Pseudomonas* are able to produce and excrete a heterogeneous mixture of biosurfactants with a glycolipid structure. These are known as rhamnolipids. In the biosynthetic process, rhamnolipid production is governed by both the genetic regulatory system and central metabolic pathways involving fatty acid synthesis, activated sugars, and enzymes. These surface-active compounds can be produced from various types of low-cost substrates, such as carbohydrates, vegetable oils, and even industrial wastes, leading to a good potential for commercial exploitation. By controlling environmental factors and growth conditions, high rhamnolipid production yields can be achieved. Rhamnolipids provide good physicochemical properties in terms of surface activities, stabilities, and emulsification activities. Moreover, these surface-active compounds exhibit anti-microbial activities against both phytopathogenic fungi and bacteria. Due to an increase in concerns about environmental protection, and the distinguishing properties of the rhamnolipids, it seems that rhamnolipids meet the criteria for several industrial and environmental applications, such as environmental remediation and biological control. Rhamnolipids have already been commercially produced, making them more economically competitive with synthetic surfactants. In the near future, rhamnolipids may be commercially successful biosurfactants.

2.1.1 Chemical Structures and Properties of Rhamnolipids

Pseudomonas strains, Gram-negative bacteria, have been reported to excrete rhamnolipids beginning in 1949 (Jarvis and Johnson, 1949). Although there are many types of rhamnolipid species, all of them possess similar chemical structures (Torrens *et al.*, 1998). Normally, rhamnolipids contain a hydrophilic head formed by one

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or two rhamnose molecules and a hydrophobic tail that contains one or two fatty acid chains (Sánchez *et al.*, 2007). Figure 2.1 shows the four general chemical structures of rhamnolipids produced by certain species of *Pseudomonas*. The two major types of rhamnolipids are found to be L-rhamnosyl-3-hydroxydecanoyl-3-hydroxydecanoate, or monorhamnolipid (Rha–C_{10}–C_{10}), and L-rhamnosyl-L-rhamnosyl-3-hydroxydecanoyl-3-hydroxydecanoate, or dirhamnolipid (Rha–Rha–C_{10}–C_{10}), but most of the biosurfactants produced by *P. aeruginosa* strains are dirhamnolipid (Mata-Sandoval *et al.*, 2001; Rahman *et al*; 2002). Only a few reports show that monorhamnolipid is the predominant component (Arino *et al.*, 1996; Sim *et al.*, 1997). The difference in types and proportion of rhamnolipids in the mixture might result from the age of the culture, bacterial strains (Déziel *et al.*, 1999), specific culture conditions, and substrate composition (Costa *et al.*, 2006).

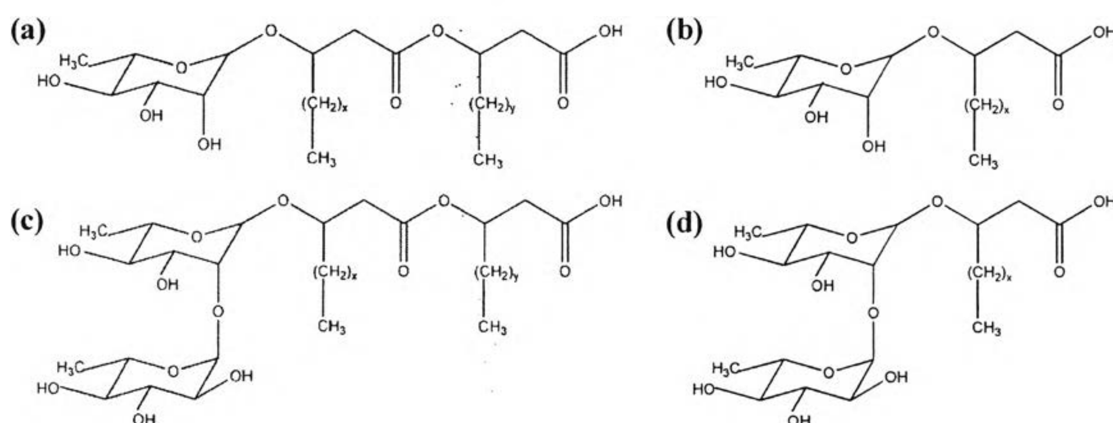

Figure 2.1 The four general chemical structures of rhamnolipid biosurfactants produced by certain species of *Pseudomonas*.

To fractionate and characterize the types of rhamnolipids in the mixture, a number of analytical methods can be used. In the past, high performance liquid chromatography (HPLC) equipped with a photodiode array detector or UV detector (Rendell *et al.*, 1990; Schenk *et al.*, 1995; Mata-Sandoval *et al.*, 1999) and gas

chromatography/mass spectrometry (GC/MS) (Mata-Sandoval *et al.*, 1999; Thanomsub *et al.*, 2006) were the most widely-used techniques; however, they are time-consuming and do not provide reliable quantification analysis. Recently, HPLC equipped with an evaporative light scattering detector (ELSD) (Noordman *et al.*, 2000; Trummler *et al.*, 2003) and liquid chromatography/mass spectrometry (LC/MS) (Déziel *et al.*, 1999; Déziel *et al.*, 2000; Chayabutra and Ju, 2001; Benincasa *et al.*, 2004) were developed as efficient techniques for the analysis of the rhamnolipid species. To identify the chemical structures of rhamnolipids, Fourier transform infrared (FT-IR) spectroscopy and nuclear magnetic resonance (NMR) analysis (Wei *et al.*, 2005; Monteiro *et al.*, 2007) were also performed. Today, nearly 30 rhamnolipid species, which differ in fatty acid chain composition and rhamnose moieties, have been reported.

Rhamnolipids exhibit free carboxylic groups and act as anions when the pH is above 4.0 (Nitschke *et al.*, 2005). These surface-active compounds are soluble in methanol, chloroform, ethyl ether, and an alkaline aqueous solution (Lang and Wagner, 1987). Rhamnolipids reduce the surface tension of pure water from 72 to below 30 mN/m, with a CMC in the range of 5-200 mg/l, depending on the components in the mixture (Finnerty, 1994). The presence of longer fatty acid chains probably increases hydrophobicity of the molecules, leading to the formation of the micellar structure at lower concentration (Mata-Sandoval *et al.*, 1999).

Another topic of interest related to the physicochemical properties of rhamnolipids is their emulsification activity. From the reported works, rhamnolipids produced from different *Pseudomonas* strains can effectively emulsify and stabilize emulsions with various types of hydrocarbons and oils. Wei *et al.* (2005) found that the biosurfactant produced by *P. aeruginosa* J4 achieved a maximum emulsion index of 70 and 78% for diesel and kerosene, respectively. Benincasa *et al.* (2004) also reported that the biosurfactant produced by *P. aeruginosa* LBI could form stable emulsions with *i*-propyl palmitate, castor oil, almond oil, crude oil, kerosene, and benzene for 21 days, suggesting potential applications of the excreted rhamnolipids in the pharmaceutical and cosmetic industries, and environmetal pollution treatment. Stable emulsions of *n*-alkanes

and aromatic compounds have also been reported; however, the emulsification activity of rhamnolipids was found to depend on the carbon sources used in the biosurfactant production (Patel and Desai, 1997).

Besides their good physicochemical properties, rhamnolipids also provide interesting biological activities, such as anti-microbial activity against phytopathogenic fungi species and bacteria. Abalos *et al.* (2001) reported that rhamnolipids produced by *P. aeruginosa* strain AT10 showed anti-fungal properties against *Gliocadium virens*, *Penicillium chrysogeum*, *Aspergillus niger*, *Chaetonium globosum*, *Aureobasidium pullulans*, *Rhizotecnia solani,* and *Botrytis cinerea.* The biosurfactant produced by *P. aeruginosa* LBI was found to be active against various phytopathogenic fungi species, such as *Penicillium funiculosum* and *Alternaria alternate* (Benincasa *et al.*, 2004). Stipcevic *et al.* (2005, 2006) found that dirhamnolipid showed differential effects on human keratinocyte and fibroblast cultures, leading to the enhancement of the burn-wound healing process. Thanomsub *et al.* (2006) reported that rhamnolipids produced by *P. aeruginosa* B189 displayed significant anti-proliferative activity against the human breast cancer cell line MCF-7 and the insect cell line C6/36, indicating potential application as anti-cancer drugs or agrochemicals.

2.1.2 Biosynthesis of Rhamnolipids

When cultivated in a liquid medium, *Pseudomonas* strains excrete mainly two types of rhamnolipids: mono-rhamnolipid and di-rhamnolipid. In the biosynthetic pathway of these two surface-active compounds, the rhamnosyl moiety and the fatty acid moiety are produced by *de novo* synthesis (Nitschke *et al.*, 2005), as shown in Figure 2.2. The donor of the rhamnosyl moiety is activated sugar, known as deoxy-thymidine-diphospho-L-rhamnose (dTDP-L-rhamnnose) (Burger *et al.*, 1963, 1966). To produce a rhamnosyl donor, D-glucose-1-phosphate is firstly synthesized by the specific reaction catalyzed by the AlgC enzyme. The production of dTDP-L-rhamnnose, which involves four sequential reactions catalyzed by the enzymes encoded by the *rml* genes, subsequently occurs (Maier and Soberón-Chavéz, 2000). The synthesis of the fatty acid

moiety of rhamnolipids is governed by the RhlG enzyme, which is responsible for draining the fatty acid precursors of rhamnolipids from the general fatty acid synthetic pathway at the level of the ketoacyl reduction (Campos-García *et al.*, 1998).

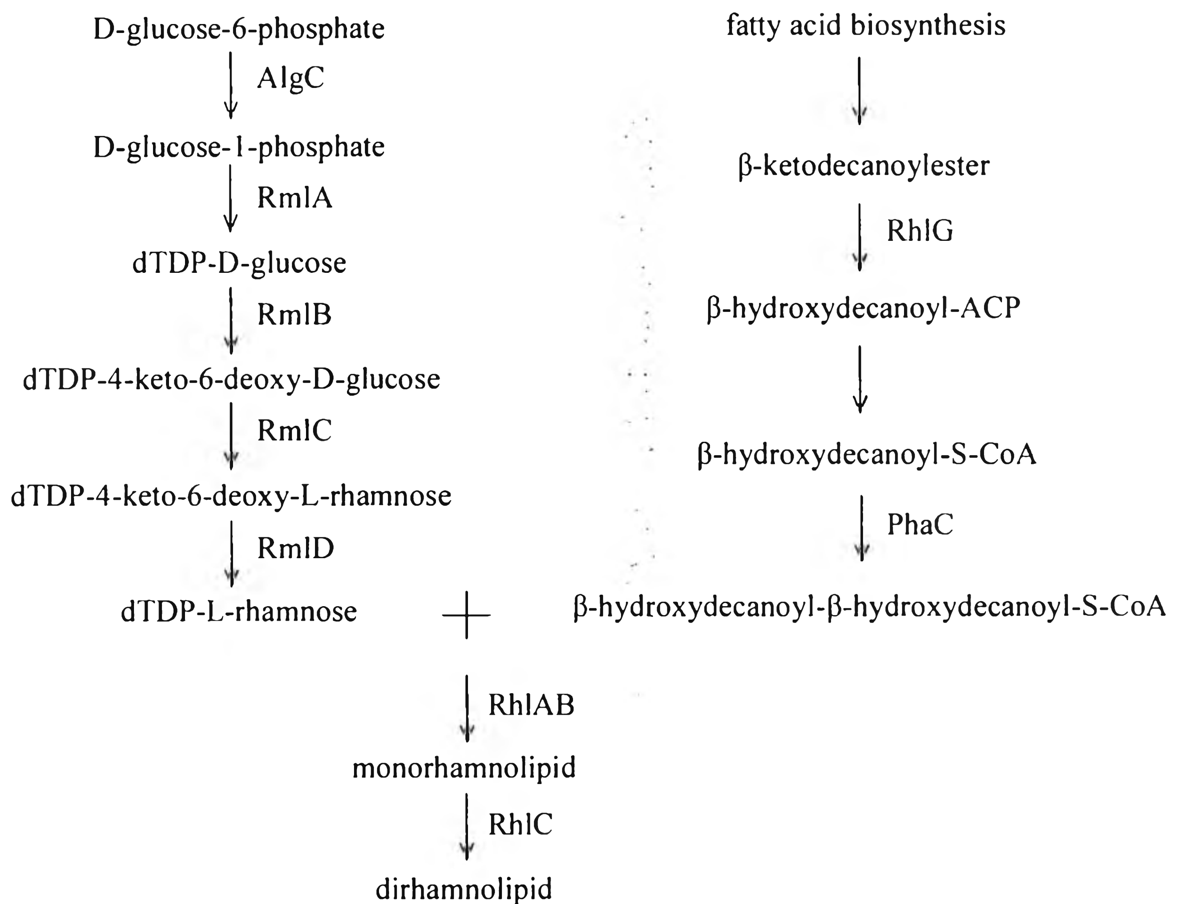

Figure 2.2 Biosynthetic pathway of rhamnolipid biosurfactants and the involved enzymes.

The biosynthesis of rhamnolipids proceeds by two sequential reactions catalyzed by the two specific rhamnosyltranferases—Rt 1 and Rt 2. The Rt 1 enzyme contains two polypeptides encoded by the *rhlA* and *rhlB* genes (Maier and Soberón-Chavéz, 2000), while the Rt 2 enzyme is encoded by the *rhlC* gene (Rahim *et al.*, 2001).

In both reactions, dTDP-L-rhamnnose acts as the rhamnosyl donor. The respective recipient in the first reaction is the fatty acid moiety of rhamnolipids, while that in the second reaction is monorhamnolipid, yielding dirhamnolipid as a product (Burger *et al.*, 1963, 1966; Maier and Soberón-Chavéz, 2000). Due to the fact that the rhamnolipid biosynthesis also involves a complex genetic regulatory system, the construction of strains with enhanced rhamnolipid production is much more difficult (Maier and Soberón-Chavéz, 2000).

2.1.3 Production of Rhamnolipid

For the production of microbial metabolites on a large scale, it is important to know the regulation mechanisms of the chosen microorganism. In general, biosurfactant production is induced by hydrocarbons or water-insoluble substrates. The production of microbial metabolites is governed by several factors: the nature of the carbon source; the concentrations of nitrogen and ions in the media; culture conditions like temperature, pH, agitation rate, and oxygen availability; the nature of the selected microorganism; and, the fermentation strategies (Nitschke *et al.*, 2005). Hence, all of these factors should be considered in the establishment of a rhamnolipid production process to achieve high rhamnolipid production yields.

The *Pseudomonas* species are able to utilize both water-soluble carbon sources (such as glycerol, glucose, mannitol, and ethanol) (Robert *et al.*, 1989; Wei *et al.*, 2005; Sim *et al.*, 1997) and water-immiscible substrates (like *n*-alkane and vegetable oils) (Syldatk *et al.*, 1985; Robert *et al.*, 1989; Wei *et al.*, 2005) for the rhamnolipid production. Normally, it seems that the water-immiscible substrates can provide a higher level of rhamnolipid production (Robert *et al.*, 1989; Wei *et al.*, 2005). It was reported that rhamnolipid production by *P. aeruginosa* UG2 was 100-165 mg of rhamnolipid per gram of substrate when hydrophobic substrates such as long chain alcohols and corn oil were used as carbon sources. Compared to hydrophilic substrates, including glucose and succinic acid, only a rhamnolipid production of 12-36 mg/g was obtained (Mata-Sandoval *et al.*, 2001). However, Wu *et al.* (2008) recently reported a different trend,

showing that glucose and glycerol used as carbon sources in the biosurfactant production by *P. aeruginosa* strain EM1 were superior to olive oil and soybean oil in terms of both rhamnolipid yield and productivity. This suggests that the carbon source preference for the rhamnolipid production depends on the bacterial strain. Table 2.1 lists rhamnolipid production by some *Pseudomonas* strains using different substrates.

Table 2.1 Rhamnolipid production by *Pseudomonas* strains using different substrates

Strain	Carbon Source	Rhamnolipid Production (g/l)	References
YPJ-80	Glucose	4.4	(Lee *et al.*, 1999)
PA1	*n*-hexadecane	1.3	(Santa Anna *et al.*, 2002)
	Babassu oil	2.0	
	Glycerol	6.9	
J4	Kerosene	0.7	(Wei *et al.*, 2005)
	Diesel	1.3	
	Glycerol	1.4-1.5	
	Grape seed oil	2.0-2.1	
	Olive oil	3.6	
LBI	Buriti oil	2.9	(Costa *et al.*, 2006)
	Cupuaçu oil	6.6	
	Babassu oil	6.8	
	Andiroba oil	8.1	
	Passion Fruit oil	9.2	
	Brazilian Nut oil	9.9	
EM1	Soybean oil	2.6	(Wu *et al.*, 2008)
	Olive oil	3.7	
	Glucose	4.9	
	Glycerol	7.5	

The type of nitrogen source is crucial to cell growth and rhamnolipid production. It was found that sodium nitrate ($NaNO_3$) was the most efficient nitrogen source for the rhamnolipid production by *P. aeruginosa* EM1 in terms of rhamnolipid yields; however, using urea and yeast extract, organic compounds, as nitrogen sources provided better cell growth (Wu *et al.*, 2008). In fact, it has been reported that the organic nitrogen source can promote cell growth, but it is unfavorable for the production of glycolipid biosurfactant (Kim *et al.*, 2006). Chen *et al.* (2007) also found that nitrate-based compounds, inorganic nitrogen sources, seemed to be good nitrogen sources for the rhamnolipid production by *P. aeruginosa* strain S2, giving a maximum rhamnolipid concentration of nearly 2300 mg/l.

Culture conditions also play a key role in the rhamnolipid production. Wei *et al.* (2005) reported that the rhamnolipid production by *P. aeruginosa* J4 increased about 80% when the agitation rate was increased from 50 to 200 rpm. Further increasing the agitation rate decreased the transfer efficiency of oxygen gas into the liquid medium, leading to unsuitable conditions for the biosurfactant production. Chayabutra and Ju (2001) found that the rate of rhamnolipid production by *P. aeruginosa* ATCC 10145 significantly increased when pH was in the range of 6.5 to 6.7. Robert *et al.* (1989) found that the best temperature for the biosurfactant production by *P. aeruginosa* 44T1 was 37°C, while the rhamnolipid production by *P. chlororaphis* NRRL B-30761 was best achieved at 23°C (Gunther *et al.*, 2005).

Based on the kinetics of biosurfactant production, fermentation strategies can be divided into four types: growth-associated production, production under growth-limiting conditions, production with precursor supplementation, and production by resting or immobilized cells. For growth-associated production, parallel relationships exist between growth, substrate utilization, and biosurfactant production. Production under growth-limiting conditions is characterized by a sharp increase in the biosurfactant level as a result of a limitation of one or more medium components. In the third fermentation strategy, the biosurfactant precursors are added to the culture medium, resulting in both qualitative and quantitative changes in the biosurfactant product (Desai

and Banat, 1997). Using resting or immobilized cells, the microorganism is separated from the culture medium after cultivation under optimal growth conditions and the wet biomass is then used for the biosurfactant production. For rhamnolipid production, the widely-used fermentation strategies are production under growth-limiting conditions and production by resting or immobilized cells (Nitschke *et al.*, 2005).

Many works have demonstrated that the limitation of multivalent ions and nitrogen causes the overproduction of rhamnolipids. It was reported that the rhamnolipid production by *P. aeruginosa* DSM2659 was promoted as the iron concentration in the culture media was reduced (Guerra-Santos *et al.*, 1984). Mulligan *et al.* (1989) found that an inorganic phosphate-limited medium provided the best yield of rhamnolipid production by *P. aeruginosa* ATCC 9027. Matsufuji *et al.* (1997) reported that a high production of rhamnolipids was achieved when *P. aeruginosa* IFO 3924 was cultivated under nitrogen-limiting conditions at a carbon to nitrogen (C/N) ratio of 18/1, while Santa Anna *et al.* (2002) found that a C/N ratio of 60/1 caused the overproduction of rhamnolipids by *P. aeruginosa* PA1. These results suggest that the effect of C/N ratio on the rhamnolipid production depends on the bacterial strains. Yateem *et al.* (2002) also reported that an increase in the nitrogen concentration caused a reduction of the rhamnolipid production by *P. aeruginosa* KISR C1; however, the bacterial growth was enhanced, leading to an increase in the bacterial number. The fermentation strategy involving production by resting or immobilized cells can be used for the continuous production of rhamnolipids. Jeong *et al.* immobilized *P. aeruginosa* BYK-2 (KCTC 18012P) in poly(vinyl alcohol) beads, and found that the relative activity of rhamnolipid production was maintained during 15 cycles in a repeated batch culture (Jeong *et al.*, 2004).

To facilitate the industrial development of rhamnolipid production, one possible method to decrease the production cost is the utilization of alternative low-cost substrates. For rhamnolipid production by *Pseudomonas*, urban and agroindustrial wastes with a high content of carbohydrates or lipids may meet the requirements for use as alternative substrates (Nitschke *et al.*, 2005). Mercadé *et al.* (1993) showed that *P.*

aeruginosa JAMM NCIB 40044 was able to grow on olive oil mill effluent as the sole carbon source. Abalos *et al.* (2001) used soybean oil refinery wastes for the rhamnolipid production by *P. aeruginosa* AT10. Wastes obtained from sunflower (Benincasa *et al.*, 2002, Benincasa and Accorsini, 2008), soybean, cottonseed, babassu, palm, and corn oil refineries (Abalos *et al.*, 2004) were tested for rhamnolipid production by *P. aeruginosa* LBI. The use of these low-cost substrates to generate a valuable product combines waste minimization in vegetable oil processing with economical biosurfactant production, hopefully leading to a reduction of pollution problems (Benincasa and Accorsini, 2008).

2.1.4 Potential Applications of Rhamnolipids

Rhamnolipids have been shown to have potential use in several applications, but most of the research has focused on environmental remediation. Currently, bioremediation is thought to be as a cost- and performance-effective technology to solve environmental pollution problems. The pollutants can range from polycyclic aromatic hydrocarbons, refined petroleum products, acid mine drainage, pesticides, industrial waste, and heavy metals to crude oil (Finnerty, 1994). With the use of rhamnolipids, the biodegradation of these pollutants can be significantly enhanced. It has been found that the biodegradation of Casablanca crude oil was accelerated in the presence of rhamnolipids produced by *P. aeruginosa* AT10 (Abalos *et al.*, 2004). Zhang *et al.* (1997) reported that rhamnolipids increased the solubility of phenanthrene (polycyclic aromatic hydrocarbons) in a test solution, resulting in the enhancement of the phenanthrene biodegration rate. In addition, rhamnolipids produced by *P. aeruginosa* UG2 was found to increase the solubilization of pesticides, resulting in the stimulation of biodegradation rate and extent (Mata-Sandoval *et al.*, 2000). The enhancement of hexadecane biodegradation by rhamnolipids has also been reported (Noordman *et al.*, 2002).

Besides their use as a pure culture, rhamnolipids can also stimulate the biodegradation of contaminated soil and water. The potential use of rhamnolipids produced by *P. aeruginosa* J4 for the biodegradation of diesel-contaminated water and

soil was reported (Whang *et al.*, 2008). Clifford *et al.* (2007) found that rhamnolipids produced by *P. aeruginosa* ATCC 9027 significantly improved the solubilization of tetrachloroethylene (PCE), a common ground water pollutant, indicating the potential use of the tested biosurfactant in surfactant-enhanced aquifer remediation (SEAR) applications. Cassidy *et al.* (2002) also suggested that rhamnolipids might be applied in intrinsic bioremediation using *in situ* rhamnolipid production at an abandoned petroleum refinery.

In some cases, biodegradation processes are too slow or infeasible, so it is necessary to remove the contaminants from the environment (Maier and Soberón-Chavéz, 2000). Urum *et al.* (2005) investigated the removal of crude oil from soil in air sparging assisted stirred tank reactors using two surfactants, sodium dodecyl sulfate (SDS) and rhamnolipids. The results indicated that rhamnolipids removed oil from the contaminated soil sample comparable to the tested synthetic surfactant. Bai *et al.* (1997) reported that monorhamnolipid produced by *P. aeruginosa* ATCC 9027 displayed efficiency in the removal of residual hexadecane from soil higher than three synthetic surfactants: SDS, polyoxyethylene, and sorbitan monooleate. Noordman *et al.* (1998) showed that rhamnolipids from *P. aeruginosa* UG2 effectively removed phenanthrene from soil. Mulligan and Wang (2006) found that rhamnolipid foam effectively removed inorganic heavy metal, including cadmium and nickel, from a contaminated soil sample. The removal of zinc, lead (Herman *et al.*, 1995), and copper (Mulligan *et al.*, 2001) by rhamnolipids has also been reported.

In soil remediation applications, one of the important considerations is the size of the surfactant microstructures. Because contaminants are often found in very small soil pores, the movement of surfactant molecules through the soil can be easily limited by the pore size. Therefore, the size of the rhamnolipid microstructures should be studied closely for their effective use. It was previously reported that rhamnolipids could form various types of microstructures in an aqueous media (including lamellar sheets, vesicles, and micelles), depending on concentration and pH (Champion *et al.*, 1995; Sánchez *et al.*, 2007). The sizes of these rhamnolipid microstructures ranged from less

than 50 nm to larger than 1 μm, while the smaller-sized soil pores was in the range of 2 μm-0.2mm. Thus, the appropriate size of rhamnolipid microstructure could be achieved by controlling the concentration and pH.

Although economic considerations limit the expansion of the biosurfactant market, rhamnolipids have recently been produced on a large scale by Jeneil Biosurfactants Corporation. The development of cost-effective bioprocesses for rhamnolipid production could perhaps lead to the widespread use of these surface-active compounds. Because of the distinguishing characteristics of rhamnolipids, several industrial applications, especially environmental remediation, may be realized in the near future. It is also interesting to study the contribution of each rhamnolipid component to the properties of the biosurfactant produced by the *Pseudomonas* in order to obtain a biosurfactant with the desired properties for specific purposes. Moreover, future research focusing on the structural modification of rhamnolipids would probably enlarge the potential use of these surface-active compounds. Knowledge of the biological activities of rhamnolipids is another key factor in introducing these surface-active compounds in high value-added exploitation, such as in cosmetics, and in the pharmaceutical industry as anti-cancer drugs. In addition, the formation of rhamnolipid vesicles may perhaps meet the criteria for drug delivery applications.

2.2 Surface Modification via Surfactant Adsorption at Solid-Liquid Interface

Much effort is used to try to design, synthesize, and fabricate materials with good mechanical properties, durability, and functionality. Although all of these properties are governed by the bulk structure of the materials, the interaction at the outermost surface of materials, or the interface, can directly affect the utility of the materials in some applications. For example, in biomedical applications, the biological media, cells, and tissues *in vivo* are in contact with implant surfaces; therefore, the biointeraction, or the bioresponse, is mainly controlled by the surface characteristics of the materials, such as surface topography, surface wettability, surface chemistry, surface

charge, and surface rigidity. To improve the properties of the materials without losing the key physical characteristics, they are surface-modified using biological, mechanical, or physicochemical methods. Normally, the surface modification techniques fall into two categories: chemically or physically altering the atoms, compounds, or molecules in the existing surface; and, overcoating the existing surface with a material having a different composition (Ratner *et al.*, 2004). The selected methods should provide the minimum thickness of the modified surface layer to get uniformity, durability, and functionality of materials. Thick surface modification can cause delamination, cracking, and the changes in the mechanical and functional properties of materials while thin treatment is difficult to ensure that the existing surface is uniformly covered. However, the modified surfaces can have a specific thickness when the amphiphilic molecules are involved in the modification process.

By using a fundamental characteristic of surfactants, which is their tendency to adsorb at the interface in an oriented fashion, an adsorbed surfactant layer can be formed resulting in surface-engineered materials (Rosen, 2004). This adsorption process is strongly affected by a number of factors, like the nature of the structural groups on the solid surface, the molecular structure of the surfactant being adsorbed or the adsorbate, and the environment of the liquid phase. These factors can determine the adsorption mechanism, the efficiency and effectiveness of adsorption.

Surfactants, both synthetic surfactants and biosurfactants, are adsorbed in the form of single ions rather than micelles, and there are many mechanisms involving the adsorption of surface-active solutes onto solid substrates, such as ion exchange, ion pairing, acid-base interaction, polarization of π electrons, dispersion forces, and hydrophobic bonding.

Ion exchange involves replacement of counterions adsorbed onto the substrates from the solution by similarly charged surfactant ions, while ion pairing causes adsorption of surfactant ions on oppositely charged sites unoccupied by counterions. For acid-base interaction, either hydrogen bond formation between substrate and adsorbate or Lewis acid-Lewis base reaction controls the adsorption process of surfactants.

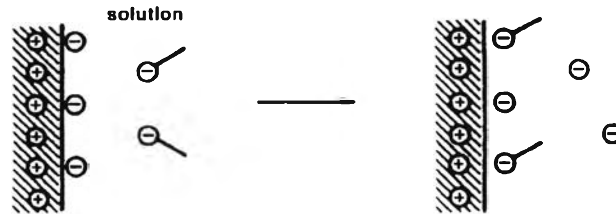

Figure 2.3 Adsorption of surfactants by ion exchange (Rosen, 2004).

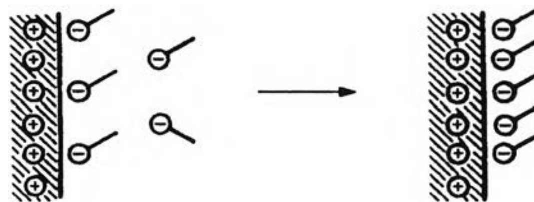

Figure 2.4 Adsorption of surfactants by ion pairing (Rosen, 2004).

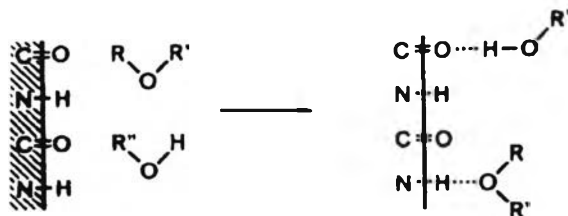

Figure 2.5 Adsorption via hydrogen bond formation (Rosen, 2004).

When the adsorbate contains electron-rich aromatic nuclei and the solid adsorbent has strongly positives sites, the attraction force will cause the adsorption via polarization of π electrons. In contrast, London-van der Waals forces acting between adsorbent and adsorbate molecules results in the adsorption via dispersion forces. In general, the adsorption by this mechanism increases with an increase in the molecular weight of the adsorbate.

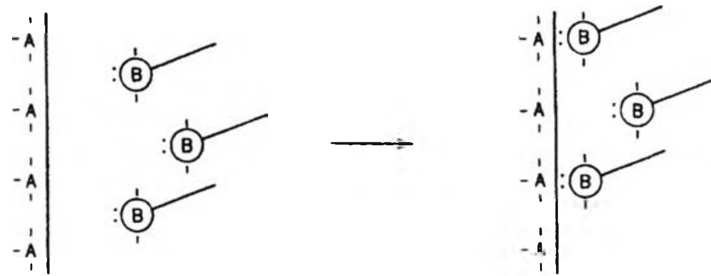

Figure 2.6 Adsorption via Lewis acid-Lewis base interaction (Rosen, 2004).

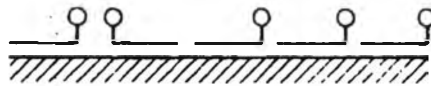

Figure 2.7 Adsorption via dispersion forces on non-polar surface (Rosen, 2004).

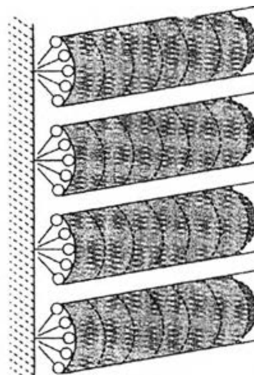

Figure 2.8 Adsorption from aqueous solution via hydrophobic bonding on an uncharged surface (Rosen, 2004).

The adsorption via hydrophobic bonding occurs when the combination of mutual attraction between hydrophobic moieties of the surfactant molecules and their tendency to escape from an aqueous environment is large enough to allow them to adsorb on the substrates by aggregating their chains, resulting in the formation of complex structure, like cylindrical admicellar structures on solid surfaces.

After the adsorption of surfactants onto substratum surfaces, the surface characteristics of the adsorbent can be modified by an orientation of the adsorbed surfactant molecules. Orientation with their hydrophobic groups predominantly away from the solid substrate will make the surface become more hydrophobic while the orientation in opposite fashion leads to an increase in surface hydrophilicity (Rosen, 2004).

During the past few decades, both synthetic surfactants and biosurfactants have been used to modify the surface characteristics of materials via the adsorption process. For example, Amiji and Park (1992) treated the surfaces of dimethyldichlorosilane-treated glass and LDPE with triblok copolymers of poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) or Pluronics®. The study showed that Pluronics® caused the ability to prevent platelet adhesion which mainly depended on the number of propylene oxide than ethylene oxide. Velraeds *et al.* (1997) investigated the potency of the biosurfactant produced by *Lactobacillus acidophilus* RC14 to reduce the initial adhesion of *Enterococcus faecalis* 1131 on a hydrophilic and a hydrophobic substratum. The results indicated that adsorbed biosurfactant layers caused an important, dose-related inhibition of the initial deposition rate and the number of adherent bacteria on both hydrophilic and hydrophobic surfaces. Rodrigues *et al.* (2005) treated silicone rubber with biosurfactant from *Streptococcus thermophilus* A in order to inhibit microbial adhesion. The obtained results indicated that the silicone rubber surface with adsorbed biosurfactant layer was more hydrophilic than bare silicon rubber, and pre-treatment with surface-active compound was effective to reduce the microbial adhesion onto silicone rubber.

2.3 Biological Responses to Surface Characteristics of Materials

Annually, millions of patients suffer from the loss or failure of an organ or tissue as a result of accidents or diseases. A general therapy to treat these patients is a transplantation of an organ from one individual to another or a transfer of tissue from

one location in the human body to the diseased site. However, these treatments are still imperfect due to a donor shortage and the limitation of donor sites. In order to solve these problems, tissue engineering is developed. This approach represents a new interdisciplinary field applying a set of tools at the interface of the biomedical and engineering sciences that use a combination of patient's own cells with materials to aid tissue formation or regeneration.

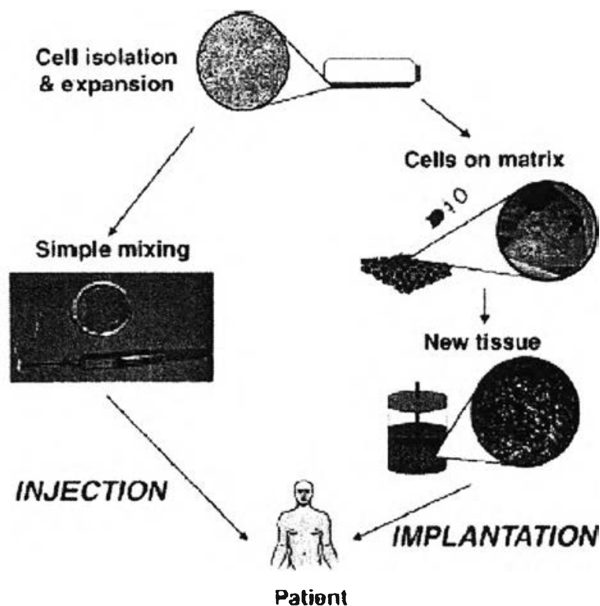

Figure 2.9 Schematic illustrations of typical tissue engineering approaches (Lee and Mooney, 2001).

For treatment of the full thickness skin loss, such as burns, skin ulcers, and deep wounds, the isolated tissue-specific cells from a small skin biopsy of the patient are harvested *in vitro* before incorporated into engineered materials that mimic the natural extracellular matrices (ECM) of the body, as shown in Figure 2.9. These materials not only provide a temporary substrate for adherence of transplanted cells but also control cell survival as well as behavior of cells—proliferation, migration, and differentiation. When materials are in contact with cells or tissues, their surfaces are rapidly covered

หอสมุดกลาง สำนักงานวิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

with proteins that are adsorbed from the surrounding body fluids. Normally, the nature of the adherent protein layer is controlled by the surface characteristics of the underlying substrates, resulting in the interaction with receptors in the cell membrane. This interaction plays an important role in the mediation of cell adhesion to materials. After cell adhesion, multiple functional biochemical signaling pathways within the cell are triggered. In general, the interaction between cells and materials is largely controlled by surface characteristics of materials such as surface topography, surface wettability, surface chemistry, surface charge, and surface rigidity.

2.3.1 Biological Responses to Surface Topography

Lampin *et al.* (1997) analyzed cell adhesion and spreading of chick embryo vascular and corneal explants cultured on rough and smooth poly(methyl methacrylate) (PMMA). The test revealed that slight roughness raised the migration area to an upper extent no matter which cell type, and cell adhesion potential was enhanced relating to the degree of roughness.

Chou *et al.* (1998) studied the topographic effect of commercially pure titanium on cell morphology in early passage human gingival fibroblasts. From scanning electron micrograph, it was found that fibroblasts on smooth titanium remained well spread and randomly oriented throughout the culture period. In contrast, cells on grooved titanium were oriented along the grooves and proliferate in this organization throughout the culture period.

Chung *et al.* (2003) reported that the increased surface roughness of biomaterial surfaces even at nano-scale could enhance the adhesion and growth of human umbilical vein endothelial cells (HUVECs) on roughness, displaying the potential use in applications of tissue engineering.

Berry *et al.* (2004) created regular arrays of pits on a two-dimensional quartz surface using micro-fabrication technology in order to examine the influence of micro-geometry on human fibroblast attachment and motility. The results clearly demonstrated that fibroblast interaction with the pit edges depended on both diameter as

well as angle of circumference, and inter pit spacing. Interestingly, the highest cell proliferation rates were observed on the smaller pits.

Vernon *et al.* (2005) fabricated thin planar collagen membranes (CMs) from air-dried hydrogels of fibrillar type I collagen. The effect of micro-grooved membranes on behavior of human dermal fibroblasts (HDFs) and human umbilical artery smooth muscle cells (HUASMCs) was observed. It was revealed that CMs supported cell attachment, spreading, and proliferation of both cell types, and cell alignments were influenced by micro-grooved pattern.

Wan *et al.* (2005) observed adhesion and proliferation of OCT-1 osteoblast-like cells on micro- and nano-scale topography structured poly(L-lactide) (PLLA). When compared to smooth surfaces of PLLA, it was found that only cell adhesion was enhanced by the created micro- and nano-scale roughness.

Schuler *et al.* (2006) studied cell adhesion and spreading patterns of epithelial cells, fibroblasts, and osteoblasts on biomimetically modified, smooth and rough titanium surfaces. The findings showed that more fibroblasts were present on smooth than on rough topographies while osteoblasts showed the opposite tendency. Epithelial cell attachment did not follow any regular pattern. The further studies indicated that footprint areas for all cell types were significantly reduced on rough compared to smooth surfaces.

2.3.2 Biological Responses to Surface Wettability

van der Valk *et al.* (1983) studied cell spreading and cell division rate of a transformed line of mouse lung fibroblasts on various polymer surfaces in the presence of serum proteins. The obtained results indicated that cell spreading appeared independent of the surface free energy while cell division rate was the same on all surfaces tested.

Schakenraad *et al.* (1986) determined the effect of surface free energy of polymeric materials on the spreading and growth of human skin fibroblasts in both the presence and absence of serum proteins. The results demonstrated the complex

relationship between cell spreading and surface free energy as well as the role of serum proteins in modifying the surface characteristics of polymers in relation to cell spreading and growth.

Lam *et al.* (1995) observed the influence of surface wettability on the inflammatory response after subcutaneous implantation in rat by comparing between poly(L-lactic acid) (PLLA) and poly(tetrafluoroethylene) (PTFE) films. The contact angle measurement indicated that PLLA was more hydrophilic than PTFE. From the obtained results, it was concluded that PLLA provoked a more intense inflammatory response than PTFE films.

Ruardy *et al.* (1995) investigated adhesion and spreading of human skin fibroblasts on gradient surfaces of dichlorodimethylsilane (DDS) coupled to glass. In the presence of serum proteins, it was found that human skin fibroblasts seeded on these gradient surfaces showed a preferential adhesion onto the steepest part of the gradient and the spread area of cells increased over the length of the gradient surface when going from the hydrophobic to the hydrophilic end.

Altankov *et al.* (1996) examined the ability of human fibroblasts to remove and recognize fibronectin bound on material surfaces as one feature of material surface biocompatibility. The effect of wettability and other traditional parameters of biocompatibility were together investigated. The study suggested that the removal/recognition process might be more sensitive to the changes in surface wettability than other parameters.

Webb *et al.* (1998) provided a range of surface wettability by preparing model surfaces with different functional groups. The behavior of NIH 3T3 fibroblasts cultured on model surfaces was studied. The obtained results found that cell attachment, spreading, and cytoskeletal organization were significantly greater on hydrophilic surfaces relative to hydrophobic surfaces. Interestingly, moderately hydrophilic surfaces promoted the highest level of cell attachment.

Khang *et al.* (1999) created the wettability chemogradient on the surfaces of poly(L-lactide-*co*-glycolide) (PLGA) films in order to investigate the interaction of

fibroblasts in terms of the surface hydrophilicity/hydrophobicity of PLGA surface. As a result, it was observed that cell adhesion, spreading, and growth were enhanced at the position with moderate hydrophilicity of the wettability chemogradient.

Tang *et al.* (2004) prepared poly (ε-caprolactone) (PCL) films from four solvent systems which are chloroform, tetrahydrofuran, acetone, and ethyl acetate. The surface properties and the influence of resulting films on behavior of murine (L929) fibroblasts were determined. It was revealed that cells favored ethyl acetate cast PCL films which had relatively low contact angle.

Kennedy *et al.* (2006) investigated the effect of surface energy on fibronectin-mediated osteoblasts adhesion, spreading, and proliferation by creating gradients in surface energy of a glass slide coated with a self-assembly monolayer (SAM) of *n*-octyldimethylchlorosilane beneath a UV lamp. From the experiments, it was found that surface energy did not affect cell adhesion as well as cell spreading. However, the rate of proliferation was linearly dependent on surface energy and increased with increasing hydrophobicity.

2.3.3 <u>Biological Responses to Surface Chemistry</u>

Jones *et al.* (1986) investigated the role of the carbohydrate residues of fibronectin concerning the specificities of that glycoprotein to interact with fibroblastic cell surfaces, gelatin, and heparin. From cell adhesion and spreading tests, the results indicated that carbohydrate residues of fibronectin acted as modulators of biological functions of the glycoprotein.

Smetana *et al.* (1990) observed the influence of hydrogel functional groups on the behavior of macrophages. It was showed that hydrogel containing alkaline groups induced a spreading of macrophages while the materials containing acidic groups inhibited spreading of the macrophages. In addition, the fusion of macrophages into multi-nucleate cells was inhibited on the surface of materials containing acidic groups and increased on the hydrogel containing alkaline groups.

Banerjee *et al.* (2000) prepared polymer latex with adhesion peptides linked to the surface of the acrylic beads in order to induce attachment and spreading of cells. As a result, coalesced films obtained from the modified latex particles promoted attachment of WT NR6 fibroblasts while the films from unmodified latex particles resisted these cells.

Tegoulia and Cooper (2000) studied the effect of surface chemistry on the adhesion of polymorphonuclear leukocytes (PMNs) under flow by using SAMs of alkanethiolate on gold surfaces. Cell adhesion was observed in the presence and absence of pre-adsorbed fibrinogen. The obtained results demonstrated that PMN adhesion depended on the terminal functionality and pre-incubation of the surfaces with fibrinogen decreased adhesion on all SAMs examined.

Webb *et al.* (2000) studied cell behavior of MC3T3-E1 osteoblasts on glass surfaces modified with different terminal silanes. The results found that MC3T3-E1 osteoblast behavior varied with individual functional groups indicating that it was surface chemistry dependent.

Shu *et al.* (2004) modified a thiol-modified hyarulonan (HA) with peptides containing the arginine-glycine-aspartic acid (RGD) sequence and then crosslinked with poly(ethylene glycol diacrylate) (PEGDA). The modified hydrogels were investigated *in vitro* and *in vivo*. The obtained results indicated that the attachment, spreading, and proliferation of cells were dramatically enhanced on RGD-modified surfaces but only modestly accerelated *in vivo* tissue formation.

Patel *et al.* (2006) modified PMMA by grafting poly(ethylene glycol) (PEG) onto its surfaces. The study found that PEG resisted cell adhesion and protein adsorption, however; the functionalization of grafted PEG molecules with RGD peptides restored cell adhesion to the surfaces as well as enhanced cell attachment and spreading.

Santiago *et al.* (2006) modified poly(carprolactone) (PCL) by covalently attaching peptide sequences derived from the extracellular matrix (ECM) protein laminin to polymer surfaces. The promoting effect of the modified PCL on attachment and proliferation of adipose-derived stem cells (ASCs) was investigated. The obtained

results indicated that PCL surfaces treated with a suitable peptide sequence had a significantly greater number of ASCs bound after cell seeding.

2.3.4 Biological Responses to Surface Charge

Kapur *et al.* (1996) investigated the electrical effect on human monocyte-surface interactions by using the nanofabrication technology to control surface charge density and maintain surface chemistry as well as surface topography. The results demonstrated that human monocytes responded *in vitro* to local surface-charge heterogeneity in the absence of substrate topography and compositional variation.

Lee *et al.* (1997) observed interaction of Chinese hamster ovary cells on chargeable functional group gradient surfaces prepared on low density polyethylene (LDPE) sheets by corona discharge treatment with gradually increasing power and graft copolymerization of different monomers. From scanning electron micrograph, it was observed that a large amount of cell attachment was presented on positively charged surfaces when compared to that on negatively charged surfaces.

Qiu *et al.* (1998) used an electrical stimulation system to investigate the interactions between rat bone marrow stromal cells and charged substrates. The results indicated that the positively charged surfaces enhanced cell attachment but suppressed cell spreading and differentiation of rat bone marrow stromal cells.

2.3.5 Biological Responses to Surface Rigidity

Choquet *et al.* (1997) studied cellular response to the rigidity of anchoring matrix by restraining movement of beads coated with fibronectin or an anti-intergrin antibody with an optical trap on fibroblasts. The results found that the strength of the intergrin-cytoskeleton linkages was dependent on matrix rigidity which served as a guidance cue in a process of mechanotaxis.

Pelham and Wang (1997) investigated responses of cells to mechanical properties of the adhesion substrate by culturing normal rat kidney epithelial and 3T3 fibroblastic cells on a collagen-coated polyacrylamide substrate in order to vary the

flexibility. The obtained results revealed that cells on flexible substrates showed reduced spreading and increased rate of motility or lamellipodial activity when compared with those on rigid surfaces.

Lo *et al.* (2000) cultured 3T3 fibroblasts on flexible polyacrylamide sheets coated with collagen which was introduced a transition in rigidity in the central region. The study demonstrated that changes in tissue rigidity controlled a number of normal and pathological processes involving cell locomotion.