CHAPTER II

THEORETICAL BACKGROUND AND LITERATURE SURVEY

2.1 Biosurfactant Classification

Microbial surfactantsor biosurfactants are commonly differentiated on the basis Major classes of .of their biochemical nature and the microbial species producing them biosurfactants include:

(1) Glycolipids

(2) Phospholipids

(3) Lipopeptides and lipoproteins

(4) Polymeric

2.1.1 Glycolips

These are compounds of a carbohydrate and a lipid; the linkage is by wat of either an ether or an ester group. As shown in Figure 2.1, the main glycolipids which are found to occur and are most often investigated are:

(a) rhamnolipids,

(b) mycolates of mono, -di and –trisaccharides,

(c) sophorolipids

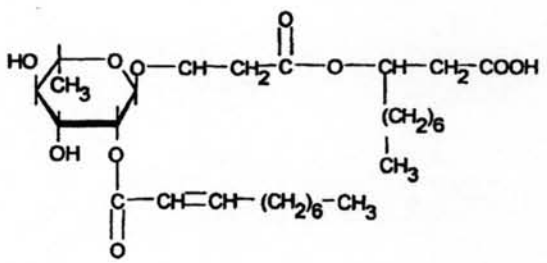

Figure 2.1 A glycolipid produced by a *Pseudomonas* stain. (Kosaric *et al.*, 2001)

2.1.2 Phospholipids

These are the esters formed between the alcohol groups on a lipid and a phosphate.

2.1.3 Lipopeptides and lipoproteins

These consist of a lipid attached to a polypeptide chain as shown in Figure 2.2.

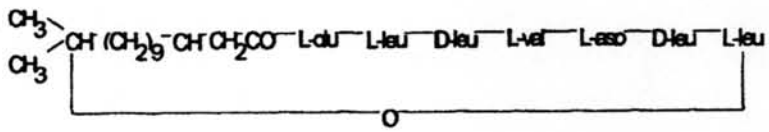

Figure 2.2 A lipopeptide structure (surfactin) produced by *Bacillus subtilis*. (Kosaric *et al.*, 2001)

2.1.4 Polymeric

These are products again formed between saccharide units and fatty acid residues; but they are polymeric in nature.

Bognolo *et al.* (1999) concluded that almost all biosurfactants are either nonionic or anionic, and there are no studies of biosurfactants with cationic structures.

Morikawa *et al.* (2000) studied arthrofactin (AF) and surfactin (SF) are the most effective cyclic lipopeptide biosurfactants ever reported. They reported that both AF and SF exhibited a higher activity under alkaline conditions than acidic conditions. AF was more resistant to acidic condition than SF and it kept high activity even under pH 0.5. Although SF drastically reduced its activity under acidic conditions, surfactin-Asp/Glu-amido ester and surfactin-Asp/Glu-methyl ester retained similar activities irrespective of the pH change. Also, AF with fatty acid chain length of C10, which was a main product of the strain, showed the highest activity.

Ron *et al.* (2002) demonstrated that the low molecular-weight molecules produced by bacteria efficiently lowered surface and interfacial tensions but the high

molecular-weight polymers were found to bind tightly to the surface of water. So, the high molecular-weight bio-surfactants are less effective in reducing interfacial tension, but are efficient at coating the oil droplets and preventing their coalescence.

2.2 Recent Analytical Methods

2.2.1 Surface Activity Measurement

Biosurfactant activities can be determined by measuring the change in surface and interfacial tensions, stabilization or destabilization of emulsions, and hydrophilic-lipophilic balance (HLB). Surface tension at the air/water and oil/water interfaces can easily be measured by using a tensiometer. The surface tension of distilled water is 72 mN/m, and an addition of surfactant lowers this value to around 30 mN/m. When a surfactant concentration increases, a reduction of surface tension is observed up to a critical level. Beyond the surfactant concentration at the minimum surface tension amphiphilic molecules associate readily to form supramolecular structures like micelles, bilayers, and vesicle. This value is known as the critical micelle concentration (CMC). CMC is defined by the solubility of a surfactant within an aqueous phase and is commonly used to measure the efficiency of a surfactant. When a microbial culture broth or biosurfactant solution is diluted several fold, surface tension is measured for each dilution, and then the CMC can be calculated from a plot between surface tension and dilution ratio, known as critical micelle dilution method.

An emulsion is formed when one liquid phase is dispersed as microscopic droplets in another liquid continuous phase. Biosurfactants may stabilize (emulsifier) or destabilize (demulsifiers) the emulsion. The emulsification activity is assayed by the ability of the surfactant to generate turbidity due to suspended hydrocarbons such as a hexadecane-2-methylnaphthalene mixture (Desai *et al.,* 1988) or kerosene (Goldenberg *et al.,* 1987), etc, in an aqueous assay system. The demulsification activity is derived by determining the effect of surfactants on a standard emulsion by using a synthetic surfactant (Rosenberg *et al.,* 1986).

The HLB value indicates whether a surfactant will promote a water-in-oil or oil-in-water emulsion by comparing it with surfactants with known HLB values and properties. The HLB scale can be constructed by assigning a value of 1 for oleic acid and a value of 20 for sodium oleate and using a range of mixtures of these two components in different proportions to obtain the intermediate values. Emulsifiers with HLB values less than 6 favor stabilization of water-in-oil emulsification, whereas emulsifiers with HLB values between 10 and 18 have the opposite effect and favor oil-in-water emulsification (Desai *et al.*, 1988).

Moreover, surface tension and critical micelle dilution (CMD-1 and CMD-2) were determined with a Krüss Processor Tensiometer (model K12 T Krüss, Germany) using the plate method. CMD-1 and CMD-2 were determined by measuring the surface tension of 10-times and 100-times diluted broth in distilled water, respectively (Desai *et al.*, 1988).

Nitschke *et al.* (2004) found the critical micelle dilution is an indirect indication of surfactant concentration. The lower the CMD values, the higher the dilution needed to cause a significant change in surface tension, thus the higher the biosurfactant concentration in the medium. The five isolates that presented the lowest surface tension values in manipueira and molasses medium were submitted to CMD measurements. The CMD-1 and especially CMD-2 data revealed a slightly increase on surface tension when manipueira medium was diluted, suggesting that a high biosurfactant concentration is present in this waste; inversely, for molasses medium the CMD-2 values showed a considerable increase. Manipueira was chosen as a potential substrate for biosurfactant production and the isolates: LB2a, LB2b, LB5a, LB262 and LBB that gave the lowest surface tension. These isolates were subsequently identified as *Bacillus sp.*

2.2.2 Oil Spreading Test

The oil spreading test is to measure the diameter of clear zones caused when a drop of a biosurfactant-containing solution is placed on an oil–water surface. The binomial diameter and biosurfactant concentration allows the determination of the cleaning efficiency of a given biosurfactant. For a general procedure of the oil spreading test, 50 ml of distilled water is first added to a large Petri dish (25 cm diameter) followed by addition of 20 ml of crude oil to the surface of water. After that, 10 ml of a biosurfactant-containing solution sample is then added to the surface of the oil. Also, a control plate is carried out by using pure water. The diameters of clear zones of triplicate assays from the same sample are determined (Morikawa *et al.*, 1993).

Rodrigues *et al.* (2006) found the clearing zones are an indirect measure of the surface activity of the tested samples against hydrocarbons, thus a higher diameter represents a higher surface active sample. For each tested sample, a linear relationship between concentration and diameter of the clearing zone is observed.

2.2.3 Establishment of Biosurfactant (BS) Screening Method

To screen an active biosurfactant-producing microorganism, there are two techniques as follows:

1) A tested medium containing microbial culture is dropped onto a layer of oil and surfactant production capacity is evaluated through the spread of water droplets due to the surfactant's surface tension reduction action.

2) A testing oil is added to the culture medium and churned; then the state of oil component emulsification resulting from surfactant emulsification, action is measured. Taking such known methods as a reference, an investigation made into simple and efficient methods of collecting diverse surfactant-producing microorganisms.

In this study, there are two methods were selected: the water droplet method, which uses surface tension reduction capacity as an index, was adopted as a simple method of selecting diverse microorganisms, and the method which uses

emulsification capacity as an index was adopted as an evaluation method closer to application purpose.

Youssef *et al.* (2004) was compared three methods to detect biosurfactants production which is drop collapse, oil spreading, and blood agar lysis. The results were shown that the oil spreading technique was better o determine the biosurfactant production than the drop collapse method. However, the blood agar lysis method was not a reliable method to detect biosurfactants production.

2.2.4 Establishment of Culture Medium Conditions

For efficient screening of all types of biosurfactants, the culture medium conditions for adjusting microorganism culture samples at the time of screening are important factors. Most of the biosurfactants known so far are ones produced when microorganisms are cultivated with normal paraffin or other oil components serving as the carbon source. To produce biosurfactants, culture medium is generally grown in conditions normal paraffins, oils or fats as carbon sources. There are also modes of biosurfactants produced using sugar as a carbon source, although its functions are unclear.

Hua *et al.* (2002) used mineral salts media consisted (per liter) of 2.0g of $NaNO_3$, 0.2g of KH_2PO_4, 0.2 g of $MgSO_4$-$7H_2O$, and 1.0 g of yeast extract. The hydrocarbon was added into the media (8% v/v) as the carbon source. The strain was incubated with hydrocarbon (8% v/v) for 6 d on a shaker table (220 rpm) at 25°C. They found that biosurfactant, BS-UC was produced by *Candida antarctica* from n-decane as the substrate. It was found that the addition of BS-UC influenced positively the emulsification and the biodegradation of a variety of n-alkanes.

Benincasa *et al.* (2002) found a new bacterial strain was isolated from petroleum contaminated soil, identified and named *Pseudomonas aeruginosa* strain LBI. The new strain produced surface-active rhamnolipids by batch cultivation in a mineral salts medium with soapstock as the sole carbon source.

2.2.5 Thin Layer Chromatography (TLC)

Thin Layer Chromatography (TLC) is a powerful technique to separate compounds based upon their polarity and interaction with silica and to assess the purifty of a sample. To perform TLC, a solution of a compound or mixture of compounds is applied to a TLC plate by using a thin capillary tube. First, a thin pencil mark is made on a TLC plate about one-quarter of an inch up from one end. A capillary tube is dripped into a tested the solution and then the tip of the capillary tube is touched the onto the line on the TLC plate. The TLC plate is then plated into a developing chamber (can be a simple beaker with some filter paper and aluminum foil cover). The developing chamber should have some developing solvent in it but the level of this solvent should not be above the pencil mark. The solvent is allowed to diffuse to move up to the top of the TLC plate. After that, the TLC plate is removed from the developing chamber and the distance from the solvent diffusion is marked. During this step, the appearance of spots can be aided by using ultraviolet light source, The TLC plate is placed in an iodine chamber to aid the location of all spots on the TLC plate. By comparing with known organic compound having certain distance (Rf), one can identify organic components in the tested sample.

The stationary phase consists of a thin layer of adsorbent like silica gel, alumina, or cellulose on a flat carrier like a glass plate, a thick aluminum foil, or a plastic sheet.The process is similar to paper chromatography with the advantage of faster runs, better separations, and the choice between different adsorbents. TLC is a standard laboratory method in organic chemistry. Because of its simplicity and speed TLC is often used for monitoring chemical reactions and for the qualitative analysis of reaction products. TLC plates are made by mixing the adsorbent with a small amount of inert binder like calcium sulfate (gypsum) and water, spreading the thick slurry on the carrier, drying the plate, and activation of the adsorbent by heating in an oven. The thickness of the adsorbent layer is typically around 0.1–0.25 mm for analytical purposes and around 1–2 mm for preparative TLC.

Several methods are available to make colorless spots visible. A small amount of a fluorescent dye is added to the adsorbent that allows the visualization of UV absorbing spots under a blacklight ("UV_{254}"). Iodine vapors are a general unspecific color reagent.

Once the spots become visible, the R_f values of the spots can be determined. These values should be the same regardless of the extent of travel of the solvent, and in theory are independent of a single experimental run. They do depend on the solvent used, and the type of TLC plate.

HPLC is a popular method of analysis because it is easy to learn and use and is not limited by the volatility or stability of sample compounds. Prior to the 1970's, few reliable chromatographic methods were commercially available. During 1970's, most chemical separations were carried out using a variety of techniques including open-column chromatography, paper chromatography, and thin-layer chromatography. However, these chromatographic techniques were inadequate for quantification of compounds and resolution between similar compounds. During this time, pressure liquid chromatography began to be used to decrease flowthrough time, thus reducing purification times of compounds being isolated by column chromatogaphy. However, flow rates were inconsistant, and the question of whether it was better to have constant flow rate or constant pressure was debated (Gross, 1990).

High pressure liquid chromatography was developed in the mid-1970's and quickly improved with the development of column packing materials and the additional convenience of on-line detectors. In the late 1970's, new methods including reverse phase liquid chromatography allowed for improved separation between very similar compounds.

By the 1980's, HPLC was commonly used for the separation of chemical compounds. New techniques improved separation, identification, purification and

quantification far above the previous techniques. Computers and automation added to the convenience of HPLC. Improvements in type of columns and thus reproducibility were made as such terms as micro-column, affinity columns, and Fast HPLC began to immerge.

During the past decade, one has seen a vast undertaking in the development of the micro-columns, and other specialized columns. The dimensions of a typical HPLC column are: XXX mm in length with an internal diameter between 3-5 mm. The usual diameter of micro-columns, or capillary columns, ranges from 3 mm to 200 mm. A fast HPLC utilizes a column that is shorter than typical columns, with a length of about 3 mm long, and they are packed with smaller particles.

Currently, one has several options of considering over 7 types of columns for the separation of compounds, as well as a variety of detectors to interface with the HPLC in order to get optimal analysis of any tested compound. We hope this review will provide a reference which all levels of HPLC users will be able to find quick answers to their HPLC problems.

Although HPLC is widely considered to be a technique mainly for biotechnological, biomedical, and biochemical research as well as for the pharmaceutical industry, these fields currently comprise only about 50% of HPLC users (Gross, 1990). Currently HPLC is used by a variety of fields including cosmetics, energy, food, and environment.

Column efficiency refers to the performance of the stationary phase to accomplish particular separations. This entails how well the column is packed and its kinetic performance (Bidlingmeyer *et al.*, 2004). The efficiency of a column can be measured by several methods which may or may not be affected by chromatographic anomalies, such as "tailing" or appearance of a "front." This is important because many chromatographic peaks do not appear in the preferred shape of normal Gaussian

distribution. For this reason, efficiency can be an enigmatic value since manufacturers may use different methods in determining the efficiency of their columns. A mobile phase in HPLC refers to the solvent being continuously applied to the column, called as the stationary phase. The mobile phase acts as a carrier for the sample solution. A sample solution is injected into the mobile phase of an assay through an injector port. As a sample solution flows through a column with the mobile phase, the components of that solution migrate according to the non-covalent interactions of the compound with the column. The chemical interactions of the mobile phase and sample, with the column, determine the degree of migration and separation of components contained in the sample. For example, those samples which have stronger interactions with the mobile phase than with the stationary phase will elute from the column faster, and thus have a shorter retention time, while the reverse is also true. The mobile phase can be altered in order to manipulate the interactions of the sample and the stationary phase. There are several types of mobile phases: isocratic, gradient, and polytyptic. A stationary phase in HPLC refers to the solid support contained within the column over which the mobile phase continuously flows. The chemical interactions of the stationary phase and the sample with the mobile phase, determines the degree of migration and separation of the components contained in the sample. For example, those samples which have stronger interactions with the stationary phase than with the mobile phase will elute from the column less quickly and thus have a longer retention time. Liquid-Solid operates on the basis of polarity. Compounds that possess functional groups capable of strong hydrogen bonding will adhere more tightly to the stationary phase than less polar compoounds. Thus, less polar compounds will elute from the column faster than compounds that are highly polar.

Liquid-Liquid operates on the same basis as liquid-solid. However, this technique is better suited for samples of medium polarity that are soluble in weakly polar to polar organic solvents. The separation of non-electrolytes is achieved by matching the polarities of the sample and the stationary phase and using a mobile phase which possesses a markedly different polarity.

Size-Exclusion operates on the basis of the molecular size of compounds being analyzed. The stationary phase consists of porous beads. Larger compounds will be excluded from the interior of the bead and thus will elute first. In contrast, smaller compounds will be allowed to enter the beads and will elute according to their ability to exit from the same sized pores they were internalized through. The column can be either silica or non-silica based. However, there are some size-exclusion that are weakly anionic and slightly hydrophobic which give rise to non-ideal size-exclusion behavior.

Normal Phase operates on the basis of hydrophilicity and lipophilicity by using a polar stationary phase and a less polar mobile phase. Thus hydrophobic compounds elute more quickly than do hydrophilic compounds.

Reverse Phase operates on the basis of hydrophilicity and lipophilicity. The stationary phase consists of silica based packings with n-alkyl chains covalently bound. For example, C-8 signifies an octyl chain and C-18 an octadecyl ligand in the matrix. The more hydrophobic the matrix on each ligand, the greater is the tendancy of the column to retain hydrophobic moieties. Thus, hydrophilic compounds elute more quickly than hydrophobic compounds.

Ion-Exchange (IE) operates on the basis of selective exchange of ions in the sample with counterions in the stationary phase. IE is performed with columns containing charge-bearing functional groups attached to a polymer matrix. The functional ions are permanently bonded to the column and each has a counterion attached. The sample is retained by replacing the counterions of the stationary phase with its own ions. The sample is eluted from the column by changing the properties of the mobile phase do that the mobile phase will now displace the sample ions from the stationary phase (ie. changing the pH).

Affinity operates by using immobilized biochemicals that have a specific affinity to the compound of interest. Separation occurs as the mobile phase and sample pass over the stationary phase. The sample compound or compounds of interest are retained as the rest of the impurities and mobile phase pass through. The compounds are then eluted by changing the mobile phase conditions.

Samples are injected into the HPLC via an injection port. The injection port of an HPLC commonly consists of an injection valve and the sample loop. The sample is typically dissolved in the mobile phase before injection into the sample loop. The sample is then drawn into a syringe and injected into the loop via the injection valve. A rotation of the valve rotor closes the valve and opens the loop in order to inject the sample into the stream of the mobile phase. Loop volumes can range between 10 µl to over 500 µl. In modern HPLC systems, the sample injection is typically automated.

There are several types of pumps available for use with HPLC analysis, they are: reciprocating piston pumps, syringe type pumps, and constant pressure pumps. Reciprocating piston pumps consist of a small motor driven piston which moves rapidly back and forth in a hydraulic chamber that may vary from 35-400 µL in volume. On the back stroke, the separation column valve is closed, and the piston pulls in solvent from the mobile phase reservoir. On the forward stroke, the pump pushes solvent out to the column from the reservoir. A wide range of flow rates can be attained by altering the piston stroke volume during each cycle, or by altering the stroke frequency. Dual and triple head pumps consist of identical piston-chamber units which operate at 180 or 120 degrees out of phase. This type of pump system is significantly smoother because one pump is filling while the other is in the delivery cycle. Syringe type pumps are most suitable for small bore columns because this pump delivers only a finite volume of mobile phase before it has to be refilled. These pumps have a volume between 250 to 500 mL. The pump operates by a motorized lead screw that delivers mobile phase to the column at a constant rate. The rate of solvent delivery is controlled by changing the voltage on the motor. For constant pressure pumps the mobile phase is driven through

the column with the use of pressure from a gas cylinder. A low-pressure gas source is needed to generate high liquid pressures. The valving arrangement allows the rapid refill of the solvent chamber whose capacity is about 70 mL. This provides continuous mobile phase flow rates.

The detector for an HPLC is the component that emits a response due to the eluting sample compound and subsequently signals a peak on the chromatogram. It is positioned immediately posterior to the stationary phase in order to detect the compounds as they elute from the column. The bandwidth and height of the peaks may usually be adjusted using the coarse and fine tuning controls, and the detection and sensitivity parameters may also be controlled (in most cases). There are many types of detectors that can be used with HPLC. Some of the more common detectors include: refractive index (RI), ultra-violet (UV), fluorescent, radiochemical, electrochemical, near-infra red (Near-IR), mass spectroscopy (MS), nuclear magnetic resonance (NMR), and light scattering (LS).

Broderick *et al.* (1994) reported how to analyze biosurfactant by using HPLC. The oily mixture of biosurfactant was suspended in methanol and the separation of the individual rhamnolipids was further carried out by gradient elution high performance liquid chromatography (HPLC) on a Shimadzu LC-9A gradient system (Shimadzu, Kyoto, Japan), equipped with a Spherisorb ODS2 column of 250 mm×4:5 mm (Teknochroma, San Cugat, Spain). The flow rate was 1 ml/min and the acetronitrile–water gradient was programmed as follows: 4 min at 70% acetonitrile, then 70–100% acetonitrile for 40 min and finally 6 min at 100–70% acetonitrile. A light scattering detector SEDEX 55 was used. Data acquisition was carried out by an HP 1000/A600 system.

2.3 Factors Affecting Biosurfactant Production

2.3.1 Carbon Source

Water-soluble carbon sources such as glycerol, glucose, mannitol, and ethanol were all used for rhamnolipid production by *Pseudomonas* spp. Biosurfactant product, however, was inferior to that obtained with water-immiscible substrates such as *n*-alkanes and olive oil (Robert *et al.,* 1989). Syldatk *et al.,* (1985) and Edmonds and Cooney (1969) demonstrated that although different carbon sources in the medium affected the composition of biosurfactant production in *Pseudomonas* spp., substrates with different chain lengths exhibited no effect on the chain lengths of fatty acid moieties in glycolipids.

Contiero *et al.,* (2006) used oils from Buriti (Mauritia flexuosa), Cupuacau (Theobroma grandiflora), Passion Fruit (Passiflora alata), Andiroba (Carapa guianensis), Brazilian Nut (Bertholletia excelsa) and Babassu (Orbignya spp.) carbon sources for rhamnolipid production by *Pseudomonas aeruginosa* LBI. The highest rhamnolipid concentrations were obtained from Brazilian Nut (9.9 g l^{-1}) and Passion Fruit (9.2 g l^{-1}) oils. Surface tension varied from 29.8 to 31.5 mN m^{-1}, critical micelle concentration from 55 to 163 mg l^{-1} and the emulsifying activity was higher against toluene (93–100%) than against kerosene (70–92%). Preliminary characterization of the surfactant mixtures by mass spectrometry revealed the presence of two major omponents showing m/z of 649 and 503, which corresponded to the dirhamnolipid ($Rha_2C_{10}C_{10}$) and the monorhamnolipid ($RhaC_{10}C_{10}$), respectively. The monorhamnolipid detected as the ion of m/z 503 is predominant in all samples analyzed.

Ilori *et al.,* (2005) found Diesel and crude oil were good sources of carbon for biosurfactant production. When diesel was mixed with acetate, the production of biosurfactant was as poor as when acetate was used alone. The organism therefore could not utilise diesel alone for growth in the presence of acetate. Probably the organism is prompted to use acetate first as carbon source before diesel when both are together. The presence of toxic metabolites might lead to death and inability to utilize

the diesel in the mixture. Glucose, a water-soluble carbon source, was found to affect biosurfactant production. This clearly indicates that the biosurfactant may be produced using non-hydrocarbon substrates. Moreover, biosurfactants have been reported to be produced on water soluble compounds such as glucose, sucrose, glycerol, or ethanol (Desai and Banat, 1997). Biosurfactants produced from water-soluble substrates have been reported to be inferior to that obtained with water immiscible substrates (Syldatk *et al.*, 1985; Robert *et al.*, 1989). Such biosurfactants may however be cheaper to produce and useful in food and pharmaceutical industries as it will not require extensive purification.

2.3.2 Nitrogen Source

Medium constituents other than carbon source also affect the production of biosurfactant. Among the inorganic salts tested, ammonium salts and urea were preferred nitrogen sources for biosurfactant production by *Arthrobacter paraffineus* (Duvnjak *et al.,* 1983), whereas nitrate supported maximum surfactant production in *P.aeruginosa* (MacElwee *et al.,* 1990) and *Rhodocoocus* spp. (Abu-Ruwalda *et al.,* 1991). Biosurfactant production by *A.paraffineus* increased by the addition of L-amino acids such as aspartic acid, glutamic acid, asparagines, and glycine to the medium. The structure of surfactin is influenced by the L-amino acid concentration in the medium to produce either Val-7 or Leu-7 surfactin (Jelmeman *et al.,* 1983). Similarly, lichenysin-A production is enhanced two-and fourfold in *B.licheniformis* BAS 50 (Yakimov *et al.,* 1996) by addition of L-glutamic acid and L-asparagine, respectively, to the medium. Robert *et al.,* (1989) and Abu Ruwalda *et al.,* (1991) reported nitrate to be the best source of nitrogen for biosurfactant production by *Pseudomonas* stain 44T1 and *Rhodococcus* stain ST-5 growing on olive oil and paraffin, respectively. The production started after 30 h of growth, when the culture reached nitrogen limitation, and continued to increase up to 58 h of fermentation. For the use of *P.aeruginosa,* a simultaneous increase in rhamnolipid production and glutamine synthetase activity were observed when the growth decreased as the culture became nitrogen limiting (Mulligan *et al.,*

1989). Similarly, nitrogen limitation caused increased biosurfactant production in *P.aeriginosa* (Rocha *et al.,* 1992), *C. tropicalis IIP-4 (*Singh *et al.,* 2004), and *Nocardia* stain SFC-D (Lee *et al.,* 1993)

Syldatk *et al.* (1985) showed that nitrogen limitation not only causes overproduction of biosurfactants but also changes the composition of the biosurfactants produced. Guerra-Santos *et al.* (1984) showed maximum rhamnolipid production after nitrogen limitation at a C:N ratio of 16:1 to 18:1 and no surfactant production below a C:N ratio of 11:1, where the culture was not nitrogen limited. According to Bai *et al.* (1997), it is the absolute quantity of nitrogen and not its relative concentration that appears to be important for optimum biomass yield, while the concentration of hydrophobic carbon source determines the conversion of carbon available to the biosurfactant.

Benincasa *et al.* (2002) found increasing the aeration rate to KLa 169:9 h^1 caused the nitrogen depletion in the first 26 h of the process. Poor cell growth was observed and at the end of the growth phase 1.75 g/l was found, whereas rhamnolipid concentration increased to 3 g/l, the cellular yield of rhamnolipid accumulation being Yp 1.71 and the productivity being calculated as 0:125 g h^{-1}. During the stationary phase of cell growth, production dramatically increased to 8.5 g/l, the productivity during this phase being 0:21 g h^{-1}. Substrate conversion rate was 33%. Matsufuji, Nakata, and Yoshimoto (1997) reached a substrate conversion ratio of 58% when cultivating P. aeruginosa on ethanol in shaker flasks.

Bednarski *et al.* (2004) evaluated the utilization of a fat waste, namely, the suitability of soap stock and post-refinery fatty acids as substrates inducing glycolipids biosynthesis by yeast. Results demonstrated that, the addition of soap stock to the culture medium or post-refinery fatty acid supplementing the culture medium have positive effect on the glycolipids synthesis by yeast (*C. antarctica* and *C. apicola*) ranged from 8.2 - 10.3 g/l and 6.5 - 10.4 g/l, respectively.

2.3.3 Other Factors

Ilori et al., (2005) found environmental factors such as pH, salinity and temperature also affected biosurfactant activity. Stability of emulsion in the presence of salt has been reported as one of the properties of the biosurfactant produced by *Bacillus licheniformis* strain JF-2. The activity of the biosurfactant produced by Aeromonas spp. was also slightly stimulated by heat. Syldatk *et al.* (1985) reported Acinetobacter radio-resistens. After exposure of the biosurfactant to a temperature of 100°C, about 77% residual activity was indicated the usefulness of the biosurfactant in food, pharmaceutical and cosmetics industries where heating to achieve sterility is of para-mount importance. The glycolipid produced by the oil degrading Aeromonas spp. represents a new type of biosurfactant with 990 M.O. Ilori ,.*et al* (2005) reported strong emulsifying ability and stable activity at high temperature, pH and salinity.

2.4 Biosurfactant Production

The kinetics of biosurfactant production are governed by many parameters and only a few generalizations can be derived. However, for convenience, kinetic parameter can be grouped into the following types: (i) growth-associated production, (ii) production under growth-limiting conditions, (iii) production by resting or immobilized cells, and (iv) production with precursor supplementation.

2.4.1 Growth-Associated Production

For growth-associated biosurfactant production, parallel relationships exist between growth, substrate utilization, and biosurfactant production. The productions of rhamnolipid by *Pseudomonas* spp. (Robert *et al.,* 1989), glycoprotein AP-6 by *P.fluorescens* 378 (Persson *et al.,* 1988), surface-active agent by *B.* cereus IAF 346, and biodispersan by *Bacillus* spp. Stain IAF-343 (Cooper *et al.,* 1987) are all examples of growth-associated biosurfactant production. The production of the cell-free emulsan by *A.calcoaceticus* RAG-1 was reported to be a mixed growth-associated and

non-growth-associated type (Eliora *et al.,* 2002). Emulsan-like substance accumulates on the cell surface during the exponential phase of growth and is released into the medium when protein synthesis decreases (Eliora *et al.,* 2002).

2.4.2 Production under Growth-Limiting Conditions

The production under growth-limiting conditions is characterized by a sharp increase in the biosurfactant level as a result if limitation of one or more medium components. A number of investigators have demonstrated an overproduction of the biosurfactant by *Pseudomonas* spp., when the culture reached the stationary phase of growth due to the limitation of nitrogen and iron (Ramana *et al.,* 1989). The productions of bioemulsifier by *Candida tropicalis* IIP-4 (Sing *et al.,* 2004), of glycolipid by *Nocardia* spp. stain SFC-D (Kosaric *et al.,* 2001), and of water-soluble biosurfactant by *Torulopsis apicola* (Cohen *et al.,* 2002) were also reported to follow this pattern.

In contrast to the observation in *P.aeruginosa* (Mulligan *et al.,* 1989), a low phosphate concentration stimulated bioemulsifier production in a gram-negative bacterium during cultivation on ethanol (Mata-Sandoval *et al.,* 1999). Phosphate, iron, magnesium, and sodium were all important elements for a biosurfactant-producing *Rhodococcus* spp. much more than either potassium or calcium (Abu-Ruwaida *et al.,* 1991). Iron was found to have a dramatic effect on the rhamnolipid production by *P.aeruginosa*, resulting in a threefold increase in the production when cells were shifted from medium containing 36 mM iron to medium containing 18 mM iron. Interestingly, under these conditions, there was no change in the biomass yield (Koch *et al.,* 1988).

2.4.3 Production by Resting or Immobilized Cells

The production of biosurfactant by resting or immobilized cells in which there is no cell multiplication. The cells nevertheless continue to utilize the carbon source for the synthesis of biosurfactants. Several examples of biosurfactant production

by resting cells were reported: the production of rhamnolipid by *Pseudomonas* spp. (Syldatk *et al.,* 1987) and *P.aeruginosa* CFTR-6 (Ramana *et al.,* 1989), sophorolipid production by *Torulopsis bombicola* (Matsuda *et al.,* 2001) and *Candida apicola* (Javaheri *et al.,* 1985), trehalose tetraester production by *Rhodococcus erythropolis* (Syldatk *et al.,* 1985), and mannosylerythritol lipid production by *Candida Antarctica* (Kinamoto *et al.,* 2001). Biosurfactant production by resting cells is important for the reduction of cost of product recovery, as the growth and the product formation phases can be separated.

2.4.4 Production with Precursor Supplementation

Many investigators have reported that the addition of biosurfactant precursors to the growth medium causes both qualitative changes in the product. For example, *T.bombicola* (Cooper *et al.,* 1984), and *T.apicola* IMET 43747 (Stuwer *et al.,* 1987) resulted in increased biosurfactant production with yields of about 120 to 150 g/liter (Lee *et al.,* 1993). Similarly, increased production of biosurfactants containing different mono-, di-, or trisaccharides was reported to occur in *Arthrobacter paraffineus* DSM 2567, *Corynebacterium* spp., *Nocardia* spp., and *Brevibacterium* spp. through supplementation with the corresponding sugar in the growth medium.

Sutthivanitchakul *et al.* (1999) proposed to produce a biosurfactants from *Bacillus licheniformis* F2.2, growth in the medium using rise straw hydrolysate as a carbon source, NH_4NO_3 as a nitrogen source and supplement with vitamin solution and trace element, isolated from fermented food. Finally, biosurfactants BF2.2 was found to have good properties; it's stability over a wide range of pH, temperature and salt concentration.

Roongsawang *et al.* (1999) focused on isolated biosurfactants *bacillus* sp.strain KP-2 from culture broth by acid precipitation and characterized by HPLC and MS. Their preliminary analytical results indicated that biosurfactant kp-2 might be a lipopotide-type biosurfactant and should to be a good surface active agent in comparison with other synthetic surfactants.

Jennings *et al.* (2000) examined the prevalence of biosurfactants producers in uncontaminated soils. Biosurfactants-producing bacteria were found to constitute a significant proportion (up to 35%) of aerobic heterotrophs. Biosurfactant producers were isolated and identified as strains of *Bacillus* and *Pseudomonas.*

Kuyukina *et al.* (2001) proposed methyl tertiary-butyl ether MTBE as a solvent for extraction of biosurfactants from *Rhodococcus* bacterial cultures. After comparison with other well known solvent systems used for biosurfactant extraction, it was found that MTBE was able to extract the crude surfactant material with a high product recovery efficiency (10g/l). The extracted surfactant was found to have the CMC of 130–170 mg/l with good functional surfactant characteristics (surface and interfacial tensions, 29 and 0.9 mN/m, respectively). The isolated surfactant complex contained 10% polar lipids, mostly glycolipids possessing maximal surface activity. Ultrasonic treatment of the extraction mixture increased the proportion of polar lipids in crude extract, resulting in increasing surfactant efficiency.

2.5 Potential Commercial Application

Kosaric *et al.* (2001) found the biodegradation of hydrocarbons in soil was able be efficiently enhanced by addition or *in situ* production of biosurfactants. It was generally observed that the degradation time, and particularly the adaptation time, the degradation of phenanthrene could be increased by adding a nonionic surfactant based on ethylene glycol. In oil-contaminated mud flats, the elimination of polycyclic aromatics from the crude oil Arabian light was due to wave action or to microbial degradation. The chemical surfactant Finasol OSR-5 doubled the initial content of aromatics and decreased the amount of aromatics removed after 6 months, whereas adding the biosurfactant trehalose-5,5-dicorynomycolates caused complete elimination within 6 months.

Eliora *et al.* (2002) demonstrated toxicities comparison of three synthetic surfactants and three microbiologically produced surfactants using three estuarine

epibenthic invertebrates, *Mysidopsis bahia* and the inland silverside, *Menidia beryllina*. The results showed the specific effect of surfactant, on the testing species. The LC50 values (nominal concentrations) for *M. bahia* ranged from 3.3 mg/l (Triton X-100) to >1000 mg/l (PES-61) and 2.5 mg/l (Triton X-100) to 413.6 mg/l (PES-61) for *M. beryllina*. The Chronic first-effect concentrations for the six surfactants ranged from 2.3 to 465.0 for *M. beryllina* and 1.0 to >1000.0 for *M. bahia* based on reductions in growth and fecundity. Few generalizations could be made concerning the results due to their variability but *M. bahia* was generally the more sensitive species and the toxicities of the biosurfactants were intermediate to those of the synthetic surfactants.

Mulligan *et al.* (2001) showed that the organic phase-associated metals can be removed from sediments by either surfactin or rhamnolipid with sodium hydroxide. Acidic conditions with sophorolipid addition were effective for removing the zinc in the oxide and carbonate phases.

Wei *et al.* (2004) studied the use of biosurfactant to remove the used oil from used polypropylene nonwoven sorbents. Washing parameters tested included sorbent type, washing time, surfactant dosage and temperature. It was found that with the biosurfactant washing, more than 95 % removal of the oil from the sorbents was achieved, depending on the washing conditions.

Rahman *et al.* (2003) examined the bioremediation of n-alkanes in petroleum sludge (2003). The sludge contained an oil and grease content of 87.4%. C8–C11 alkanes in 10% sludge were degraded 100%, as compound to 83–98 for C_{12}-C_{21}, 80–85% for C_{22}-C_{31} and 57–73% for C_{32}-C_{40} after 56 days with the use of nutrient solution containing rhamnolipids-producing bacterial consortium. Lower rates of biodegradation occurred as the chain length increased. The degradation of C_{32}-C_{40} were considerably high indicating that rhamnolipids were effective to degrade all low solubility compounds.

Straube *et al.* (2003) evaluated the effectiveness of *Pseudomonas aeruginosa* strain 64, a biosurfactant-producer, with nutrients (slow-release nitrogen) and a bulking agent (ground - rice hulls) to enhance bioremediation of a PAHs (13,000 mg/kg) and

penta-chlorophenol (PCP) (1500 mg/kg) contaminated soil. This biostimulation/bioaugmentation approach was found to total PAHs of 87% and a 67% decrease in total BaP toxic equivalents compared to 23 and 48%, respectively, for the control in microcosm studies. Larger scale pan experiments showed that decreases of 86% of the PAHs and 87% in total BaP toxicity could be achieved by biostimulation/bioaugmentation after 16 months compared to a 12% decrease in PAHs for the control. Overall, the biosurfactants were produced in the soil by the bacterial strain 64 and that this enabled PAH biodegradation to occur.

Banat *et al.*, (1991) used biosurfactants produced from a proprietary bacterial strain (Pet 1006), to clean storage-tank oil and to recover hydrocarbons from the emulsified sludge. A pilot-plant-scale fermenter with 1500 L capacity was used to produce 2 m^3 of culture broth. The biosurfactant-containing broth was used as a substitute for chemical surfactants. A Basal salt medium containing 2% w/v glucose as a readily available carbon source was used and oleic acid, a hydrocarbon source (2% v/v), was added after glucose consumption. The biosurfactant production reached a maximum after 18-19 h, as measured by the reduction in the surface and interracial tension in the broth. At the end of the production run, the culture broth was sterilized in the fermenter and stored in 200.1 sterile drums.

Shulga *et al.* (2000) examined the use of the biosurfactants in cleaning oil from coastal sand, and from the feathers and furs of marine birds and animals. The strain Pseudomonas PS-17 produces a biosurfactant and biopolymer that reduced the surface tension of water to 29.0 mN/m and the interfacial tension against heptane to between 0.01 and 0.07 mN/m. The biopolymer has a molecular weight of 3,000-4,000. The results are shown in Table 2.2 and they indicated that this biosurfactant/biopolymer could be used to remove oil from marine birds and animals.

Table 2.2 Oil removal efficiency from three types of contaminated media using the PS-17 complex (Shulga et al., 2000)

Washing agent	Oil removal efficiency (%)		
	Sand	Feat hers	Fur
1% PS-17 in fresh water	95.0	85.1	82.3
1% PS-17 in sea water	92.3	77.4	78.0
Fresh water control	0.7	2.1	0.5
Sea water control	1.2	2.4	0.8