# CHAPTER II
LITERATURE REVIEW

2.1 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is an environmental bacterium that can be isolated from many different habitats, including water, soil, and plants, but it is also an opportunistic human pathogen causing serious nosocomial infections. This bacterium was shown by Jarvis and Johnson (1949) to produce rhamnose-containing glycolipid biosurfactants called rhamnolipids, which are amphiphilic molecules composed of a hydrophobic fatty acid moiety and a hydrophilic portion composed of one or two rhamnose. While the production of rhamnolipids is characteristic of *P. aeruginosa*, some isolates of the nonpathogenic pseudomonads *P. putida* and *P. chlororaphis* as well as the pathogen *Burkholderia pseudomallei* were also recently shown to produce a variety of rhamnolipids (Soberón-Chávez *et al.*, 2005).

2.2 Rhamnolipid Biosurfactant

2.2.1 Structure

Liquid chromatography coupled to mass spectrometry (LC/MS) allows the detection of more than 28 different rhamnolipid congeners in liquid cultures. The alkyl chains of these congeners vary from C8 to C12, and some of these chains also contain one unsaturation (Soberón-Chávez *et al.*, 2005). Four different rhamnolipid homologues (Figure 2.1), produced by *Pseudomonas aeruginosa*, have been identified and characterized. The rhamnolipids consist of one or two L-rhamnose units and one or two units of β-hydroxydecanoic acid. RL1 and RL3 are the principal rhamnolipids produced. RL2 and RL4 are biosynthesized under certain cultivation conditions only (Tahzibi *et al.*, 2004).

Rhamnolipid 1   Rhamnolipid 2

Rhamnolipid 3   Rhamnolipid 4
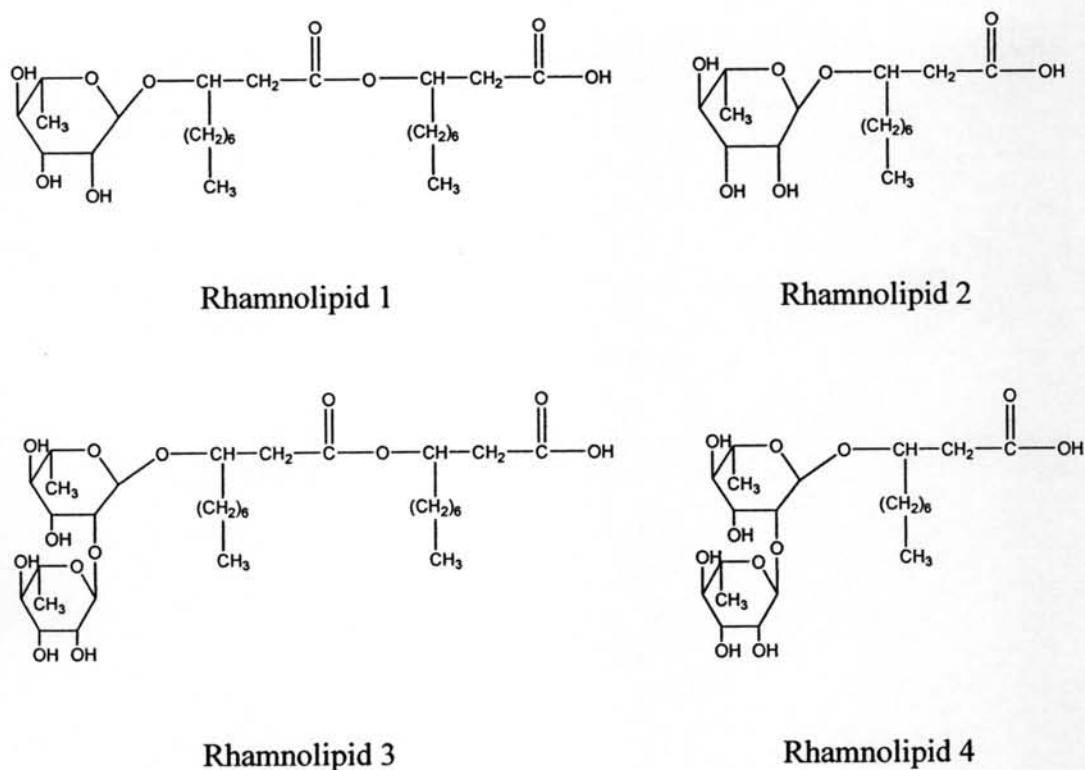
Figure 2.1 Structure of Rhamnolipids.

In liquid culture and under usual growth conditions, the two most abundant rhamnolipids observed are rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate (RhaC$_{10}$C$_{10}$), a mono-rhamnolipid, and rhamnosyl-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate (Rha$_2$C$_{10}$C$_{10}$), a di-rhamnolipid (Soberón-Chávez *et al.*, 2005).

2.2.2 Properties

It is known that the properties of rhamnolipids depend on the distribution of their homologues that vary according to the bacterial strain, culture conditions, and medium composition (Guerra-Santos *et al.*, 1984). However, little is known about the contribution of each individual homologue in the surface properties of rhamnolipid mixtures. The dirhamnolipid Rha$_2$C$_{10}$C$_{10}$ shows a critical micelle concentration (CMC) value (5 mg/L) lower than that of RhaC$_{10}$C$_{10}$, which showed a CMC of 40 mg/L, and those of the more hydrophilic rhamnolipids RhaC$_{10}$ and Rha$_2$C$_{10}$, which showed CMC values of 200 mg/L (Nitschke *et al.*, 2005a, b).

Mohan *et al.* (2006) investigated the biodegradation of rhamnolipids under aerobic, nitrate reducing, sulphate reducing and anaerobic conditions. The results indicated that rhamnolipids is biodegradable under all conditions. They hypothesized that the surfactant is degraded in two phases; in the initial phase there is a huge peak followed by a long tail, the initial peak is due to the readily biodegradable fraction and the second tail corresponds to the slowly biodegradable portion.

Rhamnolipids are completely soluble in water. These materials are also soluble in polar solvents such as alcohols, glycols and glycol ethers but not in mineral oil. The carboxylic group provides rhamnolipids with nonionic features between a pH of 1 and 5. As the pH climbs above pH 7, these biosurfactants can generate salts (Canter, 2004).
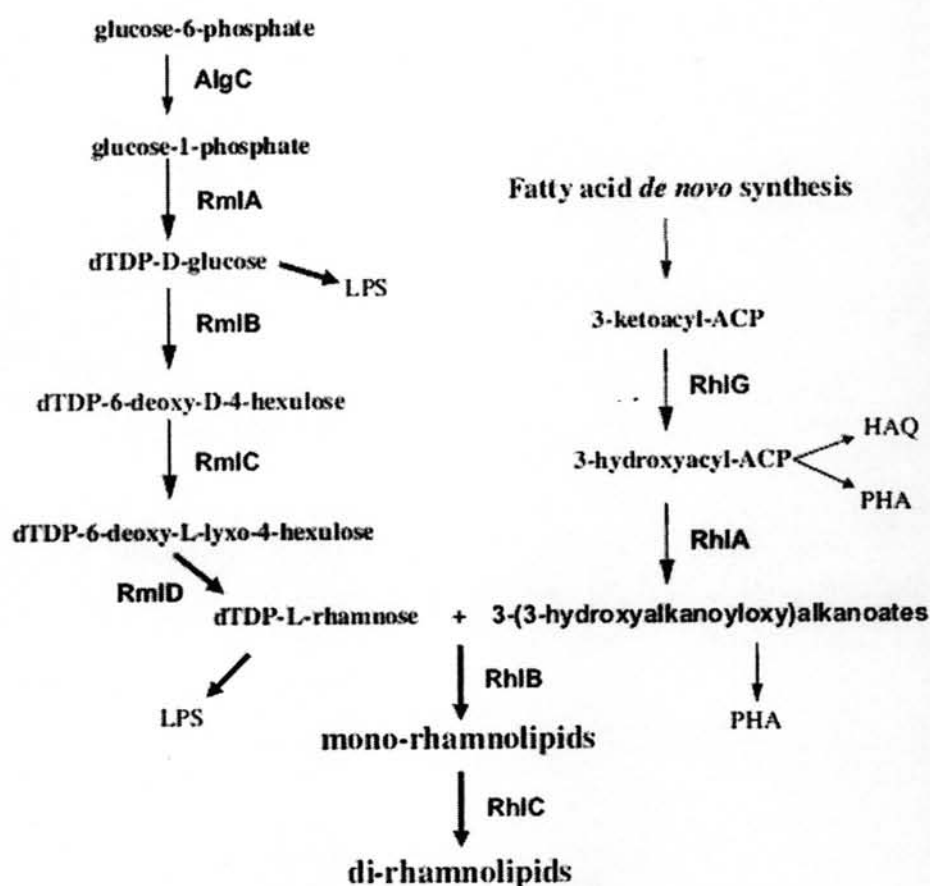
Rhamnolipids display good stability in water with a hardness of 500 ppm. This activity enables rhamnolipids to provide enhanced emulsification in hard water. These biosurfactant also display surprisingly good corrosion protection at a very low usage rate. In addition, rhamnolipids can display a foaming tendency depending on their use concentration and the pH. Another unique characteristic about rhamnolipids is their foaming properties. As the pH increases, the foam profile of rhamnolipids changes. At a pH of 5, fast foaming is observed, but it collapses quickly. When the pH is increased to 7, the foam develops more slowly but is stable for 3 to 4 hours. A further increase in the pH to 9 leads to a fairly stable foam. Furthermore, these surfactants are very heat stable (Canter, 2004).

The rhamnolipids were produced when hydrocarbons, glycerol, glucose, or peptone was the substrate. B est production was obtained with hydrocarbons or glycerol (Guerra-Santos *et al.*, 1984).

2.2.3 Biosynthesis

Rhamnolipids were secreted by *Pseudomonas aeruginosa* in the stationary phase growth as a typical secondary metabolite (Dubey and Juwarkar, 2001; Rahman *et al.*, 2002; Nitschke *et al.*, 2005a; Soberón-Chávez *et al.*, 2005; Raza *et al.*, 2006).

The biosynthesis of rhamnolipids includes two sequential reactions catalyzed by rhamnosyltransferases RhlB and RhlC (Nitschke *et al.*, 2005a), where dTDP-L-rhamnose acts as a rhamnosyl donor and the monorhamnolipid acts as a recipient (Figure 2.2). The synthesis of fatty acid moiety of rhamnolipids is separate from the general fatty acid synthetic pathway, starting with a specific reduction of a ketoacyl group (Campos-Garcia *et al.*, 1998), catalyzed by Rh1G enzyme. The sugar moiety as well as the lipid moiety of rhamnolipids are formed by de novo synthesis and are independent of the substrate used. The chain length of the carbon substrates employed has no effect on the fatty acid chain length of the rhamnolipid produced (Nitschke *et al.*, 2005a).

glucose-6-phosphate

| AlgC

glucose-1-phosphate

| RmlA

dTDP-D-glucose → LPS

| RmlB

dTDP-6-deoxy-D-4-hexulose

| RmlC

dTDP-6-deoxy-L-lyxo-4-hexulose

RmlD

dTDP-L-rhamnose + 3-(3-hydroxyalkanoyloxy)alkanoates

LPS

| RhlB

mono-rhamnolipids

| RhlC

di-rhamnolipids

Fatty acid *de novo* synthesis

3-ketoacyl-ACP

| RhlG

3-hydroxyacyl-ACP < HAQ / PHA

| RhlA

PHA

Figure 2.2 Rhamnolipids biosynthetic pathway (Soberón-Chávez *et al.*, 2005).

2.2.4 Physiological Function

Functions of rhamnolipids have been reviewed by Nitschke *et al.* (2005a) and Soberón-Chávez *et al.* (2005). Rhamnolipid surfactants play a variety of different functions in microbial cells, but there is no consensus on their real physiological role. In general, the main role attributed to biosurfactants is to permit microorganism to grow on water-immiscible substrates by reducing the surface tension of phase boundary, thus making the substrate more readily available for uptake and metabolism. In addition to emulsification of carbon sources, biosurfactants are also involved in the adhesion of microbial cells to hydrocarbons. The cellular adsorption of the hydrocarbon-degrading microorganisms to water-immiscible substrates and the excretion of surface-active compounds together allow the growth on such carbon sources (Fiechter, 1992).

Rhamnolipid surfactants can cause the cell surface to become more hydrophobic, thereby increasing the direct physical contact between the cell and the slightly soluble substrates. The mechanism involved on this increasing hydrophobicity of *Pseudomonas* cell surface is the release of membrane lipopolysaccharides (LPSs) by rhamnolipids even when they are present at low concentrations (below CMC) (Al-Tahhan *et al.*, 2000).

2.2.5 Application

Interest in microbial biosurfactants has increased for several reasons. First, biosurfactants are considered environmentally compatible since they are relatively non-toxic and biodegradable. Second, biosurfactants have unique structures that are just starting to be appreciated for their potential application to many different facets of industry, ranging from biotechnology to environmental cleanup (Maier and Soberón-Chávez, 2000). Third, biosurfactants can be produced from renewable and cheaper substrates. Biosurfactants have been tested in environmental applications such as bioremediation and dispersion of oil spills, enhanced oil recovery and transfer of crude oil, and are thought to be potential candidates to replace chemical surfactants in the future, especially in the food, cosmetic, and health care industries, industrial cleaning of products and in agricultural chemicals (Banat *et al.*, 2000).

Rhamnolipids have several potential industrial and environmental applications due to their tensio-active properties (Lang and Wullbrandt, 1999; Maier and Soberón-Chávez, 2000). These uses include the production of fine chemicals, the characterization of surfaces and surface coatings, and usage as additives for environmental remediation, and they have even been reported to be useful as a biological control agent (Soberón-Chávez et al., 2005).

The advantages of biosurfactants in the cosmetics and health care area are low irritancy or anti-irritating effects and compatibility with skin. Rhamnolipids are being used as cosmetic additives in Japan (Maier and Soberón-Chávez, 2000). In 2002, Makkar and Cameotra reported the currently patents for the use of rhamnolipids to make liposomes and emulsions, both important in the cosmetic industry. They also reported the treatment of leaves of *Nicotiana glutinosa* infected with tobacco mosaic virus and for the control of potato virus X disease by using a 1% emulsion of rhamnolipids.

Rhamnolipids have been shown to have high affinity for a variety of metals concern, including cadmium, copper, lanthanum, lead, and zinc. Their use in removing metals from soil has been demonstrated in bench-scale column experiments for both cadmium and lanthanum (Maier and Soberón-Chávez, 2000). Mulligan et al. (2001) used surfactin, rhamnolipids and sophorolipid in batch washing experiments to remove heavy metals from sediments.

Rhamnolipids have been evaluated for corrosion inhibition, emulsification, lubricity and wetting in metalworking fluids and rolling oils. These materials can also be used to provide corrosion inhibition in pickling fluids. Rhamnolipids are also used to impart detergency in cleaners (Canter, 2004).

2.3 Biosurfactant Production

2.3.1 Economic Consideration

Biosurfactants are difficult to produce in an economic manner for several reasons (Fiechter, 1992b): (i) overproducing strains of bacteria are rare and those found generally display a very low productivity. In addition complex media need to be applied; (ii) the regulation of biosurfactant synthesis is hardly understood,

seemingly it represents a secondary metabolite regulation; and (iii) an improvement of the production yield is hampered by the strong foam formation.

The cost of biosurfactant production is about 3 to 10 times higher than that of the chemical counterparts (Mulligan and Gibbs, 1993). The fermentation process holds the key to improving the overall process economics in biosurfactant production. It has been estimated that raw materials account for about 10 to 30% of the overall cost of biosurfactant production (Mulligan and Gibbs, 1993). Generally, biosurfactants are produced during growth on hydrocarbons which are usually expensive and therefore increase the overall process cost. However, other cheaper, water-soluble substrates such as glucose (Guerra-Santos *et al.*, 1984) and ethanol (Mulligan and Gibbs, 1989; Matsafuji *et al.*, 1997) are sometimes used.

2.3.2 Production From Alternative Low-Cost Substrates

In 1997, Desai and Banat have reported the search for cheaper raw materials for biosurfactant production. Industrial effluents have recently shown good promise. Striking recent developments in this area include rhamnolipid production from olive oil mill effluent by *Pseudomonas* spp. (Mercadé *et al.*, 1993), the use of agroindustrial wastes, cassava wastewater (Nitschke and Pastore, 2006), potato-processing industrial residues, the soybean curd residue okara, chicken fat residues, and wastewater pressate from fuel-grade peat processing.

Olive oil mill effluent, a major pollutant of the agricultural industry in mediterranian countries, has been used as raw material for rhamnolipid biosurfactant production by *Pseudomonas* sp. JAMM (Karanth *et al.*, 1999). Mercadé *et al.* (1993) studied the use of olive oil mill effluent (OOME) as a new substrate for production of rhamnolipids by *P. aeruginosa* sp. JAMM. OOME has a high concentration of valuable organic substances, such as sugar, nitrogen compounds, pectins, polyphenols and residual oil. For rhamnolipid production with OOME, it was only necessary to add 2.5 g/L of sodium nitrate, as nitrogen source, to support better growth and a greater subsequent drop in surface tension (from 42 to 30 mN/m).

Soapstock is the residue from oil refinery that is generated in large quantities by the vegetable oil processing industry. Sunflower oil soapstock was assayed as the carbon source for rhamnolipid production by *P. aeruginosa* LBI

strain, giving a final surfactant concentration of 12 g/L in shaker and 16 g/L in bioreactor experiments (Benincasa *et al.*, 2002).

Rasa *et al.* (2006) investigated production of biosurfactant from canola, soybean and corn oil refineries. This research revealed that canola oil refinery waste (COD=20 g l^{-1}) supplemented with sodium nitrate (at COD/N=20) showed the best microbial growth (4.50 g l^{-1}) and rhamnolipid production (8.50 g l^{-1}), at 10 d of incubation with the specific growth rate of 0.316 h^{-1} and specific product yield of 0.597 g g^{-1} h.

Rahman *et al.* (2002) studied biosurfactant production using low-cost raw materials such as soybean oil, safflower oil, and glycerol. The results showed that *P. aeruginosa* DS10-129 produced 4.31 g rhamnolipid l^{-1} with soybean oil as growth substrate at 288 h of incubation. Soybean oil supplements increased the biomass and rhamnolipid production to severalfold that obtained with safflower oil and glycerol.

Whey is a waste product from cheese production that represents a major pollution problem for countries depending on dairy economics and is normally used as animal feed. Sophorolipids production from deproteinized whey, using a two-stage batch cultivation process, was reported by Otto *et al.* (1999). In 2001, Dubey and Juwarkar reported biosurfactant production from synthetic medium and industrial waste, viz. distillery and whey wastes. The results showed that whey waste was found to be the best substrate for multiplication of *P. aeruginosa* strain BS 2 because whey waste is a very rich source of minerals like calcium, phosphorus, potassium, sodium, copper and iron. It is also a very good source of vitamins of the B-complex, riboflavin and pantothenic acid (Dubey and Juwarkar, 2001). Screening of biosurfactant-producing ability of four *Lactobacillus* strains was performed, using whey as production medium, by Rodrigues *et al.* (2006). A good substrate for biosurfactant production is lactic whey, as it is composed of high levels of lactose (75% dry matter), 12–14% protein, organic acids and vitamins.

2.3.3 Comparison of Continuous and Batch Cultivation

Guerra-Santos *et al.* (1984) discuss the advantages of the continuous production process over the production of biosurfactants in batch cultivations: (i) the

long-term incubations of several days (Cooper *et al.*, 1981; Cooper and Paddock, 1984) are avoided, yielding a much improved productivity per unit of reactor volume; (ii) there is a constant mass flow which can be adapted to the capacity of the downstream processing; and (iii) the exact control of the culture conditions which is essential for high biosurfactant formation by the cells is accomplished more easily in a continuous culture. Therefore, for the design of the continuous process, it was not possible to take over the batch data directly. When the medium which led to biosurfactant production in batch cultures was used in continuous culture, very poor formation of surface-active compounds resulted, and a medium optimization was necessary.

2.4 Bioreactor for Biosurfactant Production

2.4.1 Batch Reactor

Batch-style reactors are useful when the reaction kinetics of the degradation are first order. To optimize reactor use and reduce total project time, batch-style reactors also, necessarily, require holding tanks in which to prepare the succeeding slurry batch that can be immediately pumped into the reactor when the preceding batch is drained (RREL *et al.*, 1993).

Regarding the culture system improvements were made to design a bioprocess fit for preliminary testing of biosurfactant production without the need for sophisticated high performance systems. A simple chemostat (compact loop reactor) was used which could also be operated in a batch mode (Fiechter, A., 1992).

2.4.2 Packed column bioreactor (PCBR)

Solid state fermentation (SSF) is a simple and efficient technique to produce several interesting metabolites, one of which is biosurfactant production reported in Veenanadig *et al.* (2000). These experiments carries out in a packed column bioreactor (PCBR) have shown that the PCBR can become a more acceptable SSF system for commercial exploitation of pesticide (Fenthion) specific biosurfactant production.

2.4.3 Continuous-Flow Stirred Tank Reactor (CSTR)

With the continuous-flow reactor, a feed stream containing a high concentration of contaminants is fed continuously into steady-state slurry containing very low levels of contaminants, thereby instantaneously diluting the feed stream. The concentration of contaminants in the reactor is maintained at the same level as the concentration of contaminants in the effluent. This is done by initially charging and then operating the reactor in a recirculation mode (RREL *et al.*, 1993).

This process allows a continuous feed supply to the flourishing bacterial population but with a much lower concentration of hazardous compounds. As a matter of reaction kinetics, this process design also proceeds best with a zero order reaction. That is, the rate of the degradation of the hazardous material depends entirely on the concentration of the bacteria and not on the concentration of the hazardous material. Thus, maintaining growth conditions for a vigorous population of bacteria results in the highest possible rate of biodegradation of the hazardous compounds, regardless of the concentration of the compounds in the slurry. Several advantages accrue from reactor operation in the continuous-feed mode. This process does not require a system of holding tanks for each successive batch of slurry to be treated. There is also an instantaneous dilution of any toxic component in the feed-stream and a stable, steady-state condition that fosters a stable distribution of bacterial population levels is maintained in the reactor (RREL *et al.*, 1993).

2.4.4 Sequencing batch reactor (SBR)

The SBR is a recognized model reactor system for fill-and-draw operation (Cassidy, 2001). The SBR systems consist of a sequencing operation including the steps of fill, react, settle, decant, and idle (Ling and Lo, 2001). During the fill step, feed was introduced to the reactor. Aeration was maintained through out the react step, and was shut-off during the settle step, and the sludge and culture was allowed to settle under a quiescent condition. In the draw step, a fraction of the clarified effluent was withdrawn from the reactor. The idle step was set as a time to prepare and maintain the reactor for the next cycle (Figure 2.3).
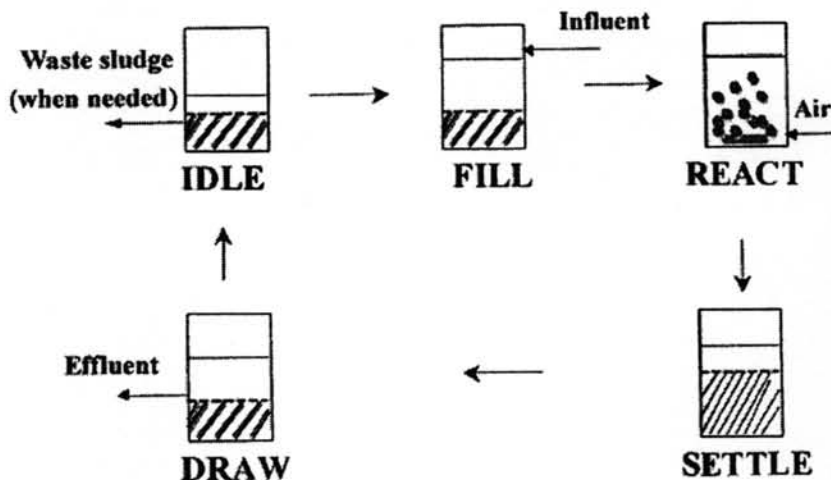
Figure 2.3 The operation cycle of the SBR (Ling and Lo, 2001).

The most important operating factors influencing the SBR removal efficiency are the hydraulic retention time (HRT), the volume of slurry replaced at the end of each cycle, the solids concentration and the mixing speed. The HRT and the volume of slurry replaced per cycle can be adjusted to face different contaminant loads to the reactor (Giordano *et al.*, 2005). Three retention times are typically sufficient to reach steady state conditions in biological reactors (Cassidy, 2001; Cassidy and Hudak, 2001; Cassidy *et al.*, 2002).

2.5 Comparison of CSTR and SBR performance

Cassidy *et al.* (2000) and Cassidy and Hudak (2001) have shown, at least for soil contaminated by hydrocarbons, that SBR are able to reach higher efficiencies than the other above-mentioned systems. They have also reported the advantages of soil slurry-sequencing batch reactor (SS-SBR) and continuous-flow stirred tank reactor (CSTR) operation. The CSTR dilutes contaminants upon entry to the reactor, which is undesirable for concentration-dependent biodegradation rates. Dilution reduces biodegradation rates if kinetics are concentration-dependent, but may be desirable for compounds that exhibit toxicity above some threshold concentration. The CSTR may require only one tank, but abrasion caused by continuous pumping

results in high operation and maintenance costs relative to the SS-SBR. For a given retention time, a CSTR provides little operational flexibility to vary the contaminant concentrations in the reactor. The fill-react-draw nature of the SS-SBR allows intermittent pumping and much greater operational flexibility than the CSTR. With a fixed retention time in the SS-SBR, the Fill volume (volume of slurry replace per treatment cycle) and cycle time can be adjusted to provide optimal concentrations of contaminants and acclimated microorganisms. A significant advantage of the SS-SBR in hazardous waste treatment is that each batch can be tested before its release. Disadvantages of the SS-SBR include a greater operation complexity resulting from multiple reaction vessels.

Cassidy *et al.* (2000) and Cassidy and Hudak, (2001) compared the performance of a CSTR and a SS-SBR treating the diesel fuel-contaminated soil, and found marked differences in biosurfactant production and reactor performance with the two modes of operation. Microbial growth was greater in the SS-SBR than the CSTR. However, significant biosurfactant production and foaming occurred in the SS-SBR, whereas none was observed in the CSTR. They found that 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. Foam was produced only after the surfactant concentration was in decline, and was removed by the end of the cycle.

Wastewater studies have shown that SBRs and CSTRs can produce different microbial consortia (Chiesa and Irvine, 1985). Cassidy and Hudak (2001) showed that the SS-SBR encouraged the growth of biosurfactant-producing species (*Candida tropicalis, Pseudomonas aeruginosa,* and *Pseudomonas fluorescens*) relative to the CSTR. The growth of *C. tropicalis* and *P. fluorescens* were favored with SS-SBR operation relative to CSTR operation. The growth of *Brevibacterium casei* and *Flavobacterium aquatile* were favored with CSTR operation relative to SS-SBR operation. Only *P. aeruginosa* had significantly greater concentrations in effluent from both modes of operation than in the feed slurry. These results explain the enhanced biosurfactant production and DF biodegradation with SS-SBR operation relative to CSTR operation. Conditions in the CSTR are relatively constant, while SBR operation exposes microorganisms to fluctuating contaminant concentrations:

high contaminant levels prevail after Fill and low levels after the contaminants are biodegraded.

Cassidy *et al.* (2000) and Cassidy and Hudak, (2001) also showed that the microorganisms related to contaminant degradation and surfactant and foam production was strictly dependent on the mode of operation and not on the reaction vessel used.

2.6 Foaming

Foaming is a common problem in the continuous production process, which is attributed to biosurfactant production. Foam production is unpredictable and occurs very rapidly in bioslurry reactors. Foaming often requires the addition of antifoaming agents and reduction of aeration rates and/or mixing speed, all of which can have a negative impact on reactor performance. Since foam contains contaminants at concentrations many times higher than the slurry, excessive foaming can require reactor shutdown to avoid contaminating areas outside the vessel (Cassidy *et al.*, 2000, 2002; Cassidy, 2001).

The amount of foam is dependent on the initial glucose concentration and on the pH of the culture. It increases with both increasing glucose concentrations and increasing pH values. Disturbances from excess foaming resulted in a decrease of biosurfactant production. Foam formation was decreased at pH values below 6, whereas the optimum for biosurfactant production was found to be at pH 6.25. Again, reliable pH control was necessary for avoiding disturbances of the steady-state production (Reiling *et al.*, 1986).

Foam production in SS-SBRs treating a diesel fuel contaminated soil was directly related to the production of biosurfactants. Reducing the diesel fuel added per cycle in the SS-SBR reduced biosurfactant production and foaming (Cassidy *et al.*, 2000). Cassidy *et al.* (2002) showed that adding feed slurry to the SS-SBRs after foaming had started immediately reduced foam thickness, and that foam reduction was proportional to the amount of DF added.

The coincidence of foam production and emulsification capacity indicates that foaming resulted from the temporary accumulation of free (i.e., not bound to

diesel fuel) biosurfactant molecules in the reactor (Cassidy *et al.*, 2000, 2002; Cassidy and Hudak, 2001; Cassidy, 2001).

2.7 Factors Affecting Biosurfactant Production

2.7.1 Nutrient Source

In 2001, Cassidy found that the control SS-SBR with no added nutrients showed considerably lower diesel fuel removal and biological activity relative to the reactor with added nutrients and did not show signs of biosurfactant production, indicating that nutrients provided a greater rate and extent of diesel fuel biodegradation in the slurry.

2.7.1.1 Carbon Source

Hydrocarbons are commonly used as the substrate for the production of biosurfactants. It has been postulated that the biological function of surface-active compounds is related to hydrocarbon uptake, and therefore a spontaneous release occurs with these substrates. Carbohydrates were rarely used as carbon and energy source for biosurfactant production with the exceptions of *Arthrobacter sp.*, *Bacillus subtilis* (Cooper *et al.*, 1981), *Torulopsis bombicola* (Cooper and Paddock, 1984), and *Pseudomonas aeruginosa*. Although less qualified for spontaneous formation, the production of biosurfactants from carbohydrate substrates offers some advantages as compared with hydrocarbons. From an engineering point of view, hydrocarbon substrates require more sophisticated equipment and more power input to achieve an adequate dispersion of the insoluble hydrocarbons. In addition, the availability of hydrocarbons is limited if applications of biosurfactants other than in enhanced oil recovery are envisaged (Guerra-Santos *et al.*, 1984).

Water-soluble carbon sources such as glycerol, glucose, mannitol, and ethanol were all used for rhamnolipid production by *Pseudomonas* spp. Rapid biosurfactant production was observed in a *Pseudomonas* strain during growth on glucose and oleic acid, when oleic acid was utilized upon the exhaustion of glucose (Desai and Banat, 1997). Among water-soluble substrates, mannitol is especially effective. In contrast to polyhydroxyalkanoates (PHAs), the carbon source

does not generally affect the composition of rhamnolipids produced presumably because their fatty acid is synthesized de novo. A noticeable exception was observed when *P. aeruginosa* 57RP was grown on the aromatic hydrocarbon naphthalene: 80% of the total rhamnolipids contained only one fatty acid moiety instead of 3-(3-hydroxyalkanoyloxy)alkanoic acids (HAAs) (Soberón-Chávez *et al.*, 2005). Biosurfactant product, however, was inferior to that obtained with water-immiscible substrates such as *n*-alkanes and olive oil (Desai and Banat, 1997). It has been thus thought that the microorganism produces rhamnolipids to emulsify these water-insoluble carbon sources for well assimilation (Matsafuji *et al.*, 1997). Oil of vegetable origin, such as soybean (Lang and Wullbrandt, 1999), corn, canola (Sim *et al.* 1997), and olive, provides the highest productivity. 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.

Matsufuji *et al.* (1997) examined the effect of different carbon sources on the rhamnolipids production by *Pseudomonas aeruginosa* IFO 3924. They showed that Rhamnolipids R1 and R2 were produced when ethanol, glucose, glycerol or rape seed oil was used. However, the highest yield was observed with ethanol. *n*-Paraffin was not substrate for rhamnolipid production in this strain probably because of poor growth. Ethanol is an attractive raw carbon source for microbial production of a variety of biochemicals. *Pseudomonas* BOP 100 has the capabilities for production of rhamnolipid and phenazine when grown on ethanol as sole carbon source.

2.7.1.2 Nitrogen Source

Medium constituents other than carbon source also affect the production of biosurfactants. In *P. aeruginosa*, a simultaneous increase in rhamnolipid production and glutamine synthetase activity was observed when growth slowed as the culture became nitrogen limiting (Mulligan and Gibbs, 1989). In 1997, Desai and Banat have reported that nitrogen limitation not only causes overproduction of biosurfactant but also changes the composition of the biosurfactant 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.

Actually, nitrogen-limiting conditions do not favor rhamnolipids production per se, but production starts with the exhaustion of nitrogen (Soberón-Chávez et al., 2005). Production of rhamnolipids is inhibited by the presence of NH^{4+}, glutamine, asparagine, and arginine as nitrogen source and promoted by NO^{3-}, glutamate, and aspartate (Mulligan and Gibbs 1989; Köhler et al. 2000). It has been repeatedly demonstrated that NO^{3-} is the best nitrogen source for rhamnolipid production (Guerra-Santos et al., 1984; Arino et al., 1996). Nitrate to be the best source of nitrogen for biosurfactant production by Pseudomonas strain 44T1 growing on olive oil (Desai and Banat, 1997). On the other hand, high levels of NH^{4+} or glutamine reduce rhamnolipid production, and this is correlated with a lower glutamine synthase activity (Mulligan and Gibbs, 1989). The basis for the preference for nitrate is unknown. One suggestion was that P. aeruginosa, which is capable of denitrification, is also using NO^{3-} as an electron acceptor even in the presence of oxygen. Interestingly, Sabra et al. (2002) recently proposed that P. aeruginosa is producing rhamnolipids to reduce oxygen transfer rate as a means to protect itself from oxidative stress, and it appears that this mechanism is activated by iron deficiency (Kim et al., 2003). However, excellent rhamnolipid production is also obtained in the absence of oxygen (Soberón-Chávez et al., 2005).

2.7.1.3 Phosphorous

Elevated C/P (Mulligan et al., 1989) ratios promote rhamnolipids production. Guerra-Santos et al. (1984) demonstrated that biosurfactant formation of the cells remained at its maximum up to a C-to-P ratio of 16. When the media of higher C-to-P ratios were applied, a decrease in biosurfactant concentration occurred. Biomass concentration did not change significantly, indicating that there was no expressed P limitation at all the phosphate concentrations tested. A certain surplus of phosphate was apparently required for P. aeruginosa biosurfactant formation.

2.7.1.4 Multivalent Cations

The limitation of multivalent cations also causes overproduction of biosurfactants (Guerra-Santos et al., 1984). A higher yield of

rhamnolipid can be achieved in *P. aeruginosa* DSM 2659 by limiting the concentrations of salts of magnesium, calcium, potassium, sodium, and trace elements (Desai and Banat, 1997). Of the trace elements, iron had a major influence on *P. aeruginosa* biosurfactant production. Iron limitation stimulates biosurfactant production in *P. fluorescens* (Persson *et al.*, 1990) and *P. aeruginosa* (Guerra-Santos *et al.*, 1984).

2.7.2 Environmental Factors

Environmental factors and growth conditions such as pH, temperature, agitation, and oxygen availability also affect biosurfactant production through their effects on cellular growth or activity (Desai and Banat, 1997).

2.7.2.1 pH

Rhamnolipid production in *Pseudomonas* spp. was at its maximum at a pH range from 6 to 6.5 and decreased sharply above pH 7 (Guerra-Santos *et al.*, 1984). In contrast, Powalla *et al.* (1989) showed that penta- and disaccharide lipid production in *N. corynbacteroides* is unaffected in the pH range of 6.5 to 8. In addition, surface tension and CMCs of a biosurfactant product remained stable over a wide range of pH values, whereas emulsification had a narrower pH range (Desai and Banat, 1997).

2.7.2.2 Temperature

In *A. paraffineus* and *Pseudomonas* sp. strain DSM-2874, temperature causes alteration in the composition of the biosurfactant produced. A thermophilic *Bacillus* sp. grew and produced biosurfactant at temperatures above 40°C. Heat treatment of some biosurfactants caused no appreciable change in biosurfactant properties such as the lowering of surface tension and interfacial tension and the emulsification efficiency, all of which remained stable after autoclaving at 120°C for 15 min (Desai and Banat, 1997).

2.7.2.3 Agitation Speed and Aeration Rate

An increase in agitation speed results in the reduction of biosurfactant yield due to the effect of shear in *Nocardia erythropolis*. While studying the mechanism of biosurfactant production in *A. calcoaceticus* RAG-1, the cell-bound polymer/dry-cell ratio decreases as the shear stress increases. On the other

hand, in yeast, biosurfactant production increases when the agitation and aeration rates are increased (Desai and Banat, 1997).

The bioreactor had been earlier found to be a better system with increase in air flow rates (Gowthaman *et al.*, 1993). Oxygen transfer is one of the key parameters for the process optimization and scale-up of surfactin production in *Bacillus subtilis* (Desai and Banat, 1997). Veenanadig *et al.* (2000) investigated the production of a pesticide (Fenthion) dispersing biosurfactant by *B. subtilis* FE-2, in a PCBR using various flow rates. They showed that oxygen transfer is enhanced at larger flow rates resulting in efficient fermentation, enhanced surfactant production and hence, further reduction in surface tension.

2.7.2.4 Salt

Salt concentration also affected biosurfactant production depending on its effect on cellular activity. Some biosurfactant products, however, were not affected by salt concentrations up to 10% (w/v), although slight reductions in the CMCs were detected (Desai and Banat, 1997).

2.7.3 Volumetric Loading

The volumetric loading (i.e., fill volume per cycle time) can be adjusted to provide different contaminant concentrations at the beginning of React with the same retention time. For example, loadings of 10% per 1-day cycle and 90% per 9-day cycle both use a 10-day retention time but, assuming similar effluent concentrations, 90% loading achieves a higher concentration after fill (i.e., at the beginning of React) to take advantage of concentration-dependent degradation kinetics.

Cassidy *et al.* (2002) found that the effects of increased contaminant loading on the performance of SS-SBRs can be both positive (contaminant emulsification) and negative (foaming). This result also showed that culturable concentrations of *Candida tropicalis, Brevibacterium casei, Flavobacterium aquatile,* and *Pseudomonas fluorescens* varied significantly with loading, except *P. aeruginosa*. The total microbial counts did not vary significantly with loading. Culture-based counts of surfactant-producing species (*C. tropicalis, P. aeruginosa,* and *P. fluorescens*) relative to total counts increased from 21 to 86% as loading

increased from 5 to 50%. This explains the enhanced biosurfactant production and diesel fuel biodegradation observed with increase loading.