ENHANCED BIODEGRADATION OF PERSISTENT ORGANIC POLLUTANTS IN INDUSTRIAL WASTEWATERS BY BIOSURFACTANTS

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by

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İZMİR

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We certify that we have read this thesis and that in our opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science.

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ABSTRACT

The release of specific industrial wastewaters, including nonbiodegradable and/or toxic pollutants, to receiving environments is today one of the most significant type of environmental pollution by hazardous wastes. Although biological treatment methods have been generally found most effective alternatives in the removal of persistent compounds, they require some enhancements due to the presence of refractory or toxic compounds in the wastewaters. For this reason, the use of surfactants in the removal of persistent organic pollutants by biological treatment was investigated as an enhancement technique for the biological treatment process.

Surfactants, which are amphipathic molecules with both hydrophilic (water-soluble) and hydrophobic (water-insoluble) functional groups, act at the surface, or interface, between polar and nonpolar phases to modify the surface properties of both phases due to presence of the hydrophobic group. Surfactants reduce surface or interfacial tension and form colloidal sized aggregates called micelles. In contrast to synthetic surfactants, microbial surfactants (biosurfactants), which are a structurally diverse group of surfactants produced by various microorganisms under highly aerobic conditions, have several advantages over the synthetic counterparts such as biodegradability, low or no toxicity, acceptable production economics, and use in environmental control.

The effectiveness of both synthetic and microbial surfactants in the remediation of contaminated soil and groundwater environments, oil spill remediation at sea and inland, and oil tank clean-up has been proven by many activities including researches, demonstrates and field-scale applications of in-situ surfactant enhancement as a technology. The application of surfactants can enhance soil and groundwater remediation in three ways:

- by increasing contaminant mobility and solubility to improve the performances of conventional remediation technologies such as pump-and-treat;
- by decreasing the mobility of contaminants to prevent their migration; and
- to speed the rate of biodegradation of contaminants in soil.

There are not any results about the use of surfactants in the removal of persistent organic pollutants in industrial wastewaters. The proven effectiveness of surfactants in soil and groundwater and oil spill remediation has been considered reasonable to expect that surfactants can also enhance the removal of persistent organic pollutants in industrial wastewaters. From this point of view, the effects of sophorolipid type biosurfactant on the removal of persistent organic pollutants in industrial wastewaters have been examined in this study.

To determine the effectiveness of sophorolipids used in the experimental studies both synthetic wastewater samples, prepared by gasoline and kerosene, and real wastewater samples taken from a dye industry were used. Moreover, different sophorolipid concentrations (0 to 100 mg/L) were examined to optimize biosurfactant-enhanced degradation of persistent pollutants in industrial wastewaters.

The results of this study indicate that the biodegradation of persistent organic pollutants in industrial wastewaters is enhanced by the use of biosurfactants. The principal mechanism that enhances the biodegradation is the increasing solubility of poorly soluble compounds in the wastewater. According to the results of this study, it can be expected that biosurfactant-enhanced degradation would result in faster and more complete degradation.

ÖZET

Biyolojik olarak parçalanamayan ve toksik kirleticiler içeren endüstriyel atıksuların çevresel ortamlara tam olarak arıtılmadan verilmesi, günümüzde tehlikeli atıklardan kaynaklanan çevre kirliliklerinin en önemli sebeplerinden biridir. Biyolojik arıtma yöntemleri, genellikle bu tür kirleticileri içeren atıksuların arıtımında en etkin alternatif olarak kabul edilse de, atıksuda zor ayrışabilir ve toksik maddelerin varlığı biyolojik arıtmada temel mekanizma olan biyolojik parçalanmayı engellerler. Bu durum, biyolojik arıtma sürecinde parçalanmayı hızlandıracak modifikasyonları gerektirir. Bu çalışmada, zor ayrışabilir kirleticileri içeren atıksuların biyolojik olarak arıtımında alternatif bir hızlandırıcı yöntem olarak surfaktant kullanımı incelenmiştir.

Hem hidrofilik (suda çözünebilir) hem de hidrofobik (suda çözünmeyen) fonksiyonel grupları içeren moleküller olan surfaktantlar, çok fazlı sistemlerde polar ve polar olmayan fazlar arasındaki yüzeyde ya da yüzeylerarasında etkin olurlar; ve hidrofobik grupun varlığı nedeniyle her iki fazın yüzey özelliklerini değiştirirler. Bu etkinlikleri yoluyla bulundukları ortamın yüzey gerilimini ya da yüzeylerarası gerilimi düşürürler.

Surfaktantlar kimyasal olarak ya da mikrobiyolojik olarak üretilebilirler. Kimyasal yöntemlerle üretilen surfaktantlar sentetik surfaktantlar, mikrobiyolojik olarak birçok farklı mikroorganizma tarafından çoğunlukla oksijenli ortam koşullarında üretilenler ise mikrobiyal surfaktantlar (biyosurfaktantlar) olarak isimlendirilirler. Yapısal olarak surfaktantların farklı bir grubu olan biyosurfaktantlar, sentetik surfaktantlara göre biyolojik olarak parçalanabilirlikleri, düşük toksisiteye sahip olmaları ya da toksik olmamaları, ekonomik olarak üretilebilirlikleri, ve çevresel uygulamalarda kullanılmaları gibi birçok avantaja sahiptirler.

Gerek sentetik gerekse mikrobiyal surfaktantların kirletilmiş toprak ve yeraltısuyu rehabilitasyonunda, deniz ve toprak ortamına petrol dökülmelerinde ve petrol tanklarının temizlenmesinde etkin olduğu birçok araştırma ve alan çalışmasında ortaya konmuştur. Surfaktant kullanımı, klasik toprak ve yeraltısuyu rehabilitasyonu yöntemlerini, kirleticilerin çözünürlüklerini arttırarak, kirleticilerin taşınımlarını engellemek için hareketliliklerini azaltarak ya da toprak ortamında kirleticilerin biyolojik parçalanma hızını arttırarak hızlandırmaktadır.

Günümüzde kalıcılığı yüksek kirleticileri içeren endüstriyel atıksuların arıtımında surfaktantların kullanımına dair herhangi bir araştırılma sonucu mevcut değildir. Surfaktantların toprak ve özellikle yeraltısuyu rehabilitasyonunda ispatlanan etkinlikleri gözönüne alınarak, atıksulardaki zor ayrışabilir kirleticilerin biyolojik olarak gideriminde de etkin olarak bir hızlandırma sağlayacakları kabul edilmiştir. Bu noktadan hareketle bu çalışmada sophorolipidlerin endüstriyel atıksulardaki zor ayrışabilir kirleticilerin giderimine etkileri araştırılmıştır.

Sophorolipidlerin etkinliğini belirlemek için, deneysel çalışmalarda benzin ve gazyağı kullanılarak hazırlanan sentetik atıksu örnekleri ve bir boya sanayi tesisinden alınan gerçek atıksu numuneleri kullanılmıştır. Ayrıca endüstriyel atıksulardaki zor ayrışabilir kirleticilerin biyosurfaktantlar kullanılarak hızlandırılmış biyolojik parçalanması sürecini optimize etmek için farklı sophorolipid konsantrasyonları (0-100 mg/L) incelenmiştir.

Bu çalışmanın sonuçları, biyosurfaktant kullanımının endüstriyel atıksulardaki zor ayrışabilir yada kalıcılığı yüksek kirleticilerin biyolojik parçalanmasını hızlandırdığını göstermektedir. Biyolojik parçalanmayı hızlandıran etkin mekanizma, atıksudaki çözünürlüğü düşük kirleticilerin çözünürlüklerinin arttırılmasıdır. Çalışma sonuçlarına göre, biyosurfaktantlarca hızlandırılan biyolojik parçalanma süreci ile klasik biyolojik arıtma yöntemlerinde elde edilenlerden daha hızlı ve tam bir parçalanma sağlanacağı beklenmektedir.

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CHAPTER ONE
INTRODUCTION

1.1 PERSISTENT POLLUTANTS PROBLEM

In contemporary world, accidental or intentional release of hazardous wastes to natural environments is one of the most significant pollution types threatening environmental sustainability and human health, and industrial wastes constitute the primary source of hazardous wastes. Increase in the quantities of chemical substances used in industrial processes together with rapid developments in industrial sectors, and especially inadequacy of disposal methods applied for industrial wastes have led to deliberate accumulation of huge quantities of wastes, many of which are hazardous, in environmental mediums. Although numerous industrial pollution control efforts have been performed, environment has been polluted and continues to being polluted today, because these efforts generally aim to control only conventional pollutants.

Specific industrial waste streams whose releases to receiving environments cause water and soil pollution generally include nonbiodegradable and/or toxic pollutants. Recent regulations limiting the discharges of these types of wastewaters to receiving environments are becoming more restrictive each year and require source control and advanced pre- or post-treatment alternatives. Consequently, in the last years new treatment methods such as chemical oxidation, granular activated carbon adsorption, powdered activated carbon treatment, wet-air oxidation, and anaerobic treatment have been improved for the treatment of wastewaters containing refractory compounds. However, many of these methods have limited effectiveness in the removal of refractory pollutants or high capital and operational costs. In addition,

environmentally acceptable and cost-effective treatment options. The presence of refractory or toxic pollutants in the wastewaters often hinders treatment of these wastewaters through the biological treatment processes. These pollutants are often co-metabolized, thus not completely mineralized due to their low aqueous solubilities, and strong sorption properties. Low dissolution rates, which limits the bioavailability of these compounds to degradative organisms, and toxicity, directly inhibiting biodegradation, extend the persistence of these pollutants.

1.2 PURPOSE OF THE STUDY

The rapidly increasing costs of new wastewater treatment technologies and/or their limited effectiveness in the removal of persistent pollutants and regulations becoming more restrictive each year are fostering interest in the development and the use of alternative cost-effective and environmentally acceptable approaches. One approach being investigated in this study is the use of surfactants to enhance the biodegradation of persistent pollutants in industrial wastewaters.

The use of surfactants has been found as an effective and feasible alternative in the bioremediation of contaminated soil and groundwater environments and oil spill clean up at sea and inland. However, the use of -both synthetic and microbial type- surfactants in the removal of persistent organic pollutants in industrial wastewaters has not been investigated. Based on the knowledge about the effectiveness of surfactants in the bioremediation of persistent organics-contaminated soil and especially groundwater environments by increasing the bioavailability of these contaminants to microorganisms, it was considered that surfactants might have an effect on the biodegradation of persistent organic contaminants in industrial wastewaters.

The primary objective of this study is to investigate the effectiveness of biosurfactants in the biodegradation of persistent organic compounds in industrial wastewaters, and to determine enhancement level of biosurfactants in microbial

degradation. Additionally, it was aimed to yield preliminary data on the use of biosurfactants in wastewater treatment.

1.3 SCOPE OF THE STUDY

In this study, microbial type surfactants having biodegradable and non-toxic characteristics were used, because synthetic surfactants have been often found toxic and/or recalcitrant or pose threat of additional contamination and inhibit biodegradation. Sophorolipid type biosurfactant produced by *Torulopsis Bombicola* ATCC 22214 was used in experimental studies. In many studies on environmental applications of biosurfactants, sophorolipids has been found effective in contaminated soil and groundwater remediation and oil spill clean-up studies.

The effectiveness of sophorolipids in persistent organic pollutants removal was examined by studying with synthetic wastewater samples prepared with gasoline and kerosene; and dye industry wastewater samples. Gasoline and kerosene were selected as model petroleum contaminants, whereas dye industry wastewater was considered as problematic waste stream in terms of high toxic or oily (hydrophobic) characteristics.

This study is the first - published - study about the use of biosurfactants in wastewater treatment in Turkey. Up to now, the effectiveness of synthetic or microbial surfactants has not been investigated. The presence of some ongoing studies in this area has been stated by Prof. Dr. N. Kosaric, but, any ongoing project proposal, research report, article or other types of publications have not been found, although many engineering indexes or databases such as Current Contents, ERIC, and EBSCO and general indexes have been searched.

CHAPTER TWO
BIOSURFACTANTS

2.1 CHARACTERISTICS AND STRUCTURAL FEATURES OF BIOSURFACTANTS

A ***surface active agent*** or ***surfactant*** can be defined as any substance, which exhibits the characteristic of affecting intermolecular forces and modifying interfacial interactions with adsorbing onto the surface or interface of a solution, when present at low concentrations (Sutton, 1992; Connell, 1997). The surfactant molecule is typically composed of a strongly ***hydrophilic*** (water-soluble) group, or moiety, and a strongly ***hydrophobic*** (water-insoluble) moiety. The entire surfactant monomer is often referred to as amphiphilic because of its dual nature. The hydrophobic portion of the surfactant monomer is generally a long hydrocarbon chain, called as the "***tail***" of the molecule. The hydrophilic "***head***" group often includes anions or cations such as sodium, chloride, or bromide (Desai & Banat, 1997; Myers, 1992). "The surfactant monomer is illustrated as a tadpole structure in Figure 2.1. The molecular weight of surfactants under consideration for environmental applications typically ranges from 200 g/mol to 2000 g/mol" (AATDF, 1997).

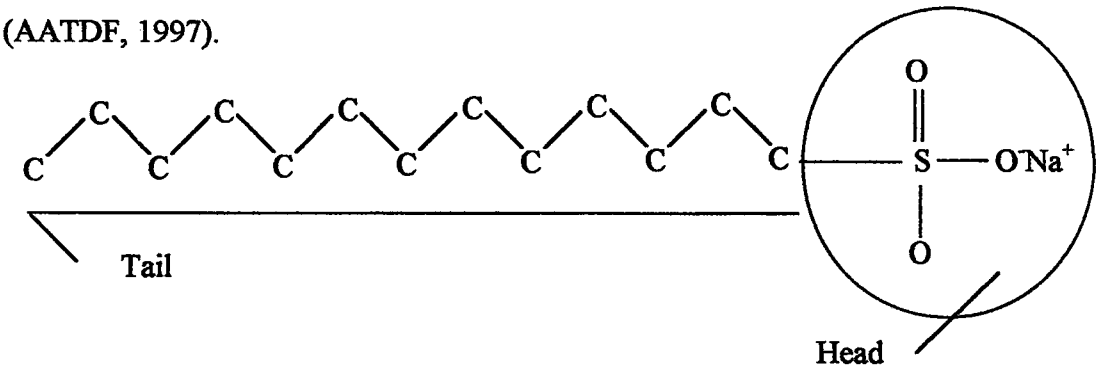

Figure 2. 1 Sodium dodecyl sulfide surfactant monomer (AATDF, 1997)

A ***surface*** or an ***interface*** is the boundary between at least two phases. An interface boundary exists between two immiscible phases with having polar and nonpolar properties, whereas a surface boundary is an interface where one phase is a gas, commonly air. In a system having two immiscible phases, the boundaries between the phases are of primary importance in terms of determining the characteristics and behavior of the whole system, although the various bulk characteristics of each phase is theoretically unaffected (Desai & Banat, 1997; Sutton, 1992; Myers,1992).

Interfacial tension is a measure of the interfacial free energy per unit area between two phases. The adjacent layers of two immiscible fluids have potential energies greater than those of similar molecules in its bulk phases. The amount of work required expanding the interface by unit area and equals to the potential energy difference is a measure of the interfacial tension. Similarly, ***surface tension*** is a measure of the surface free energy per unit area between the liquid and the air above it. Molecules at the surface have higher potential energies than molecules in the liquid bulk. To expand the surface by unit area, molecules in the bulk must be brought to the surface. Surface tension measures the amount of work required to expand the surface by unit area (Sutton, 1992; Myers, 1992).

A surfactant added to an aqueous solution will concentrate at the surface, due to presence of the hydrophobic group (Sutton, 1992). *Surfactant molecules gather together, orienting themselves with the hydrophilic part in the water and the hydrophobic tail projecting into the air or grease. This* ***aggregation*** *is so efficient that the surfaces can be completely covered with a surfactant monolayer, even though the surfactant concentration in the bulk water may be as low as* 10^{-4} *molar* (Connell, 1997). Consequently, less work is required to bring a surfactant molecule to surface than a water molecule. The presence of surfactants and their aggregation reduce surface or interfacial tension, since less work is needed to bring molecules to surface. "The amphipathic structure of surfactants results in three phenomena;

- concentration of the surfactant at the surface
- reduction of surface tension

- orientation of the surfactant molecule with its hydrophilic end towards the aqueous solution and the hydrophobic end away from aqueous solution" (Sutton, 1992, p.2394).

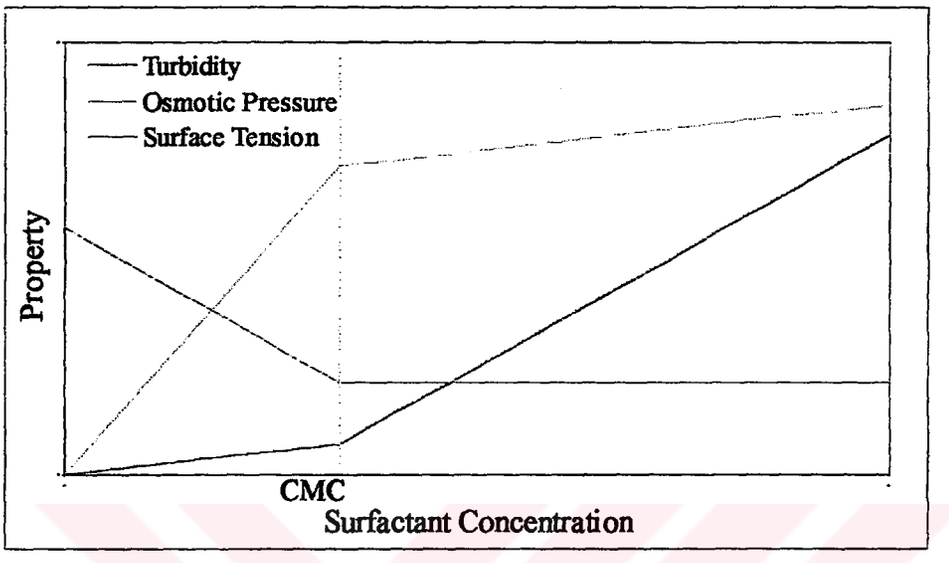

Figure 2. 2 Solution properties changes versus the surfactant concentration (Connell, 1997)

In addition to absorbing at the surfaces or interfaces of solutions and reduction of surface and interfacial tension, surfactants have the unique property of forming colloidal sized aggregates called ***micelles***. When surfactants are present in dilute numbers, they have monomeric form micelle clusters. The concentration of a surfactant at which micelle formation begins is called the ***Critical Micelle Concentration (CMC)*** (Sutton, 1992). CMC is defined by the solubility of a surfactant within an aqueous phase and is commonly used to measure the efficiency of a surfactant. Microbial culture broth or biosurfactants are diluted severalfold, and surface tension is measured for each dilution. Surface tensions are plotted against the logarithm of the biosurfactant concentration. The biosurfactant concentration at which the surface tension no longer decreases significantly is the CMC (Desai & Banat, 1997). At surfactant concentrations exceeding the CMC, all additional surfactant monomers form micelles and the surface tension remains essentially constant, as shown in Figure 2.2. This is because that only the monomeric form of surfactants is surface-active, micelles are not. Micelles are structured with the hydrophilic groups in contact with the aqueous solution and the hydrophobic group

allows the surfactant molecule enough solubility to attain the CMC value. In general, the more surface-active the surfactant monomer is, the greater the tendency to form micelles and the lower the CMC value (Sutton, 1992). The surfactant molecules are most stable at interfaces such as air-water or oil-water surfaces (Connell, 1997).

Microbial surface-active agents or biological surfactants (***biosurfactants***) are a structurally diverse group of surfactants mainly produced by hydrocarbon-utilizing microorganisms during growth on insoluble substrates or produced on water-soluble compounds such as glucose, sucrose, glycerol, or ethanol (Banat, 1995; Desai & Banat, 1997; Thangamani & Shreve, 1994).

The hydrophilic component of biosurfactants generally consists of either carbohydrate, a hydrophilic amino acid such as glutamate, aspartate, lysine or arginine, or a hydrophilic peptide. The hydrophobic or water-insoluble portion frequently consists of either a lipid structure, an isoprenoid structure such as a cholesterol, or a hydrophobic amino acid or peptides including amino acids such as phenylalanine, leucine, iso leucine, valine, or alanine (Thangamani & Shreve, 1994).

Biosurfactant molecules are cell wall associated and are secreted into the surrounding media. Such extracellular and cell wall associated molecules have the potential to promote cellular attachment to hydrophobic surfaces, to affect the distribution of cells between oil and water phases, to emulsify water-insoluble substances, and to mediate transport of hydrophobic substances into the cell (Thangamani & Shreve, 1994). Biosurfactants, which are high molecular weight lipid complexes, share the same properties with synthetic surfactants and have both hydrophilic and hydrophobic functional groups (Kosaric, 1992; Radosevich *et al.*, 1997). In some cases, microbial surfactants are more effective in creating microemulsions in which micelle formation occurs and hydrocarbons can solubilize in water. Biosurfactants mainly reduce surface tension, interfacial tension, and CMC in both aqueous solutions and hydrocarbon mixtures, therefore they increase the aqueous concentrations of poorly soluble compounds, which leads to improving the accessibility of these substrates to microorganisms (Banat, 1995).

2.2 CLASSIFICATION OF SURFACTANTS

2.2.1 SYNTHETIC SURFACTANTS CLASSIFICATION

Chemically synthesized surfactants (***synthetic surfactants***) are classified according to the nature of their polar grouping. The hydrophilic portion of a surfactant may ionize or it may not. Surfactant molecules having ionizing hydrophilic portions are ***ionic surfactants***, however those having non-ionizing hydrophilic portions are called as ***non-ionic surfactants***. Polar group of a non-ionic surfactant molecule is not electrically charged. An ionic surfactant molecule that can dissociate to yield a surfactant ion, whose polar group is negatively charged, is called as ***anionic surfactant***; and that whose polar group is positively charged is known as ***cationic surfactant***. However, a surfactant molecule that may contain both negatively and positively charged groups and the ionic character of the polar group of the surfactant molecules depends on solution pH, these type of surfactants are known as ***zwitterionic*** or ***amphoteric surfactants*** (Desai & Banat, 1997; Myers, 1992; Connell, 1997). General classification of surfactants and examples for each group is given in Table 2.1.

2.2.2 CLASSIFICATION AND MICROBIAL ORIGINS OF BIOSURFACTANTS

Unlike chemically synthesized surfactants, biosurfactants are differentiated mainly on their biochemical compositions or nature, structures and microbial origins (Desai & Banat, 1997; Desai & Desai, 1993). The major classes of biosurfactants include:

(1) glycolipids
(2) lipopeptides and lipoproteins
(3) phospholipids and fatty acids
(4) polymeric surfactants and
(5) particulate surfactants (Desai and Banat, 1997).

The major types of biosurfactants with their general properties and biosurfactant-producing microbial species are listed in Table 2.2.

Table 2. 1 Synthetic Surfactants Classification

Surfactant Class	Surfactant
Amphoteric surfactants	• Acyl-amino acids • N- acyl-amino acids
Ionic surfactants	
▫ Anionic surfactants	• Acyl-amino acids and derivatives • Carboxylic acids • Ester and ether carboxylic acids • Phosphoric acid esters • Sulfonic acids (acyl isethionates, alkylaryl sulfonates, alkyl sulfonates, sulfosuccinates) • Sulfuric acid esters
▫ Cationic surfactants	• Alkylamines • Alkyl imidazolines • Ethoxylated amines • Quarter-naries
Non-ionic surfactants	• Alcohols • Alkonlamides • Amine oxides • Esters

2.2.2.1 GLYCOLIPIDS

Glycolipids, the most known and commonly isolated and studied biosurfactants, are carbohydrates in combination with long-chain aliphatic acids or hydroxyaliphatic acids. Among the glycolipids, the best-known types are:

- rhamnolipids
- trehalolipids
- sophorolipids. (Desai & Banat, 1997; Desai & Desai, 1993).

Table 2. 2 Microbial Sources and Properties of Biosurfactants (Desai & Banat, 1997)

Biosurfactant	Organism	Surface Tension mN/m[1]	CMC mN/m[1]	Interfacial Tension mN/m[1]
Glycolipids				
Rhamnolipid	*P.aeruginosa*	29		0.25
	Pseudomonas sp.	25-30	0.1-10	1
Trehalolipids	*R. erythropolis*	32-36	4	14-17
	N. erythropolis	30	20	3.5
	Mycobacterium sp.	38	0.3	15
Sophorolipids	*T. bombicola*	33		1.8
	T. apicola	30		0.9
	T. petrophilium			
Cellobiolipids	*U. zeae, U. maydis*			
Lipopeptides and Lipoproteins				
Peptide-lipid	*B. licheniformis*	27	12-20	0.1-0.3
Serrawettin	*S. marcescens*	28-33		
Viscosin	*P. fluorscens*	26.5	150	
Surfactin	*B. subtilis*	27-32	23-160	1
Subtilisin	*B. subtilis*			
Gramicidins	*B. brevis*			
Polymyxins	*B. polymyxa*			
Fatty acids, Neutral lipids, and Phospholipids				
Fatty acids	*C. lepus*	30	150	2
Neutral lipids	*N. erythropolis*	32		3
Phospholipids	*T. thiooxidans*			
Polymeric Surfactants				
Emulsan	*A. calcoaceticus*			
Biodispersan	*A. calcoaceticus*			
Mannan-lipid-protein	*C. tropicalis*			
Liposan	*C. tropicalis*			
Carbohydrate-protein-lipid	*P. fluorescens*	27	10	
	D. polymorphis			
	P. aeruginosa			
Particule Biosurfactants				
Vesicles and fimbriae	*A. calcoaceticus*			
Whole cells	*Variety of bacteria*			

[1] miliNewton/meter (1 mN/m ≅ 0,00102 g/cm)

2.2.2.1.1 Rhamnolipids

Rhamnolipid type biosurfactants can be defined as glycolipids containing rhamnose molecules. Certain species of *Pseudomonas* are known to produce large amounts of glycolipids containing one or two molecules of rhamnose. These rhamnolipids, which are the principal glycolipids produced by *Pseudomonas aeruginosa*, are called as rhamnolipid type 1 (Figure 2.3a) and type 2. Jarvis and Johnson first described the production of rhamnose-containing glycolipids in *Pseudomonas aeruginosa*. It has been also shown that depending on the pH and salt concentration, pure rhamnolipids from *Pseudomonas* spp. can lower the interfacial tension and surface tension to around 1mN/m and to 25 to 30 mN/m, respectively. They also emulsify alkanes and stimulate the growth of *P. aeruginosa* on hexadecane (Desai & Banat, 1997; Desai & Desai, 1993).

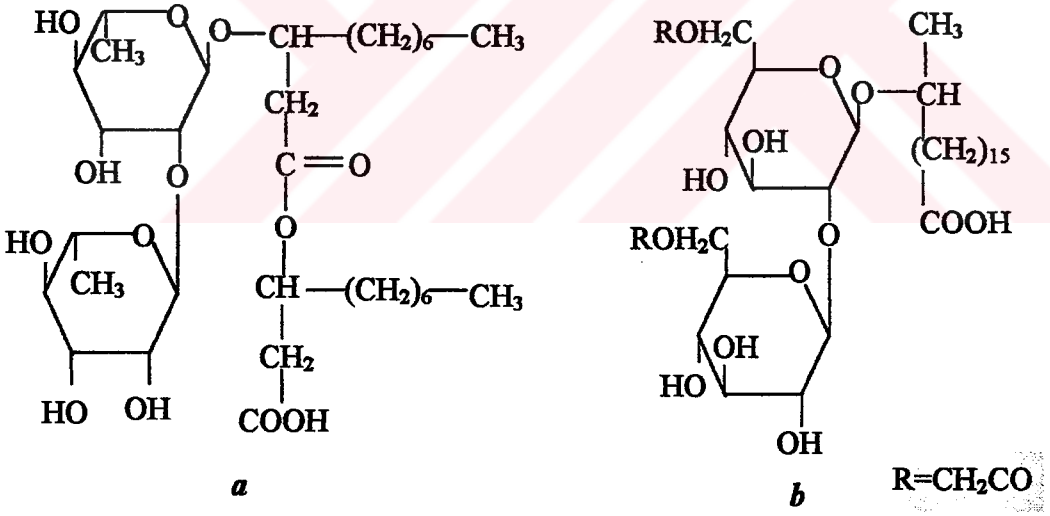

Figure 2. 3 Structure of some common glycolipid biosurfactants (Desai & Banat, 1997)

(a) Rhamnolipid type 1 from Pseudomonas aeruginosa in which two rhamnose subunits are linked to two β-hydroxydecanoic acids in a side chain. (b) Sophorolipid from Trulopsis bambicola in which dimeric sophorose is linked to a long chain (C_{18}) hydroxy fatty acid.

2.2.2.1.2 Trehalolipids

Several structural types of trehalolipids or trehalose-containing biosurfactants have been reported. Disaccharide trehalose linked at C-6 and C-6' to mycolic acids, long-chain fatty acids, is associated with most species of *Mycobacterium*, *Nocardia*, and *Corynebacterium*. Trehalolipids from different organisms differ in the size and structure of mycolic acid, the number of carbon atoms, and the degree of unsaturation. Trehalose dimycolate produced by *Rhodococcus erythropolis* has been extensively studied. It has been also found that *R. erythropolis* synthesizes a novel anionic trehalose lipid. Trehalose lipids from *R. erythropolis* and *Arthrobacter* sp. lowered the surface and interfacial tensions in the culture broth to 25 to 40 mN/m and 1 to 5 mN/m, respectively (Desai & Banat, 1997; Desai & Desai, 1993).

2.2.2.1.3 Sophorolipids

Sophorolipids or sophorose-containing type biosurfactants, which are produced mainly by yeasts such as *Torulopsis bombicola*, *Torulopsis petrophilum*, and *Torulopsis apicola*, consist of a dimeric carbohydrate sophorose linked to a long-chain hydroxy fatty acid (Figure 2.3b). These biosurfactants are a mixture of at least six to nine different hydrophobic sophorosides. Similar mixtures of water-soluble sophorolipids from several yeasts have also been reported. Cutler and Light showed that *Candida bogoriensis* produces glycolipids in which sophorose is linked to docosanoic acid diacetate. *T. petrophilum* has been reported to produce sophorolipids on water-insoluble substrates such as alkanes and vegetable oils. It has been determined that these sophorolipids, which have been chemically identical to those, produced by *T. bombicola*, have not emulsified alkanes or vegetable oils. When *T. petrophilum* was grown on a glucose-yeast extract medium, however, sophorolipids have not been produced, but an effective protein-containing alkane emulsifying agent has been formed. These results appear to contradict the conventional belief that microbial emulsifiers and surfactants are produced to facilitate the uptake of water-insoluble substrates. Sophorolipids can lower surface and interfacial tension, for example both lactonic and acidic sophorolipids lowered the interfacial tension

between n-hexadecane and water from 40 to 5 mN/m and showed remarkable stability toward pH and temperature changes (Desai & Banat, 1997; Desai & Desai, 1993).

2.2.2.2 LIPOPEPTIDES AND LIPOPROTEINS

A large number of cyclic lipopeptides including decapeptide antibiotics (gramicidins) and lipopeptide antibiotics (polymyxins), produced by *Bacillus brevis* and *B. polymyxa*, respectively, possess remarkable surface-active properties. Ornithine-containing lipids from *P. rubescens* and *Thiobacillus thiooxidans*, cerilipin an ornithine- and taurine-containing lipid from *Gluconobacter cerinus* IFO 3267, and lysine-containing lipids from *Agrobacterium tumefaciens* IFO 3058 also exhibit excellent biosurfactant activity. An aminolipid biosurfactant called serratamolide has been isolated from *Serratia marcescens* NS.38. Studies on serratamolide-negative mutants have showed that the biosurfactant increased cell hydrophilicity by blocking the hydrophobic sites on the cell surface.

The cyclic lipopeptide surfactin, produced by *B. subtilis* ATCC 21332, is one of the most powerful biosurfactants. It lowers the surface tension from 72 to 27.9 mN/m at concentrations as low as 0.005%. *B. licheniformis* produces several biosurfactants which act synergistically and exhibit excellent temperature, pH, and salt stability. The biosurfactant BL-86, produced by B. *licheniformis* 86, is capable of lowering the surface tension of water to 27 mN/m and the interfacial tension between water and n-hexadecane to 0.36 mN/m. Each molecule contains seven amino acids and a lipid portion, which is composed of 8 to 9 methylene groups and a mixture of linear and branched tails.

Recently, the production of a new lipopeptide surfactant, lichenysin-A, by *B. licheniformis* BAS50 containing long chain fatty acids have been shown by Yakimov *et al.* Lichenysin-A reduces the surface tension of water from 72 to 28 mN/m with a CMC of as little as 12 μM, comparing favorably with surfactin (24 μM) (Desai & Banat, 1997).

2.2.2.3 Fatty Acids, Phospholipids, and Neutral Lipids

Microbial fatty acids are usually of a narrow range of chain lengths, C_{16} and C_{18} with relatively small amounts of shorter (C_{12} and C_{14}) and longer (C_{20}) acids (Desai & Desai, 1993). Several bacteria and yeasts produce large quantities of fatty acid and phospholipid surfactants during growth on n-alkanes. The quantitative production of phospholipids has also been detected in some *Aspergillus* spp. and *Thiobacillus thiooxidans*. *Arthrobacter* strain AK-19 and *P. aeruginosa* 44T1 accumulate up to 40 to 80% (wt/wt) of such lipids when cultivated on hexadecane and olive oil, respectively. Phosphatidylethanolamine produced by *R. erythropolis* grown on n-alkane caused a lowering of interfacial tension between water and hexadecane to less than 1 mN/m and a CMC of 30 mg/L (Desai & Banat, 1997).

2.2.2.4 Polymeric Biosurfactants

The best-studied polymeric biosurfactants are emulsan, biodispersan, liposan, alasan, mannoprotein and other polysaccharide-protein complexes.

It has been shown that Acinetobacter calcoaceticus RAG-1 produces a potent extra-cellular polymeric bioemulsifier called emulsan. Emulsan, which has been characterized as a polyanionic amphipathic heteropolysaccharide, does not appreciably reduce interfacial tension, but it is a effective emulsifying agent for hydrocarbons in water even at a concentration as low as 0.001 to 0.01%. In the de-emulsification of an emulsion, emulsan separates emulsion into two layers. The upper cream layer contains approximately 70 to 75% oil in the bulk aqueous phase, and can remain stable for months.

Biodispersan, which is an anionic heteropoiysaccharide, is an extracellular, nondialyzable dispersing agent produced by *A. calcoaceticus* A2. The active component of biodispersan is an anionic heteropolysaccharideand and four reducing sugars, glucosamine, 6-methly aminohexose, galactosamine uronic acid, an undefined amino sugar.

Liposan is an extracellular water-soluble emulsifier synthesized by *Candida lipolytica* and is composed of 83% carbohydrate and 17% protein. The carbohydrate portion is a heteropolysaecharide consisting of glucose, galactose, galactosamine, and galacturonic acid. It has been also demonstrated by Sar and Rosenberg that polysaccharide has no emulsification activity alone but may become a potent emulsifier when combined with some proteins released during growth on ethanol.

It has been recently described the isolation of alasan, an anionic alanine-containing heteropolysaccharide-protein biosurfactant from *Acinetobacter radioresistens* KA-53. It has been found 2.5 to 3 times more active after being heated at 100 °C under neutral or alkaline conditions.

The production of large amounts of mannoprotein by *Saccharomyces cerevisiae* has been recently reported. This protein showed excellent emulsifier activity toward several oils, alkanes, and organic solvents. The purified emulsifier contains 44% mannose and 17% protein. A mannan-fatty acid complex isolated from alkane-grown *Candida tropicalis* stabilized hexadecane-in-water emulsions. *Schizonella malanogramma* and *Ustilago maydis* produce biosurfactant, which has been characterized as erythritol- and mannose-containing lipid. Recently, the production of two kinds of mannosyletythritol lipids in *Candida antarctica* T-34 has been demonstrated. The production of a peptidoglycolipid bearing 52 amino acids, 11 fatty acids, and a sugar unit by *P. aeruginosa* P-20 has been described previously. An emulsifying and solubilizing factor containing protein and carbohydrate from hexadecane-grown *Pseudomonas* spp. has also been reported. Desai *et al.* demonstrated the production of bioemulsifier by *P. fluorescens* during growth on gasoline. This bioemulsifier is composed of 50% carbohydrate, 19.6% protein, and 10% lipid. Trehalose and monoglycerides have been the major components of the carbohydrate and lipid, respectively. Similarly, an extracellular bioemulsifier-composed of carbohydrate, protein, and lipids have been isolated from *C. tropicalls* and *Phornidium* strain J1 (Desai & Banat, 1997).

2.2.2.5 PARTICULATE BIOSURFACTANTS

Accumulation of extracellular membrane vesicles having 20 to 50 nm diameters and a density of 1.158 g/cm^3 has been reported in *Acinetobacter* sp. cells. The vesicles partition hydrocarbons in the form of microemulsion, and play an important role in alkane uptake by microbial cells. The purified vesicles are composed of protein, phospholipid, and lipopolysaccharide. The membrane vesicles have about phospholipid 5 times higher and a polysaccharide contents about 350 -fold higher than that observed in the outer membrane of the same organism.

A large variety of microorganisms such as most hydrocarbon degrading and pathogenic bacteria possess surfactant activity. Their surface components that contribute to the surfactant activity include certain structures such as M-protein and lipoteichoic acid on *Streptococci* group-A, protein-A of *Staphylococcus aureus*, layer-A of *Aeromonas salmonicida*, prodigiosin of *Serratia* spp., gramicidins in *Bacillus brevis* spores and thin fimbriac in *A. calcoaceticus* RAG-1 (Desai & Banat, 1997; Desai & Desai, 1993).

2.3 FACTORS AFFECTING BIOSURFACTANT ACTIVITY

2.3.1 EMULSIFYING ACTIVITY OF BIOSURFACTANTS

In addition to surface and interfacial tension, stabilization of an oil-water emulsion is another indicator, commonly used to determine surface activity. Abu-Ruwaida, Banat, and Haditirto have examined emulsifying activity of biosurfactant isolated from ST-5 bacterial culture in 1991. Table 2.3 presents experimental results of the emulsifying activity of the whole broth of the isolate ST-5 with various short- and long-chain hydrocarbon substrates.

Highest emulsion values of about 78% and 70% have obtained using motor oil and corn oil respectively. Pentane, heptane, and cyclohexane produced emulsions (oil-in-water) comparable to that of light-grade kerosene. Light crude oil, hexane and

toluene have not emulsified effectively. Abu-Ruwaida *et al.* reported that these results indicate that the biosurfactant produced by the isolate ST-5 has a high emulsification specificity towards motor oil and corn oil, and a rather low efficiency with crude oil and some short-chain hydrocarbons. It was also reported by Abu-Ruwaida *et al.* that this results is similar to Kaplan and Rosenberg results, which indicate that the emulsifier obtained from *Torulupsis petrophilum* is more effective towards short-chain than the longer-chain alkanes. "These findings suggest that the activity of emulsifiers depends on its affinity for hydrocarbon substrates which involves a direct interaction with the hydrocarbon itself rather than an effect on the surface tension of the medium" (Abu- Ruwaida *et al.*, 1991).

Table 2. 3 Emulsification of Various Hydrocarbons by Whole Broth Cultures of the Isolate ST-5 (Abu- Ruwaida *et al.*, 1991)

Hydrocarbon	Emulsification Index, E_{24}, %	Emulsion Type
Heptane	36	oil-in-water
Pentane	37	oil-in-water
Hexane	20	oil-in-water
Toluene	30	oil-in-water
Cyclohexane	42	oil-in-water
Kerosene	40	oil-in-water
Light crude oil	6	oil-in-water
Motor oil	78	water-in-oil
Corn oil	70	water-in-oil

2.3.2 Effect of Sodium Chloride

The effects of sodium chloride on the surface and emulsification activity have been investigated by Abu-Ruwaida *et al.* (1991). Abu-Ruwaida *et al.* have used the critical micelle dilution (CMD), which is defined as the corresponding dilution of culture broth, instead of biosurfactant concentration in their study, because CMC is proportional to the total amount of surface active compound present, and can be used

as an approximate measure for biosurfactant concentration. Figure 2.4 adopted from Abu-Ruwaida *et al.* show the effect of sodium chloride addition on surface tension and critical micelle dilution of ST-5 biosurfactant. Little changes have been observed in either parameter with the addition up to 10% [w/v] sodium chloride, although CMD values decreased slightly with increasing salt concentration, indicating increased biosurfactant activity in the presence of sodium chloride (Abu-Ruwaida *et al.*, 1991).

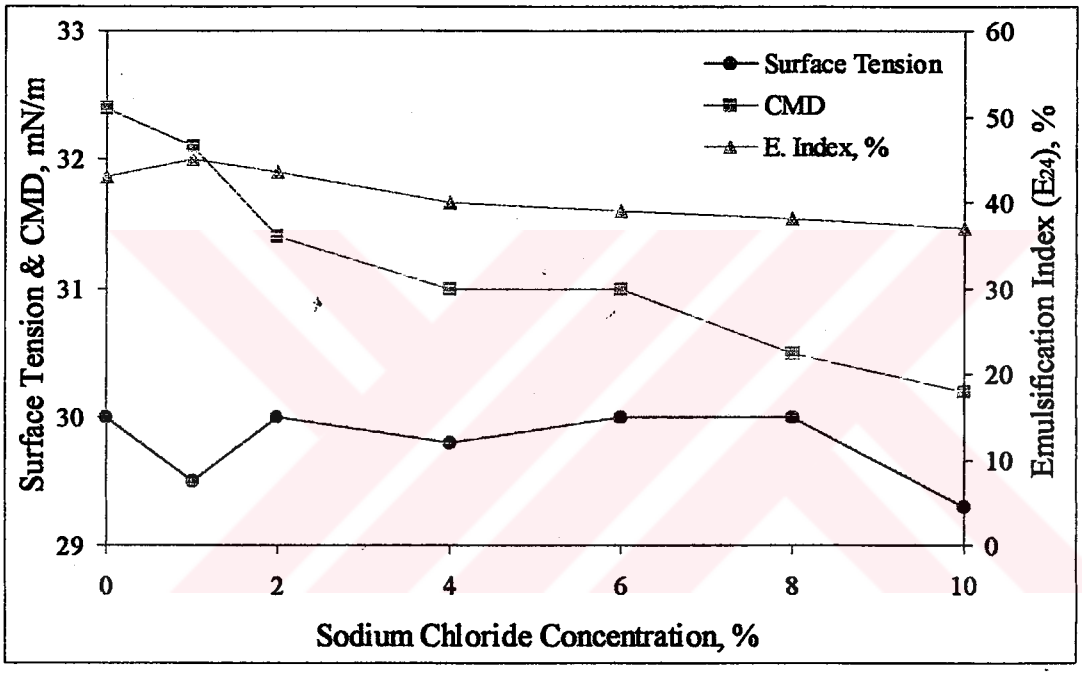

Figure 2. 4 Effect of sodium chloride on the surface and emulsifying activity of whole broth of the isolate ST-5 (Abu-Ruwaida, 1994).

2.3.3 pH EFFECT

Figure 2.5 shows the results of the study about the effect of pH on the biosurfactant properties, conducted by Abu-Ruwaida *et al.* These results indicate that pH variation has no appreciable effect on surface tension and concentration, which is expressed as CMD.

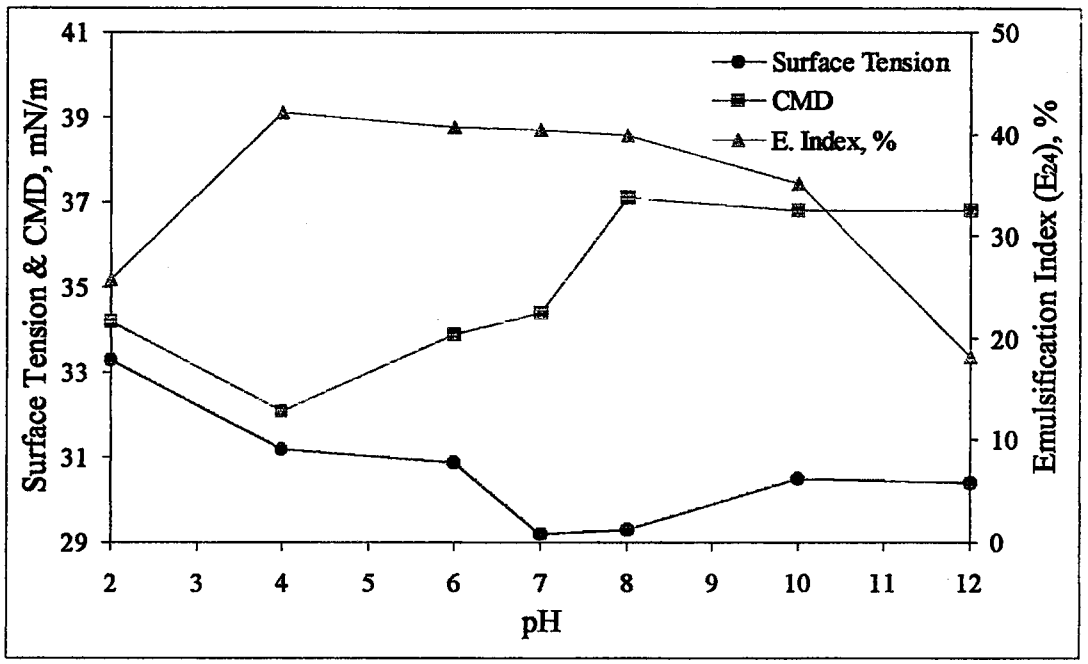

Figure 2. 5 Effect of pH on the surface and emulsifying activities of whole broth of the isolate ST-5 (Abu-Ruwaida *et al.*, 1991)

2.3.4 EFFECT OF TEMPERATURE

Abu-Ruwaida *et al.* demonstrated that no applicable change in biosurfactant properties occurred, if the culture broth has been heated. The effect of heat treatment on the biosurfactant activity of ST-5 culture can be shown in Table 2.4.

Table 2. 4 Heat Stability of the Biosurfactant Produced from ST-5 Culture (Abu-Ruwaida *et al.*, 1991)

Biosurfactant Property	Unit	Before Heat Treatment	After Heat Treatment	
			100°C/15 min	120 °C/15 min
Surface Tension	mN/m	31.8	31.8	30.5
CMD[1]	mN/m	32.7	32.5	32.3
CMD[2]	mN/m	40.8	43.9	42.9
E_{24}	%	45.0	45.0	45.0

[1] CMD in which broth has been diluted 1:10

[2] CMD in which broth has been diluted 1:100

It has been found that the biosurfactant properties measured as surface tension, CMD, and E_{24} have been remained stable after exposure to high temperatures of 100-120 °C for 15 min., and also found no change in biosurfactant activity at lower temperatures (0-4 °C) (Abu-Ruwaida *et al.*, 1991).

2.4 PRODUCTION OF BIOSURFACTANTS

Biosurfactants are produced by microbial biosynthesis with using organic matter, containing carbon and oil sources. Most of the biosurfactants are high molecular weight lipid complexes, which are normally produced under highly aerobic conditions. The production of microbial surfactants can be achieved in their ex-situ production in aerated bioreactors. When their large-scale application is encountered, their in-situ production or action (production of biosurfactants in the application site directly) would be advantageous. Low oxygen availability in their in-situ production conditions requires maintenance of anaerobic microorganisms and anaerobic biosynthesis of biosurfactants (Kosaric, 1992).

2.4.1 BIOSURFACTANT PRODUCING MICROORGANISMS

Microorganism used in the biosurfactant synthesis is the most significant factor determining the type of produced biosurfactant because the production of biosurfactants is based on microbial activity. It has been noted by many investigators that in most cases, a biosurfactant type can be produced by only a certain microbial culture. Other factors affecting biosurfactants production have some effects on the quality and the quantity of the biosurfactant produced. Most common biosurfactant producing-microorganisms, and biosurfactants produced by these microbial cultures are listed in Table 2.5.

Table 2. 5 Biosurfactants Producing Microorganisms (Banat, B. Tech., 1995)

Microorganism	Biosurfactant
Arthrobacter RAG-1	Hetropolysaccharides
Arthrobacter MIS38	Lipopeptide
Arthrobacter sp.	Trehalose, sucrose, and fructose lipids
Bacillus licheniformis JF-2	Lipopeptides
Bacillus licheniformis 86	Lipopeptides
Bacillus subtilus	Surfactin
Bacillus pumilus A1	Surfactin
Bacillus sp. AB-2	Rhamnolipids
Bacillus sp. C-14	Hydrocarbon-lipid-protein
Candida antarctica	Mannosylerthritol lipid
Candida bombicola	Sophorolipids
Candida tropicalis	Mannan fatty acid
Candida lipolytica Y-917	Sophorolipids
Clostridium pasteurianum	Neutral Lipids
Cornyebacterium hydrocarbolastus	Protein-lipid-carbohy
Cornyebacterium insidiosum	Phospholipids
Cornyebacterium lepus	Fatty acids
Strain MM1	Glucose, lipid and hydroxydecanoic acid
Nocardia erythropolis	Neutral lipids
Ochrobactrum anthropii	Protein
Penicillium spiculisporum	Spiculosporic acid
Pseudomonas aeruginosa	Rhamnolipid
Pseudomonasfluorescens	Lipopeptide
Phaffia rhodozyma	Carbohydrate-lipid
Rhodococcus erythropolis	Trehalose-dicorynomycolate
Rhodococcus sp. ST-5	Glycolipid
Rhodococcus sp. H13-A	Glycolipid
Rhodococcus sp. 33	Polysaccharide
Torulopsis bombicola	Sophorolipids

2.4.2 FACTORS INFLUENCING BIOSURFACTANTS PRODUCTION

The type, quantity, and quality of a large variety of biosurfactants are influenced by following factors:

- microbial culture producing biosurfactant (discussed in above section)
- the nature of the carbon source
- the concentration of N, P, Mg, Fe, and Mn ions in the medium
- culture (environmental) conditions, including pH, temperature, agitation, and dilution rate (Banat, Biores. Tech, 1995; Banat, Acta Biotech., 1995; Desai & Desai, 1993).

2.4.2.1 CARBON SOURCE

Beside the microorganisms used in the biosurfactants production, another important factor affecting biosynthesis of biosurfactants is the carbon source. Desai & Desai have reported an important difference between the composition of biosurfactants produced using water-soluble carbon sources such as glycerol, glucose, and ethanol and water-immiscible substrates such as *n*-alkanes and olive oil. These investigators also reported that Syldatk *et al.* and Edmonds & Cooney demonstrated that different carbon sources in the medium affected the composition of biosurfactant products in *Pseudomonas* spp. However, there is a conflict in the literature about the effects of carbon sources with different chain lengths on biosurfactant production. Syldatk *et al.* and Edmonds & Cooney reported that substrates with different chain lengths exhibited no effect on the chain lengths of fatty acid moieties in glycolipids. On the other hand, Finnerty & Singer and Neidleman & Gelgert showed evidence for qualitative variation, reflecting the carbon number of alkane for biosurfactant production in *Acinetobacter* sp. strains. Moreover, Desai & Desai have stated that the chain length of the hydrocarbon used has an affect on biosurfactant production.

To reflect the effect of carbon source on the biosurfactant production biosurfactants produced with using different carbon sources by the same microbial cultures have been listed by Banat. The surface tension of the microbial culture broth has been measured as the indicator of the biosurfactant properties. The observed changes in surface tensions have been considered as the effect of carbon sources. The effect of different carbon sources on the production of biosurfactants is shown in Table 2.6 (Desai & Desai, 1993; Banat, Acta Biotech., 1995).

Table 2. 6 The Effect of Carbon Sources on the Production of Biosurfactants in Some Strains As Measured by the Surface Tension of the Culture Broth (Banat, Acta Biotech., 1995)

Carbon Source	Concentration, %	Surface Tension, mN/m
Pseudomonas species		
Alkane	2	26
Alkane + glycerol	2	25
Glycerol	2	25
Corynebacterium hydrocarboclastus		
Kerosene	N.A.	35
Fructose	N.A.	60.5
Heptadecane	N.A.	39.5
Corynebacterium fascians		
Yeast extract + hexadecane	N.A.	27.5
Kerosene	N.A.	33
Torulopsis species		
Glucose + alkane	N.A.	39
Alkane	N.A.	50
Ustilago maydis		
Glucose	1	30
Coconut oil	1	50
Strain ST5		
Glucose	3	46.3
Kerosene	2	29.2
Yeast extract	3.5	42
n-Paraffin	2	27.6
Peptone	3	36.8
Tetradecane	2	28

N.A.: Not Available

2.4.2.2 NITROGEN SOURCE AND LIMITATION

Medium constituents other than carbon source also affect the production of biosurfactants. Among the inorganic salts, ammonium salts and urea have been preferred as nitrogen sources for biosurfactant production by *Arthrobacter paraffineus*, whereas nitrate has been found to supported maximum surfactant production in *P. aeriginosa* and *Rhodococcus* spp. It has been also reported that biosurfactant production by *A. paraffineus* has increased by the addition of some amino acids such as aspartic acid, glutamic acid, asparagine and glycine to the medium.

Desai & Banat have been reported that Robert *et al.* and Abu-Ruwaida *et al.* observed nitrate to be the best source of nitrogen for biosurfactant production by *Pseudornonas* strain 44T1 and *Rhodococcus* strain ST-5 growing on olive oil and paraffin, respectively. The production has started after 30 h of growth when the culture has reached nitrogen limitation, and continued to increase up to 58 h of fermentation. In *P. aeruginosa*, an increase in rhamnolipid production has been observed when growth slowed as the culture became nitrogen limiting. Similarly, nitrogen limitation has been determined to be increased biosurfactant production in *P. aeruginosa*, *C. tropicalis* IIP-4, and *Nocardia* strain SFC-D. Desai & Banat have been also reported that nitrogen limitation not only causes overproduction of biosurfactant but also changes the composition of the biosurfactant produced. Guerra-Santos *et al.* showed minimum 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 Hommel *et al.*, the absolute quantity of nitrogen - not its relative concentration appears to be important for optimum biomass yield, while the concentration of hydrophobic carbon source determines the conversion of carbon available to the biosurfactant.

2.4.2.3 ENVIRONMENTAL FACTORS AND GROWTH CONDITIONS

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. For example, the pH of the medium plays an important role in sophorolipid production by T. *bombicola*. Rhamnolipid production in *Pseudomonas* spp. has been found to be at its maximum level at a pH range from 6 to 6.5 and decreased sharply above pH 7. In contrast, penta- and disaccharide lipid production in *N. corynbacteroides* has unaffected in the pH range of 6.5 to 8.

In A. paraffineus and *Pseudomonas* sp. strain DSM-2874, temperature causes alteration in the composition of the biosurfactant produced. A thermophilic *Bacillus* spp. has been found to produce 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.

Salt concentration also has important effect on biosurfactant production, depending on its effect on cellular activity. It has been also reported that some biosurfactant products, however, have not been affected by salt concentrations up to 10% (wt/vol), although slight reductions in the CMCs were detected (Desai & Banat, 1997).

2.4.3 BIOSYNTHESIS OF BIOSURFACTANTS

Biosynthesis of biosurfactants is composed of the synthesis of hydrophilic and hydrophobic moieties and their linkage. Metabolic pathways involved in the synthesis of hydrophilic and hydrophobic group are diverse and utilize a specific set of enzymes. Therefore, in spite of the existence of wide spectrum of surface active compounds, there are some common features for biosynthesis of biosurfactants and their regulation. Existing possibilities for the biosynthesis of biosurfactants are:

1. *De novo* synthesis hydrophilic and hydrophobic groups by two independent pathways followed by their linkage to form a complete biosurfactant molecule
2. *De novo* synthesis of the hydrophilic moiety and the substrate-dependent synthesis of hydrophobic moiety and their linkage.
3. *De novo* synthesis of the hydrophobic moiety and the substrate-dependent synthesis of hydrophilic moiety followed by their linkage.

The synthesis of both hydrophilic and hydrophobic groups depends on the substrate used for biosurfactant production. In general, microorganisms produce biosurfactants when they grow at the expense of water immiscible substrates. It has been found that both lipid and peptide domains can be directly synthesized from carbohydrates. Addition of amino acids or fatty acids into the growth medium affects the yield, but not the structure of the surfactant. Hydrophilic moieties in biosurfactants show a great degree of complexity and involve a number of biosynthetic pathways.

2.4.4 PRODUCTION OF BIOSURFACTANTS VIA THE FERMENTATION

Depending upon the nature of the biosurfactant and the biosurfactant-producing microorganism, there are four possible types of biosurfactant production pattern by fermentation:

1. Growth associated production
2. Production under growth limiting conditions
3. Production by resting/nongrowing cells
4. Production by microbial cells and addition of precursors (Desai & Desai, 1993).

2.4.4.1 GROWTH ASSOCIATED PRODUCTION

In the growth associated production case, there is a parallel relationship between the substrate utilization, growth, and biosurfactant production (Figure 2.6-a). It has been reported by many investigators that two carbon sources, one of which is used

for the microbial growth and the other carbon source for the production of biosurfactant, have been generally used. In the biosurfactant production by *Arthrobacter paraffeniem* ATCC 19558, when *A. paraffeniem* grown on glucose, added hexadecane into the medium during the stationary phase of growth has been found to caused a significant increase in production. *T. bombicola* grown on glucose has produced substantial amounts of glycolipid by addition of vegetable oils during the growth in the late exponential phase (Desai & Desai, 1993).

2.4.4.2 PRODUCTION BY GROWING CELLS UNDER GROWTH-LIMITING CONDITIONS

The sharp increase in the biosurfactant level as a result of limitation of one or more medium components, as depicted in Figure 2.6-b, is the unique feature of this category of biosurfactant production. Overproduction of rhamnolipid by *P. aeruginosa* has been demonstrated only when the culture reaches the stationary phase of growth due to limitation of the nitrogen source. Recently, the production of glycolipid by *Nocardia* SFC-D under nitrogen limitation has been reported, and the correlation between the substrate consumption and biosurfactant production has been observed. Sodium nitrate and olive oil have also been reported as the best nitrogen and carbon sources, respectively, in the rhamnolipid production by *Pseudomonas* 44T1. It has been determined that the production started soon after the culture reached nitrogen limitation at 30 h and continued to increase up to 58 h of fermentation. When *P. aeruginosa* CFTR-6 was grown on glucose, similar observations have been also noted. The production of pentasaccharide lipid by *N. corynebacteriods* SM-1 has been found to be favored by inorganic nitrogen sources and sodium nitrate gave maximum specific surfactant production. Moreover, it has been observed that during growth, the initial yield of glycolipid increases greatly after the exhaustion of the nitrogen sources and after attaining the stationary growth phase. The production of water-soluble biosurfactant by *T. apicola* has been studied extensively. The secretion of exolipid during the growth on *n*-hexadecane started in the middle of the exponential phase and rose sharply in the late exponential growth phase, when nitrogen is nearly exhausted.

Furthermore, it has been shown by Desai & Desai that the C:N ratio in the medium plays an important role in biosurfactant production, and a large amount of *n*-hexadecane is found to be incorporated into the biosurfactant at a higher C:N ratio. This effect is different from those proposed by several other investigators, where nitrogen exhaustion is believed to switch on biosurfactant production. Desai & Desai noted that according to Hommer *et al.*, the absolute quantity of nitrogen (not its relative concentration) appears to be important in determining an optimum concentration of biomass, whereas the concentration of hydrophobic carbon source determines the conversion degree of available carbon into the biosurfactant. It has been observed that rhamnolipid production from glucose in *P. aeruginosa* by continuos culture has been increased 7- to 10-fold after nitrogen limitations.

Nitrogen limitation not only causes the overproduction of biosurfactant but also changes the composition of the biosurfactant produced. Growth of R. erythpolis under normal conditions produces only nonionic trehalose corynomycolates, whereas under nitrogen limitations it produced only anionic trehalose tetraesters. The limitation of multivalent cations like Fe^{++}, Mg^{++}, and Ca^{++}, has been also found to increase the rhamnolipid production in *Pseudomonas* sp (Desai & Desai, 1993).

2.4.4.3 PRODUCTION BY RESTING CELLS

In this category, the cells used in the biosurfactant production are harvested from the biosurfactant-producing state and maintained in the same state. Thus, biosurfactant-producing cells do not multiply, but continue to utilize carbon source for the synthesis of biosurfactants as illustrated in Figure 2.6c. It has been shown that rhamnolipid can be produced by resting free and immobilized cells of *Pseudomonas* sp. Other examples of biosurfactant production by resting cells are the production of sophorolipids by *T. bombicola*, cellobioselipid production by *Ustilago Maydis*, and trehalose tetraester production by *R. erythropolis*. Using resting cells of *Arthrobacter* sp. and various mono-, di-, or tri-saccharides as the carbon source, the production of corresponding glycolipids have been also observed. The highest yield of rhamnolipid from *n*-alkanes has been reported by the resting cells of *Pseudomonas* sp. at pH 6.6

and 37°C. In contrast to rhamnolipids (type 1 and type 2) synthesized by growing cells, two new rhamnolipids called type 3 and type 4 have been synthesized by the resting cells. The production of these biosurfactants is dependent on both carbon sources in the medium and the incubation temperature. It has been determined that determined biosurfactant production level has been much lower as compared to that with growing cells although by incubating resting cells in phosphate buffer, repeated use of cells for rhamnolipid production was possible. However, Desai & Desai have been reported that a twofold increase in rhamnolipid production when *P. aeruginosa* CFTR-6 was transferred after the growth phase into a medium devoid of phosphate. Immobilizing the resting cells of *Pseudomonas* sp. in hydrophilic polymer, better stability of the cells has been demonstrated. Using this technique, rhamnolipid production in *Pseudomonas* sp. has been increased by almost 5- to 6-fold. The enhancement in the production of cell-bound biosurfactant by immobilized *A. calcoacetius* RAG-1 has been reported. On the other hand, in the sophorolipid production no difference has been determined between the growing cells and the resting cells (Desai & Desai, 1993; Desai & Banat, 1997).

2.4.4.4 PRODUCTION WITH ADDITION OF PRECURSORS

Many investigators have reported that the addition of biosurfactant precursors to the growth medium causes both qualitative and quantitative changes in the product (Desai & Banat, 1997; Desai & Desai, 1993). For example, addition of lipophilic compounds to the culture medium of *T. magnoliae* and *T. bombicola* resulted in the higher production of biosurfactants. A large number of observations have provided the conclusion that the carbon source in the medium, particularly the carbohydrate, has great bearing on the type of glycolipid formed (Desai & Desai, 1993).

When *Arthrobacter paraffineus* ATCC 19558 was grown on glucose, supplementation with hexadecane in the medium during the stationary growth phase resulted in a significant increase in biosurfactant yield. It has been also shown that the presence of large amounts of biosurfactant bound to *Corynebacterium lepus* cells when grown on glucose, and addition of hexadecane facilitated the release of

surfactant from cells. Others observed little biosurfactant production when cells were growing on a readily available carbon source; however, biosurfactant production triggered only when all the soluble carbon was consumed and when water-immiscible hydrocarbon was available. Glycolipid production by *T bombicola* has been stimulated by the addition of vegetable oils during growth on 10% glucose medium, giving a yield of 80 g/L.

Using *T. apicola* IMET 43747 Stuwer *et al.* achieved glycolipid yields as high as 90 g/L with a medium containing glucose and sunflower oil. In another study, Lee & Kim reported that in batch culture, 37% of the carbon input has been channeled to produce 80 g of sophorolipid per liter by *T. bombicola.* However, in fed-batch cultures about 60% of the carbon input were incorporated into biosurfactant increasing the yield to 120 g/L (Desai & Banat, 1997).

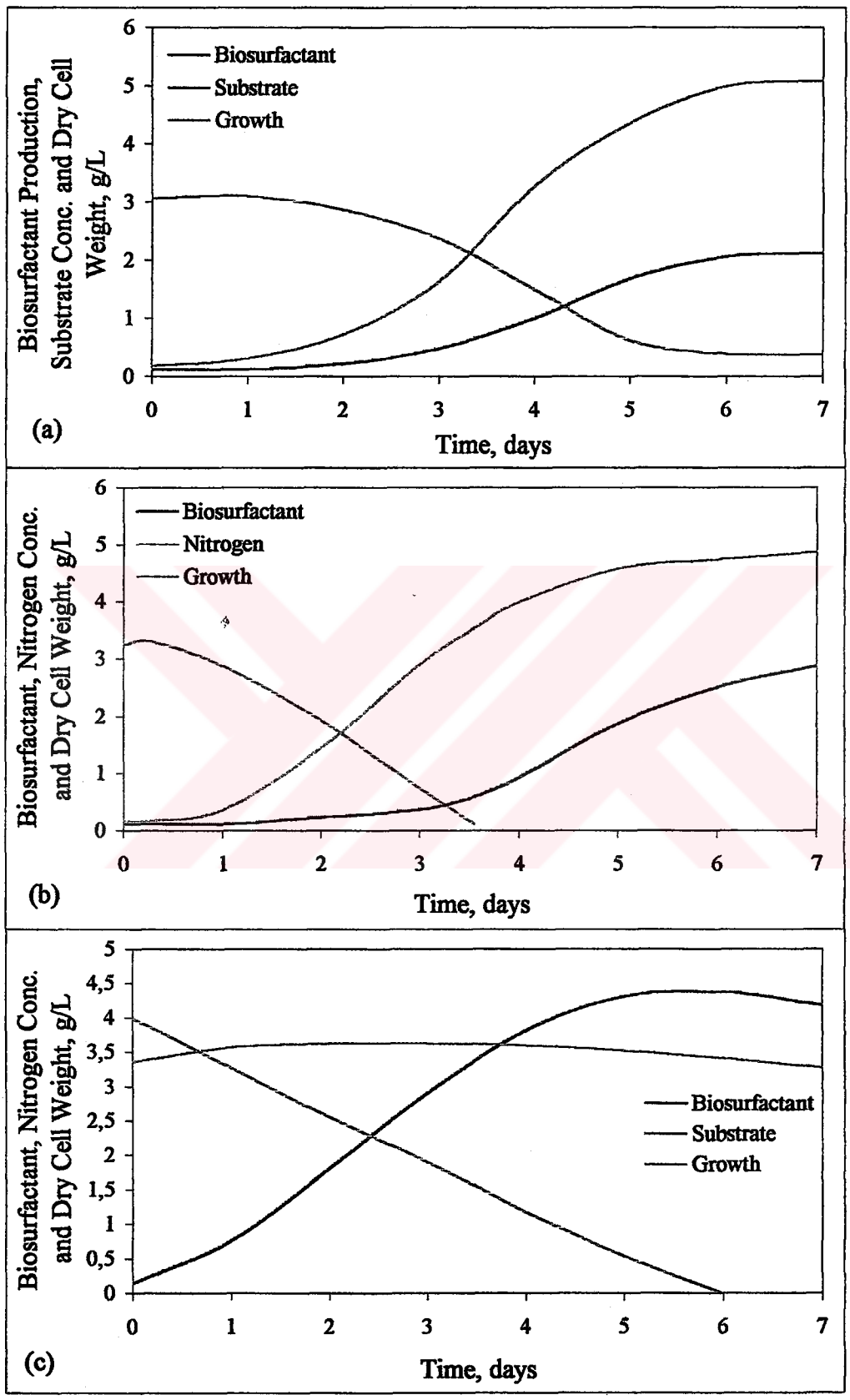

Figure 2. 6 Pattern of biosurfactant production (a) growth associated (b) under growth limiting conditions (c) by resting cells (Desai & Desai, 1993).

CHAPTER THREE
ENVIRONMENTAL APPLICATIONS OF BIOSURFACTANTS

3.1 ADVANTAGES AND LIMITATIONS OF BIOSURFACTANTS

3.1.1 ADVANTAGES AND DIFFERENCES OF BIOSURFACTANTS OVER SYNTHETIC SURFACTANTS

Over the last decades, surfactants have been found and applied to be useful tools in enhancing the apparent water solubilities and bioavailabilities of poorly soluble organic compounds and increasing the rates of contaminants desorption from soil to water by forming micelles and reducing surface and interfacial tension. However, in some pilot and field scale studies, microbial degradation of hydrocarbons by synthetic surfactants has been inhibited due to their toxic and non-biodegradable characteristics (Radosevich et al., 1997; Banat, 1995)

Almost all surfactants currently in use are chemically derived from petroleum; however, interest in microbial surfactants has been steadily increasing in recent years due to their diversity, environmentally acceptable nature, the possibility of their production through fermentation, and their potential applications in the environmental protection, crude oil recovery, health care, and food-processing industries (Desai & Banat, 1997). Biosurfactants having the same properties with synthetic surfactants, such as forming macro- or micro-emulsions and reducing surface and interfacial tension, additionally have unique properties, which allow their use and possible replacement of chemically synthesized surfactants in numerous industrial operations (Kosaric, 1992).

The principal advantages of biosurfactants compared with synthetic surfactants are:

a. ***Biodegradability.*** In contrast to synthetic surfactants, biosurfactants have biodegradable characteristic, and can be readily and fully degraded if released to the environment after its function is completed.

b. ***Generally low or no toxicity.*** Because biosurfactants are produced by living organisms on environmentally acceptable substrates (hydrocarbons and/or carbohydrates) biosurfactants show no toxicity during and after their applications. The biodegradable and nontoxic characteristics render biosurfactants effective and useful in microbial degradation.

c. ***Biocompatability.*** Many biosurfactants and particularly those produced by yeast such as sophorolipids are compatible with living tissues. This property of biosurfactants allows their applications in the many industrial production processes such as especially food processing, pharmaceuticals, and cosmetics industry.

d. ***Availability of raw materials.*** Biosurfactants can be produced from cheap raw materials, which are available in large quantities. The carbon source may come from hydrocarbons, carbohydrates and/or lipids, which may be used separately or in combination with each other.

e. ***Acceptable production economics.*** Recent studies have been shown that depending on the application, biosurfactants can also be produced from industrial wastes and by-products. This properties and especially in-situ production of biosurfactants balance the overall biosurfactant application costs.

Industrial and or municipal wastewaters, which contain organic pollutants, can be utilized as substrate for the production of biosurfactants. With using wastewaters as organic matter source, a double benefit is expected:

(i) the wastewaters, utilized for the biosurfactant production, are treated and

(ii) a valuable product is resulted.

This approach reduces the cost for wastewater treatment with even a potential of generating a profit through the sale of biosurfactant (Kosaric, 1992). An extensive study about the biosurfactant production using municipal wastewater approach has been performed by Kosaric in 1992. Kosaric has examined the approach through producing glycolipids with both classical organic substrate and waste glucose. Kosaric has selected *Torulopsis bombicola* as a model microorganism, which is effectively used in the sophorolipid biosynthesis. *Torulopsis bombicola* has been generally reported as giving high concentrations of 67 g/L of sophorose lipid with a yield of 0.347 g/g substrate. In addition, a yield of 137 g/L sophorolipid has been obtained by Kosaric.

In first part of the study, sophorose lipids have been synthesized by *Torulopsis bombicola* with using molasses and soybean oil as inexpensive and commercially available substrates. It has been calculated that the substrates alone would place biosurfactant production cost at about $0.70/kg, and total production cost about $2.00/kg. The price of synthetic surfactants of the non-ionic alcohol ethoxylate and alkylphenoletoxylate types for use in enhanced oil recovery has been estimated to $1.0-1.2/kg. This clearly shows that, when molasses are used as the substrate in the sophorolipid synthesis, the biosurfactant becomes more expensive than synthetic surfactants (Kosaric, 1992).

In second part of the study, 'waste glucose' has been selected as the substrate, which may come from food processing, agricultural or from a variety of industrial operations (e.g. starch waste liquors, domestic waste, potato processing waste, waste liquors from rice production, etc.). An appropriate lipid accumulating microorganism (lipogenic bacterium or yeast), that could be used to produce triglyceride precursors to biosurfactant, and the appropriate lipogenic algae (Chlorella or other) have been co-cultured to produce microbial single cell oil in the form of triglyceride. Kosaric has

been used lipomyces as lipid accumulating microorganism and chlorella as lipogenic algae. The microbial triglycerides and the same sugar have been used by *T. bombicola* to produce sophorolipid type glycolipid, and the yield of 0.2 g biosurfactant/g waste glucose has been obtained (Figure 3.1). Kosaric has concluded that this process can produce a 600% increase in sophorolipid yield from sugar alone as compared with a single organism strategy, which produces only 0.032 g biosurfactant/g sugar.

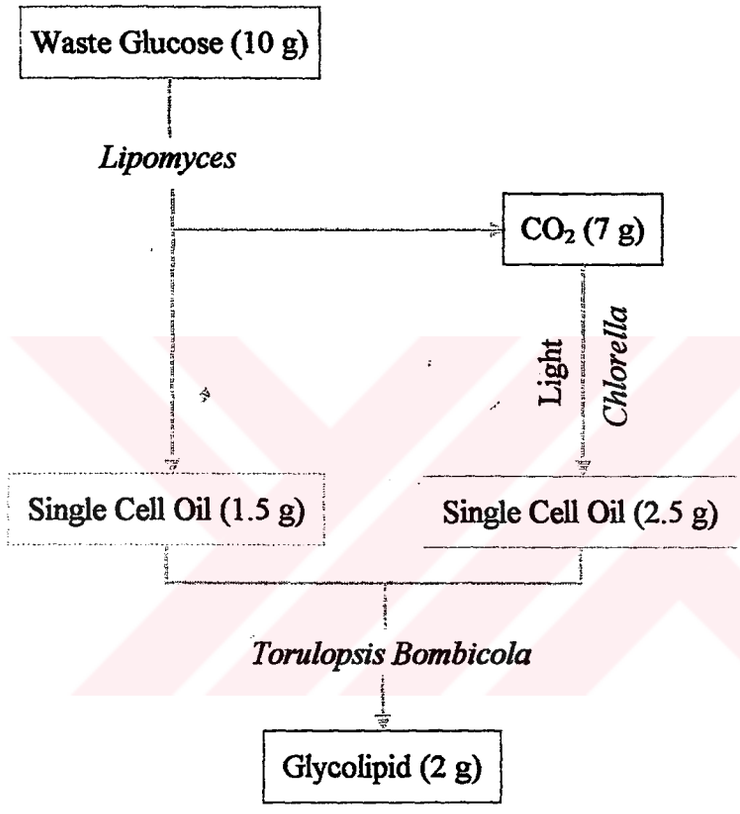

Figure 3. 1 Production of sophorolipid from 'waste glucose' (Kosaric, 1992)

Kosaric has also stated that for cheaper production municipal waste sludge could be used as substrate in an anaerobic treatment process, followed by partial hydrolysis of anaerobic sludge on which lipogenic microbes could be grown. The strategy using waste organic pollutants as substrate in the biosurfactant production, has several advantages:

- *Only one feedstock (municipal waste sludge) is used.*
- *In-situ production of biosurfactant is possible*
- *There is a cost credit for environmental benefit.*

- *The feedstock is available year-around.*
- *Energy requirements can be met by the production of methane.*
- *Process is relatively simple* (Kosaric, 1992).

f. ***Use in environmental control.*** Biosurfactants can be effectively used in handling industrial emulsions, control of oil spills, biodegradation and detoxification of industrial effluents and in bioremediation of contaminated soil and groundwater resources.

g. ***Specificity.*** Biosurfactants, being complex organic molecules with specific functional groups, are often specific in their action. This would be of particular interest in detoxification of organic or inorganic pollutants, de-emulsification of industrial emulsions, and other specific food, pharmaceutical applications (Kosaric, 1992).

h. ***Extreme temperature, pH, and salinity tolerance.*** Biosurfactants have been found effective at extreme temperatures, pH values, and salinity in which synthetic surfactants cannot work effectively (Thangami & Shreve, 1994).

3.1.2 POTENTIAL LIMITATIONS OF BIOSURFACTANT APPLICATIONS

Concerning disadvantages or limitations of biosurfactants, the existing of two most common problems about biosurfactants have been reported by Kosaric. *One of the problems is related to large scale and cheap production of biosurfactants. Large quantities are particularly needed in petroleum and environmental applications, which, due to the bulk use, may be expensive. To overcome this problem, processes should be coupled to utilization of waste substrates combating at the same time their polluting effects, which balances the overall costs.* If wastes processed or wastewater treated is used for the organic substrate in the in-situ biosurfactant production with the strategy mentioned above, the production costs might be decreased.

Another problem may be encountered in obtaining pure substances, which is of particular importance in pharmaceutical, food and cosmetic applications (Kosaric, 1992). This problem seems to have very low or no effects on environmental applications, because biosurfactants are used as an enhancement tool in the ***contaminated*** soil and groundwater bioremediation, or ***oil spill*** clean-up.

3.2 ENHANCED BIOREMEDIATION OF CONTAMINATED SOIL AND GROUNDWATER ENVIRONMENTS

3.2.1 CONTAMINATION OF SOIL AND GROUNDWATER ENVIRONMENTS

Contamination of soil and groundwater environments has become an increasing threat to the safety of human health and the sustainability of environment by especially affecting drinking water sources (Thangamani & Shreve, 1994). The main sources of soil and groundwater contamination can be categorized as follows.

a. Facilities for the direct disposal of hazardous wastes
    - Landfills
    - Surface impoundments
    - Waste piles
    - Land treatment
    - Underground injection
    - Illegal dumping

b. Sites of indirect disposal of hazardous wastes
    - Leaks and spills from storage facilities
    - Leaks and spills from transport facilities
    - Leaks and spills from treatment plants
    - Leaks and spills from process facilities

c. Non-point sources

- Urban infiltration
- Agricultural activities
- Mining activities
- Oil and gasoline exploration (Şirin, 1998).

Soil and groundwater contaminants can be classified in terms of their structural features, which are the most significant factors determining their fate and transport in soil and subsurface environments. A simplified classification of soil and groundwater contaminants was given in Figure 3.2.

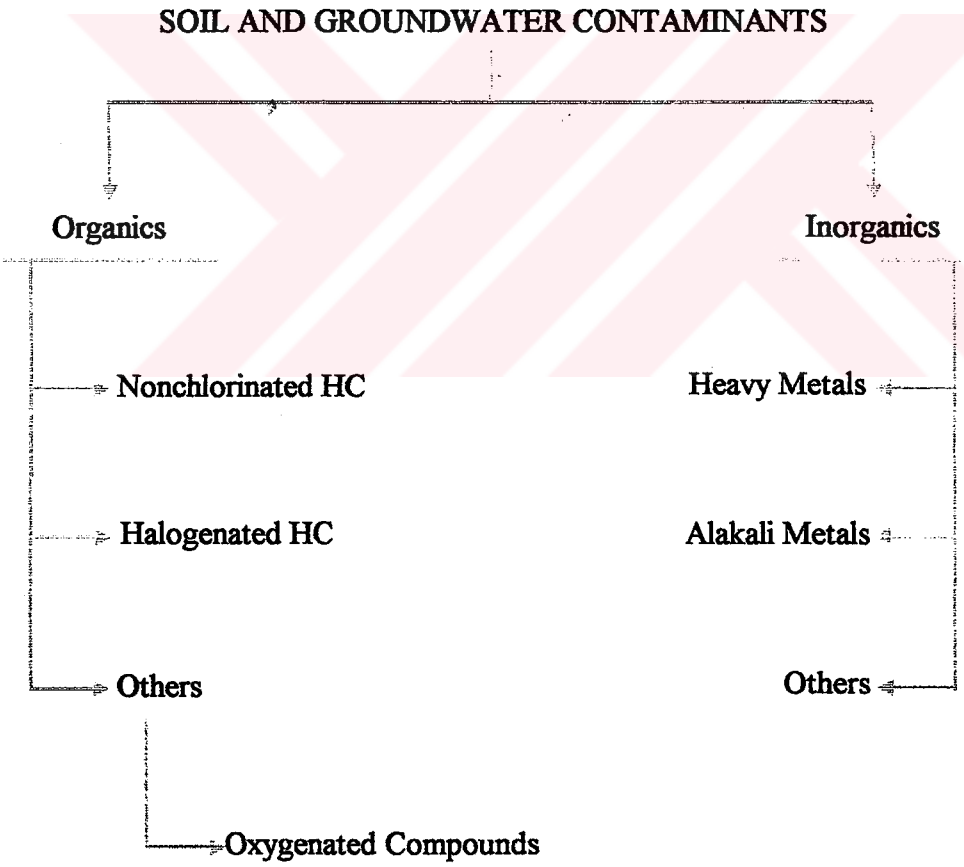

Figure 3. 2 Soil and groundwater contaminants classification (Şirin, 1998)

Organic contaminants cause soil pollution by entering the subsurface generally in liquid phase as a dilute aqueous solution such as a concentrated leachate or a ***nonaqueous phase liquid*** (NAPLs). Common types of organic contaminants are chlorinated hydrocarbons (trichloroethylene (TCE), perchloroethylene (PCE)), fuel hydrocarbons (benzene, toluene), oxygenated compounds (phenol), polyaromatic hydrocarbons (PAHs), pesticides, polychlorinated biphenyls (PCBs), other organic mixtures such as crude oil and gasoline. However in some cases, contaminants can present in solid or gas phase.

NAPLs, which are the most widely using chemical products and wastes derived from the use of these chemicals, are the chlorinated solvents and the petroleum hydrocarbons. Chlorinated solvents are denser than water, and are called as ***dense nonaqueous phase liquids*** (DNAPLs), whereas petroleum hydrocarbons are less dense than water, and are called as ***light nonaqueous phase liquids*** (LNAPLs).

The fate of organic contaminants in subsurface environments is determined by their subsurface behavior characteristics including migration, mobility and persistence, which depend on their chemical nature and release mechanism. Moreover, retention, transformation and migration (transportation) of a contaminant, depending on its solubility and NAPL type, are the key variables for subsurface behavior of the contaminant. To change the fate of organic contaminants in subsurface environments, remediation efforts deal with changing their subsurface behavior characteristics because their chemical natures and release mechanisms cannot be changed. So, the principal purposes of a remediation effort are to enhance the transformation of organic contaminants such as enhanced degradation in bioremediation or transportation of organic contaminants from soil to water such as in the soil-washing remediation method and to decrease their retention time and persistence.

3.2.2 REMEDIATION OF SOIL AND GROUNDWATER CONTAMINATED BY ORGANIC CONTAMINANTS

Organic contaminants entering to soil or aquifer environments tend to partition to non-polar phases, such as soil, sediment, and other organic-containing materials. Sorption of organic contaminants to soil or sediment is the critical process affecting contaminant mobility, toxicity, and persistence in soil and subsurface environment (Robinson, Ghosh, Shi, 1996). Sorption and/or diffusion through the soil matrix reduce the contaminant bioavailability to degradative microorganisms (Radosevich *et al.*, 1997). In addition, the rates of desorption and degradation processes, which occur naturally at contaminated sites, are very slow due to their hydrophobicity, stability, and low solubility (Robinson, Ghosh, & Shi, 1996). It has been proven in many soil and groundwater remediation studies that sorption of contaminants to soil or sediment, and low desorption and degradation rates of contaminants hinder remediation efforts (Radosevich *et al.*, 1997). Due to slow desorption and degradation rates of contaminants, commonly applied contaminated soil and groundwater remediation methods including pump-and-treat, vapor extraction, excavation followed by off-site treatment, and bioremediation require high design and operation costs and/or consume long periods of time to remediate contaminated sites.

Implementation of pump-and-treat remediation technique usually involves groundwater extraction from withdrawal wells screened in the contaminated aquifer and treatment of contaminated water at land surface. The treated water is then returned to the subsurface by injection wells. However, pump-and-treat remediation system has been proven impracticable in many instances especially for treating NAPLs in aquifers, and several reasons have been suggested to explain the ineffectiveness of this technology (Deitsch & Smith, 1995).

1. When aquifers contaminated with NAPLs, remediation is limited by the rate of dissolution of the organic liquid into the surrounding groundwater. Their very low solubility rates in water leading to very slow rates of removal by pumping.

NAPLs tend to exist in pockets at the subsurface location to which they have migrated. (USEPA Tech. Inn. Off., 1995).

2. When subsurface contamination continues, organic pollutants are transported into low-permeability lenses in aquifers. During remediation, the low advection velocities in these formations limit removal of these contaminants.

3. Groundwater remediation may be limited by the slow desorption rate of contaminants from soil to water.

It has now become obvious that available physicochemical remediation methods cannot reach the required contaminant concentrations in most aquifers. Even with extended operating periods, physicochemical methods have never remediated contaminated aquifers completely, and removed the organic and heavy metal compounds at high enough rates (Nyer, *et al.*, 1996). In many laboratory-scale, and pilot plant studies and full-scale applications of available physicochemical remediation techniques, it has been demonstrated that all methods have similar limitations and insufficiencies in the contaminated soil and groundwater remediations. For these reasons, bioremediation is a viable alternative to physicochemical methods of soil and groundwater treatment. It has been proven effective in both the laboratory, pilot plant studies and in full-scale commercial applications (Müller, Wagner, Blaszczyk, Kosaric, 1993). However, reduced bioavailability of contaminants to degradative microorganisms due to sorption and/or diffusion through the soil matrix hinder degradation of organic contaminants, and reduce the effectiveness of biological remediation techniques (Radosevich, 1997).

New technologies are being developed to improve removal efficiency through enhanced mobilization or solubilization of organic contaminants. Some studies, recently applied, have shown that the application of synthetic surfactants can improve physicochemical remediation performance by increasing the mobility of contaminants and enhance biological remediation of contaminated soil and groundwater by

increasing the solubility of contaminants to enhance their biodegradation rate (Radosevich, 1997; USEPA Tech. Inn. Off., 1995). Increased mobility promotes a significant increase in desorption, while increased solubility cause an enhancement in the degradation process. Surfactants may also be used to reduce interfacial tension between the water and the contaminant. This requires greater surfactant concentrations than those needed for increasing solubility, but results in direct mobilization of NAPLs, which may allow them to be extracted more efficiently. However, if uncontrolled, increasing the mobility of the NAPLs also increases the risk of increasing the contaminant plume (USEPA Tech. Inn. Off., 1995).

While synthetic surfactants have been shown to enhance desorption in soils and sediments, these compounds themselves are often toxic and recalcitrant. In addition to this, many other studies about synthetic surfactants have shown an inhibitory effect or mixed results. However, very few studies have addressed the effects of biosurfactants on the bioavailability of soil-sorbed substrates (Radosevich, 1997). The uses of biosurfactants to improve desorption and to enhance degradation process of contaminants recently receive increasing attention.

Biosurfactants may influence these systems in several ways. Soil solution biosurfactant concentrations above CMC may enhance the overall rate of hydrocarbon degradation by;

1. enhancing the apparent solubility of hydrocarbon resulting in higher aqueous phase concentrations and thus higher rates of degradation,
2. altering the distribution of the contaminant between sorbed and solution phases, or
3. enhancing the mass transfer rate of the contaminant from the sorbed to the solution phase .

Preliminary experiments have shown that trehalose micelle-water partition coefficients for toluene, xylene, and trimethyl benzene were higher than those observed for either sodium dodecyl sulfate or soil organic matter. Therefore, it is anticipated that the

presence of biosurfactant will enhance the overall rate of hydrocarbon biodegradation via enhanced desorption and solubility. This result has been demonstrated at the laboratory scale, and it provides the basis for developing economically and technically feasible remediation techniques based on flushing the contaminated area with biosurfactant or stimulating biosurfactant production in situ (Rodosevich, 1997).

Thangamani & Shreve have studied on hexadecane partioning in multiphase systems to determine and to compare the effectiveness of both synthetic and microbial surfactants on the remediation of contaminated sites. They have used *n*-hexadecane, which is essentially insoluble in water, as a useful model compound for examining the role of biosurfactants in hydrophobic organic compounds mobilization and microbial degradation, alkyl benzyl sulfonate as synthetic anionic surfactant, and rhamnolipid as anionic biosurfactant. The soil used in the study has been obtained from Environmental Protection Agency (EPA), and designated as EPA-22. EPA-22 has been defined as sediment (21.2% clay, 52.7% silt, and 26.1% sand) possessing an organic carbon content of 1.67%. Organic phase constituents have been selected as benzene, toluene and xylene (a mixture of *o*-, *m*-, and *p*- isomers).

The CMC values of alkyl benzyl sulfonate and rhamnolipid in water have been found to be 160 mg/L (0.424 mM) and 18 mg/L (0.0342mM), respectively, using a conventional plot of the surface tension versus the logarithm of the surfactant concentration.

The partioning of hexadecane in multiphase system containing aqueous, organic and soil phases has been examined in the presence of varying concentrations of rhamnolipid and alkyl benzyl sulfonate. The experimental study has been conducted in two different phase systems: two-phase system and three-phase system.

Hexadecane partioning in two-phase systems consisting of an organic and an aqueous phase or a soil and an aqueous phase has been examined to determine the effect of the addition of synthetic or microbial surfactants on contaminant partioning

and solubilization into the aqueous phase. And the solubilization of hexadecane into the aqueous phase from the organic or soil phases in the presence of alkyl benzyl sulfonate and rhamnolipid has been compared.

In the hexadecane partioning in aqueous and organic two-phase system, firstly addition of BTX (benzene-toluene-xylene) organic phase has been found to enhanced the solubility of hexadecane in the aqueous phase to approximately $4.7 \cdot 10^{-9}$ mol/L presumably the cosolvency effect of dissolved aqueous phase BTX molecules. The quantity of hexadecane in the aqueous phase has been dramatically enhanced by the addition of sufficient quantities of surfactant. This surfactant-mediated micellar solubilization has been considered to represent the dissolution of the compound by reversible interaction and solubilization within the micellar phase of the surfactant solution. For experimental systems containing either alkyl benzyl sulfonate or rhamnolipid, it has been observed that transportation of more contaminant into the aqueous phase due to the addition of surfactant has started after the system attained the CMC. Transport of contaminant has been also found to be proportional to the volume of aqueous micellar phase. Thangamani & Shreve have noted that there has been not much change in the amount of aqueous phase contaminant at concentrations below the critical micelle level when no micellar phase exists. It has been observed that below the CMC, hexadecane concentration in the aqueous phase remained constant at approximately $4.7 \cdot 10^{-9}$ mol/L. At surfactant concentrations above the CMC, hexadecane has been increasingly solubilized into the aqueous micellar phase. Thangamani & Shreve have defined the molar solubilization ratio (MSR), which is the ratio of the number of moles of organic compound solubilized per mole of surfactant added to solution, to reflect the effectiveness of surfactant in hexadecane partioning. MSR may be calculated as

$$MSR = (S_{HD,mic} - S_{HD,CMC}) / (C_{surf} - CMC) \qquad -3.1$$

where $S_{HD,mic}$ is the total apparent aqueous solubility of the hexadecane (in mol/L) in micellar solution at a particular surfactant concentration; $S_{HD,CMC}$ is the apparent

solubility of hexadecane compound (in mol/L) at the critical micellar level; and C_{surf} is the surfactant concentration at which $S_{HD,mic}$ is evaluated.

MSR values for alkyl benzyl sulfonate and rhamnolipid have been determined as $6.2 \cdot 10^{-7}$ moles of hexadecane partitioned into aqueous phase per mole of alkyl benzyl sulfonate added and $1.35 \cdot 10^{-5}$ moles of hexadecane partitioned into the aqueous phase per mole of rhamnolipid added, respectively.

A similar effect has been observed in the soil and aqueous two-phase system. For both surfactants, at concentrations below the CMC the aqueous phase hexadecane concentrations has been approximately constant at $4.5 \cdot 10^{-9}$ mg/mL. After the critical micelle level, as the surfactant concentration has increased, increasing amounts of hexadecane has been mobilized from the soil to the aqueous phase. The MSR values for alkyl benzyl sulfonate and rhamnolipid obtained in the soil and aqueous two-phase system has been reported as $7.9 \cdot 10^{-7}$, and $1.33 \cdot 10^{-5}$, respectively.

The solubilizing abilities of the alkyl benzyl sulfonate and rhamnolipid surfactants have been compared by evaluating their MSR values for the multiphase systems examined. Thangamani & Shreve have concluded that the magnitude of the MSR values reflects the effectiveness of a particular surfactant in solubilizing the contaminant. In addition, the MSR of the rhamnolipid has been determined to be approximately 20 times greater than that of alkyl benzyl sulfonate. "This indicates that on a per mole basis the rhamnolipid is capable of solubilizing about 20 times more hexadecane than the synthetic surfactant"(Thangamani & Shreve, 1994).

In the hexadecane partioning and solubilization in aqueous, organic, and soil three-phase system, contaminant solubility has been found to show a linear dependence on surfactant concentration at surfactant concentrations above the CMC. There has not been determined a significant change in the amount of contaminant in the aqueous phase at surfactant concentrations below the CMC. It has been also found that the

amount of contaminant solubilized again showed a linear dependence on the concentration of surfactant added.

The hexadecane MSR values for the rhamnolipid and the alkyl benzyl sulfonate has been determined as $1.38 \cdot 10^{-5}$, and $5.9 \cdot 10^{-7}$, respectively, in the three-phase systems. Thus, the MSR of rhamnolipid has been evaluated as approximately 23 times greater than that of alkyl benzyl sulfonate.

Thangamani & Shreve have evaluated the low MSR value and effectiveness of alkyl benzyl sulfonate for both two-phase and three-phase systems with applying known generalizations about micellar formation to the alkyl benzyl sulfonate surfactant micelles. According to these generalizations, the principal reason of the low MSR values of alkyl benzyl sulfonate has resulted from the formation of spherical micelles under the conditions used in the study. "The formation of spherical or cylindrical micelles by surfactants is determined by the ratio of the micelle hydrocarbon core volume to the product of the optimum surface area and the critical length of the hydrocarbon chain" (Thangamani & Shreve, 1994). This ratio for alkyl benzyl sulfonate has been estimated lower than 0.333 at which the transition from spherical to cylindrical micelles begins.

It has been noted by Thangamani & Shreve that since hexadecane is fully miscible in the presence of BTX mixture, their presence may enhance the hexadecane solubility.

The greater capacity of the rhamnolipid to solubilize the hexadecane contaminant has been found most likely due to the greater volume of the micellar phase in rhamnolipid systems, which allow for increased hexadecane solubilization. Also since the hexadecane has a very long chain, solubilization of hexadecane into a spherical alkyl benzyl sulfonate micelle may be very limited whereas a much larger elliptical rhamnolipid micelle structure may more favorably accommodate the hexadecane molecule. Therefore, because the rhamnolipid structure forms a micelle with a lower

likelihood of being spherical, rhamnolipid has been evaluated more effective in the hexadecane solubilization (Thangamani & Shreve, 1994).

In addition to the differences between the effectiveness of alkyl benzyl sulfonate and rhamnolipid above critical micelle level, the higher CMC value of alkyl benzyl sulfonate is its another significant disadvantages. To obtain effective desorption and degradation enhancement by using alkyl benzyl sulfonate at least the concentration of 160 mg/L, which is its CMC value, must be applied. However, applied rhamnolipid concentrations above the 18 mg/L, which is the CMC value of rhamnolipid, can yield effective enhancements in desorption and degradation. The higher concentration requirements of alkyl benzyl sulfonate cause high operational costs, and can inhibit biodegradation or pose threat of additional contamination because synthetic surfactants have been often found toxic and/or recalcitrant especially when applied at high concentrations.

Bai, Brusseau, and Miller have investigated the potential of an anionic monorhamnolipid biosurfactant produced by *P. aeruginosa* to remove residual hexadecane from sand columns. The molecular weight of used rhamnolipid has been determined as 504 g/mol. All surfactant solutions have been prepared with deionized, distilled water and adjusted to pH 7.0, at which the predominant surfactant structure is micellar. The CMC of rhamnolipid has been determined as 50 mg/L from a plot of surface tension vs. biosurfactant concentration.

n-hexadecane, whose solubility has been determined as 0.029±0.009 mg/L, has been selected to represent LNAPLs with low volatility such as jet fuel. It is representative because the interaction between NAPLs and surfactants depends on the alkane carbon number of the NAPLs. Most NAPLs detected in the contaminated sites have an equivalent the alkane carbon number ranging from 10 to 22. For example, the equivalent alkane carbon number of jet fuel-4 is 16. The alkane carbon number of hexadecane is 16 (Bai et al., 1997). Accusand containing 0.04% of total organic carbon and 0.03% of iron oxide has been used as the solid phase.

Table 3. 1 Physical Parameters of Columns Used in Residual Hexadecane Removal Experiments by Bai *et al.* (Bai *et al.*, 1997)

Parameter	Column A	Column B
Diameter, cm	2.12	2.54
Length, cm	7	5
Volume, cm^3	24.70	25.32
Bulk density, g/cm^3	1.76	1.81
Pore volume, cm^3	9.4	8.8
Porosity, %	38.1	34.8
Residual Saturation, %	24.2	19.3
Packed with	40/50 mesh sand	20/30 mesh sand

Removal of hexadecane by rhamnolipid (500 mg/L) from both Column A and Column B have been determined in duplicate and obtained results are shown in Figure 3.4. As seen in this figure, the recovery of hexadecane from the column packed with larger diameter (20/30 mesh) has been much higher (83.6% after 120 pore volumes) than recovery from the 40/50 mesh sand column (22%).

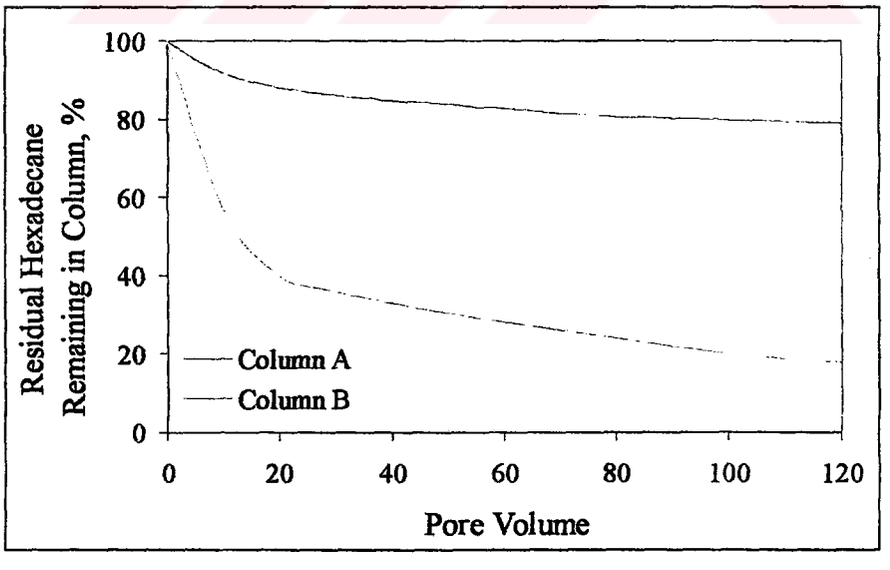

Figure 3. 4 Comparison of removal of residual hexadecane from Column A and B (Rhamnolipid concentration 500 mg/L) (Bai *et al.*, 1997)

Bai *et al.* have concluded that rhamnolipid surfactant has been effective in removing hexadecane residual from sand columns. Removal efficiency has been found to be dependent on both the average particle size of the sand and on biosurfactant concentration applied. Mobilization has been considered as the predominant mechanism of removal, with solubilization playing an insignificant role. It has been also noted that these laboratory-scale results indicate potential for rhamnolipid in remediation, however, many other factors will have to be considered in application of rhamnolipid on a field-scale. These factors include ionic strength of the soil solution, the type of cations present and porous medium properties such as clay and organic matter content.

3.2.3 REMEDIATION OF SOIL AND GROUNDWATER CONTAMINATED BY INORGANIC CONTAMINANTS

Contamination of the soil and groundwater environments by heavy metals is of growing concern because of health risks posed by human and animal exposure. According to the results of a survey conducted at 395 remedial action sites in the USA, heavy metals (cadmium, copper, lead, zinc, etc.) have been considered the most prevalent class of soil and subsurface contaminants. Conventional methods for the remediation of soil and groundwater environments contaminated by heavy metals involve the excavation and transport of contaminated soils to hazardous waste sites for landfilling. However, the rapidly increasing costs of excavation and landfilling today stimulate interest in the development and use of alternative, cost-effective remediation approaches (Tan *et al.*, 1994).

Two widely used approaches for remediating contaminated soil or subsurface environments are in situ soil washing, which is used primarily for surface soil contamination, and pump-and-treat technology, which is used at sites with deep soil or aquifer contamination. The time required for the remediation of heavy metals contaminated soil or subsurface environments by soil washing or pump-and-treat

to the sorption interactions of the contaminants with the soil. An environmentally and economically acceptable strategy to enhance the mobilization of heavy metals adsorbed to soil must be developed to shorten the time required for the soil washing or pump-and-treat remediation technologies (Tan *et al.*, 1994). In recent years, growing attention has been directed toward the use of microbial surfactants for the remediation of heavy metals contaminated soils as a viable alternative.

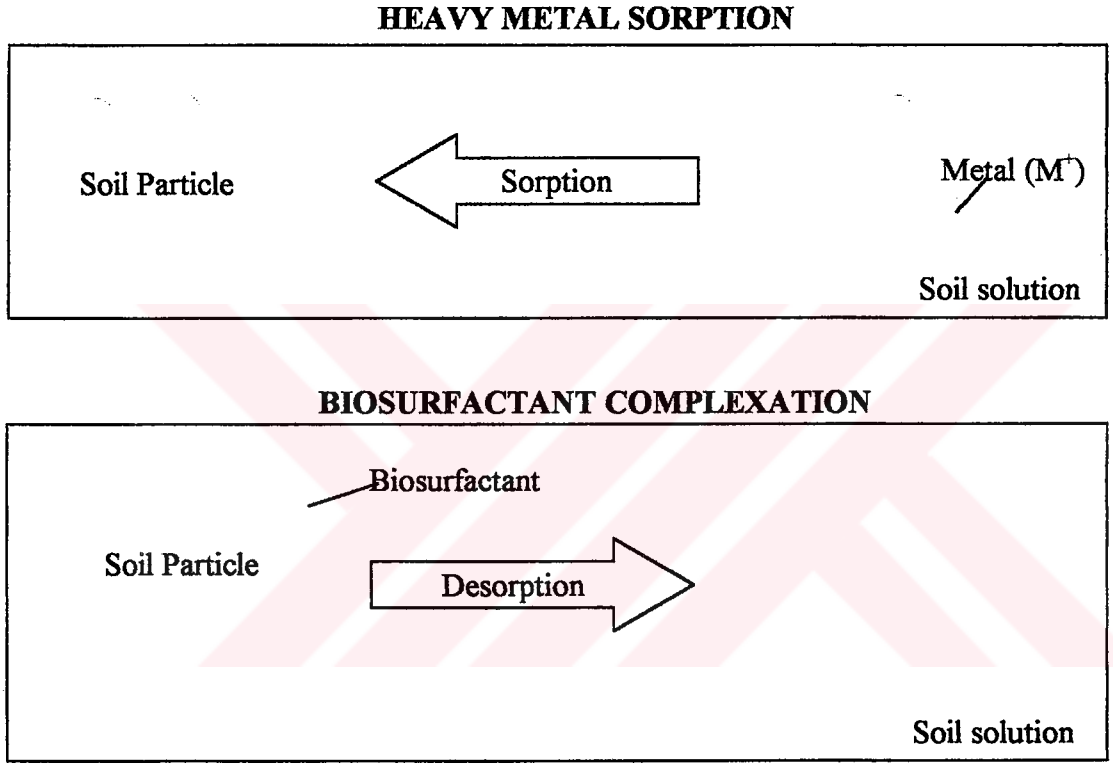

Figure 3. 5 A schematized diagram of heavy metal sorption to soil and biosurfactant complexation.

Complexation of a heavy metal with microbial surfactants has been firstly examined by Tan *et al.* in 1994. A schematized diagram of heavy metal sorption to soil which is the principal mechanism causing soil contamination by heavy metals and biosurfactant complexation is given in Figure 3.5. Complexation of cadmium, as a model metal, by an anionic monorhamnolipid type biosurfactant produced by *Pseudomonas aeruginosa* has been studied. Tan *et al.* have aimed to determine both the effectiveness of rhamnolipid type biosurfactant and the effects of cadmium

concentration, pH, and temperature on cadmium complexation. The CMC of rhamnolipid used in the study has been determined as approximately 0.24 mM, and the pH of the rhamnolipid-Cd^{2+} solution has been found ranging from 6.0 to 6.9. So a buffer system has been used to maintain a constant pH in the biosurfactant-metal complexation experiments to minimize the effect of pH. Pipes buffer determined as having negligible effects on cadmium complexation in the presence of rhamnolipid has been selected as buffer system. All experiments have been performed at a concentration of 0.1 M Pipes buffer to obtain maximal buffering capacity, and adjusted pH of 6.8. To determine the effects of rhamnolipid and cadmium concentration on complexation two separate series of batch experiments have been performed. In the first series of experiments, concentrations of rhamnolipid ranging from 0 to 7.3 mM have been added to solution, and the effectiveness of rhamnolipid has been determined. In the second series of experiments, similarly various cadmium concentrations (0.005-5 mM) have been added to solution to determine the effects of cadmium concentration on cadmium complexation with rhamnolipid. These experiments have been performed using two initial cadmium concentrations of 0.72 and 0.36 mM.

The complexation of cadmium (Cd^{2+}) with rhamnolipid has been found to be dependent on both rhamnolipid and cadmium concentrations (Figure 3.6). In the 0.72 mM Cd^{2+} solution, 78% of the cadmium (0.56 mM) has been complexed by 3.9 mM rhamnolipid whereas in the 0.36 mM Cd^{2+} solution, 97% of the cadmium (0.35 mM) has been complexed by the same amount of rhamnolipid. According to these results, although the complexed Cd^{2+} amount increases with increasing initial Cd^{2+} concentration, increasing initial Cd^{2+} concentration decreases the complexation efficiency and effluent Cd^{2+} concentration. However these results indicate that total complexed Cd^{2+} concentration is dependent on the concentration of rhamnolipid present. As shown in Figure 3.7, rhamnolipid type biosurfactant has complexed cadmium rapidly and complexation has been found to reached equilibrium within minutes, which has been the first sampling time in the experiments. In addition, complex has remained stable for over 27 hours (data have not been given). So,

period of 24 h used for all other experiments has been considered as more than sufficient to allow equilibration. To indicate the effectiveness of rhamnolipid complexation capacity, which is the ratio of the concentration of cadmium complexed and the rhamnolipid concentration used to complex cadmium, has been described. Maximum cadmium complexation capacity determined in the study has been 0.2 Cd^{2+}/rhamnolipid. As rhamnolipid concentrations increased and the free Cd^{2+} in solution decreased, the complexation capacity of rhamnolipid has been decreased from 0.2 to 0.08 Cd^{2+}/rhamnolipid. Complexation efficiencies of up to 92% (7.3 mM rhamnolipid) and 97% (3.6 mM rhamnolipid) have been achieved in solutions containing 0.72 mM and 0.36 mM cadmium, respectively. Stability constant (K) and an equilibrium partioning coefficient (K_p) have been calculated for complexation as a function of cadmium concentration. As shown in Figure 3.8 and Table 3.2 the complexation reaction has been independent of pH in the range 6.0-7.0, buffer concentration and temperature between 18 °C and 37 °C.

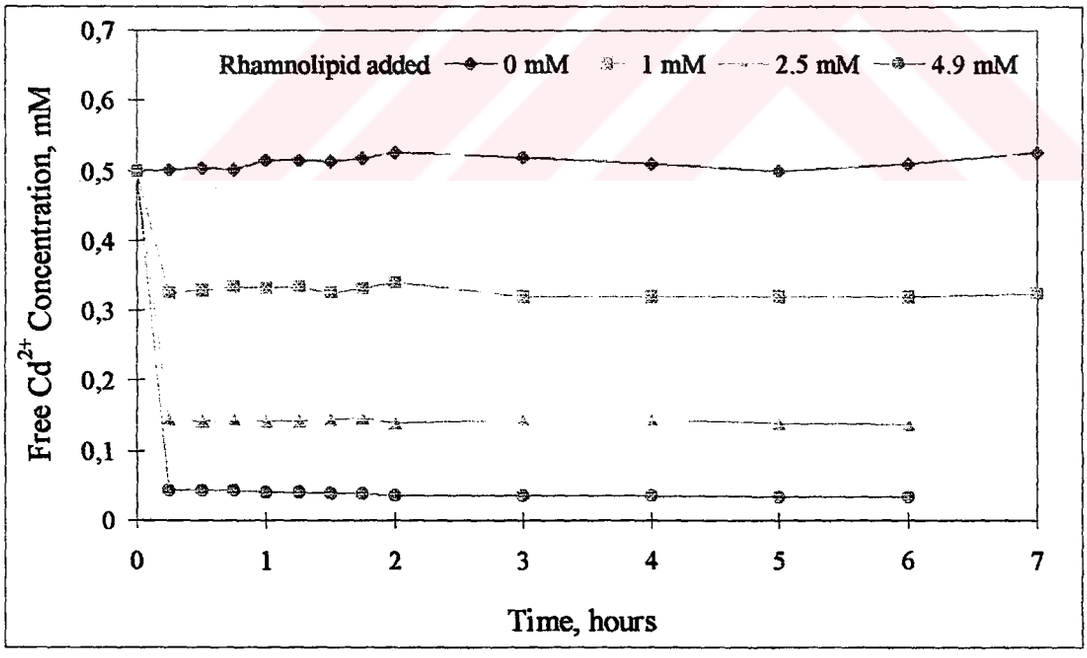

Figure 3. 6 Effect of time and different rhamnolipid concentrations on complexation of Cd^{2+} with rhamnolipid (Tan *et al.*, 1994)

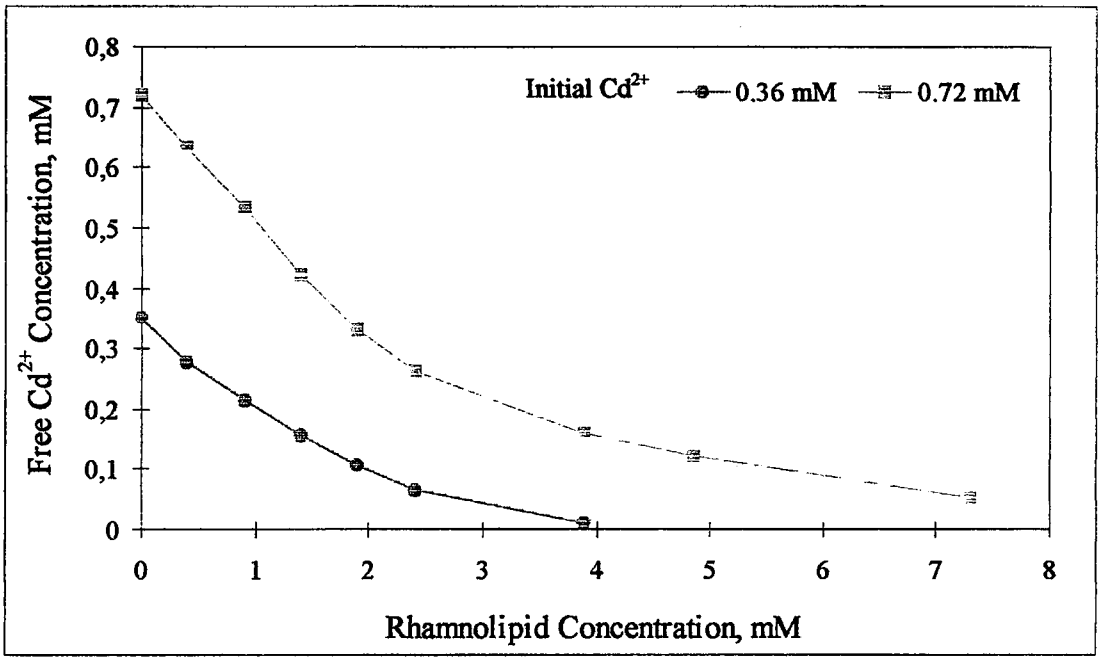

Figure 3. 7 Effect of rhamnolipid concentration on complexation of Cd^{2+} (Tan *et al.*, 1994)

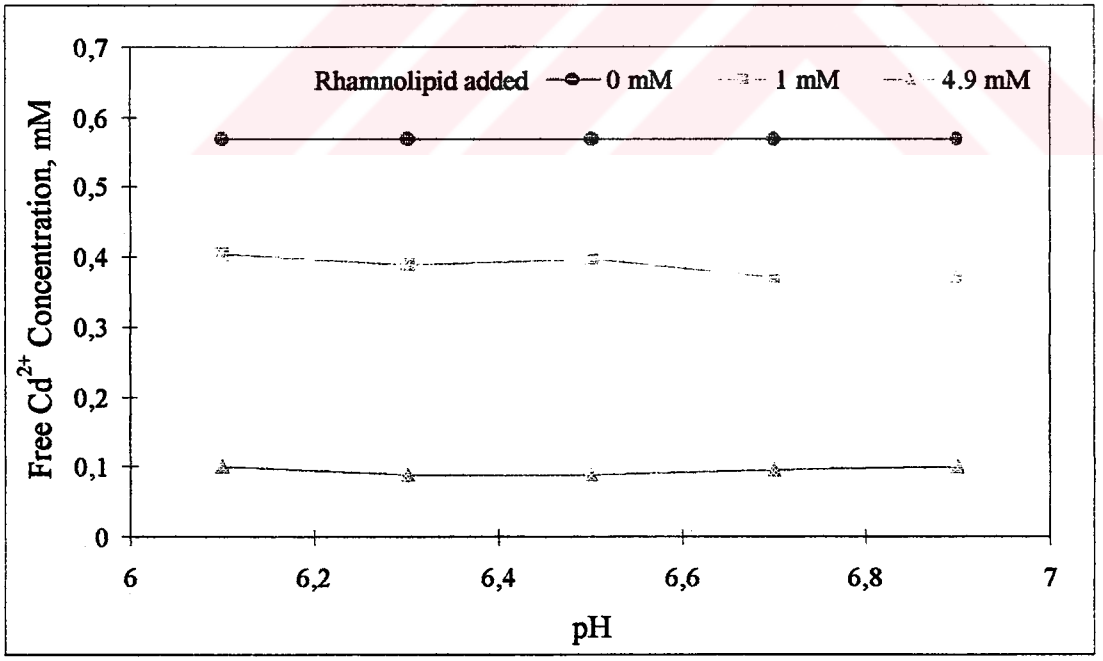

Figure 3. 8 Effect of pH on complexation of Cd^{2+} (Tan *et al.*, 1994)

The calculated cadmium-rhamnolipid stability constant (log K = -2.47 L/μmol) has been evaluated as higher than those reported for cadmium-sediment (-6.08 to -5.03 L/μmol) and cadmium-humic acid systems (-6.02 to -4.92 L/μmol) at pH 6.5. It has been also concluded from these data that the cadmium-rhamnolipid complex is much stronger than the cadmium-sediment or cadmium-humic acid complex. The calculated cadmium-rhamnolipid equilibrium partition coefficient (log K_p= 4.89 L/kg) has been found being similar to values reported for sorption of organic contaminants by dissolved organic matter. "Thus, a comparison of K and K_p values with literature values indicates a strong complexation between cadmium and rhamnolipid".

Table 3. 2 Effect of Temperature on Cd^{2+} Complexation with Rhamnolipid (Tan *et al.*, 1994)

Temperature, °C	Free Cd^{2+}, mM	Complexation, %
18	0.0937	81.3
25	0.0898	82.0
37	0.0908	81.8

The amount of Cd^{2+} complexed with rhamnolipid on a weight basis has been similar to levels reported for several bacterial exopolymers. Complexation at the maximum complexation ratio of 0.2 Cd^{2+}/rhamnolipid obtained in the experimental study can be expressed on a weight basis as 45 mg of Cd^{2+}/g of rhamnolipid assuming an average molecular weight of 504 g for monorhamnolipid. This complexation ratio has been reported as 14-fold higher than the cadmium complexation capacity for *Arthrobacter* exopolymer (3.3 mg of Cd^{2+}/g of rhamnolipid) and 4-fold higher than the complexation capacity for a *Klebsiella* exopolymer (11 mg of Cd^{2+}/g of rhamnolipid) by Tan *et al.*

In addition to high complexation capacity of rhamnolipid, rhamnolipid has been easily and efficiently separated from Cd^{2+} and recycled using a two-step procedure: acid precipitation and centrifugation (Table 3.3). These results suggest that using biosurfactants to facilitate the removal of cadmium from contaminated soils and

aquifers have a high potential and this method can be considered as an cost-effective remediation enhancement alternative (Tan *et al.*, 1994).

Table 3. 3 Separation of Rhamnolipid-Cd^{2+} Complex (Tan *et al.*, 1994)

Rhamnolipid, mM	Free Cd^{2+} after complexation, mM	Complexed Cd^{2+}, % [a]	Free Cd^{2+} after precipitation, mM	Separated Cd^{2+}, % [b]
0.0	0.657 ± 0.0006	0.0	0.636 ± 0.0016	100.0
1.0	0.446 ± 0.0006	32.1	0.646 ± 0.0009	101.6
4.9	0.0698 ± 0.00007	89.4	0.612 ± 0.0009	96.2

[a] Complexed Cd^{2+} percentage has been calculated as :

Complexed Cd^{2+} = [(Free Cd^{2+}) – (Free Cd^{2+} after complexation) / (Total Cd^{2+})]

[b] Separated Cd^{2+} percentage has been calculated as:

Separated Cd^{2+} = [100*{(Free Cd^{2+} after precipitation)/(Free Cd^{2+} after precipitation in the sample with no rhamnolipid)}]

Herman, Artiola, and Miller have conducted another study about heavy metal contaminated soil and groundwater remediation by biosurfactants in 1995. Herman *et al.* have aimed to determine biosurfactant complexation selectivity for metals, both singly and in mixtures, as well as to investigate biosurfactant-facilitated desorption of metals from soil, based on the results of previous work described above.

A monorhamnolipid type biosurfactant, produced by *P. aeruginosa* ATCC 9027 and characterized by a CMC of 0.1 mM, has been used in the study. Herman *et al.* have selected cadmium ($Cd(NO_3)_2$), lead ($Pb(NO_3)_2$), and zinc ($Zn(SO_4)_2$) as model metals. And a sandy loam soil with 0.11% total organic carbon content has been used for all metal desorption studies (Herman *et al.*, 1995).

The transfer of resin-bound metals into the aqueous phase has indicated rhamnolipid complexation by Herman *et a.* In the study of rhamnolipid complexation of metals, an ion-exchange resin has been used to compare rhamnolipid complexation of a single metal with metals in a mixture. In the first stage of the experiment, Cd^{2+},

Pb^{2+}, and Zn^{2+} have been exposed to ion exchange resin either separately (with 0.1 mM concentration) or in a mixture (0.1 mM of each metal). It has been noted by Herman *et al.* that according to the results given in Table 3.4, binding of each metal to the ion exchange resin in the absence of rhamnolipid has been found to be the same whether metals were present separately or in a mixture.

Table 3. 4 Rhamnolipid Complexation of Individual Metal or Metals Present in a Mixture (Herman *et al.*, 1995)

Metal	Percentage of Metal in Aqueous Phase, %			
	In the Absence of Rhamnolipid		In the Presence of Rhamnolipid [a]	
	Individual metal	Metal in mixture	Individual metal	Metal in mixture
Cd^{2+}	29.6 ± 2.0	32.3 ± 3.7	69.3 ± 1.4	57.6 ± 1.4
Zn^{2+}	34.9 ± 0.5	36.6 ± 0.4	73.9 ± 0.5	59.4 ± 0.9
Pb^{2+}	15.3 ± 1.2	14.9 ± 2.1	95.3 ± 0.6	73.2 ± 4.2

[a] 2 mM rhamnolipid

In the next stage of the experiment, after the addition of 2 mM of rhamnolipid, the increase in the metal concentration in the aqueous phase has been determined. Rhamnolipid complexation of single metals has been found to followed the order Pb^{2+} (95.3% complexed) > Zn^{2+} (73.9% complexed) > Cd^{2+} (69.3% complexed). Rhamnolipid complexation of metals in a mixture have been found to followed the same order of preference as for metals alone; however, the total amount of each metal complexed has been reduced by 17-23%. Herman *et al.* have stated that the reduction in rhamnolipid complexation when metals presented in the mixture may be a result of competition for rhamnolipid binding sites since for single metals the metal concentration has been 0.1 mM and for metal mixtures the total metal concentration has been 0.3 mM. Rhamnolipid complexation of Cd^{2+} as a single metal is shown in Figure 3.9 (Herman *et al.*, 1995).

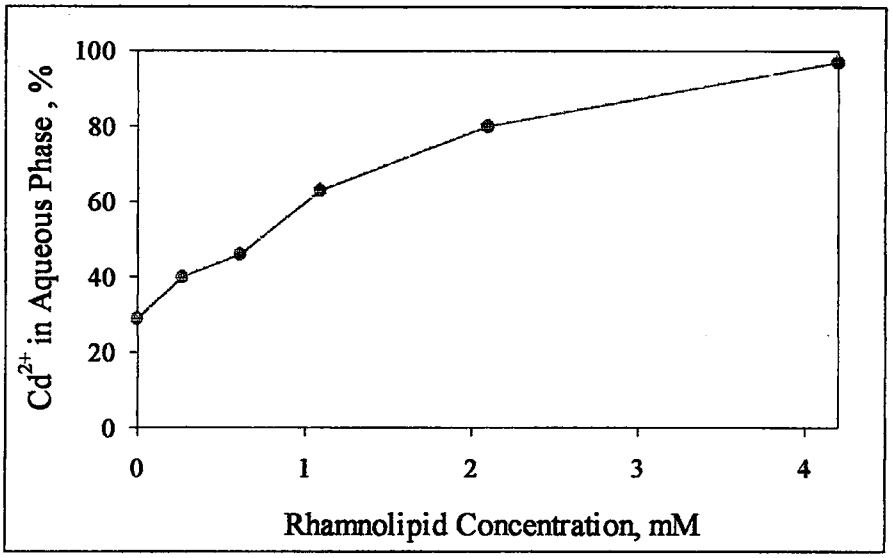

Figure 3. 9 Rhamnolipid complexation of Cd^{2+} as individual metal (Herman *et al.*, 1995)

In the first stage of the experiment of rhamnolipid desorption of metals from soil, soil has been load with metal. After exposure of the soil to a 1 mM concentration of two single metals, Cd^{2+} and Pb^{2+}, the amount of sorbed metal has been determined. It has been determined that 73% of the Cd^{2+} (1.46 mmol/kg) and 98% Pb^{2+} (1.96 mmol/kg) was sorbed by soil.

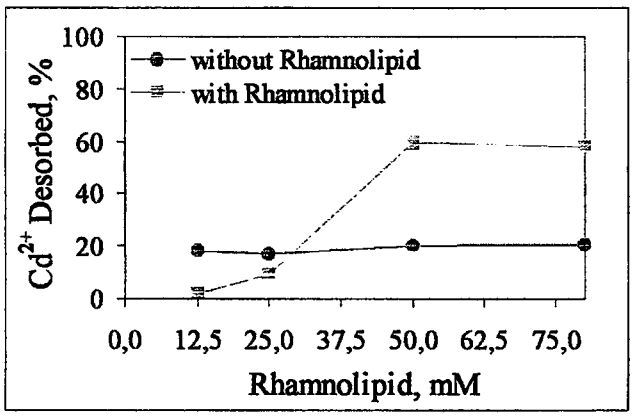

Figure 3. 10 Cd^{2+} desorption from the soil exposed to Cd^{2+} (Herman *et al.*, 1995)

The second stage of this experiment has been to determine desorption of soil-bound metals by rhamnolipid solutions. Desorption of both metals has been found to be dependent on the amount of rhamnolipid added (Herman *et al.*, 1995). In Figure 3.10 and Figure 3.11, the changes in the desorbed metal percentage versus applied rhamnolipid concentration are given.

For example, only 2.2% of the Cd^{2+} has been removed by a 12.5 mM rhamnolipid solution, but 55.9% of the Cd^{2+} has been removed by a 80 mM of rhamnolipid solution (Herman *et al.*, 1995).

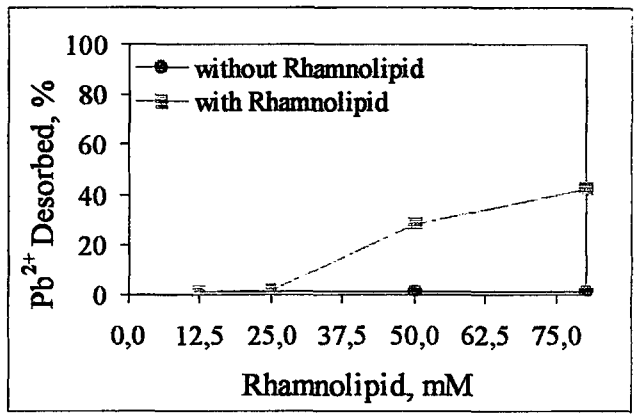

Figure 3. 11 Pb^{2+} desorption from the soil exposed to Pb^{2+} (Herman *et al.*, 1995)

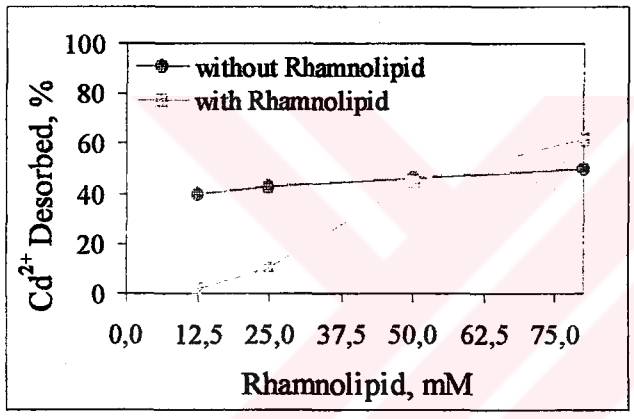

Figure 3. 12 Cd^{2+} desorption from soil exposed to metals in a mixture (Herman *et al.*, 1995)

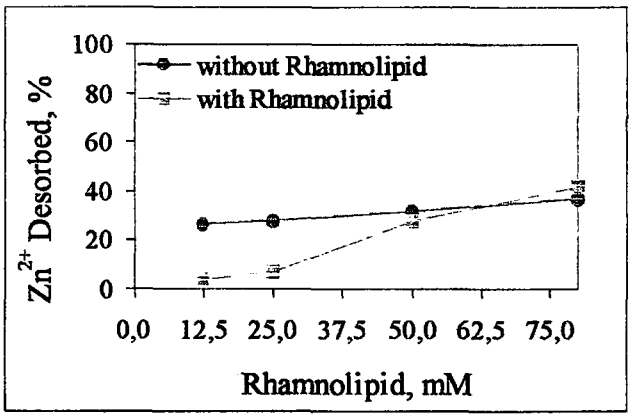

Figure 3. 13 Zn^{2+} desorption from soil exposed to metals in a mixture (Herman *et al.*,

Rhamnolipid mobilization of soil-bound metals has been also examined using a mixture of Cd^{2+}, Pb^{2+}, and Zn^{2+} (1mM each metal). When applied as a mixture, Herman *et al.* determined that approximately 94% of Pb^{2+}, 44% of Cd^{2+} and 32% of Zn^{2+} (a total of 3.4 mmol/kg) has been bound to the soil. They have also found that exposure of the metal-containing soil to rhamnolipid solutions resulted in a concentration-dependent removal of the metals (Herman *et al.*, 1995). The results of Cd^{2+}, Zn^{2+} and Pb^{2+} desorption by rhamnolipid are given in Figures 3.12, 3.13, 3.14, respectively.

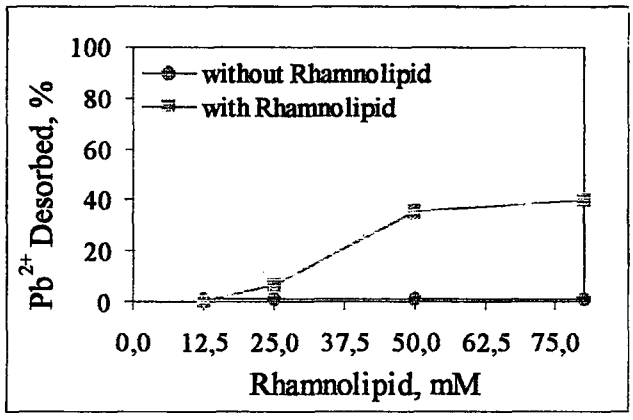

Figure 3. 14 Pb^{2+} desorption from soil exposed to metals in a mixture (Herman *et al.*,

Herman *et al.* have also concluded that all metals had a similar complexation ratio of approximately 2 moles of rhamnolipid/mol of metal, and this complexation ratio seems reasonable, since the rhamnolipid has been a monovalent anion and the metals studied have been predominantly divalent cations.

The principal reason of low effectiveness of rhamnolipid in desorption and complexation up to 50 mM has been evaluated as sorption of rhamnolipid to soil. At rhamnolipid concentration less than 50 mM, sorption of rhamnolipid to soil has been found greater than 70% of the total rhamnolipid added. Two possible mechanisms for rhamnolipid sorption, stated by Herman *et al.*, are:

- cation bridging between the anionic polar head group of rhamnolipid and sorbed cations on soil
- hydrophobic interactions between the nonpolar tails of rhamnolipids and hydrophobic regions in the soil organic matter.

Since the total organic carbon content of the soil used in experimental studies was low (0.11%), Herman *et al.* have considered cation bridging to be the most likely sorption mechanism. The presence of metals in the soil prior to addition of the rhamnolipid has been also found to affect the sorption of rhamnolipid. Herman *et al.* have also noted that due to the strong sorption of rhamnolipid by soil, high levels of rhamnolipid application have been required in order to successfully mobilize soil-bound metals (Herman *et al.*, 1995). As a result, it has been concluded that rhamnolipids may be particularly effective in the remediation of contaminated soils, however, in application of rhamnolipid to metal-contaminated soils, the efficiency rhamnolipid must be improved with minimizing rhamnolipid sorption.

3.3 OIL SPILL REMEDIATION

Petroleum, which continues to be principal energy source in the world today, is a leading contaminant in both prevalence and quantity in environment because of its wide scale production, transport, use and disposal globally. Each year 3.2 billion tons of petroleum is produced and as an estimate 3.2 million tons of petroleum enter the marine environment (Chhatre, Purohit, Shanker, & Khanna, 1996). The importance of the oil spill problem depends on quantity of spilled oil, frequency of oil spill accidents, and physical and chemical properties of spilled oil. Some early attempts to clean up the oil from sea for aesthetic reasons caused more ecological damage and safety hazards. The traditional physicochemical methods to cope with oil spills include mechanical containment and collection, natural removal (no clean up action), and shoreline clean-up methods based on mitigation mechanism or other methods such as burning, gelling, and sinking. Biological methods have an advantage over the physico-chemical methods in removing spills as they offer in-situ biodegradation of oil fractions by the microorganisms.

Due to inadequacies of traditional methods to overcome oil spills, use of biosurfactants has been investigated for oil spill clean up in both soil and marine environments as a novel remediation technology. Crude oil that is a complex mixture of hydrocarbons cannot be degraded completely by pure cultures. For this reason, the strategy to clean up oil spills has been directed towards the enhancement of indigenous mixed populations wherein the individual species are expected to have the ability to consume different components of crude oil. The mixed culture approach has several disadvantages as a process particularly as the rate of degradation is a crucial factor in oil spill clean up. Since a multi-species system like mixed culture is not fully characterized, the organisms being present in varying ratios undergo uncontrolled selective population growth and ultimately inhibit the degradative activity leading to accumulation of various components of the multi-substrate system like crude oil (Chhatre, 1996).

Biosurfactants are known for their ability to emulsify hydrocarbon-water mixtures, and emulsification properties of biosurfactants can enhance the degradation of hydrocarbons in the environment potentially making biosurfactants useful tools for the pollution control of oil spills. Many studies have been shown that hexadecane, heptadecane, octadecane and monodecane incubated for a 28-day period under laboratory conditions with the Pseudomanas aeruginosa that produces by 47%, 58%, 73%, and 60%, respectively (Banat, 1995).

Numerous observations on oil spill remediation have proven that the use of biosurfactants is a better approach over the use of pure or mixed cultures only for remediating oil spills. With biosurfactant addition, effective degradation of various components of crude oil has been achieved, even the relatively recalcitrant aromatic fraction shows substantial reduction.

3.4 OIL TANK CLEAN-UP

A major cost component in crude oil production is the cost of cleaning of sludges and waste oil deposits from storage and holding tanks. Sludges and heavy oil deposits accumulate in the tanks because they are not lifted by conventional pumps. For this reason, tanks must be cleaned periodically, usually by manual means, although the oil tanks cleaning procedure is hazardous, time-consuming labor-intensive and expensive.

Numerous studies, aimed to determine the suitability of surfactants, have been carried out to resolve the oil tank-cleaning problem in crude oil production. As the result of these investigations, it has been recognized that oil storage tank sludges can be cleaned-up, and a significant percentage of the oil can be recovered from the sludge layer when treated with biosurfactants (Banat *et al.*, 1991). Use of biosurfactants in oil storage tank cleaning is another in-situ application of biosurfactants (Banat, 1995 -Acta Biotech.).

In tank cleaning, the principal mechanism of biosurfactant aided oil tank clean-up operation involves dispersion of heavy oil components by reducing the viscosity of sludge by forming either macro- or micro-emulsions of either oil-in-water or water-in-oil. The less viscous emulsion is easily pumped and the oil fraction can be easily separated after breaking the emulsification (Banat *et al.*, 1991).

In a pilot field investigation, the ability of a biosurfactant, produced by a proprietary bacterial strain (Pet 1006), to clean oil storage tank and recover hydrocarbon from the emulsified sludge has been tested by Banat *et al* in 1991. Their principal purpose has been to assess the suitability of this biosurfactant for cleaning a large oil storage tank in order to evaluate the effectiveness of such a cleaning procedure and to estimate the amount of oil that can be recovered from the sludge.

The process details of biosurfactant production by Pet 1006 and the results of laboratory fermenter experiments and pilot-plant production have been described by Banat *et al.* in Banat *et al.*, 1991.

In the Banat *et al.* study, 2 tons of a biosurfactant-containing whole cell culture broth has been produced in a 1500 L up-lift fermenter and heat sterilized before use in oil tank clean-up. Oil storage tank cleaning procedure has been planned as four steps. In the first step, called as surveying, the initial survey has been carried out by dipping in 17 points at the bottom of the proposed floating roof tank with 44 m diameter, belonging to Kuwait Oil Company to determine the sludge topography and sludge volume. The sludge quality and the optimum biosurfactant requirements to lift the sludge have been also determined in first step. According to the results of the tank survey, sludge analysis and laboratory tests on the sludge it has been determined two main sludge areas about 1 to 1.5 m high in the tank, presence of 850 m^3 sludge, which could be potentially easily emulsified. In addition it has been considered that 1 t biosurfactant would be sufficient to obtain the necessary emulsification and recover the hydrocarbon trapped in the sludge. In second step, mobilizing, all necessary equipments (pumps, hoses, and circulation boxes) to provide circulation and

biosurfactant mixing in the tank have been installed. After the installation of the equipments, in desludging step, which is the third step of the oil tank clean-up procedure, a blend of the produced biosurfactant, brackish water and fresh crude oil have been introduced into the tank. The mixture in the tank, which had consisted of 850 m^3 of sludge, 1.5 t biosurfactant, 850 m^3 crude oil (1:1 crude oil to sludge), and 2125 m^3 of brackish water [1.25:1 brackish water to total hydrocarbon (sludge + fresh crude oil)], has been circulated for 24 hours per day for 10 days at ambient temperatures during July until sampling had showed that most of the sludge has been processed (lifted from the bottom of the tank) and resuspended as a result of its emulsification. Although 1 t of biosurfactant has been considered sufficient for proposed cleaning, 0.5 t of additional biosurfactant has been used to allow for any inaccuracies in the sludge sampling or any activity loss of the product due to the long storage or unforeseen delay in its application. However, 5 days has been found enough to achieve the required results, due mainly to the use of 1.5 t as opposed to the calculated 1 t of biosurfactant and to the high summer temperatures (40 to 50°C). After circulation, the emulsion has been allowed to separate into oil and water layers using an emulsion breaker, and the two layers have been easily extracted from the tank to be reused; the water in future clean-up operations and the oil hydrocarbon layer for blending and release as crude oil. The oil layer then have been pumped out and transferred to a storage tank and the water layer has been removed for disposal. In the last step, the remaining impurities (sand, gravel, and non-hydrocarbon materials) have been removed manually before tank inspection.

The clean-up has been found as successful; and nearly 91% of the sludge has been recovered, as crude oil, while 76 m^3 non-hydrocarbon materials remaining as impurities at the bottom of the tank. The characteristics of the recovered crude oil compared with Kuwait crude oil characteristics are shown in Table 3.5. The recovered crude oil had an API value of 28.6, which has been within the API range for the standard Kuwait crude oil range of 27 to 29, and had a 100% hydrocarbon content. The recovered crude oil of 774 m^3 (=5550 barrels, at US $20/barrel) has worth $110000. This can turn the currently expensive clean-up operations, costing

from $100000 to $150000 per tank, into a financial rewarding process. At worst, the value of recovered hydrocarbons should virtually cover the clean-up costs (Banat *et al.*, 1991; Banat, 1995- Acta Biotech.).

Table 3. 5 The Comparison of Biosurfactant-Recovered Crude Oil and Control Kuwait Crude Oil in terms of Their Characteristics.

Property	Unit	Recovered Crude Oil	Control[1]
Specific gravity	g/mL	0.883	0.890
API gravity	API	28.6	27-29
Kinematic viscosity at 20°C	cSt	166.6	154-196
Asphaltic material	% wt	2.09	<2
Oil content	% vol	100	100

[1] Kuwait Crude Oil

The advantages of biosurfactants use for oil sludge cleaning include:

1. The biosurfactant-aided oil tank clean-up process uses nonhazardous, biodegradable, and environmentally acceptable compounds, when compared to the environmentally unacceptable chemicals now widely used.
2. The process, when optimized, may significantly reduce the operational time lost for oil storage tank cleaning derived from current manual cleaning methods.
3. The process also reduces the amount of residual sludge to be disposed of by burning or land farming.

CHAPTER FOUR
EXPERIMENTAL STUDY

4.1 GENERAL

Experimental study was performed to determine whether biosurfactants have any effects on the microbial decay of persistent organic chemicals in industrial wastewaters. For this purpose, both normal activated sludge system (called as control) and biosurfactant-aided activated sludge system were performed simultaneously in the experimental study to compare the organic pollutants removal efficiencies in both systems. It was considered that the removal efficiency difference between these two systems would reflect the effects of biosurfactants, and simultaneous operation would obliterate the effects of other effective parameters such as temperature, aeration, and wastewater sample composition on activated sludge system.

In order to determine the effect of biosurfactants on microbial degradation of persistent organic compounds, the type and the concentration of biosurfactant, pollutant type, and the retention time in activated sludge system were investigated as effective parameters on biosurfactant-aided activated sludge system. For this reason, two different sophorolipid types of biosurfactants were used, and all experiments were performed with different initial sophorolipid concentrations. In addition to these, different retention times were applied in activated sludge systems to determine both its effect and biosurfactant effectiveness vs. time. The applied interval for retention time was selected as acceptable range up to 24 hours, because conventional activated sludge systems and its modifications are generally applied for approximately 6 h to 36 h. In addition, because biosurfactants were expected as

effective compounds in degradation the time required for complete degradation would decrease with the addition of biosurfactants.

The experimental study was conducted in two stages. In the first stage, synthetic wastewater samples prepared with compounds, which are difficult to decay, and both sophorolipids were used. In this stage, two different experimental studies designated as study A and study B were conducted. Two different types of sophorolipids were used in each study. In the second stage, real wastewater samples taken from the wastewater treatment plant of a dye industry facility were used. In this stage only one study called as study C was performed. Experimental procedures for each study (A, B, and C) are given separately following sections.

4.2 MATERIALS

4.2.1 BIOSURFACTANTS

Sophorolipid type biosurfactants were selected as a representative biosurfactants due to its ability to enhance solubilization of organic compounds in soil and groundwater remediation and its availability. With a similar approach, it was expected that sophorolipids would enhance the solubility of poorly soluble compounds like oil in wastewater.

Two different sophorolipids were used in this study. The production and purification of both sophorolipids has been described previously by Pekin. (Pekin, 1999). Sophorolipids, which were designated as sophorolipid-I and sophorolipid-II, have been produced by *Candida (Torulopsis) bombicola* ATCC 22214 in Biotechnology Department of Graduated School of Natural and Applied Sciences of Ege University, and in the Stuttgart University, respectively.

Çaylak and Sukan have aimed to examine different carbon sources and production methods in the ongoing study about the production of sophorolipids. When sophorolipid-I was received, studies have been continued on the purification of

produced sophorolipids. So sophorolipid-I, used in experimental study A, had low purification level. However, it has been stated that in the production of sophorolipid-II, high purification performance obtained. As the result, the purification level of sophorolipid-II is higher than that of sophorolipid-I.

All sophorolipid solutions used in the experimental studies were prepared using deionized, distilled water. Because the CMC values and molecular weights of sophorolipids cannot be obtained, the concentrations of sophorolipid solutions were measured on the 'mg/L' basis. Generally reported CMC values for sophorolipids are from 10 to 30 mg/L, in some studies, however, different CMC values have been reported. So, in this study, a wide surfactant concentration range (0 to 100 mg/L) was investigated.

4.2.2 CHEMICALS

Generally both synthetic and microbial surfactants have been proven to be effective especially in the remediation of petroleum hydrocarbon-contaminated soil and groundwater. Petroleum hydrocarbons-containing wastewaters from specific industrial processes, especially oil refinery wastewaters and petrochemical industry wastewaters, are also one of the most problematic waste streams. For these reasons, gasoline and kerosene were used as model wastewater pollutants in the first two experimental studies (called as study A, and study B), performed with using synthetic wastewater samples, because they were considered to represent petroleum hydrocarbons. Gasoline, which comprises primarily light hydrocarbons, alkenes, and benzene, toluene, ethyl benzene, and xylenes (BTEX), is representative because it is a mixture of over 200 petroleum-derived chemicals and a few synthetic products that are added to improve fuel performance. Gasoline totally contains approximately 280 different hydrocarbons in the range of C4 to C12. Hazardous characteristics of gasoline derived from its components such as BTEX compounds, methanol, ethanol, MTBE (Methyl Tertiary Butyl Ether), aromatic hydrocarbons, PAHs, and alkyl PAHs. Kerosene is also representative because it has been reported that the major constituents of kerosene are alkanes and cycloalkanes (68.6%); benzene and

substituted benzene (13.7%); and naphthalene and substituted naphthalenes. The average chemical composition of kerosene by weight is 35% paraffins, 60% naphthenes, and 15% aromatics (Irwin, VanMouwerik, Stevens, Seese, Basham, 1997).

Although gasoline was used as the model pollutants in the first stage of the experimental study (study A), in study B kerosene was preferred to minimise the effect of volatility of pollutant on experimental procedure. The volatility of kerosene is lower than that of gasoline, as reported by Irwin *et al.* in 1997.

4.2.3 ACTIVATED SLUDGE SAMPLES

Microbial culture used in the activated sludge treatment models must be acclimated to petroleum hydrocarbons because studying with petroleum hydrocarbon-containing wastewaters was proposed. For this reason, the use of activated sludge samples from biological treatment processes of wastewater treatment plants treating petroleum hydrocarbon-containing wastewaters was considered favorable. Activated sludge samples used in the experimental study A were obtained from the biological treatment process in the wastewater treatment plant of a petrochemical facility. In experimental study B, activated sludge of the biological treatment process in the wastewater treatment plant of an oil refinery industry was used.

4.2.4 WASTEWATER SAMPLES

Surfactants have been shown that they have capabilities to enhance the removal of hydrophobic organic compounds from soil and groundwater environments. Based on this knowledge, the investigation of the use of biosurfactants in hydrophobic organic compound-containing wastewater treatment was also aimed. For this reason, in the experimental study C, dye industry wastewater was used.

Liquid effluents resulting from dye industry production processes (especially equipment cleaning after batch operation) contain persistent and toxic organic residues. In addition, because hydrophobic compounds are used in the production of wide variety of oil based dyes, dye industry wastewaters also contain hydrophobic pollutants. Toxic organic pollutants in wastewaters generally inhibit the biodegradation in the biological wastewater treatment process, whereas persistent and hydrophobic compounds cannot be treated effectively in conventional treatment units.

The dye industry facility from where wastewater samples were taken produces both water and oil based dyes. So wastewater samples were taken when hydrophobic organic compounds containing wastewaters were released to wastewater treatment plant after oil based dyes production. After wastewater samples taken from the biological treatment process influent including both domestic and industrial wastewaters, all samples were taken to the laboratory for experimental study in a time of approximately one-hour.

4.2.5 EXPERIMENTAL APPARATUS

In this study, the use of biosurfactants to enhance conventional activated sludge system was aimed. So, all experiments were performed with a classical activated sludge system model. The activated sludge system model including two -or mostly four- flasks (reactors) were used as aeration tanks. Oxygen was supplied to flasks by an air pump to obtain air (oxygen) required for aeration. Experimental apparatus is illustrated in Figure 4.1.

The first flask was used as control reactor, and the others were used as biosurfactant reactors. These reactors were cooperated to obtain simultaneous operation to eliminate the effects of temperature, aeration, different wastewater composition and etc. on the activated sludge system.

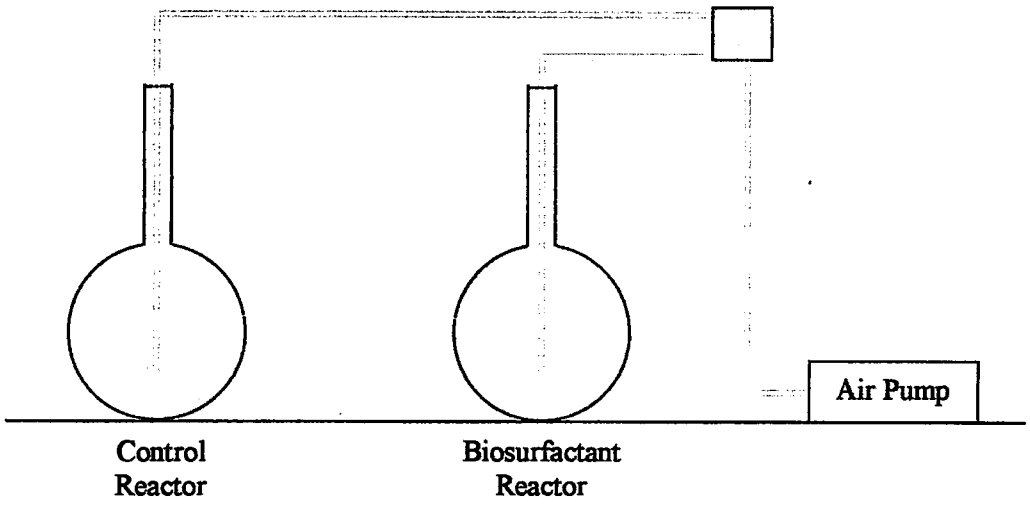

Figure 4. 1 Experimental apparatus

Although only one biosurfactant reactor was shown in the figure, experiments were generally performed using more than one biosurfactant reactor.

4.3 EXPERIMENTAL METHODS

4.3.1 GENERAL

Experimental study was conducted in two stages: In the first stage synthetic wastewater samples prepared with using gasoline and kerosene, and in the second stage real wastewater samples taken from a dye industry facility were used. In addition, in the first stage both sophorolipids (-I and -II) were used whereas only sophorolipid-II was used in the second stage. Experiments were also categorized in terms of sophorolipids used. To differentiate both wastewater type and sophorolipid used, experiments were also categorized as study A, B and C. The experimental study categorization can be seen in Table 4.1.

Table 4. 1 Experimental Studies

Stage	Study	Sophorolipid	Wastewater
Stage I	A	Sophorolipid-I	Synthetic wastewater prepared with gasoline
	B	Sophorolipid-II	Synthetic wastewater prepared with kerosene
Stage II	C	Sophorolipid-II	Dye industry wastewater samples

4.3.2 ENHANCED BIODEGRADATION OF PERSISTENT ORGANICS IN SYNTHETIC WASTEWATER SAMPLES BY SOPHOROLIPID-I (STUDY A)

4.3.2.1 EXPERIMENTAL PROCEDURE IN STUDY A

In this study, it was aimed to determine the enhancement effect of sophorolipid-I in the treatment of prepared synthetic wastewater sample containing gasoline. After the synthetic wastewater sample was prepared sophorolipid-I at a certain concentration was added to biosurfactant reactor. With COD measurements at the start-up (0^{th} h), and 6^{th} h (showed as COD_6) and 24^{th} h (showed as COD_{24}) of the process, the effectiveness of sophorolipid-I on the persistent organics removal was examined. Varying concentrations of sophorolipid-I were used in different experiments. Comparing control reactor and biosurfactant reactor results the actual effectiveness of sophorolipid-I was determined.

4.3.2.2 PREPARATION OF SYNTHETIC WASTEWATER SAMPLES

Activated sludge was brought from the wastewater treatment plant of a petrochemical industry. Average oil and grease concentration of influent of the plant is approximately 100 mg/L. For this reason, 100 mg/L oil and grease containing-solutions were prepared using commercially available gasoline.

In preliminary experiments, it was determined that 700 mg/L gasoline solution, prepared by distilled water and gasoline, contained approximately 100 mg/L oil and grease. In addition, activated sludge samples were analyzed in terms of suspended solids concentration to provide required SS concentration in synthetic wastewater samples for sufficient microbial growth. It was determined that 100 mL activated sludge sample and 500 mL distilled water mixture (totally 600 mL) contained approximately 2500 mg/L SS. For this reason the volumetric ratio of activated sludge sample to synthetic wastewater sample was considered as favorable around 1/5 or 1/6.

In conducting the experimental study, firstly, a stock gasoline solution of 50 g/L was prepared by mixing 50 g gasoline and enough distilled water to obtain a volume of 1 L. Using stock gasoline solution, 700 mg/L gasoline solution was prepared with sequential dilution. 600 mL gasoline solution then was added to each reactor (control and biosurfactant), Synthetic wastewater samples were prepared by adding of 100 mL activated sludge sample to each reactor.

4.3.2.3 PREPARATION OF SOPHOROLIPID SOLUTION

Sophorolipid-I solution used in the experimental study A was prepared at the concentration of 70 mg/L to be added required concentrations. Since 1 mL can be considered the minimal measurable and usable volume, the addition of 1 mL of sophorolipid-I solution to reactor containing 700 mL of wastewater sample must give 0.1 mg/L that was assumed the minimum appropriate concentration of sophorolipid in the reactor. For this reason, 70 mg/L was selected as sophorolipid solution concentration.

Firstly, a stock sophorolipid-I solution of 10 g/L was prepared with mixing 10 g sophorolipid-I and enough amount of distilled water to obtain a volume of 1 L. Using stock solution of 10 g/L, 70 mg/L sophorolipid-I solution was prepared with sequential dilution. Because of the observed structural damage in sophorolipid solution when the solution was kept at room temperature conditions, all prepared sophorolipids solutions kept in a refrigerator at 4-8 °C.

4.3.3 ENHANCED BIODEGRADATION OF PERSISTENT ORGANICS IN SYNTHETIC WASTEWATER SAMPLES BY SOPHOROLIPID-II (STUDY B)

4.3.3.1 EXPERIMENTAL PROCEDURE IN STUDY B

In study B, it was aimed to determine the enhancement effect of sophorolipid-II in the treatment of kerosene containing-synthetic wastewaters. After the synthetic

wastewater sample was prepared, 500 mL synthetic wastewater sample was added to each reactor. Varying sophorolipid-II concentrations (2-100 mg/L) to biosurfactants reactors were added to biosurfactant reactors. With COD measurements at the start-up (0^{th} h), and 3^{rd}, 6^{th} h and 24^{th} h of process, the sophorolipid-II effectiveness on the persistent organics removal was examined. Comparing control reactor and biosurfactant reactors results the actual effectiveness of sophorolipid-II in was determined.

4.3.3.2 PREPARATION OF SYNTHETIC WASTEWATER SAMPLES

Activated sludge samples taken from a oil refinery industry wastewater treatment plant were analyzed in terms of suspended solids concentration to provide required SS concentration in synthetic wastewater samples for activated sludge system. It was determined that 100 mL activated sludge sample and 500 mL distilled water mixture contained approximately 3500 mg/L SS. To obtain 2000 – 2500 mg/L SS in synthetic wastewater samples the volumetric ratio of activated sludge sample to synthetic wastewater sample was considered as favorable around 1/3 - 1/5. Moreover, it was determined that kerosene solution of 750 mg/L contained approximately 100 mg/L oil and grease.

In study B, kerosene solution of 750 mg/L was prepared using commercial kerosene. Firstly, a stock kerosene solution of 50 g/L was prepared with mixing 50 g gasoline and enough amount of distilled water to obtain a volume of 1 L. Using stock solution, 750 mg/L gasoline solution was prepared with sequential dilution.

In this study, more biosurfactant reactors were used to compare the effectiveness of different biosurfactant concentrations. In each experiment, 400 mL kerosene solution and 100 mL activated sludge sample was added to each reactor (control and biosurfactant).

4.3.3.3 PREPARATION OF SOPHOROLIPID SOLUTION

In study B, the use of higher concentrations of sophorolipid was proposed in order to determine the effect of sophorolipid concentration. For this reason, sophorolipid-II solution used in this stage was prepared at the concentration of 1 g/L. Firstly, a stock sophorolipid-II solution of 10 g/L was prepared with mixing 10 g sophorolipid-II and enough amount of distilled water to obtain a volume of 1 L. Using stock solution of 10g/L, 1 g/L sophorolipid-I solution was prepared with sequential dilution.

4.3.4 ENHANCED BIODEGRADATION OF PERSISTENT ORGANICS IN DYE INDUSTRY WASTEWATER SAMPLES BY SOPHOROLIPID-II (STUDY C)

4.3.4.1 EXPERIMENTAL PROCEDURE IN STUDY C

In the experimental study C, firstly pH and SS values of dye industry wastewater samples were analysed. In all samples pH values ranged from 6.5 to 8 and SS values were determined as about 2500 mg/L.

In study C, four reactors, one of which was control, were used. 500 mL of wastewater sample was added to each reactor. In the first four experiments, the sophorolipid-II concentrations were applied as 25 mg/L, 50 mg/L and 100 mg/L. After the sophorolipid-II solutions were added, COD concentrations at the start-up (0^{th} h), 3^{rd} h, and 6^{th} h of process for first two experiments and at the start-up (0^{th} h), 6^{th} h and 24^{th} h of process for the next two experiments were measured. According to the results of the first four experiments, the use of 10 mg/L and 20 mg/L sophorolipid-II concentrations were considered for later experiments, because lower sophorolipid-II concentrations have gave higher COD reduction at 6^{th} h of the process and higher sophorolipid-II concentrations have increased COD concentration to higher values than lower sophorolipid-II concentrations in the start-up. It was assumed that the higher COD values for higher sophorolipid-II concentrations have arisen due to sophorolipid added.

Later experiments were conducted by using 10 mg/L and 20 mg/L sophorolipid-II, COD measurements were performed at the start-up (0^{th} h), 3^{rd} h, and 6^{th} h of process or at the start-up (0^{th} h), 6^{th} h and 24^{th} h of process. The experimental results have been compared to determine the effect of sophorolipid concentration and time.

4.3.4.2 PREPARATION OF SOPHOROLIPID SOLUTION

In study C, the examining similar sophorolipid concentrations range (0 to 100 mg/L) with in study B was proposed. During the experiments, it has been decided that application of low sophorolipid concentrations was not necessary to investigate because higher sophorolipid concentration gave greater COD removal efficiencies in experimental study B. For this reason, the preparation of sophorolipid-II solution of 5 g/L was proposed. Sophorolipid-II solution of 5 g/L was prepared with directly mixing 5 g sophorolipid-II and enough amount of distilled water to obtain a volume of 1 L. Sophorolipid-II solution was also kept in the refrigerator at 4-8 °C.

4.4 ANALYTICAL METHODS

4.4.1 CHEMICAL OXYGEN DEMAND (COD)

Chemical oxygen demand (COD) was determined according to potassium dichromate ($K_2Cr_2O_7$) reflux method given in *Water and Wastewater Analyses*, 1998 (Şengül & Türkman, 1998). Oxidation of most organic compounds is 95 to 100% of the theoretical value when potassium dichromate is used. All samples used to determine the COD were filtrated before standard potassium dichromate reflux method performed, and COD testing results were thus evaluated as soluble COD.

CHAPTER FIVE
RESULTS AND DISCUSSIONS

5.1 ENHANCED BIODEGRADATION OF PERSISTENT ORGANICS IN SYNTHETIC WASTEWATERS BY SOPHOROLIPID-I

In study A, three experiments were performed and COD parameter was observed to determine the effects of sophorolipid-I. The results of these experiments are given in Table 5.1, 5.2 and 5.3. In the first experiment (A-1) a very high COD value (400000 mg/L) was observed in the biosurfactant reactor, to which 10 mg/L sophorolipid-I was added, at start-up, whereas only 5120 mg/L COD value was detected in the control reactor at the same time. Since synthetic wastewater sample in the control reactor contains only 5120 mg/L COD and the unique difference between both reactors was the addition of sophorolipid-I to biosurfactant reactor, the increase in the COD of wastewater in biosurfactant reactor was resulted from the addition of sophorolipid-I solution to the biosurfactant reactor. This result indicates that the COD value of sophorolipid-I solution may be high. It was presumably evaluated that the high COD content of sophorolipid-I solution derived from impurities in sophorolipid-I including extraction compounds used in the biosurfactant recovery from microbial culture and non-removed microorganisms, because sophorolipid-I originally has not been completely purified.

After obtaining this result, later experiments were performed using 0.1 mg/L sophorolipid-I solutions. These two experiments also gave conflicting results. In the second experiment (A-2), 0.1 mg/L sophorolipid-I solution was added to the biosurfactant reactor. The start-up COD values were 480 mg/L for the control reactor and 160 mg/L for the biosurfactant reactor. However, in the third experiment (A-3),

in which also 0.1 mg/L sophorolipid-I solution was used, the start-up COD values were determined as 240 mg/L for the control reactor and 720 mg/L for the biosurfactant reactor. And COD value of wastewater in the biosurfactant reactor reached the 80 mg/L at the 24th hour of the process while this value was reached at 6th hour of the process in the control reactor. However, in the second experiment 6th hour COD effluent values were found to be 80 mg/L for the biosurfactant reactor and 320 mg/L for the control reactor.

Table 5. 1 The Results of Experiment A-1

Sophorolipid-I Concentration, mg/L	COD_0, mg/L at t=0 h	CODe, mg/L at t=6 h
0 (Control)	5120	2040
10	400 000	288 000

Table 5. 2 The Results of Experiment A-2

Sophorolipid-I Concentration, mg/L	COD_0, mg/L at t=0 h	CODe, mg/L at t=6 h
0 (Control)	480	320
0.1	160	80

Table 5. 3 The Results of Experiment A-3

Sophorolipid-I Concentration, mg/L	COD_0, mg/L at t=0 h	COD_6, mg/L at t=6 h	CODe, mg/L at t=24 h
0 (Control)	240	80	80
0.1	720	240	80

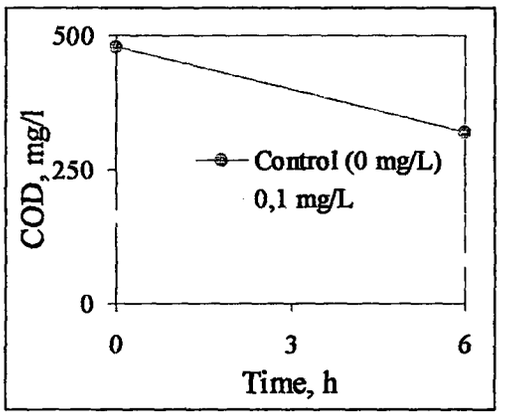

Figure 5. 1 COD reduction vs. time for different sophorolipid-I concentrations

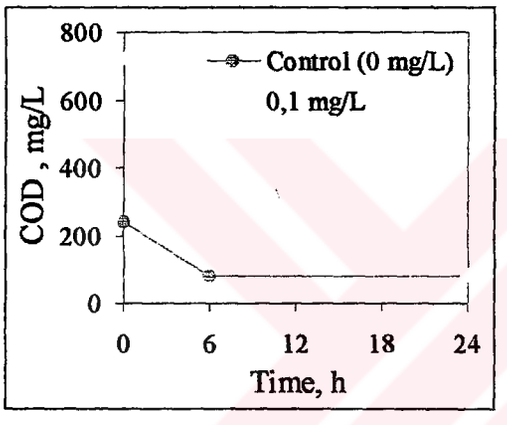

Figure 5. 2 COD reduction vs. time for different sophorolipid-I concentrations

These mixed results indicate the existence of a significant factor affecting sophorolipid activity. It is a strong possibility that this factor is the low purification level of sophorolipid-I. Another possibility may be the activated sludge-sorbed pollutants. If the activated sludge used in the study A contained solid phase-bound pollutants, these pollutants may be desorbed from sludge to water medium, and increased the COD value of the wastewater in the biosurfactant reactor. However, this mechanism cannot explain the results obtained in the experiment A-2.

5.2 ENHANCED BIODEGRADATION OF PERSISTENT ORGANICS IN SYNTHETIC WASTEWATERS BY SOPHOROLIPID-II

In experimental study B, five experiments were performed and COD parameter was also observed to assess the effect of sophorolipid-II on the treatment of synthetic wastewaters prepared by kerosene and oil refinery industry activated sludge. Experimental results are given in Table 5.4- Table 5.8. It can be seen in these tables that similar COD values were obtained in each experiment. For this reason these results were evaluated together.

Table 5. 4 COD Changes vs. Sophorolipid-II Concentration in the Experiment B-1

Sophorolipid-II Concentration, mg/L	COD_0, mg/L	COD_6, mg/L	COD Removal Efficiency, %
0	780	250	67.9
2	1500	220	85.0
10	1570	204	87.0
100	2460	186	92.4

Table 5. 5 COD Changes vs. Sophorolipid-II Concentration in the Experiment B-2

Sophorolipid-II Concentration, mg/L	COD_0, mg/L	COD_6, mg/L	COD Removal Efficiency, %
0	800	260	67.5
2	1520	224	85.3
10	1600	200	87.5
100	2560	192	92.5

Table 5. 6 COD Changes vs. Sophorolipid-II Concentration in the Experiment B-3

Sophorolipid-II Concentration, mg/L	COD_0, mg/L	COD_6, mg/L	COD Removal Efficiency, %
0	840	280	66.7
2	1540	216	85.0
10	1720	196	88.6
100	2620	188	92.8

Table 5. 7 COD Changes vs. Sophorolipid-II Concentration in the Experiment B-4

Sophorolipid-II Concentration, mg/L	COD_0, mg/L	COD_6, mg/L	COD Removal Efficiency, %
0	890	265	70.2
2	1550	216	86.1
10	1680	205	87.8
100	2640	200	92.4

Table 5. 8 COD Changes vs. Sophorolipid-II Concentration in the Experiment B-5

Sophorolipid-II Concentration, mg/L	COD_0, mg/L	COD_6, mg/L	COD Removal Efficiency, %
0	960	280	70.8
2	1610	216	86.6
10	1720	196	88.6
100	2700	188	93.0

COD_0 : COD concentration at start-up (t=0 h)

COD_6 : COD concentration at 6th hour of the process (t=6 h)

In Figure 5.3, the COD concentrations measured at start-up and 6th hour of process -as effluent- are given as the average values of five experimental results. A certain increase in the COD concentrations in all biosurfactants reactors was observed in each case. However, the COD increase amounts for each biosurfactant reactor were found to be different. After the addition of 1 mL of sophorolipid-II solution to synthetic wastewater sample in the first biosurfactant reactor of 500 mL to obtain sophorolipid-II concentration of 2 mg/L, COD increased from 854 mg/L to 1544 mg/L as average values of all experiments. The addition of 5 mL of sophorolipid-II solution to second biosurfactant reactor of 500 mL to obtain sophorolipid-II concentration of 10 mg/L increased COD value to only 1658 mg/L as average values of al experiments. Also, the addition of 50 mL of sophorolipid-II

solution to last biosurfactant reactor of 500 mL to obtain sophorolipid-II concentration of 100 mg/L increased COD value to 2596 mg/L at start-up.

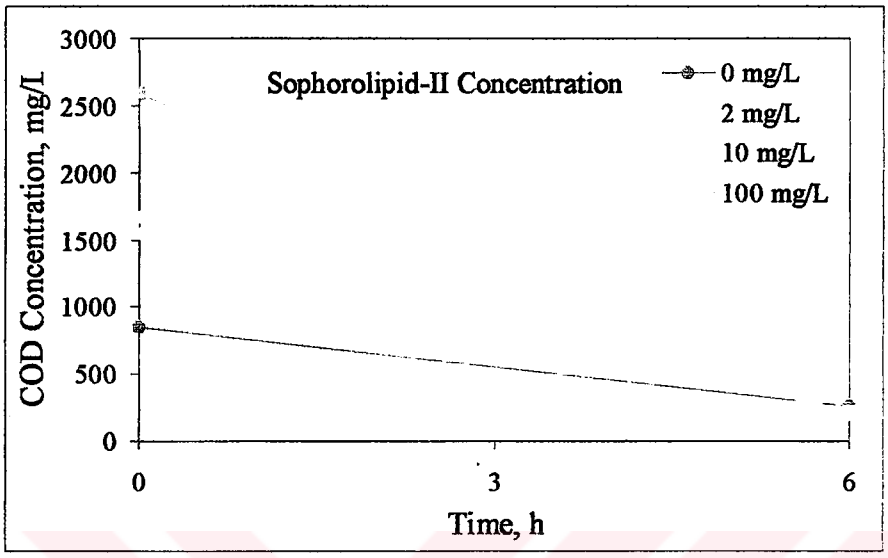

Figure 5. 3 COD reduction vs. time for different sophorolipid-II concentrations. ***(Each point indicates the average of the results of five experiments)***

First possibility caused the increase in COD concentration at the start-up can be shown as the addition of sophorolipid-II solution, because sophorolipid itself is an organic compound. To determine whether the increase in COD concentrations at the start-up caused completely by sophorolipid-II addition, a ratio called as COD increasing ratio was defined as follows.

$$\text{COD Increasing Ratio} = \frac{COD_{new} - COD_0}{V_B}$$

where COD_0 is the COD concentration (mg/L) of wastewater sample before biosurfactant addition at the start-up, COD_{new} is the COD concentration (mg/L) of wastewater sample after biosurfactant addition at the start-up, and V_B is the volume (mL) of the sophorolipid added to wastewater. In the COD increasing ratio definition, it was aimed to determine the increased COD concentration by the

addition of 1 mL of sophorolipid-II solution. It was assumed that if COD increase at the start-up caused by the addition of sophorolipid-II solution, COD increasing ratios must increased with increasing volumes of sophorolipid-II solution.

Although the increasing amounts of sophorolipid-II solution added seem to increase risen the increase of COD concentration at the start-up, the COD increasing ratios given in Table 5.9 indicates opposite results. These ratios show that the addition of 50 mL of sophorolipid-II solution of 1 g/L to wastewater sample produced only a COD increasing ratio of 34.8, whereas the addition of 1 mL of sophorolipid-II solution of 1 g/L to wastewater sample produced a COD increasing ratio of 690.

Since the same sophorolipid solution (sophorolipid-II) was used for each reactor, this difference cannot be attributed to only sophorolipid-II solution. If sophorolipid-II have been the unique reason of COD increase at the start-up, these increases should have been increased proportional with added sophorolipid-II volume (similarly concentration).

Table 5. 9 COD Increasing Ratio for the Results Obtained in Study B

C_B, mg/L	V_B, mL	C_0, mg/L	C_{new}, mg/L	COD Increase, mg/L	COD Inc. Ratio, mg/L per 1 mL
2	1	854	1544	690	690
10	5	854	1658	804	160.8
100	50	854	2596	1742	34.8

Even if it is considered that sophorolipid itself contributed the COD increase in the cases of the addition of 5 and 50 mL of sophorolipid-II, the rapid increase in COD concentration with the addition of only 1 mL of sophorolipid-II solution (2 mg/L) indicates another mechanism. The same sharp increase can be also shown in influent COD concentration changes line in Figure 5.4.

The principal mechanism causing rapid increase in COD with the addition of sophorolipid-II at the start-up was considered as desorption of solid phase-bound (sorbed to activated sludge or suspended solids) pollutants, having hydrophobic characteristics, from sludge to water.

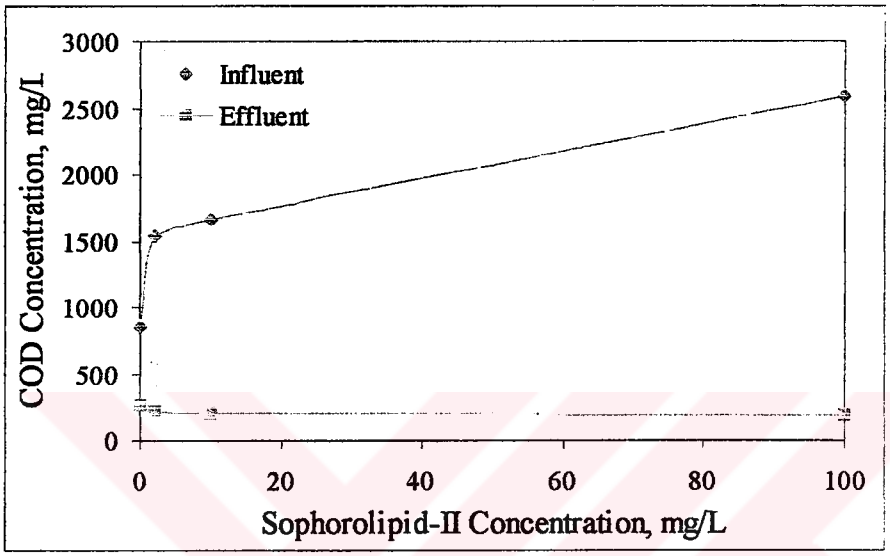

Figure 5. 4 The change in influent and effluent COD concentrations vs. sophorolipid-II concentration ***(Each point indicates the average of the results of five experiments)***

The change in COD removal efficiency versus the concentration of sophorolipid-II added can be seen in Figure 5.5. This figure reflects that COD removal efficiency increases with increasing sophorolipid-II concentration. However, these increases caused by the increase in influent COD concentration with the addition of sophorolipid-II. This was also confirmed by the rapid increase in COD removal efficiency at the level of sophorolipid-II concentration of 2 mg/L.

Figure 5.5 also reflects that the CMC value of sophorolipid may be about 2 mg/L. Consequently, the use of high concentrations of sophorolipid-II like 100 mg/L may not be necessary in most cases because the addition of 10 times more sophorolipid-II solution (in this case 50 mL instead of 5 mL) can increase the COD removal efficiency only 3 or 4 %.

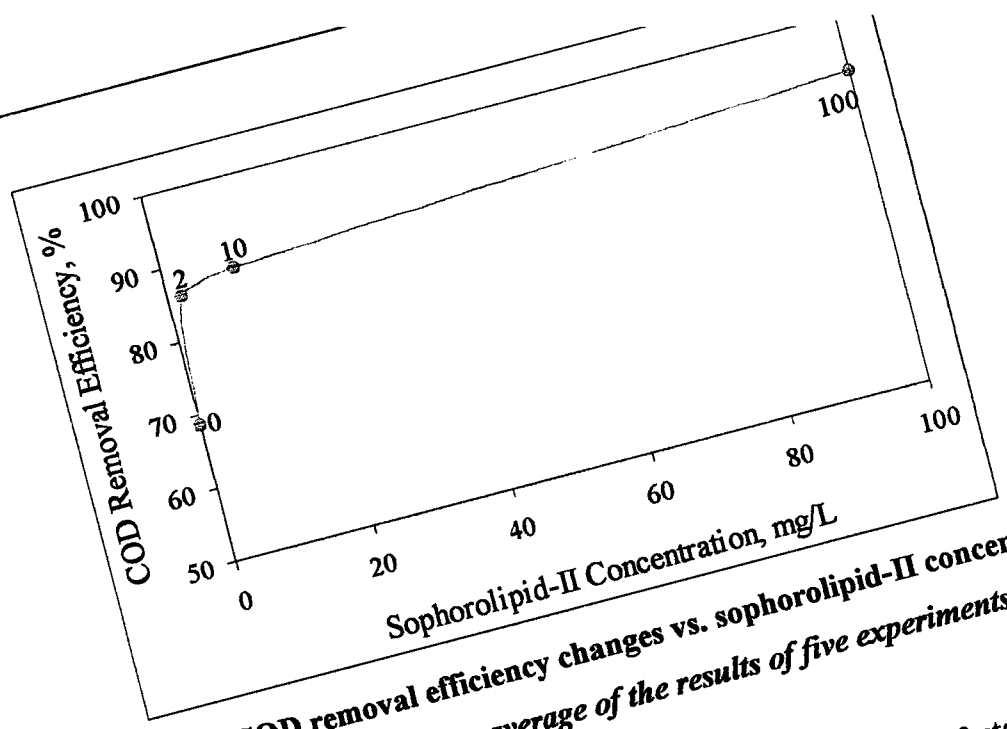

Figure 5. 5 COD removal efficiency changes vs. sophorolipid-II concentration ***(Each point indicates the average of the results of five experiments)***

Although the addition of sophorolipid to wastewaters in biosurfactant reactors firstly increased the COD concentrations, effluent COD values of biosurfactant reactors were always lower than that of control reactor. This shows that pollutants in water phase and present as solid phase-bound can be removed with sophorolipid-enhanced biodegradation of persistent organic pollutants. This result is the most important outcome of the present study.

5.3 ENHANCED BIODEGRADATION OF PERSISTENT ORGANICS IN DYE INDUSTRY WASTEWATER SAMPLES BY SOPHOROLIPID-II

In experimental study C, seven experiments were performed and COD param was also observed in all reactors to assess the effect of sophorolipid-II or treatment of dye industry wastewater samples. In the study C, a broad range f concentration of sophorolipid-II solution was used to determine ef concentration range for dye industry wastewaters specifically. Experimenta are given in Table 5.4- Table 5.8. Experiments were applied with

sophorolipid-II concentrations or the same retention time (C-1 and C-2, C-3 and C-4, C-5 and C-6) and the results compared.

Table 5. 10 COD Changes vs. Sophorolipid-II Concentration in the Experiment C-1

Sophorolipid-II Concentration, mg/L	COD_0, mg/L	COD_3, mg/L	COD_6, mg/L	Total COD Removal Efficiency, %
0	13200	11200	6200	53.0
25	10800	7600	5000	53.7
50	11600	8800	5400	53.5
100	11600	10800	7500	35.4

Table 5. 11 COD Changes vs. Sophorolipid-II Concentration in the Experiment C-2

Sophorolipid-II Concentration, mg/L	COD_0, mg/L	COD_3, mg/L	COD_6, mg/L	Total COD Removal Efficiency, %
0	12800	11000	7100	44.5
25	10200	6800	5200	49.0
50	11600	8000	6000	48.3
100	10800	9600	7200	33.3

Table 5. 12 COD Changes vs. Sophorolipid-II Concentration in the Experiment C-3

Sophorolipid-II Concentration, mg/L	COD_0, mg/L	COD_6, mg/L	COD_{24}, mg/L	Total COD Removal Efficiency, %
0	14400	5600	116	99.2
25	9800	4800	124	98.7
50	15200	7200	88	99.4
100	13000	7200	80	99.4

Table 5. 13 COD Changes vs. Sophorolipid-II Concentration in the Experiment C-4

Sophorolipid-II Concentration, mg/L	COD_0, mg/L	COD_6, mg/L	COD_{24}, mg/L	Total COD Removal Efficiency, %
0	15200	7400	145	99.1
25	11200	5600	144	98.7
50	13800	7000	110	99.2
100	14200	6800	96	99.3

Table 5. 14 COD Changes vs. Sophorolipid-II Concentration in the Experiment C-5

Sophorolipid-II Concentration, mg/L	COD_0, mg/L	COD_3, mg/L	COD_6, mg/L	Total COD Removal Efficiency, %
0	12600	9800	6400	49.2
10	9600	6800	3800	60.4
20	10200	7600	4800	52.9

Table 5. 15 COD Changes vs. Sophorolipid-II Concentration in the Experiment C-6

Sophorolipid-II Concentration, mg/L	COD_0, mg/L	COD_3, mg/L	COD_6, mg/L	Total COD Removal Efficiency, %
0	13800	10600	7400	46.4
10	10200	8800	5400	47.1
20	11600	9400	6200	46.6

Table 5. 16 COD Changes vs. Sophorolipid-II Concentration in the Experiment C-7

Sophorolipid-II Concentration, mg/L	COD_0, mg/L	COD_3, mg/L	COD_6, mg/L	COD_{24}, mg/L	Total COD Removal Efficiency, %
0	14600	11000	8200	320	97.8
10	9800	8400	6800	180	98.2
20	11200	10200	7800	260	97.7

COD_0 : COD concentration at the start-up (t=0 h)

COD_3 : COD concentration at 3rd hour of the process (t=3 h)

COD_6 : COD concentration at 6th hour of the process (t=6 h)

COD_{24} : COD concentration at 24th hour of the process (t=24 h)

In contrast to the results of the study-B, some decreases in COD concentrations of wastewater samples in the biosurfactant reactors at the start-up were observed during the study-C. It can be seen in Figure 5.6 that the COD concentrations in biosurfactants reactors were determined as 10500 mg/l, 11600 mg/L and 11200 mg/L at the start-up although the COD concentration for control reactor was 13000 mg/L.

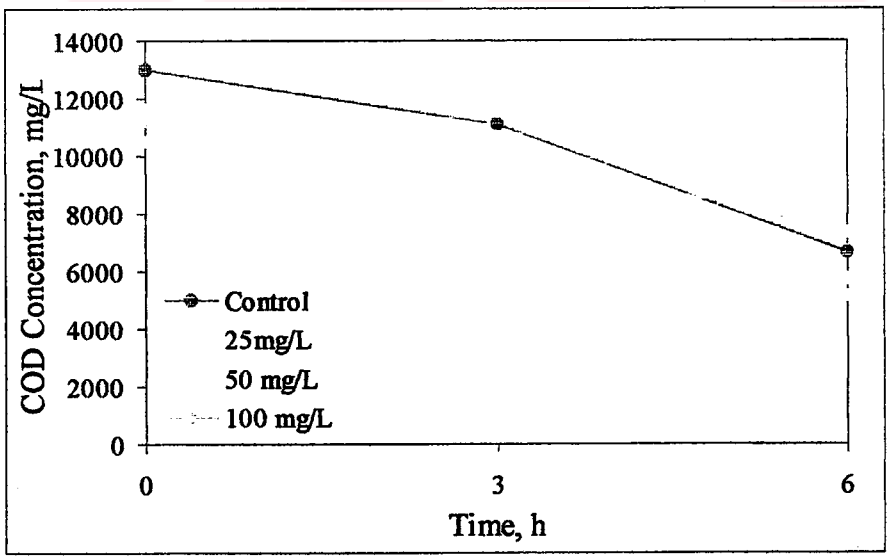

Figure 5. 6 COD concentration change vs. time for different sophorolipid-II concentrations (*Based on the results of experiments C-1 and C-2*)

Similar results were obtained other experiments also. Average COD concentrations determined in experiments C-3 and C-4 were 14800 mg/L for control reactor and 10500 mg/L, 14500 mg/L, and 13600 mg/L for biosurfactant reactors (Figure 5.9). The sorption of organic pollutants to sludge or suspended solids derived from characteristics of pollutants in wastewater samples was considered the principal mechanism causing COD decreases at the start-up.

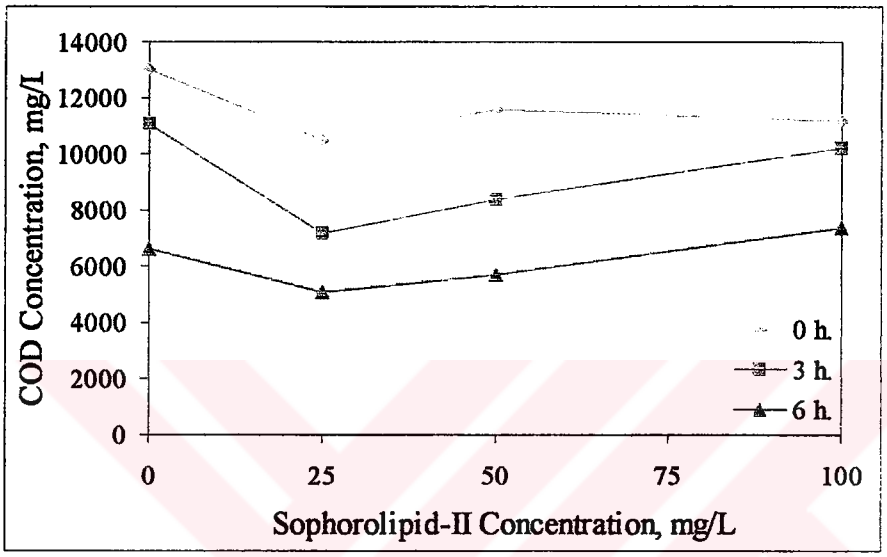

Figure 5. 7 COD concentration change vs. sophorolipid-II concentration applied in different reactors (*Based on the results of experiments C-1 and C-2*)

When 25 mg/L and 50 mg/L sophorolipid-II solution were applied to the biosurfactant reactor 1 and reactor 2, respectively, the highest decreases in COD values at the start-up were always observed in the biosurfactant reactor 1 (Table 5.10 – 5.13). In the biosurfactant reactor 2 and 3, when 50 mg/L and 100 mg/L sophorolipid-II solution was applied, respectively, higher COD concentrations were measured at the start-up. It was assumed that after COD concentrations in the biosurfactant reactor 2 and 3 decreased up to the same level as that in the biosurfactant reactor 1, the high concentrations of sophorolipid-II caused the increase of COD concentrations again.

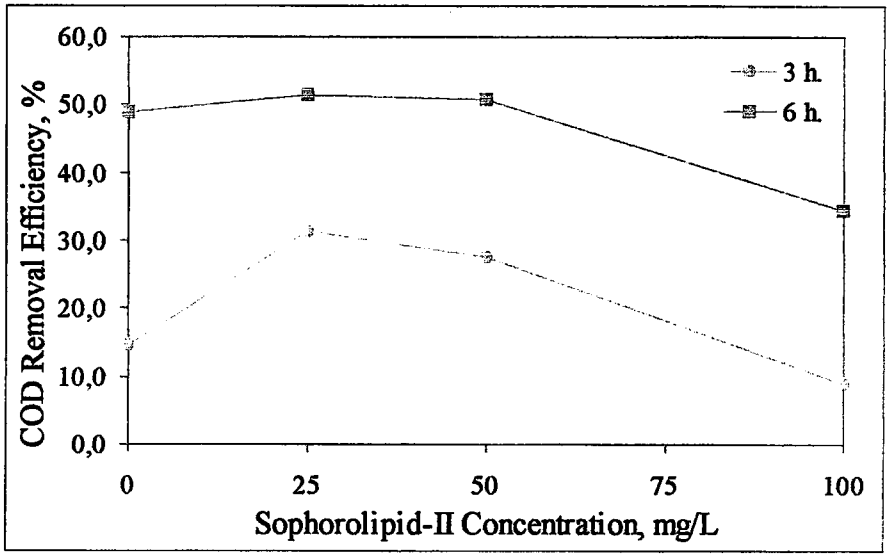

Figure 5. 8 COD removal efficiency vs. the concentration of sophorolipid-II added in different reactors (*Based on the results of experiments C-1 and C-2*)

In addition to the maximum decrease in COD concentration at the start-up in the biosurfactant reactor 1, it was also determined that the highest COD removal efficiencies for both 3rd hour and 6th hour of the process were obtained at this reactor (Figure 5.8). Figure 5.11, however, shows that COD removal efficiencies for all biosurfactant reactors (all sophorolipid-II additions) had similar values in experiments C-3 and C-4. These results indicate that the CMC value of sophorolipid-II (effective concentration) was lower than expected. For this reason, further experiments were performed by using 10 mg/L and 20 mg/L effective sophorolipid-II concentrations.

It can be seen in Figure 5.7 and 5.10 that a significant decrease in the effectiveness of sophorolipid was observed at 25 mg/L concentration. The principal reason for efficiency decrease may be explained by overdose of sophorolipid-II. Although surfactants have effectiveness when their aqueous concentrations exceed the their CMC values, the very high concentrations have been reported have some inhibitory effects.

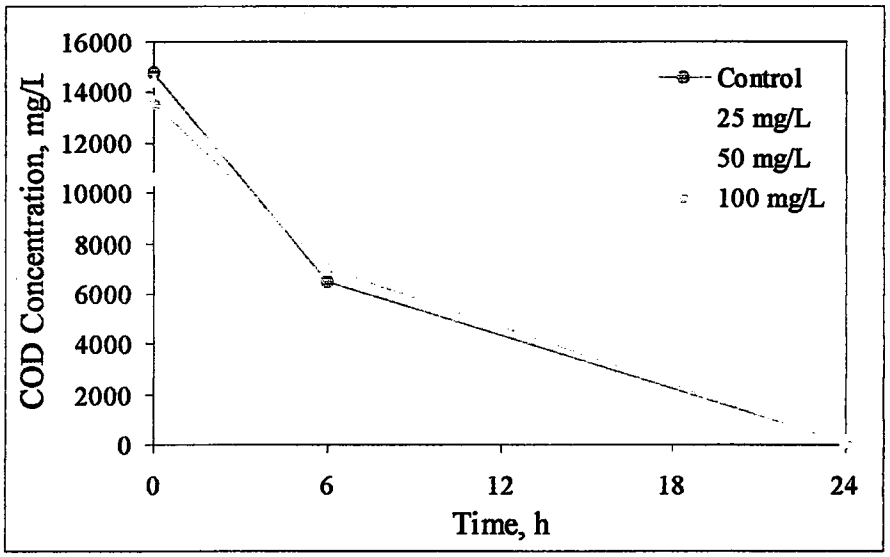

Figure 5. 9 COD concentration change vs. time for different sophorolipid-II concentrations (*Based on the results of experiments C-3 and C-4*)

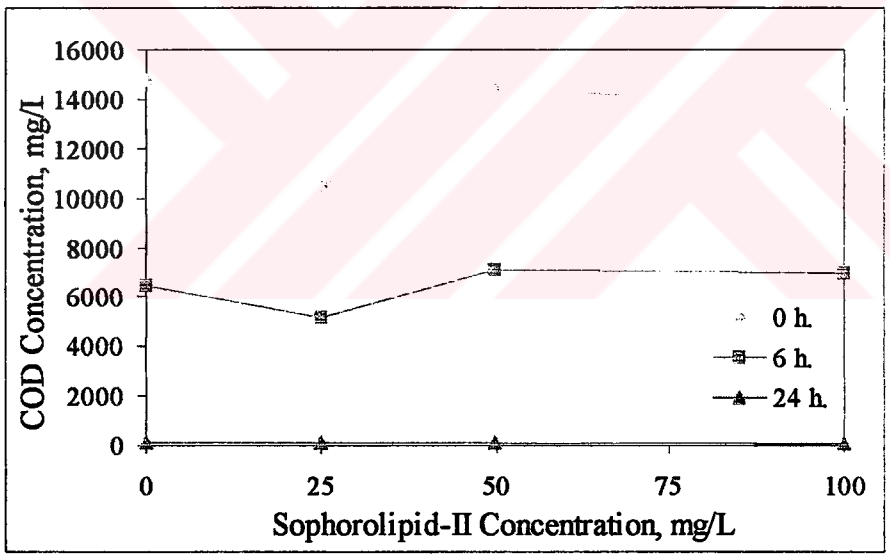

Figure 5. 10 COD concentration change vs. sophorolipid-II concentration applied in different reactors (*Based on the results of experiments C-3 and C-4*)

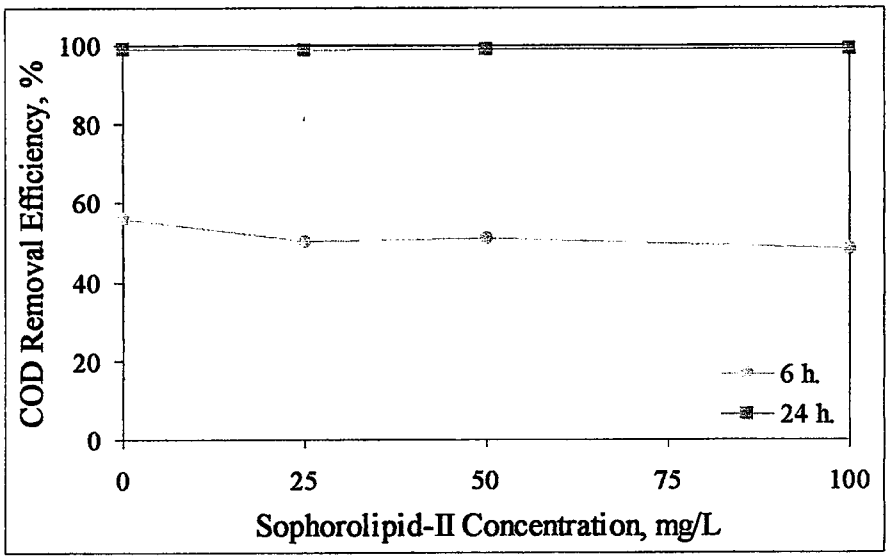

Figure 5. 11 COD removal efficiency vs. the concentration of sophorolipid-II added in different reactors (*Based on the results of experiments C-3 and C-4*)

In experiments C-5, C-6 and C-7 similarly a significant decreases in COD concentrations in the biosurfactant reactors were observed at the start-up. As expected according to the results of the first experiments of study C, COD decrease at the start-up in biosurfactant reactor 1 (10 mg/L) have higher than that in biosurfactant reactor 2 (20 mg/L). In experiments C-5 and C-6, COD decreased as 3300 mg/L in the biosurfactant reactor 1 whereas a COD decrease of 2300 mg/L in the biosurfactant reactor 2 was observed. Therefore, the higher COD removal efficiencies were also determined in biosurfactant reactor 1 in both 6^{th} hour and 24^{th} hour of the process (Table 5.14, 5.15, and 5.16).

It can be clearly seen in Figure 5.12 and 5.15 that the use of 10 mg/L sophorolipid-II resulted in lower COD values throughout the process. Figure 5.13 , 5.14, 5.16 and 5.17 also confirm the results of study B that sophorolipid-II have the maximum effectiveness about the 10 mg/L.

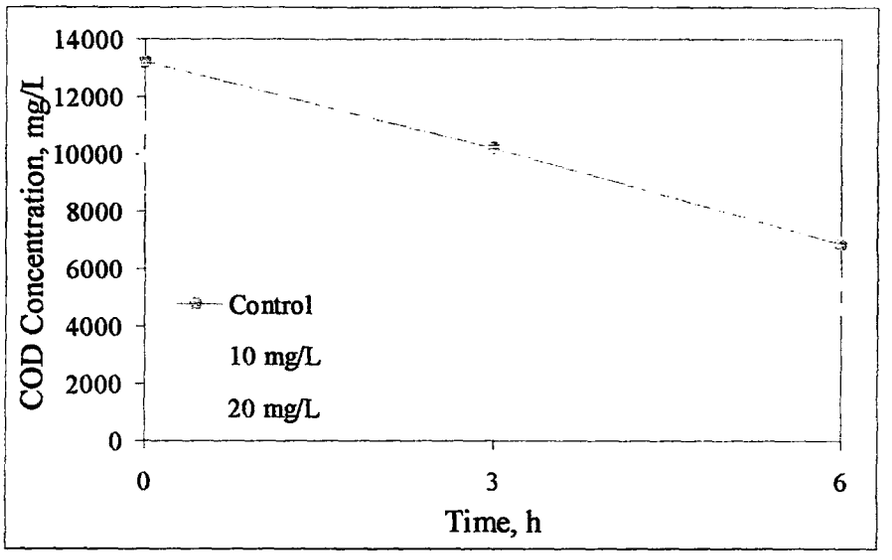

Figure 5. 12 COD concentration change vs. time for different sophorolipid-II concentrations (*Based on the results of experiments C-5 and C-6*)

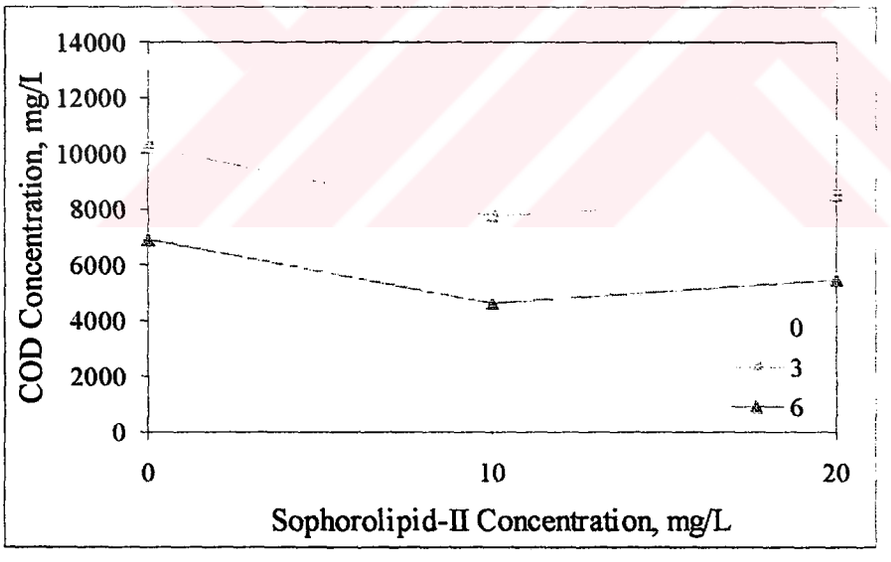

Figure 5. 13 COD concentration change vs. sophorolipid-II concentration applied in different reactors (*Based on the results of experiments C-5 and C-6*)

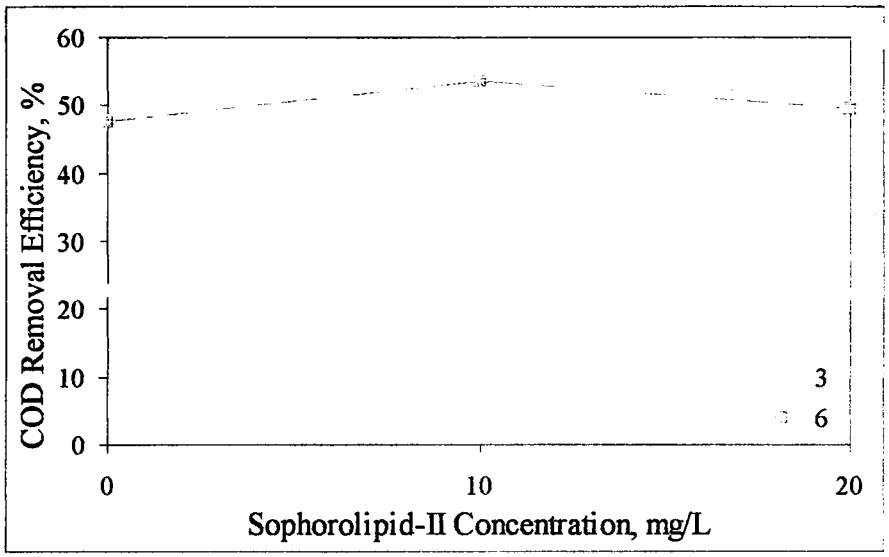

Figure 5. 14 COD removal efficiency vs. the concentration of sophorolipid-II added in different reactors (*Based on the results of experiments C-5 and C-6*)

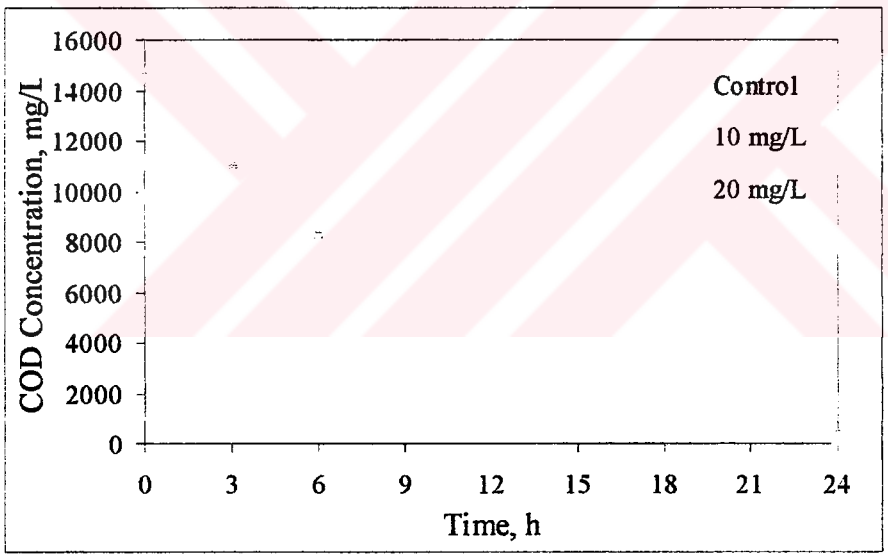

Figure 5. 15 COD concentration change vs. time for different sophorolipid-II concentrations

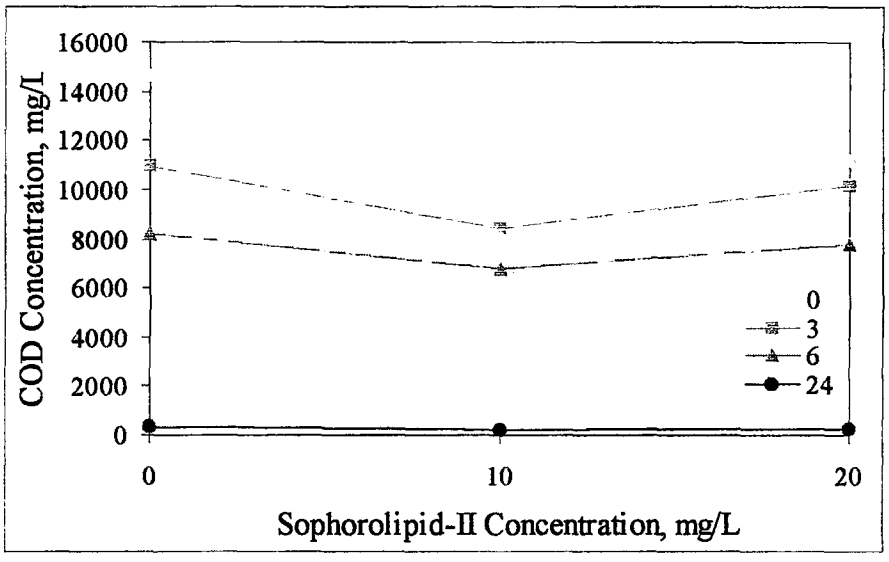

Figure 5. 16 COD concentration change vs. sophorolipid-II concentration applied in different reactors

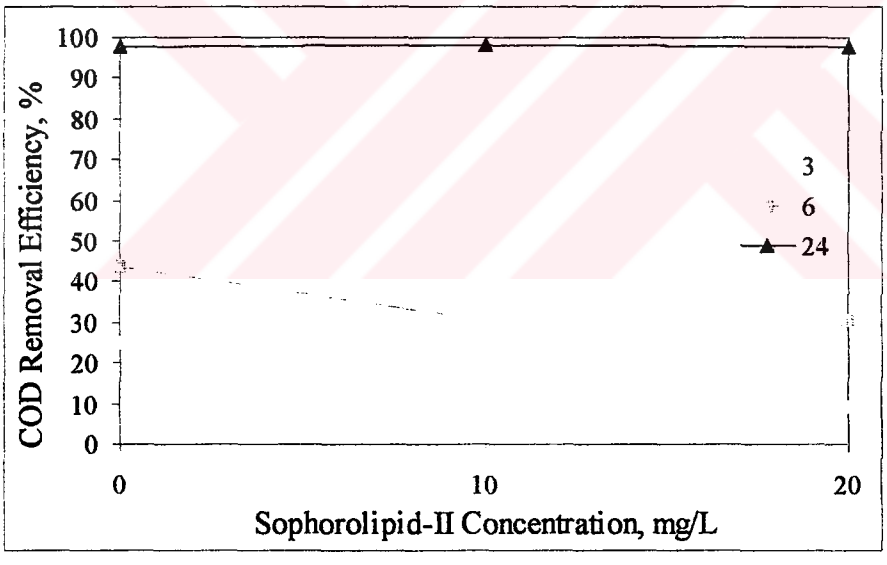

Figure 5. 17 COD removal efficiency vs. the concentration of sophorolipid-II added in different reactors

CHAPTER SIX
CONCLUSIONS AND RECOMMENDATIONS

The following conclusions may be derived from the study:

- Application of biosurfactants in the treatment of industrial wastewaters containing persistent hydrophobic organic compounds like oil and grease enhances the microbial decay by increasing the solubility of poorly soluble compounds in the wastewater to speed the rate of biodegradation of these pollutants. Therefore, enhanced removal of persistent organic pollutants by biosurfactants will facilitate meeting effluent standards for industries having difficulties in the removing these types of pollutants.

- The type, structural characteristics and concentration of the biosurfactant, and pollutant type proposed to remove are the effective parameters on the biosurfactant-aided removal of persistent organic compounds in wastewaters. These parameters should also be investigated with other biosurfactants because each different biosurfactant may have different properties. In addition to biosurfactant concentration and pollutant type, other parameters, which might affect biosurfactant-aided microbial decay, should be examined to determine optimum conditions and better removal efficiencies.

- According to the results of this study, it can be completely proven by further investigations that biosurfactant-enhanced degradation would result in faster (reduced treatment times) and more complete degradation (the more recalcitrant compounds would be more likely to mineralize leaving no additional byproducts), improved water quality, and overall cheaper treatment costs.

- The interaction between biosurfactant and the pollutants in wastewater is a very complex phenomenon and is not the subject of this study. But it is possible to say that biosurfactants are very efficient in the removal of some certain compounds as indicated in this study. So, the effectiveness of different biosurfactants in the removal of more pollutants should be investigated in the future studies in order to determine the best combination.

- The overall production cost of the biosurfactants, which consists of biosynthesis cost and purification cost, varies very much depending on the biosurfactant type and its purity. Producing more pure biosurfactant products increases overall costs of the biosurfactants. The advantage of biosurfactants in wastewater treatment applications is that high purity is not an obligation although high purity (99% or more) is always required in the food and cosmetic applications. However, less pure biosurfactants contain some impurities, which will be further transferred into the wastewater in the wastewater treatment applications. It has been reported by some investigators that these impurities might also include some toxic compounds. Of course additional toxic substances originating from biosurfactants are not desired in the wastewater; but very small concentrations of toxic substances may be allowed since the application is wastewater treatment, and the concentration will decrease due to dilution. To prevent the transfer of the toxic substances into the wastewater, biosurfactants containing toxic impurities should be determined and necessary purification levels for different biosurfactants should be investigated in the future studies.

- It has been reported by Kosaric that the cost of biosurfactant production vary from $10/kg to $10000/g in 1988 (Appendix 1). However the rapid development in biosurfactant production and purification technologies decrease these costs. In addition, a significant part of the production costs belongs to the purification required by food and cosmetic applications. Biosurfactants, which are suitable for the environmental applications, either have lower costs or can be produced by using cheaper production techniques.

- Economical analysis should be performed in the industrial applications. Although an economical analysis is not performed in this study, it is possible to say that the application of biosurfactants will not be very expensive. Just to give an idea for the dye industry wastewater treatment considering 10 mg/L biosurfactant application, a yearly consumption of 365 kg/year of biosurfactant will be necessary for a daily flowrate of 100 m^3/d ($36500/year or $0.010 additional cost/$m^3$ treated water). This value indicates that biosurfactants have moderate costs compared with the costs of other chemicals used in the wastewater treatment.

- The economical loss in the industrial production due to the application of biosurfactants in the wastewater treatment will be very low as compared to economical gain obtained. This economical loss may be removed by using recent production technologies such as in-situ production of biosurfactants using available organic wastes.

Application of biosurfactants in industrial wastewater treatment seems promising. In order to obtain better information about the enhancement of biosurfactants, the following studies are recommended.

- Special biosurfactant types such as especially rhamnolipids and surfactin should be investigated in terms of the effectiveness in the enhanced removal of persistent organic pollutants.

- The effect of biosurfactants in the persistent organic compounds removal should also examined for single pollutants such as pesticides, and other non-aqueous phase liquids.

- Biosurfactants should be also investigated in the treatment of certain industrial wastewaters such as petrochemical, petroleum refinery, and oil industry wastewaters in order to determine industrial wastewaters in which biosurfactant may be effectively applied.

- As an alternative biosurfactant application method, the direct addition of biosurfactant-producing microorganisms into the wastewater, which would lead to in-situ production of biosurfactants in the wastewater, may also be investigated. The application of this method would also decrease the production cost of the biosurfactants.

- Gradual addition of biosurfactant solution into the wastewater should also examined in order to optimize the decay conditions. With gradual addition, the rapid increase in the amount of organic substances (COD) observed in this study would prevent.

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APPENDIX 1

Table A-1. 1 Production of Biosurfactants in Japan (December 1988)

Biosurfactant	**Price**
Spiculisporic acid	$25/kg
Rhamnolipid	$50/g
Surfactin	$1000/100 mg
Sophorolipid	Cosmetic use
N-acyl amino acid	$25/kg
Sherac	$15/kg
Sodium casein	$10/kg
Synthetic lecithin	$100/g
Chirayasaponin	$80/kg
Glycyrrhizin	$1000/kg