Biosurfactant Producing Bacteria from Mangrove Sediment: Isolation, Screening and Optimization

Dina Riska

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Biotechnology

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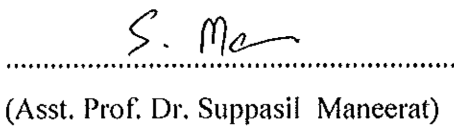
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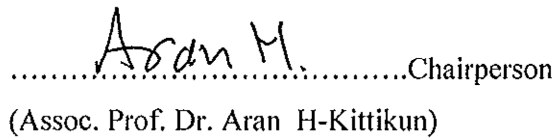
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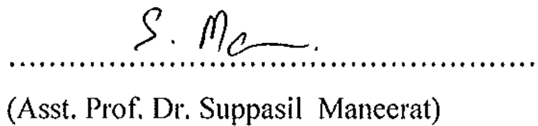
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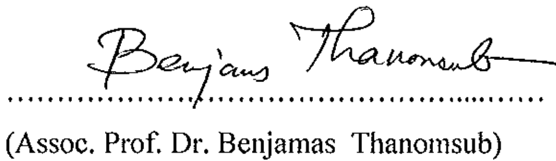
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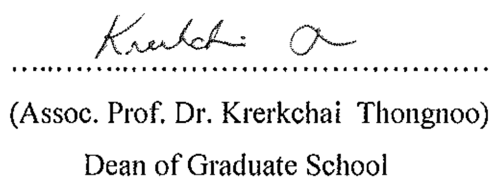
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ABSTRACT

Biosurfactant-producing bacteria were isolated from mangrove sediment collected in Thungwa District, Satun Province, Thailand. Screening of bacteria consortia degrading the WLO was carried out by a conventional enrichment-culture technique. Twenty bacterial consortia were obtained after five times transfer in minimal salt medium (MSM) containing WLO as sole carbon source. Eighty strains were isolated as WLO-utilizing bacteria after streak-plated of each bacterial consortia on MSM agar coated with WLO. Oil displacement area test and emulsification activity toward *n*-hexadecane were used to determine biosurfactant producing activity of isolated bacteria. Among those strains, 24 biosurfactant producing bacteria were obtained. The isolated bacteria strain 207-4B, showed the highest biosurfactant activity and was identified as *Achromobacter xylosoxidans* 207-4B based on 16s rDNA sequencing.

The optimum condition for biosurfactant production by *A. xylosoxidans* 207-4B was MSM containing 1% (w/v) WLO as carbon source, 0.1% (w/v) of $(NH_4)_2SO_4$ as nitrogen source with shaking speed at 200 rpm, initial pH of 7.0 and 5% of inoculum. It showed the maximum surface tension reduction (11.83 mN/m) at 1 day cultivation.

Crude biosurfactant was recovered from the culture supernatant by chloroform/methanol (2:1) extraction with a yield of 20.55 mg/mL and had critical micelle concentration of 0.025 mg/mL. The crude biosurfactant was capable to reduce surface tension in a broad pH range (2-12), temperature (30-121°C) and in the presence of NaCl up to 15% (w/v). It was stable in salt solution ranging from 0 to 0.2% (w/v) of $MgCl_2$ and $CaCl_2$. The crude biosurfactant was capable of reducing surface tension when dissolved in seawater. The broad range of pH stability,

thermostability and salt tolerance suggested that the biosurfactant from *A. xylsoxidans* 207-4B could be useful in environmental application. Preliminary chemical characterization of crude biosurfactant by TLC indicated that *A. xylosoxidans* 207-4B produced glycolipid biosurfactant.

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LIST OF ABBREVIATION

MSM	Minimal Salt Medium
CMC	Critical Micelle Concentration
EA	Emulsification Activity
mN/m	millinewton per meter
ODA	Oil Displacement Area
PAHs	Polycyclic Aromatic Hydrocarbon
Rpm	round per minute
SDS	Sodium Dodecyl Sulphate
WLO	Waste Lubricating Oil

CHAPTER 1

INTRODUCTION

Introduction

Biosurfactants are surface-active substances synthesized by living cells. Various types of biosurfactants are synthesized by a number of microbes particularly during their growth on water-immiscible substrates and the majority of biosurfactants are produced by bacteria (Carrillo *et al.*, 1996; Das *et al.*, 2008; Rismani *et al.*, 2006; Bicca *et al.*, 1999). The microbial biosurfactant is a group of amphiphilic molecules produced on living surfaces, mostly microbial cell surface or excreted extracellularly and contains hydrophobic and hydrophilic moieties, which reduce surfaces tension and interfacial tension between individual molecules at the surface and interface (Gautam and Tyagi, 2005). Biosurfactants enhance the emulsification of hydrocarbons, have the potential to solubilize hydrocarbon contaminants and increase their availability for microbial degradation. These compounds have been used to enhance oil recovery (Bordoloi and Konwar, 2008), food, pharmaceutical and cosmetic industries (Karanth *et al.*, 1999). Interest in microbial surfactants has been steadily increasing in recent years due to their diversity, environmentally friendly nature, possibility of large-scale production, selectivity, performance under extreme conditions and potential applications in environmental protection (Banat *et al.*, 2000).

Almost all surfactants currently in use are chemically derived from petroleum. However, in recent years, the interest in chemical surfactants has been substituted by the increase of microbial surfactants (Desai and Banat, 1997; Kim *et al.*, 2000). The used of synthetic surfactant to enhanced the contaminant solubility are often toxic and representing an additional source of contamination (Bognolo, 1998), but microbially produced surface-active compounds have similar properties but provide less toxic, biodegradable and can be produced *in situ* (Cha, 2000). In order to spread the application of microbial surfactants, methods of possible cost reductions have been sought.

Waste lubricating oil (WLO) is a substance that can be a threat to the environment since it can bind to organic matter, mineral particles and organisms. It

could play important role in the persistence and toxicity of oil components and also could be potentially eliminated by microbial degradation. Bacteria are the most active agents in petroleum biodegradation and play an essential role as primary degraders of spilled oil in environment (Rahman *et al.*, 2002). Many oil-degrading bacteria are capable of emulsifying hydrocarbons in solution by producing surface-active agents such as biosurfactants, which increase the adhesion of cells to the substrate (Bredholt *et al.*, 1998). In some case, the production of biosurfactant is cell-associated and facilitates hydrocarbon degradation by direct cell contact (Hommel, 1990). Biosurfactants and other natural emulsifying agents are important tools for biotreatment of hydrocarbon-polluted environments (Banat *et al.*, 2000). Bioaugmentation with degrader and/or surface-active bacteria can be efficiently applied for the bioremediation of petroleum hydrocarbon contaminated site. However, the microorganisms must adapt and be able to produce biosurfactants under the environmental conditions of the site to be remediated. Alternatively, an exogenously produced biosurfactant can be used (Banat, 1995; Desai and Banat, 1997). In addition, surface tension reduction, emulsion forming and stabilizing capacity are important properties of biosurfactant screening for potential application.

This study aimed for isolation, screening and identification of isolated bacteria from mangrove sediment that could produce surface-active compound from waste lubricating oil. The optimization of biosurfactant production by selected strain and the characteristic of the biosurfactant were also studied.

Literature Review

1. Biosurfactant

Biosurfactants, heterogeneous groups of surface-active molecules, are surfactants that are produced extracellularly or as part of the cell membrane by microorganisms. They are gaining prominence and have already taken over for a number of important industrial uses, due to their advantages of biodegradability, production on renewable resources and functionality under extreme conditions. 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 and acceptable production economics depending upon application. Biosurfactants can also be produced from industrial wastes and by-products and this is of particular interest for bulk production (Kosaric, 2001).

Most of biosurfactants are amphiphilic in nature containing hydrophobic and hydrophilic moieties, which reduce surface tension and interfacial tension between individual molecules at the surface and interface, respectively. The combinations of these hydrophobic and a hydrophilic group are very elegant in biosurfactants. The whole structures of biosurfactants have been refined and optimized during the integration process of microbial evolution (Kitamoto *et al.*, 2002).

1.1 Biosurfactant classification

Biosurfactants are categorized mainly by their chemical composition and their microbial origin. They classified according to the nature of their polar grouping. Generally, the structure of biosurfactants includes a hydrophilic moiety consisting of amino acids or peptides anions or cations; mono-, di-, or polysaccharides; and a hydrophobic moiety consisting of unsaturated, saturated, or fatty acids. Accordingly, the major classes of biosurfactant include: (Guerra-Santos *et al.*, 1986; Cooper and Goldenberg, 1987)

1.1.1 Glycolipids

Glycolipids are the type of biosurfactant that well known. They are carbohydrates in combination with long-chain aliphatic acids or hydroxyaliphatic

acids. The major glycolipid contains a disaccharide, glycerol, an amino sugar and fatty acids. The best known of glycolipids are rhamnolipids, trehalolipids and sophorolipids.

Rhamnolipid biosurfactants (Fig. 1), specifically produced by *Pseudomonas aeruginosa* in particular, offer special advantages because of their potent emulsifying activity and low critical micelle concentration (CMC) and also has a strong potential to be used in industrial and bioremediation purposes (Chayabutra *et al.*, 2001). *Pseudomonas aeruginosa* produces two types of glycolipids both containing rhamnose as the carbohydrate moiety.

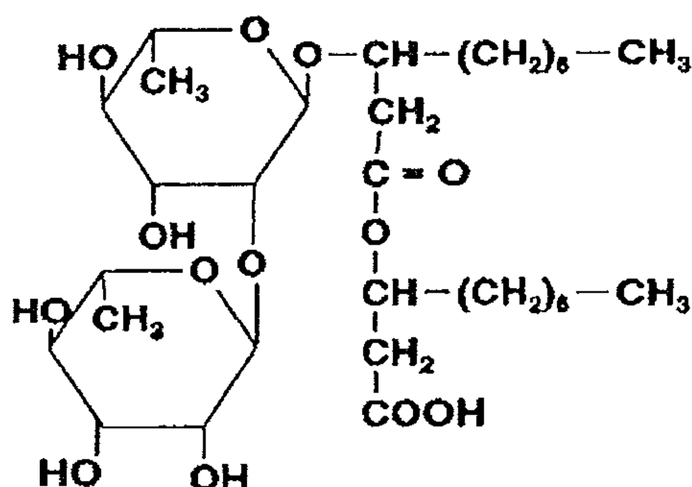


Figure 1. Structure of rhamnolipid.

Source: Desai and Banat (1997)

Trehalose lipids (Fig. 2), which are cell wall-associated biosurfactant, are certainly involved in cellular adaptation to the presence of *n*-alkanes. Trehalolipids render the cell surface hydrophobic, which may then facilitate the attachment and subsequent passive transport of the substrate into the cell (Kitamoto *et al.*, 2002). Disaccharide trehalose linked at C-6 and C-6′ to mycolic acids is associated with most species of *Mycobacterium, Nocardia* and *Corynebacterium.* Mycolic acids are long-chain, α-branched-β-hydroxy fatty acids. Trehalolipids from different organisms differ in the size and structure of mycolic acid, the number of carbon atoms, and the degree of unsaturation.

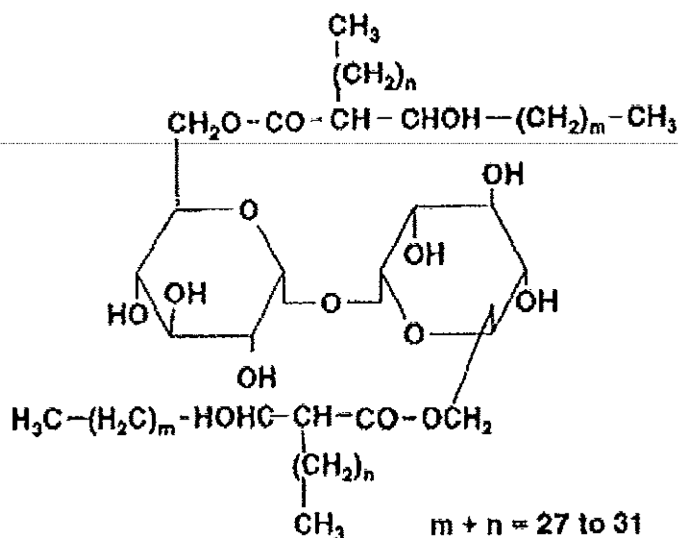


Figure 2. Trehalose dimycolate from *Rhodococcus erythropolis*.

Source: Desai and Banat (1997)

Sophorolipids (SLs) are surfactant molecules produced and secreted into the medium by the yeast *Torulopsis bombicola*, which is now classified under the genus *Candida* (Lang and Wagner, 1993). Based on the observation that the presence of n-alkanes stimulates the growth of C. *bombicola* whereas other lipophilic substrates do not, a theory that has been forwarded is that the sophorolipids are produced expressly to aid in the assimilation of alkanes (Ito *et al.*, 1980; Ito *et al.*, 1982). These compounds contain the dimeric sugar sophorose (β-1,2) glucose linked to one long chain carboxylic acid, with a hydroxyl function on the penultimate or terminal carbon. The lipid portion is connected to the reducing end through a glycosidic linkage (Rosenberg and Ron, 1999) (Fig. 3). Sophorolipids obtained by fermentation are not used as emulsifiers because they are not effective at stabilizing watering-oil emulsions. They can be modified into various derivatives having a different hydrophilic-hydrophobic balance. Their derivatives show a wide range of surface activities such as emulsifying, wetting, cleaning, and solubilizing (Fujii, 1998).

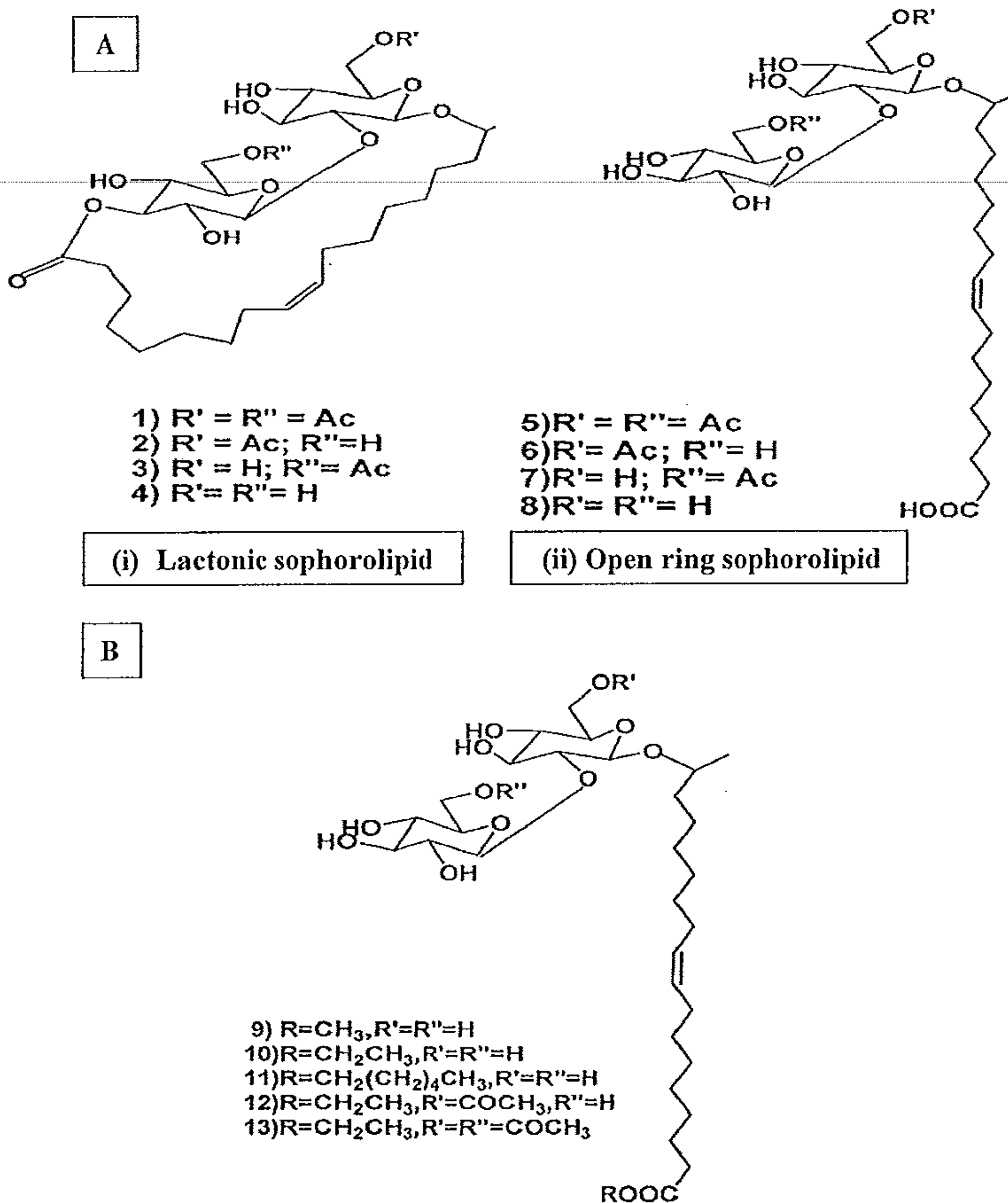


Figure 3. Structures of natural mixture sophorolipids (a) and SL analogs (b). The common names of SLs shown in the figure are as follows: mixture of compounds 1 to 8, natural mixture SL; mixture of compounds 1 to 4, lactonic SL; compound 8, open-ring nonacetylated SL; compound 9, methyl ester SL; compound 10, ethyl ester SL; compound 11, hexyl ester SL; compound 12, monoacetate ethyl ester SL; compound 13, diacetate ethyl ester SL. Ac, acetyl.

Source: Shah *et al.* (2005)

1.1.2 Lipopeptides and lipoproteins

The best-characterized lipopeptide surfactants are surfactin (Fig. 4), one of the most powerful biosurfactants, which has been isolated from several strains of *Bacillus subtilis* and *Bacillus pumilus* (Morikawa *et al.*, 1992). All of these surface-active lipopeptides consist of several amino acids covalently bound with the carboxy and hydroxy groups of β-hydroxy fatty acid. They vary in amino acid composition, position of the lactone ring, and lipid portion. The solubility and surface activity of surfactin depend on the arrangement of the amino acid residues to produce two domains, a minor hydrophilic domain and a major hydrophobic domain (Bonmatin *et al.*, 1994). Surfactin can exhibits an antiviral and antibacterial action could be the prototype of a new generation of antitumor drug because of its excellent interfacial properties (Peypoux *et al.*, 1999).

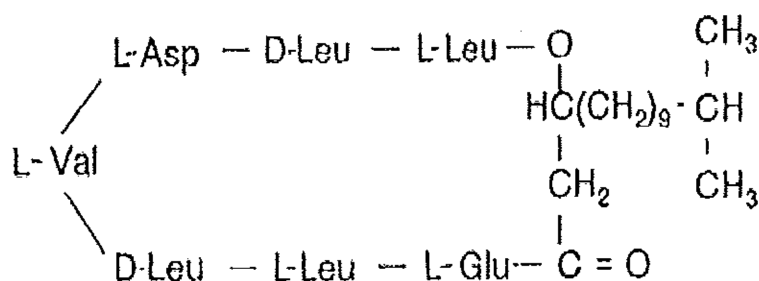


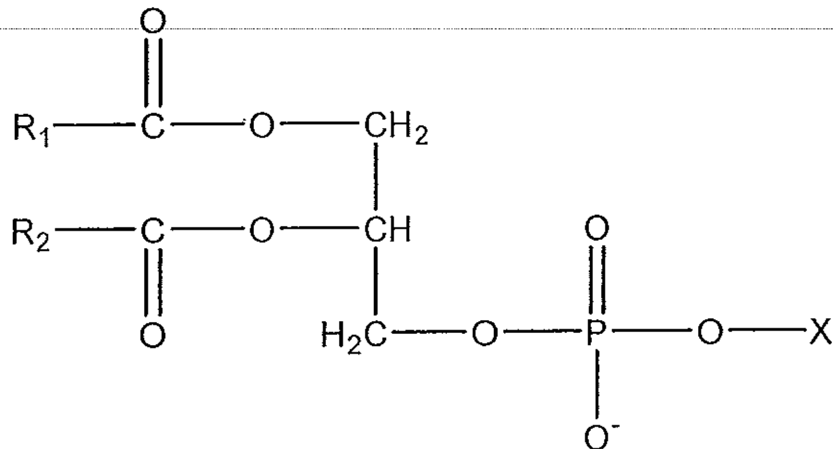
Figure 4. Structure of cyclic lipopeptide surfactin produced by *Bacillus subtilis.*

Source: Desai and Banat (1997)

1.1.3 Fatty acids, phospholipids, and neutral lipids

These biosurfactants are able to produce optically clear microemulsions of alkanes in water (Desai and Desai, 1993). The hydrophilic lipophilic balance (HLB) of fatty acids was found clearly related to the length of the hydrocarbon chain. Example of microorganisms produced these types of biosurfactant is sulphur-reducing bacteria, *Thiobacillus thiooxidans* (Desai and Banat, 1997) and *Corynebacterium lepus* (Rosenberg and Ron, 1999). Phospholipids are major components of microbial membranes. When certain C_xH_y-degrading bacteria or yeast are grown on alkane substrates, the levels of phospholipids increase greatly. Phosphatidylethanolamine (Fig. 5) is a phospholipid produced by *Rhodococcus*

erythropolis which grown on *n*-alkane that caused a lowering of interfacial tension between water and hexadecane to less than 1 mN/m and a critical micelle concentration of 30 mg/L (Kretschmer *et al.*, 1982).



R1, R2 = alkyl groups
X = hydrogen, ethylamine, inositol

Figure 5. Structure of phospholipid biosurfactant.

Source: Desai and Banat (1997)

1.1.4 Polymeric Biosurfactants

The best-studied polymeric biosurfactants are emulsan, liposan, mannoprotein, and other polysaccharide-protein complexes. Most of these are polymeric heterosaccharide containing protein. *Pseudomonas nautica* produced polymeric biosurfactant that the major constituents were proteins, carbohydrates and lipid at the ratio of 35:63:2, respectively (Husain *et al.*, 1997). *Acinetobacter calcoaceticus* RAG-1 produces a potent polyanionic amphipathic heteropolysaccharide bioemulsifier (Fig. 6) called emulsan (Rosenberg *et al.*, 1979).

Liposan is an extracellular water-soluble emulsifier synthesized by *Candida lipolytica* and composed of 83% carbohydrate and 17% protein (Cirigliano and Carman, 1984).

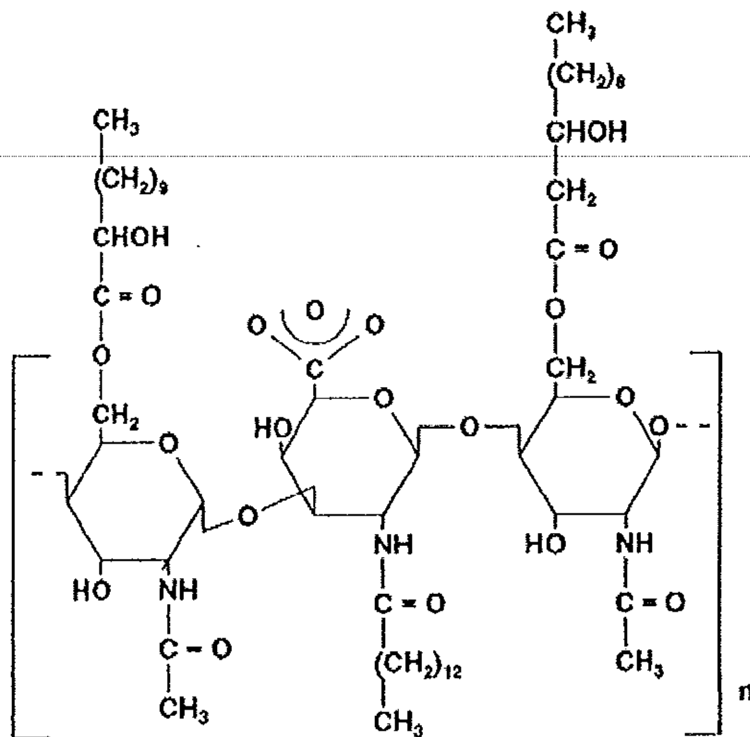


Figure 6. Structure of emulsan produced by *Acinetobacter calcoaceticus,* in which fatty acids are linked to a heteropolysaccharide backbone.

Source: Desai and Banat (1997)

1.1.5 Particulate biosurfactants

Extracellular membrane vesicles partition hydrocarbons to form a microemulsion, which plays an important role in alkane uptake by microbial cells. Microbial cell with high cell surface hydrophobicities: most hydrocarbon-degrading microorganisms, many nonhydrocarbons degrade some species of *Cyanobacteria* and some pathogens have a strong affinity for hydrocarbon-water and air-water interfaces. In such cases, the microbial cell itself is a surfactant.

The biosurfactant-producing microbes are distributed among a wide variety of genera. The major types of biosurfactants, their properties and microbial species of origin are listed in Table 1.

Table 1. Types of microbial surfactants

Type of biosurfactants		Microbial sources
Glycolipids	Rhamnolipids	*Pseudomonas* sp.
		P. aeruginosa
	Trehalolipids	*Rhodococcus erythropolis*
	Sophorolipids	*Torulopsis apicola*
Lipopeptides and lipoproteins	Peptide-lipid	*Bacillus licheniformis*
	Serrwattin	*Serratia marcescens*
	Viscosin	*P. flourescens*
	Surfactin	*B. subtilis*
	Subtilisin	*B. subtilis*
	Gramicidins	*B. brevis*
	Polymyxins	*B. polymyxa*
Fatty acids, neutral lipids and phospholipids	Fatty acids	*Candida lepus*
	Neutral lipids	*Nocardia erythropolis*
	Phospholipids	*Thiobacillus thiooxidans*
Polymeric surfactants	Emulsan	*Acinetobacter calcoaceticus*
	Biodispersan	*A. calcoaceticus*
	Mannan-lipid-protein	*C. tropicalis*
	Liposan	*C. lipolytica*
	Carbohydrate-protein-lipid	*P. fluorescens*
	Protein PA	*P. aeruginosa*

Source: Desai and Banat (1997)

1.2 Screening of potential biosurfactant-producing microorganisms

Recent advances in the field of microbial surfactants are largely attributed to the development of quick, reliable methods for screening biosurfactant-producing microbes and assessing their potential. The diverse of applications

necessitate an easy, rapid, and reliable method to detect biosurfactant production with a minimum number of false positives and/or negatives. Many assays can use to screen biosurfactant-producing microbes. Screening for biosurfactant production can be done by measuring hemolysis of red blood cells, lysis of filamentous bacteria (Jain *et al.*, 1991), reduction in surface tension (Van der Vegt *et al.*, 1991; Willumsen and Karlson, 1997), reduction in interfacial tension, the presence of emulsifying activity (Willumsen and Karlson, 1997), cell surface hydrophobicity (Neu and Poralla, 1990) and by measurement of contact angles or the wetting of water repelling materials (Jain *et al.*, 1991). Carrillo *et al.* (1996) found an association between hemolytic activity and surfactant production, and they recommended the use of blood agar lysis as a primary method to screen for biosurfactant activity since it can be used to limit the number of samples that are subsequently subjected to biosurfactant activity tests in the liquid media. However, only 13.5% of the hemolytic strains lowered the surface tension below 40 mN/m. In addition, other microbial products such as virulence factors lyse blood agar and biosurfactants that are poorly diffusible may not lyse blood cells. Thus, it is not clear whether blood agar lysis should be used to screen for biosurfactant production.

The reduction of interfacial or surface tension by biosurfactants can also determined with no specialized equipment and on a small scale, which enables facile screening of a large number of cultures (Jain *et al.*, 1991; Bodour and Miller-Maier, 1998). The reduction of surface tension and emulsifying activity do not necessarily correlate (Willumsen and Karlson, 1997). The use of methods that measure properties other than the surface activity can be problematic. The measurement of surface tension is time-consuming, which makes it inconvenient to use for screening of a large number of isolates. Development of other simple methods because they are rapid, easy to perform, reproducible and requires little specialized equipment include the following: (i) a rapid drop-collapsing test (Jain *et al.*, 1991), in which a drop of a cell suspension is placed on an oil-coated surface, and drops containing biosurfactants collapse whereas non-surfactant-containing drops remain stable; (ii) a direct thin-layer chromatographic technique for rapid characterization of biosurfactant-producing bacterial colonies as described by Matsuyama *et al.* (1991); (iii) colorimetric methods described by Siegmund and Wagner (1991) and Hansen *et*

al. (1993) for screening of rhamnolipid-producing and hydrocarbon-degrading bacteria, respectively; and (iv) estimation of the emulsification index value (E-24) by vigorously shaking culture broth samples with an equal volume of kerosene and measuring the percent emulsification after 24 h by the method of Cooper and Goldenberg (1987), which is most suitable for emulsifying biosurfactants.

1.3 Biosurfactant Production

Various researchers using different microorganisms and carbon sources (hydrocarbons, carbohydrates and vegetables oils) have synthesized biosurfactant. Olive oil mill effluent, whey from cheese making, and cassava flour water, used vegetable oils, molasses, which are industrial waste, can also be used for biosurfactant production as other carbon sources to reduce production costs.

There is a wide variety of microorganisms able to produce different kinds of biosurfactants such as bacteria, yeasts and fungi, which type and amount produced, depend on several factors. Although, there are a number of reports on the synthesis of biosurfactants by hydrocarbon-degrading microorganisms, some biosurfactants have been reported to produce on water-soluble compounds such as glucose, sucrose, glycerol, or ethanol (Passeri, 1992). Others produced biosurfactants on both water soluble and hydrophobic substrate such as *Pseudomonas aeruginosa* and *Torulopsis bombicola.*

1.3.1 Factors affecting biosurfactant production

- **Carbon source**

Organisms show great variation in their carbon requirements. Variation in the nature of the carbon sources required by living cells probably accounts for the flora of specific environments. In biosurfactant production, carbon source have important role. There are three categories of carbon source that generally use in biosurfactant production: carbohydrate, hydrocarbon and vegetable oils. Some microorganisms produce biosurfactant only using hydrophobic carbon source, hydrocarbon or vegetable oils, others only carbohydrates and still other using several carbon sources in combination or individually (Kim *et al.*, 1997). Soluble carbon source usually give effect on growth of microorganism and the amount of

biosurfactants. It also happens when insoluble carbon source was used and also can detect which material that can degrade by the microorganism. Soluble carbon sources such as glycerol, glucose and ethanol were all used for rhamnolipid production by *Pseudomonas* spp. (Desai and Banat, 1997). Sometimes combination of two types carbon source in a complex medium that used to produce biosurfactant was necessary.

Moussa *et al.* (2006) used different carbon source to produce biosurfactant by *Nocardia amarae*. They used castor oil, corn oil, olive oil, hexadecane and sunflower oil (insoluble carbon source), glycerine and sucrose were used as soluble carbon source. They found that olive oil was the best carbon source due to show the low surface tension (37 mN/m) of culture broth (Table 2).

Table 2. Effect of carbon source on biosurfactant production by *Nocardia amarae*

Carbon Source	Biosurfactant (g/L)	Surface tension (mN/m)
Hexadecane	1.0	39
Castor oil	1.0	45
Corn oil	33	49
Glycerin	9.0	46
Olive oil	1.5	37
Sucrose	1.5	47
Sunflower oil	10	42

Source: Moussa *et al.* (2006)

Benincasa *et al.* (2002) assayed different oily as carbon source for rhamnolipid production from *Pseudomonas aeruginosa* in mineral salts medium (Table 3) and all of different substrates allowed good bacterial growth and resulting different amounts of rhamnolipid. The best result showed by the residues containing soapstock substrate with 12 g/L of rhamnolipids followed by soapstock + wastewater (7.2 g/L) and olive oil (5.4 g/L). Sunflower oil, soybean oil and olein gave 4.9, 4.8 and 4.5 g/L of rhamnolipids, respectively.

Table 3. Effect of the carbon source on rhamnolipid production by *Pseudomonas aeruginosa* LBI

Carbon source	Rhamnolipid concentration (g/L)
Sunflower oil	4.9
Olive oil	5.4
Soybean oil	4.8
Olein	4.5
Soapstock	12
Soapstock + wastewater	7.2

Source: Benincasa *et al.* (2002)

High yields of sophorolipids were obtained by overcoming product inhibition in *Candida bombicola* CBS6009 through the addition of ethyl esters of rapseed oil fatty acids in D-glucose medium (Davila *et al*, 1992). Banat (1993) isolated biosurfactant-producing bacteria from 35 bacteria strain and found that only two strains AB-2 and Y12-B had a capability to reduce surface tension in their culture broth to values below 35 mN/m. Strain AB-2 grew on glucose and other organism media in addition to hydrocarbon. On 5 g/L of glucose and hydrocarbon (oleic acid 2%, v/v) which added after 8 h fermentation, the culture grew rapidly. Emulsification index (E-24) value also increased rapidly once the hydrocarbon was added to the culture broth and reached 95% after 14 h fermentation.

Kim *et al.* (1997) produced biosurfactant by *Bacillus subtilis* C9 using a carbohydrate as carbon source while a hydrocarbon substrate inhibited the production of biosurfactant. Lipopeptide biosurfactant emulsified hydrocarbons, vegetable oils and crude oils. The optimum medium for the production of C9-BS was as follows (g/L): glucose, 40; NH_4HCO_3, 13.5; K_2HPO_4, 10.5; NaH_2PO_4, 1.5; $MgSO_4 .7H_2O$, 0.5; $MnSO_4.4H_2O$, 0.05; yeast extract, 0.5. The biosurfactant C9-BS was obtained by collection of the foam that overflowed in the fermentor culture. The biosurfactant C9-BS lowered the surface tension of water to 28.5 dyne/cm, and the CMC was 40 mM. C9-BS was stable from pH 5.0 to pH 9.5, in incubation at 100°C for 1h.

Makkar and Cameotra (1997) studied the utilization of molasses for biosurfactant production by two strains of *Bacillus subtilis* (MTCC 2423 and MTCC 1427). Two strains grew and produced biosurfactant when cultured on molasses as substrate. Biosurfactant production started after 24 h and continued until 96 h of fermentation. The surface tension of medium was lowered to 23 and 31 dyne/cm, respectively, because of biosurfactant accumulation. *B. subtilis* MTCC 2423 produced biosurfactant higher than *B. subtilis* MTCC 1427. Maximal biosurfactant production in both strains was achieved in the late stationary phase.

- **Nitrogen source**

Nitrogen source is a medium constituent other than carbon source also affect the production of biosurfactants because changing the nitrogen source in the medium for the production of biosurfactant was greatly affected on the amount produced. Nitrogen source also can be an important key to the regulation of biosurfactant synthesis. Interesting observations relate to the effect of nitrogen limitation that appears to stimulate biosurfactant production and overproduction by some microorganisms has implications for bioremediation as systems are often supplemented with nitrates and phosphates to alleviate N and P limitation and enhance microbial activity.

Robert *et al.* (1989) and Abu-Ruwaida *et al.* (1991) revealed that nitrate was the best source of nitrogen for biosurfactant production by *Pseudomonas* strain 44T1 and *Rhodococcus* strain ST-5 growing on olive oil and paraffin, respectively. The production started after 30 h of growth, when the culture reached nitrogen limitation, and continued to increase up to 58 h of fermentation. Nitrogen limitation also caused increase of biosurfactant production in *P. aeruginosa*, *C. tropicalis* and *Nocardia* strain SFC-D (Ramana and Karanth, 1989; Singh *et al.*, 1990; Kosaric *et al.*, 1990).

Ilori *et al.* (2005) showed that soybean containing protein and starch was the best nitrogen source for biosurfactant production because it gave the highest concentration and stimulates the growth of *Aeromonas* spp. This observation is noteworthy for possible biosurfactant production from agricultural materials. They

also proved that among the inorganic salts tested, ammonium nitrate was the preferred nitrogen source by *Aeromonas* spp.

Moussa *et al.* (2006) studied the effect of nitrogen source on biosurfactant production by *Nocardia amarae*. They found that nitrogen source was greatly affected on the amount of biosurfactant production. The highest biosurfactant production was obtained when ammonium nitrate combination with yeast extract was applied.

1.3.2 Environmental factors

Growth conditions and environmental factors such as temperature, pH, agitation and oxygen availability also affect the production of biosurfactant.

- **Temperature**

Batista *et al.* (2005) isolated biosurfactant-producing bacteria from terrestrial and marine samples collected in areas contaminated with crude oil or its by product and they found nineteen isolates were identified as biosurfactant-producing bacteria. They found that isolate 58 produced biosurfactant at 40 h when cultivated at 25°C. The production time was reduced to 15 h when incubation temperature was increased to 35°C.

Thavasi *et al.* (2008) studied biosurfactant production by *Bacillus megaterium* in different temperature (28°C - 46°C). They found the highest biosurfactant production was obtained when temperature was 38°C and upper or lower from this point, the production of biosurfactant was decreased.

- **pH**

The pH of the medium gives an important role in biosurfactant production. The effect can be apportioned to its effect on substrate as well as its direct influences on the bacteria growth. Different microorganisms have different initial pH for medium of growth.

Bacillus subtilis MTCC 2423 could produce biosurfactant at 45°C and within the pH range of 4.5-10.5. The optimal production of biosurfactant was observed at pH 7 (Makkar and Cameotra, 2002).

Thavasi *et al.* (2008) studied the production of biosurfactant from *Bacillus megaterium* at different pH (5.0 – 9.5). They found that the higher amount of biosurfactant was obtained at pH 8.0 (1.4 mg/mL) at early stationary phase and after that it decreased.

- **Agitation rate**

Shaking culture affected biosurfactant production more than static. Rate of shaking have positive linear with biosurfactant production, on the other hand, biosurfactant production increase when the agitation rate increased. Moussa *et al.* (2006) studied the effect of agitation rate on biosurfactant production by *Nocardia amarae*. It showed that by increasing rate of shaking, the amount of biosurfactant production increased greatly. They found that the highest amount was recorded at 150 rpm.

Guilmanov *et al.* (2001) studied the effect of agitation rate on sophorose lipid (SL) production from *Candida bombicola* to diversify of the oxygenation condition and different volume of cultures. They found that using higher agitation speed between days 6 to 10 did not significantly influence SL production for the 70, 110 and 150 mL cultures. On the other hand, increasing in agitation for the 30 mL culture resulted in decreased of biosurfactant production. The increasing agitation speed also affected to the profile of biomass concentrations.

1.4 Recovery of Biosurfactants

Biosurfactant recovery depends on its ionic charge, water solubility and location (intracellular, extracellular or cell bound). The most commonly used biosurfactants recoveries listed in Table 5.

Table 4. Biosurfactants recovery methods

Downstream recovery procedure	Biosurfactant property	Instrument/ setup required	Advantages
Acid precipitation	Biosurfactants become insoluble at low pH values	No set-up required	Low cost, efficient in crude biosurfactant recovery
Organic solvent extraction	Biosurfactants are soluble in organic solvent due to the presence of hydrophobic end	No set-up required	Efficient in crude biosurfactant recovery and partial purification, reusable nature
Ammonium sulfate precipitation	Salting-out of the polymeric or protein rich biosurfactant	No set-up required	Effective in isolation of certain type of polymeric biosurfactants
Centrifugation	Insoluble biosurfactants get precipitated because of centrifugal force	Centrifuge required	Reusable, effective in crude biosurfactant recovery
Foam fractionation	Biosurfactants, due to surface activity, form and partition into foam	Specially designed bioreactors that facilitate foam recovery during fermentation	Useful in continuous recovery procedures, high purity of product
Ion-exchange chromatography	Charged biosurfactants are attached to ion-exchange resins and can be eluted with proper buffer	Ion-exchange resins packed in columns	High purity, reusability, fast recovery
Membran ultrafiltration	Biosurfactants form micelles above their critical micelle concentration (CMC), which are trapped by polymeric membranes	Ultrafiltration units with porous polymer membrane	Fast, one-step recovery, high level of purity

Table 4. (cont.)

Downstream recovery procedure	Biosurfactant property	Instrument/ setup required	Advantages
Adsorption on polystyrene resin	Biosurfactants are adsorbed on polymer resins and subsequently desorbed with organic solvent	Polystyrene resin packed in glass columns	Fast, one-step recovery, high level of purity, reusability
Adsorption on wood-activated carbon	Biosurfactants are adsorbed on activated carbon and can be desorbed using organic solvent	No setup required, can be added to culture broth, can also be packed in glass columns	Highly pure biosurfactants, cheaper, reusability, recovery from continuous culture
Solvent extraction	Biosurfactants dissolve in organic solvents owing to the hydrophobic ends in the molecule	No set-up required	Less toxic than conventional solvents, reusable, cheap

Source: Mukherjee *et al.* (2006)

The most widely used technique in batch mode process is extraction with chloroform-methanol, dichloromethane-methanol, butanol, ethyl acetate, pentane, hexane, acetic acid, ether, etc. The commonly used technique in continuous mode process is by centrifugation.

Glycolipid produced by *Ustilago zeae* and mannosylerythritol lipid produced by *Candida* spp. that sedimented as heavy oils upon centrifugation and then extracted in either ethanol or methanol (Kim *et al.*, 1999). Tabatabaee *et al.* (2005) extracted glycolipid produced by bacterial strains by chloroform-methanol.

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Kuyukina *et al.* (2001) proposed to use methyl tertiary-butyl ether (MTBE) for extraction of biosurfactant from *Rhodococcus* culture. MTBE had high efficiency to extract crude surfactant material with high product recovery (10 g/L), efficiency (CMC) 130-170 mg/L and good functional surfactant characteristics (surface and interfacial tension, 29 and 0.9 mN/m, respectively).

Nocardia sp. L-417 strain grown with *n*-hexadecane as a carbon source produced two types of biosurfactant that had different characteristics. These biosurfactants were purified by procedures including ammonium sulphate fractionation, chilled acetone and hexane treatments, silica-gel column chromatography and Sephadex LH-20 gel filtration (Kim *et al.*, 2000).

Downstream processing in many biotechnological processes is responsible for up to 60% of the total production cost and so it can greatly increase the cost of biosurfactants. Due to economic considerations, most biosurfactant would have to involve either whole-cell spent culture broths or other crude preparations. In addition, other materials present in these preparations may affect biosurfactant activity.

1.5 Hydrocarbon degrading microorganism from mangrove sediment

In recent years, pollution on that cause by hydrocarbon needs a particular attention. Mangrove ecosystems are important inter-tidal estuarine wetlands along the coastline of tropical and sub-tropical regions, and they are frequently contaminated by PAHs generated from human activities (Bernard *et al.*, 1996). Because of inter-tidal reason, mangrove have biodiversity of microorganism, that caused by influence of land and sea. Oil spill, that containing large amount of hydrocarbon, is one of the pollution that carcinogenic and dangerous because it one of the most serious threats to marine environments, particularly in tidal flats, and gives negative impact to the ecology and human. It is absorbed and accumulated in the environment, particularly in soil. To minimizing adverse effect of environment pollution caused by PAH compound, it needs to remove quickly, effectively and safely. Mangrove sediments contained a diverse group of indigenous bacterial species capable of degrading PAHs efficiently (Ke *et al.*, 2002).

Tam *et al.* (2002) studied phenanthrene degradation by bacteria isolated from mangrove sediments in Hong Kong. They found that different surface sediments have different degree of PAH contamination and different indigenous phenanthrene-degrading bacterial consortia. These bacteria have capability to utilize phenanthrene as carbon source and degrade it. Significant positive relationship found between bacterial growth and percentages of phenanthrene degradation. Olivera *et al.* (2003) also proved that biosurfactant have capability to increasing the bioavailability of poorly soluble PAH such as phenanthrene.

Commonly PAH-degrading bacteria enriched from mangrove sediment could be used for PAHs bioremediation either as a mixed culture or as a single isolate. It proved by Guo *et al.* (2005) by isolation of PAH-degrading bacteria from mangrove sediments. The surface sediment samples collected from seven mangrove swamps in Hong Kong SAR with different degree of contamination. In each swamps, they found three bacterial consortia were enriched using phenanthrene as sole carbon source and energy source. The bacteria consortia that isolated from Sai Keng and Ho Chung sediments can degrade 90% phenanthrene and fluoranthrene in 7 days.

Kolar *et al.* (2007) isolated bacteria from marine sediments in seawater mineral salts medium with biphenyl as the only carbon source. They found that all enriched mixed cultures shown the capability to use biphenyl and it is indication that biphenyl-utilizing bacteria are widespread in coastal marine sediments. These bacteria (six out of seven isolates belonged to the genus *Rhodococcus* and one to the genus *Sphingomonas*) showed little taxonomy diversity and only *Rhodococcus* strain showed substantial polychlorinated biphenyls (PCB)-degrading activity. These bacteria might play an important role in PCB degradation in polluted marine sediment.

Petroleum is a complex mixture of many compounds such as alkanes, aromatics, resins and asphaltenes that could be potentially eliminated by microbial degradation. Polycyclic aromatic hydrocarbons (PAH) constitute an important fraction of petroleum hydrocarbons and they are considered cytotoxic, mutagenic and potentially carcinogenic among the major contaminants in soil and water environment. Biodegradation of petroleum hydrocarbons is a complex process that depends on the nature and on the amounts of the hydrocarbons present.

Biodegradation of a given hydrocarbon depends on its dispersion state. Optimal process can be obtained when the water-insoluble substrate is dissolved, solubilized, or emulsified. Biosurfactants are either extracellular compounds or localized on the cell surface. In the latter case, the microbial cell itself is a biosurfactant and adheres to hydrocarbon. By that ways, biosurfactants are capable of increasing the bioavailability of poorly soluble polycyclic aromatic hydrocarbons such as phenanthrene, resins, etc. Bacteria are the most active agents in petroleum biodegradation and play an essential role as primary degraders of spilled oil in environment (Rahman *et al.*, 2002). Many oil-degrading bacteria are capable of emulsifying hydrocarbons in solution by producing surface-active agents such as biosurfactants, which increase the adhesion of cells to the substrate (Bredholt *et al.*, 1998). In some case, the production of biosurfactant is cell-associated and facilitates hydrocarbon degradation by direct cell contact (Hommel, 1990). Biosurfactants and other natural emulsifying agents are important tools for biotreatment of hydrocarbon-polluted environments (Banat *et al.*, 2000). Bioaugmentation with degrader and/or surface-active bacteria can be efficiently applied for the bioremediation of petroleum hydrocarbon contaminated site. However, the microorganisms must adapt and be able to produce biosurfactants under the environmental conditions of the site to be remediated. Alternatively, an exogenously produced biosurfactant can be used (Banat, 1995; Desai and Banat, 1997).

1.6 Applications of biosurfactants in bioremediation

Biosurfactant have wide range of application because of it properties compare with chemical surfactant. All surfactants are chemically synthesized. The rise in popularity of this family of chemicals is chiefly attributed to the personal care sector because of increasingly innovative formulations that are focused on functionality, efficacy and desirable textures. The chemical surfactant usually toxic and non-biodegradable. It may bio-accumulate and its production, processes and by-products can be environmentally hazardous. To protect ecosystem, the use of biosurfactant performing had better result than chemical surfactant. Biosurfactants have been tested in environmental applications such as bioremediation and dispersion of oil spills, enhanced oil recovery and transfer of crude oil, are thought to be

potential candidates to replace chemical surfactants in the future, especially in the food, cosmetic, and health care industries, industrial cleaning of products and in agricultural chemicals (Banat *et al.*, 2000).

Usually a result of spillage of oil and byproducts of coal treatment processes is polycyclic aromatic hydrocarbons (PAHs). Its released in environment as a pollutants and threaten the ecology because they toxicity, carcinogenic and mutagenic. PAHs have low water solubility and the use of biosurfactant will increase the bioavailability of these hydrophobic compounds. That is why biosurfactant have potential application in bioremediation. In addition, the advantages of in situ bioremediation that done by using biosurfactant would be more cost effective and visibly more acceptable with less transport or labour.

- **Bioremediation**

One of the important ecological problems today is bioremediation of waste sites with crude oil and its processing products. The general aims of bioremediation also providing effective of cost and contaminant-specific treatments to reduce the concentration of individual or mixed environmental contaminants (Head, 1998). The common application of biosurfactant in the environmental application is especially bioremediation of oil spill.

Biosurfactant also potential in remediated of metal-contaminated sites because it can be considered environmentally benign washing agents that are capable of removing the readily extractable (soluble and exchangeable) metal fractions (Neilson *et al.*, 2003).

A rhamnolipid biosurfactant has been shown to be capable of removing Cd, Pb, and Zn from soil. Polysaccharide high molecular weight emulsifier interacts with metal by binding them, as has been shown for the binding of uranium by emulsan of *A. calcoaceticus* (Ron and Rossenberg, 2001).

Whang *et al.* (2007) investigated the potential application of two biosurfactant, surfactin and rhamnolipid for enhanced biodegradation of diesel-contaminated water and soil with a series of bench-scale experiment. They found that with addition 40 mg/L of surfactin significantly enhanced biomass growth (2500 mg VSS/L) as well as increased diesel biodegradation percentages (94%) when compared

to batch experiments with no surfactin addition (1000 mg VSS/L and 40% biodegradation percentages). Addition of rhamnolipid to diesel/water systems from 0 to 80 mg/L was increased biomass growth and diesel biodegradation from 1000 to 2500 mg VSS/L and 40 to 100%, respectively.

Nakata (2000) applied two bacterial glycolipids for the bioremediation of mono-halogenated aromatic compounds in soil. He found that a bacterial glycoglycerolipid, di-*O*-12-methyl-tetradecanoyl-3-*O*-β-D-galactopyranosyl-*sn*-glycerol could decrease the level of cellular damage induced by the effects of oxygen radical of the intermediate metabolites of 4-chloro-1-naphthol (CN) and 3-chlorodiphenylamine (CDA) for bioremediation in the aqueous phase. The cellular resistance to those CN and CDA approximately increased three fold at 100 mg/L of glycoglycerolipid concentration.

Wattanaphon *et al.* (2008) evaluated the ability of biosurfactant produced by *Burkholderia cenocepacia* BSP3 to enhance pesticide solubilization for application in environmental remediation. Glucolipid produced by this strain, have a good emulsion stability and at the concentration below and above its CMC (316 mg L^{-1}) could enhance the apparent water solubility of three pesticides, i.e. methyl parathion, ethyl parathion and trifluralin. They concluded that this glucolipid-type biosurfactant noticeably enhanced pesticide solubilization suggesting its role in environmental remediation and it could be used as a solubilizing agent for environmental remediation and synergistic treatment with bioremediation of pesticide-contaminated soil.

- **Oil Industry**

Biosurfactants are used to enhance oil recovery. Due to the potential use in the oil industry with minimum purity specification, whole-cell broth could be used. Compared with chemical surfactants, they are very selective and required in small quantities and are effective under broad ranges of oil and reservoir.

An area of considerable potential for biosurfactant application is microbial enhanced oil recovery (MEOR). In MEOR, microorganisms in reservoir are stimulated to produce polymers and surfactants, which aid MEOR by lowering interfacial tension at the oil–rock interface. To produce microbial surfactant *in situ,*

microorganisms in the reservoir are usually provided with low-cost substrates, such as molasses and inorganic nutrients, to promote growth and surfactant production. To be useful for MEOR *in situ,* bacteria must be able to grow under extreme conditions encountered in oil reservoirs such as high temperature, pressure, salinity, and low oxygen level.

Ilori *et al.* (2005) found that *Aeromonas* spp. isolated from tropical estuarine water grew on crude oil and produced biosurfactant that could emulsify hydrocarbon. The organism grew on a range of hydrocarbons that include crude oil and hexadecane while no growth was recorded on some hydrocarbons that include benzene. The biosurfactant produced by *Aeromonas* spp emulsified a range of hydrocarbons with diesel ($E_{24} = 65$) as the best substrate and hexane ($E_{24} = 22$) as the poorest.

Objectives

- To isolate and screen biosurfactant producing bacteria from mangrove sediment
- To optimize conditions for growth and biosurfactant production by selected strain
- To recover crude biosurfactant produced by selected strain
- To characterize the crude biosurfactant produced by selected strain

Scope of study

Biosurfactant producing bacteria were isolated from mangrove sediment in the Thungwa District, Satun Province, Thailand. The selected strain was identified by 16S rDNA. Then, the optimization for growth and biosurfactant production of selected strain was studied including type and concentration of carbon and nitrogen sources, initial pH, temperature, agitation rate and inoculation concentration. In addition, time course of biosurfactant production under optimized condition was studied. The obtained crude biosurfactant was characterized in order to study the stability of biosurfactant activity to pH, temperature and salts.

CHAPTER 2

MATERIALS AND METHODS

Materials

1. Sampling of mangrove sediments

Mangrove sediment from two different sites (each site collected 10 samples) in Thungwa District, Satun Province, Thailand were collected at a level of 0-5 cm in depth. The samples were stored at 4°C until used for isolation of biosurfactant producing bacteria.

2. Chemicals

Waste lubricating oil (WLO) was collected from motorcycle garage near Prince of Songkla University, Thailand. Used palm oil (UPO) was obtained from Sea Wealth factory in Songkhla Province, Thailand.

The chemicals and solvents used were analytical grade and purchased from various suppliers as follows:

Chemical	Supplier
Ammonium nitrate	Lab Scan Asia Co., Ltd., Thailand
Ammonium sulphate	Lab Scan Asia Co., Ltd., Thailand
Ammonium hydrogen carbonate	Asia Pasific Specialty Chemicals Ltd., Australia
Calcium chloride	Lab Scan Asia Co., Ltd., Thailand
Chloroform	Lab Scan Asia Co., Ltd., Thailand
D-Glucose	Ajax Finechem Pty., Ltd., Australia
di-Potassium hydrogen orthophosphate	Ajax Finechem Pty., Ltd., Australia
Ethyl acetate	Lab Scan Asia Co., Ltd., Thailand
n-Hexadecane	Fluka, Switzerland
Hexane	Lab Scan Asia Co., Ltd., Thailand
Iron (III) chloride	Ajax Finechem Pty., Ltd., Australia
Magnesium chloride	Ajax Finechem Pty., Ltd., Australia
Methanol	Lab Scan Asia Co., Ltd., Thailand

Chemical	Supplier
Nutrient Broth	HiMedia Laboratories Pvt., Ltd., India
Potassium dihydrogen orthophosphate	Ajax Finechem Pty., Ltd., Australia
Sucrose	Ajax Finechem Pty., Ltd., Australia
Sodium chloride	Lab Scan Asia Co., Ltd., Thailand
Sodium sulfate	Ajax Finechem Pty., Ltd., Australia
Yeast extract	HiMedia Laboratories Pvt., Ltd., India

3. Equipments

Scientific equipments used in this research consist of instruments as mentioned in the following list:

Equipments	Series	Supplier
Autoclave	SS-325	Tomy Seiko Co., Ltd
Centrifugation	5415 R	Eppendorf AG, Germany
Rotary evaporator	SB-XL 651	Tokyo Rikakikai, Japan
Hot air oven	UM 200	Memmert, Germany
Laminar air flow	Hotpack	Scientific Promotion Co., Ltd., Thailand
Light microscopy	YS100	Nikon Corporation Japan
pH meter	420A	Orion Research Inc, USA
Spectrophotometer	GENESYS^{TM}10	Thermo Electron Corporation, USA
Tensiometer	OS	Torsion balance supplier
Vortex mixer	MS 1	IKA-Works, Inc., USA

4. Medium

Samples were grown in Minimal Salt Medium (MSM) containing (g/L): K_2HPO_4, 0.8; KH_2PO_4, 0.2; $CaCl_2$, 0.05; $MgCl_2$, 0.5; $FeCl_3$, 0.01; $(NH_4)_2SO_4$, 1.0; NaCl, 5.0 and distilled water to 1000 mL. The pH was adjusted to 7.0 (modified from Yin *et al.*, 2005).

5. Culture condition

The bacteria were grown in MSM (as described in section 4) with 1% (w/v) of WLO as sole carbon source (isolation part) and either WLO or glucose (screening

part) in 250 mL flask and shaking (150 rpm) and incubated at room temperature for 5 days or oil emulsion is observed.

6. Analytical Methods

6.1 Growth measurement

Growth was determined from cells in the culture by total cell protein measurement using Lowry method (Lowry *et al.*, 1951). Prior to analysis, cells was collected by centrifugation at 10,000 g for 30 min then wet cells was resuspended in 0.1 N NaOH, 30 min, at 100°C (Shabtai, 1990).

6.2 Emulsification activity (%EA)

Emulsification activity was measured according to the method of Cooper and Goldenberg (1987) with a slightly modification. To 1 mL of culture supernatant, 1 mL of *n*-hexadecane was added and vortexed at high speed for 2 min. The mixture was allowed to stand for 10 min prior to measurement. The emulsification activity was defined as the height of the emulsion layer divided by the total height and expressed as percentage.

6.3 Oil displacement area (ODA)

Ten μL of weathered crude oil was placed on the surface of distilled water (40 mL) in a petri dish (250 mm in diameter). Then, 10 μL of the culture supernatant was gently put on the centre of the oil film. The diameter of clear halo area visualized under visible light was measured and calculated as described by Morikawa *et al.* (2000).

6.4 Surface tension measurement

The surface tension of samples was measured with Ring Tensiometer (Kim *et al.*, 2002). Ten mL of culture supernatant was put into 50 mL beaker and placed onto the Tensiometer plateform. To measure the surface tension (mN/m), a platinum wire ring was submerged into the solution and then slowly pulled through the liquid-air layer interface. During each measurement, the platinum wire ring was rinsed three times with distilled water, three times with acetone and allowed to dry before measuring.

7. Statistical analysis

Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple-range test. Statistical analysis was performed using Statistical Package fro m Science (SPSS 10.0 for Windows, SPSS Inc, Chicago, IL).

Methods

1. Isolation and screening of biosurfactant producing bacteria consortia

Enrichment and isolation of biosurfactant producing bacteria was carried out using WLO as a sole carbon and energy source. Initially, a bacterial consortium was enriched by adding 1 g of soil sample to 50 mL MSM containing WLO (1%, w/v) in 250 mL flask and shaking (150 rpm) at room temperature for 5 days or until oil emulsion is observed. Then 1 mL aliquot of the culture broth was transferred to fresh 50 mL MSM added with WLO (1%, w/v) in 250 mL flask and incubated for 5 days or until oil emulsion is observed. The subculturing was repeated 5 times. The biosurfactant producing bacterial consortia were stored at -20°C in MSM medium containing 25% glycerol.

The dominant bacteria of biosurfactant producing bacteria was isolated by spreading 0.1 mL of ten fold dilutions on Petri dished containing WLO in MSM agar. The various colonies were randomly picked and isolated in pure cultures.

One loop full of pure culture was inoculated in 5 mL MSM containing WLO in test tube and shaking (200 rpm) at room temperature for 3 days. Then 1 mL aliquot of the culture broth was transferred to fresh 50 mL MSM containing WLO in 250 mL flask and incubated for 5 days or until oil emulsion is observed. Culture supernatant was obtained by centrifugation (8500 rpm, 15 min, and 4°C). WLO residue was eliminated from culture supernatant by hexane extraction. Then emulsification activity and ODA was measured.

For obtained cell pellet, cell was washed twice with normal saline (0.85% NaCl) and then washed cells were finally resuspended in normal saline with the equal volumes to supernatant. Cell-bound biosurfactant activity was measured by emulsification activity and ODA.

The strain that exhibited either the highest emulsification activity or the lowest surface tension was selected for further experiment.

2. Identification of selected strain

The selected strain was characterized by microscopic observation, Gram stain, catalase test and by 16S rDNA sequence. The BLAST program (http://www.ncbi.nlm.nih.gov/BLAST) was used to analyze sequence homology (Thompson *et al.*, 1994).

3. Optimization for biosurfactant production

Starter culture preparation: One loop full of pure culture was inoculated in 5 mL WLO-containing MSM in test tube and shaking (200 rpm) at room temperature for 3 days. Then 1 mL aliquot of the culture broth was transferred to fresh 50 mL MSM containing WLO in 250 mL flask and incubated with the same condition as previously described in section 1.

3.1 Effect of carbon source on biosurfactant production

The 1% of different soluble carbon sources (molasses, glucose and sucrose) and 1% of different insoluble carbon sources (WLO and UPO) were used. MSM with different carbon source were inoculated with 5% of starter culture. Growth and biosurfactant production studies were performed in 50 mL cultures in 250 mL shaken flasks (200 rpm) at room temperature for 3 days.

Growth was monitored by measuring total cell protein. Biosurfactant production was monitored by measuring emulsification activity and surface tension.

The carbon source and the day of incubation rendering the highest biosurfactant production were chosen for further study.

3.2 Effect of carbon source concentration on biosurfactant production.

The MSM containing different concentrations of carbon source (0.5, 1.0, 3.0 and 5.0%) chosen from section 3.1 were used for this study.

Concentration of carbon source which gave the highest biosurfactant production was chosen for further study.

3.3 Effect of nitrogen source on biosurfactant production

The MSM consisting of carbon source with proper concentration from section 3.2 in the presence of 0.1% of inorganic nitrogen source (ammonium sulfate, ammonium hydrogen carbonate and sodium nitrate) were used for this study.

Nitrogen source showing the highest biosurfactant production was chosen for further study.

3.4 Effect of nitrogen source concentration on biosurfactant production

The MSM consisting of carbon source with proper concentration from section 3.2 in the presence of nitrogen source (0.1, 0.2, 0.3%) from section 3.3 was prepared. Different MSM was inoculated with 5% of starter culture.

Concentration of nitrogen source which gave the highest biosurfactant production was chosen for further study.

3.5 Effect of yeast extract addition on biosurfactant production

The MSM consisting of carbon source and nitrogen source with proper concentration from section 3.2 and 3.4, respectively were prepared and inoculated with combination of inorganic nitrogen source with yeast extract in the different ratio (w/v) (1:0, 1:0.5, 1:1, 0.5:1, 0:1). Different MSM was inoculated with 5% of starter culture.

The ratio of yeast extract addition which gave the highest biosurfactant production was chosen for further study.

3.6 Effect of initial pH on biosurfactant production

The MSM consisting of carbon source and combination nitrogen source with proper concentration from section 3.4 with different initial pH (5.5, 6.0, 6.5, 7.0, 7.5 and 8.0) were prepared and inoculated with 5% of starter culture.

The initial pH which gave the highest biosurfactant production was chosen for further study.

3.7 Effect of shaking speed on biosurfactant production

Starter culture 5% was inoculated in MSM comprising carbon source and nitrogen source at appropriate concentration, initial pH from section 3.7 with different shaking speeds (100, 150, 200 and 250 rpm).

Shaking speed exhibiting the highest biosurfactant production was chosen for further study.

3.8 Effect of inoculum size on biosurfactant production

Starter culture at various concentrations (1, 3, 5 and 10%) were inoculated in MSM containing carbon source and nitrogen source at appropriate concentration, initial pH and shaking speed from section 3.8.

Inoculum concentration which gave the highest biosurfactant production, was chosen for further study.

4. Time course of biosurfactant production

Starter culture at the obtained concentration from section 3.9 was inoculated in MSM consisting of carbon source and nitrogen source at appropriate concentration. Growth and biosurfactant studies were performed in 50 mL MSM in shake flask with shaking speed selected from section 3.8, respectively. Growth and biosurfactant production measurement were conducted as previously described at time interval (0, 12, 24, 48, 72, 96, and 120h). The cultivation time exhibiting the highest growth and biosurfactant production was chosen for further study.

5. Recovery of biosurfactant produced by the selected bacteria strain

The selected bacterial strain was cultivated in MSM under the optimized conditions (section 3). Culture broth at chosen time from section 4 was centrifuged at 8,500 rpm for 15 min at 4°C to get cell-free broth. The extraction methods of culture supernatant were compared as follows:

5.1 The culture supernatant was acidified with 2 N HCl to pH 2 and subsequently extracted two times with an equal volume of ethyl acetate (Rau *et al.*, 2005). The solvent layer was treated with anhydrous Na_2SO_4 to eliminate water

residual. After evaporation of ethyl acetate, the remaining material was dissolved in methanol.

5.2 The culture supernatant was extracted twice with an equal volume of mixtures of chloroform/methanol (2:1, v/v) (modified from Mercade *et al.*, 1996). The organic solvent phase was treated with anhydrous Na_2SO_4 to remove residual water. The combined extracts were concentrated to dryness with a vacuum rotary evaporator.

5.3 The supernatant was subjected to acid precipitation by adding concentrated HCl to achieve a final pH of 2.0 and allowing the precipitate to form at 4°C overnight. The pellet was colected by centrifugation (10,000 rpm, 20 min, 4°C) and washed several times with acidic water (pH 2.0 with concentrated HCl), dissolved in distilled water (pH 7.5), and lyophilized (Yakimov *et al.*, 1995).

The weight of the crude biosurfactant from 3 methods was measured and biosurfactant activity was monitored by measuring critical micelle concentration (CMC). The extraction method which gave the lowest CMC value was chosen for further characterization.

6. Characterization of crude biosurfactant

The crude biosurfactant obtained from extraction method which give the lowest CMC value was used for characterization.

6.1 Effect of pH on biosurfactant activity

The effect of pH on biosurfactant activity was done by dissolving crude biosurfactant (0.5 mg/mL) in different pH buffer; phosphoric acid buffer (pH 2.0), acetate buffer (pH 3.0-6.0), Tris-HCl buffer (pH 7.0-9.0), glycine-NaOH buffer (pH 10.0-12.0). Biosurfactant activity was tested by measuring the surface tension.

6.2 Effect of temperature on biosurfactant stability

Crude biosurfactant (0.5 mg/mL) was dissolved in distilled water and the sample was incubated at various temperatures (30, 40, 50, 60, 70, 80, 90, 100, 110 and 121°C) for 1h, except for temperature 110 and 121°C, the samples was kept for 15 min. Biosurfactant activity was then determined as described in section 6.1.

6.3 Effect of salts on biosurfactant activity

Crude biosurfactant (0.5 mg/mL) was dissolved in distilled water and NaCl was added to obtain the different final concentrations (0, 3, 6, 9, 12, 15, 18, and 21%). Biosurfactant activity was measured. $MgCl_2$ and $CaCl_2$ was added with samples to obtain the various concentration (0, 0.05, 0.1, 0.15 and 0.2%). The effect of sea water on biosurfactant activity was also investigated. Crude biosurfactant (0.5 mg/mL) was dissolved in seawater. Biosurfactant activity was tested as previously described in section 6.1.

7. Identification of crude biosurfactant composition

The obtained crude biosurfactant with the best recovery method from section 5 was used for identification of biosurfactant composition by Thin-layer chromatography (TLC). The crude biosurfactant was dissolved in methanol. Two μL of crude biosurfactant were applied to silica gel plate (silica gel 60 F_{254}, Merck, Darmstadt, Germany) and developed in the solvent system hexane/ethyl acetate (7:3, v/v). After air drying a spot from TLC plate was visualized by staining with ninhydrin, rhodamine B and *p*-anisaldehyde. The plate stained with ninhydrin and anisaldehyde were placed at 100°C for 4-5 min and then viewed directly for the presence of amino acids (Wilkinson, 1972) and sugar (Schulz *et al.*, 1991), respectively. The plate sprayed with rhodamine B was observed under ultraviolet light for the presence of lipids (McInerney *et al.*, 1990).

CHAPTER 3

RESULTS AND DISCUSSION

1. Isolation of biosurfactant producing bacterial consortia from mangrove sediment

The pH of mangrove sediment samples were 6.07 – 7.85 (Table 6). The result was in accordance with previous study that pH of mangrove sediment was 7.71 (Suprayogi and Murray, 1999).

In the enrichment culture technique, WLO was used as a sole carbon and energy sources. Microorganisms in the mangrove sediment were able to utilize WLO as carbon source since the turbidity and emulsion of WLO was observed after 3 days of incubation. After five times transferred, 20 WLO degrading consortium from 20 mangrove sediment samples were obtained.

Table 5. The pH of each mangrove sediment soil samples

Sample	pH	Sample	pH
101	7.04 ± 0.15	201	7.82 ± 0.07
102	6.67 ± 0.25	202	7.63 ± 0.04
103	6.61 ± 0.18	203	7.72 ± 0.07
104	6.56 ± 0.21	204	6.07 ± 0.05
105	7.85 ± 0.06	205	7.67 ± 0.06
106	7.06 ± 0.17	206	6.21 ± 0.45
107	7.56 ± 0.11	207	7.78 ± 0.07
108	7.68 ± 0.32	208	7.77 ± 0.08
109	7.07 ± 0.11	209	6.97 ± 0.31
110	6.76 ± 0.15	210	6.73 ± 0.59

2. Screening of biosurfactant producing bacteria

From 20 WLO degrading consortium, 80 isolates were isolated as WLO-utilizing bacteria on MSM agar coated with WLO as carbon source. Those 80 bacterial isolates were cultivated in MSM containing WLO broth and MSM containing glucose broth. Then, the emulsification activity (EA) and oil displacement area (ODA) were examined for screening of biosurfactant producing bacterial strain.

It was found that supernatant of 15 isolates exhibited ODA activity when cultivated in MSM added with WLO as carbon source (Fig. 7A). Strain 207-4B showed the highest ODA activity at 0.356 cm^2. Supernatant from only 6 isolates exhibited ODA activity when glucose was used as carbon source in MSM medium and strain 210-4B exhibited the highest ODA activity with 0.283 cm^2 (Fig. 7A). In addition, supernatant of 2 strains (207-4B and 210-4B) expressed ODA activity when cultivated in either WLO or glucose as carbon source. For emulsification activity test, supernatant from only 3 isolates cultivated in MSM containing glucose exhibited emulsification activity and strain 207-4B showed the highest activity about 50% (Fig. 7B).

Since supernatant of 19 isolates from 80 isolates exhibited biosurfactant activities, cell-bound biosurfactant was also determined. It was found that cell suspension of 12 isolates showed biosurfactant activities. Contrast to supernatant, higher number of isolates cultivated in MSM containing glucose exhibited biosurfactant activities than cultivated in MSM containing WLO (Fig. 8). In addition, both cell suspension and supernatant of isolate 207-4B, 207-5B and 209-4A had emulsification activity when cultivated in MSM containing glucose as carbon source.

Twenty-four isolates were further characterized for morphology, Gram staining and catalase test (Table 7). Most of the strains were gram-negative rod (75%) and only one isolate was catalase negative. Generally, gram-negative bacteria were isolated from hydrocarbon-contaminated soil (Bicca *et al.*, 1999; Bodour *et al.*, 2003; Batista *et al.*, 2005) because gram-negative bacteria have outer membrane which acts as biosurfactant. Singh and Desai (1986) referred to two modes of initial interaction of hydrocarbon with the microbial cells: (a) direct contact of microorganisms with insoluble substrate (unmediated interaction) or (b) by the contact through a mediator (mediated interaction). Biosurfactants are present in various pools inside cells: as intracellular molecules, extracellularly secreted compounds or as compounds located at the cell surface (Prabhu and Phale, 2003). Together they have been known to enhance degradation by alteration in cell hydrophobicity and enhancement of dispersion of water immiscible compounds (Zang and Miller, 1992; Zang and Miller, 1994; Patricia and Jean-Claude, 1999). Biosurfactants exhibit properties as emulsifying or dispersing agents, favoring the release of hydrophobic contaminants

absorbed to organic matter or increasing the surface area of the contaminant available as substrate (Mercade *et al*, 1996).

From the supernatant results, strain 207-4B was selected for further studied according to it exhibited the highest biosurfactant activity.

Table 6. Gram stain and microscopic observation of isolates

Sample	Shape	Gram staining	Catalase test
101 -4A	Rod	-	+
101 -4B	Rod	-	+
102 -4B	Rod	-	+
103 -4B	Rod	+	+
105 -4B	Rod	-	+
106 -4B	Rod	-	+
106 -5B	Short rod	+	+
107 -4A	Short rod	+	+
107 -4B	Short rod	-	+
108 -4A	Short rod	-	+
108 -4B	Rod	-	+
108 -5A	Rod	+	+
109 -4A	Rod	-	+
109 -4B	Rod	-	+
202 -4A	Short rod	+	+
202 -4B	Short rod	-	+
205 -4B	Rod	-	+
206 -5B	Rod	-	+
207 -4B	Rod	-	+
207 -5B	Rod	-	+
209 -4A	Short rod	-	-
210 -4A	Rod	-	+
210 -4B	Short rod	+	+
210 -5A	Rod	-	+

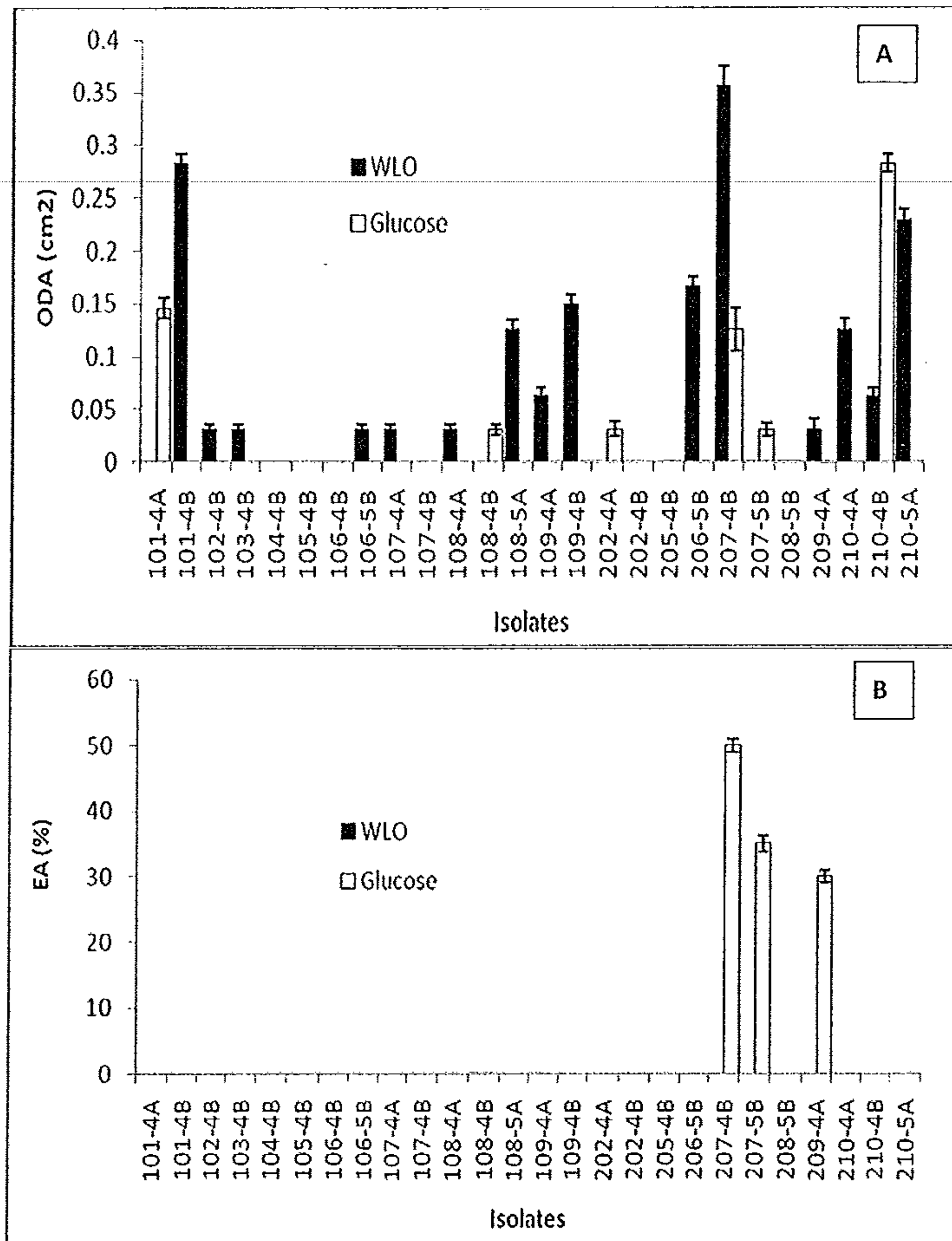

Figure 7. Oil displacement area (A) and emulsification acitivity (B) from supernatant when cultivated in MSM (pH 7.0) containing glucose or WLO as carbon source at room temperature, 200 rpm for 2 days (oil emulsion was observed).

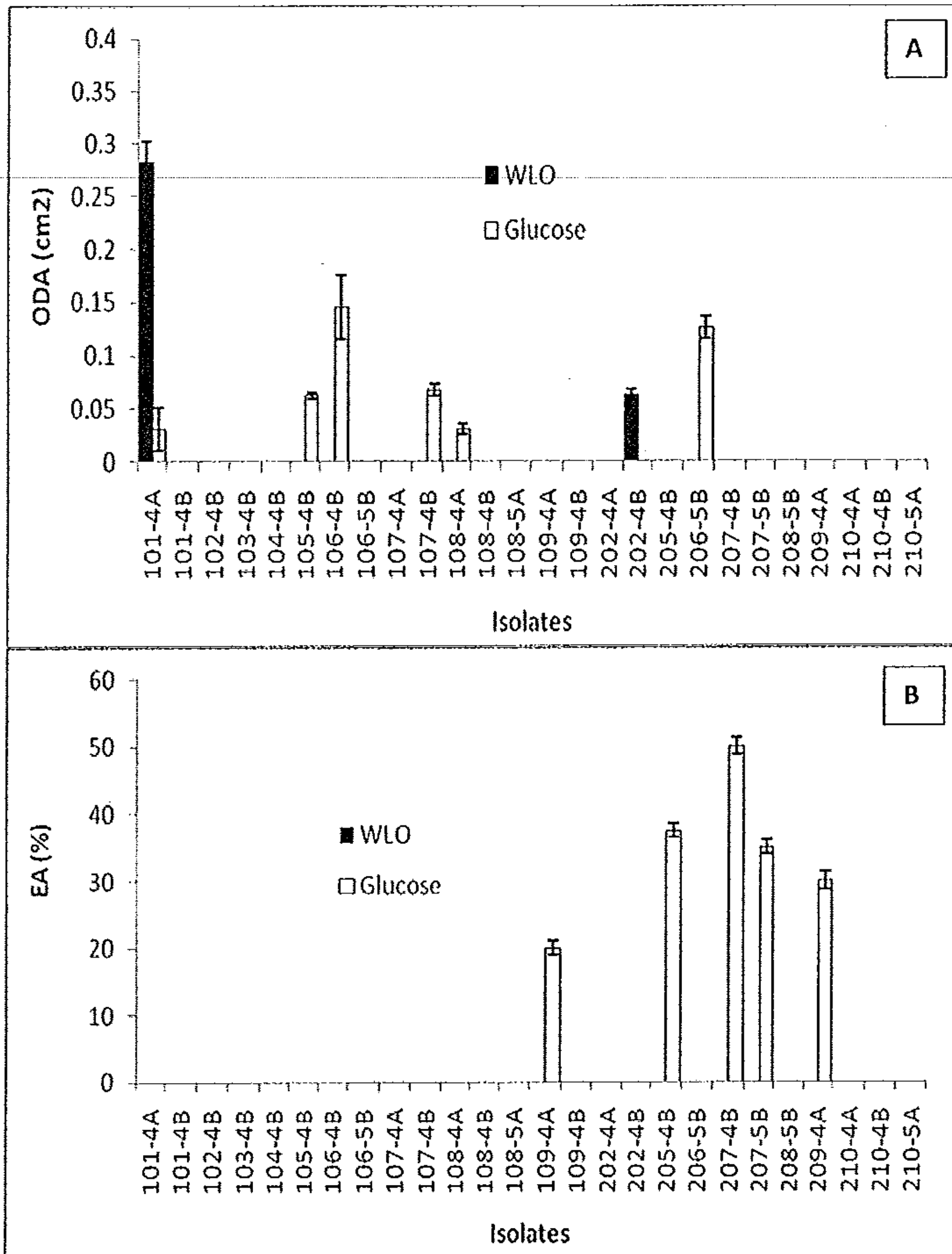

Figure 8. Oil displacement area (A) and emulsification acitivity (B) from cell-bound when cultivated in MSM (pH 7.0) containing glucose or WLO as carbon source at room temperature, 200 rpm for 2 days (oil emulsion was observed).

3. Identification of selected strain

The strain 207-4B was a Gram-negative bacilli and catalase positive. The result obtained from the sequencing of the 16S rDNA gene of the genomic DNA revealed that strain 207-4B was *Achromobacter xylosoxidans* (99%) (Fig. 9). The rDNA sequence was deposited in DDBJ/EMBL/GenBank as accession number AB481106. *A. xylosoxidans* usually isolated from hydrocarbon contaminated environments (Pavlu *et al.*, 1999; Adreoni *et al.*, 2004; Wan *et al.*, 2007; Ilori *et al.*, 2008; Pantaroto *et al.*, 2009) and degraded many types of contaminated hydrocarbon (Jencova *et al.*, 2004; Wan *et al.*, 2007; Nielsen *et al.*, 2007; Zhang *et al.*, 2007). Hydrocarbon degrading bacteria usually produced biosurfactant which improved the solubilization of hydrophobic compounds from soil or sediments (Van Dyke *et al.*, 1993; Scheibenbogen *et al.*, 1994; Herman *et.*, 1997). However, there is no biosurfactant production from *A. xylosoxidans* reported so far.

A. xylosoxidans strain AE4 grew well with ethanesulfonate (Erdlenbruch *et al.*, 2001). Yann *et al.* (2004) reported that *A. xylosoxidans* remarkably inhibited production of norsolorinic acid, a precursor of aflatoxin, potent carcinogenic and toxic substance, that produced by *Aspergillus parasiticus*. *A. xylosoxidans* also regarded as more important in the pathogenesis of lung disease in patients with cystic fibrosis (Doring and Hoiby, 2004). A case of *Achromobacter xylosoxidans*-induced pacemaker lead endocarditis has been reported by Ahn *et al.* (2004).

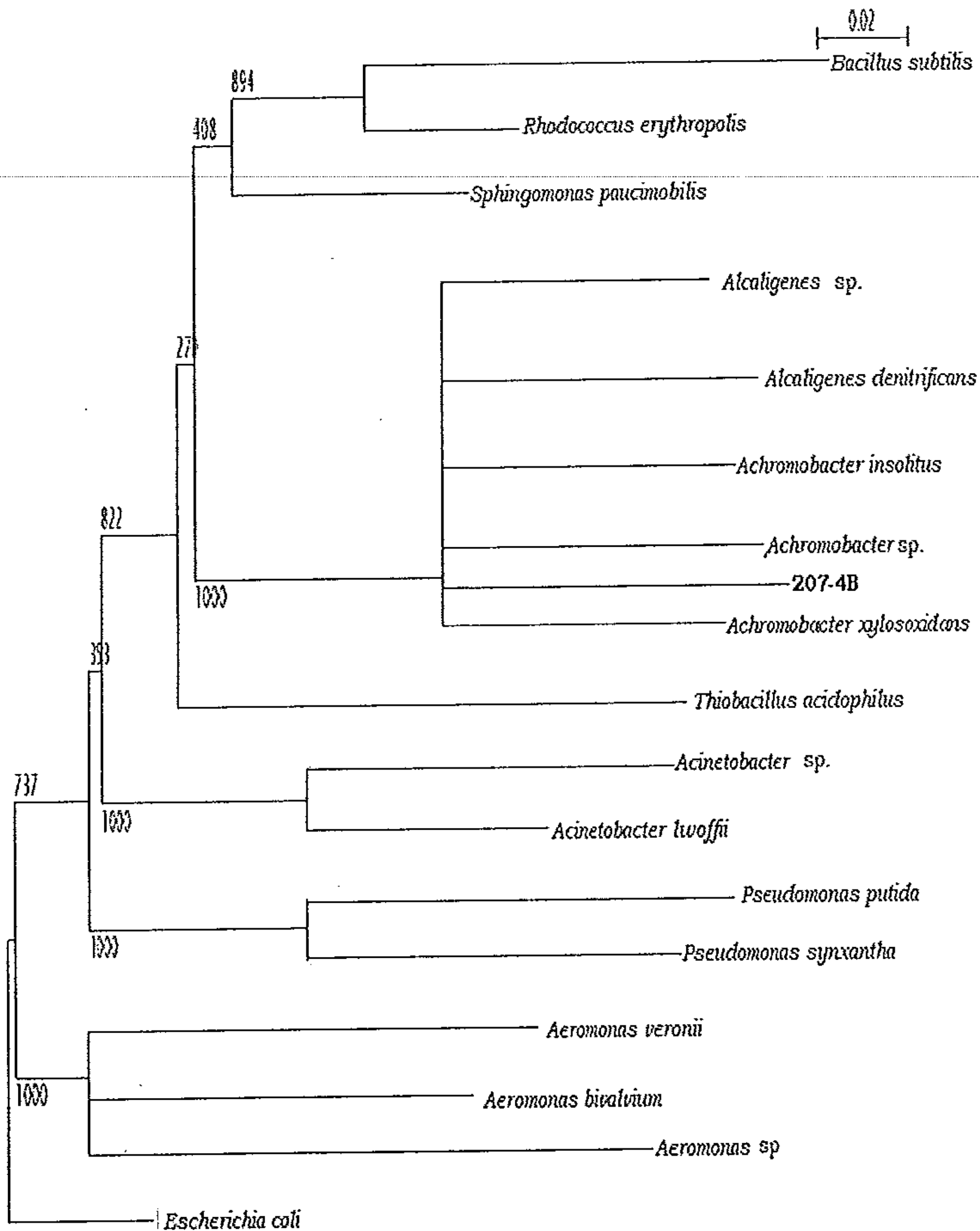

Figure 9. Phylogenetic analysis of 16s rDNA of selected bacterial isolate 207-4B.

4. Optimization of biosurfactant production

4.1 Effect of carbon source on biosurfactant production

The effect of various carbon sources on growth and biosurfactant production by *Achromobacter xylosoxidans* 207-4B is shown in Figure 10. Cultivation was performed in MSM with initial pH about 7.0 and 200 rpm at room temperature. *A. xylosoxidans* 207-4B could grow in MSM containing both soluble and insoluble carbon sources tested. However, the growth of *A. xylosoxidans* 207-4B was higher when soluble substrates was used particularly molasses (Fig. 10a). *A. xylosoxidans* 207-4B exhibited emulsification activity toward *n*-hexadecane when only glucose, molasses and sucrose were used. The emulsification activity about 62%, 40% and 50% were obtained, respectively (Fig. 10c). Although *A. xylosoxidans* 207-4B showed the highest emulsification activity, the surface tension reduction of the culture broth was slightly reduced (Fig. 10d). *A. xylosoxidans* 207-4B did not show emulsification activity when WLO was used as carbon source. However, *A. xylosoxidans* 207-4B exhibited the highest surface tension reduction of MSM from 65 mN/m to 52 mN/m (Fig. 10d) in 1 day of cultivation as well as the highest ODA activity when WLO was used (Fig. 10b). *A. xylosoxidans* 207-4B could grow in MSM containing UPO however, it did not produce biosurfactant. Therefore, WLO was selected as a carbon source for further study.

From the results indicated that types of carbon sources affected on growth and biosurfactant activity of *A. xylosoxidans* 207-4B. Generally, different substrates will generate different effect on biosurfactant activity with the same strain. In some case, addition of water-immiscible substrates resulted in induction of biosurfactant production (Pruthi and Cameotra, 2003; Thaniyavarn *et al.*, 2006; Thavasi *et al.*, 2008). However, some have been produced on water-soluble substrates such as sucrose (Makkar and Cameotra, 2002), molasses and whey (Solaiman *et al.*, 2004; Joshi *et al.*, 2008). *Bacillus circulans* isolated from marine environment as a extracelullar biosurfactant producer when water soluble substrates (glucose, sucrose, starch and glycerol) were used (Das *et al.*, 2009).

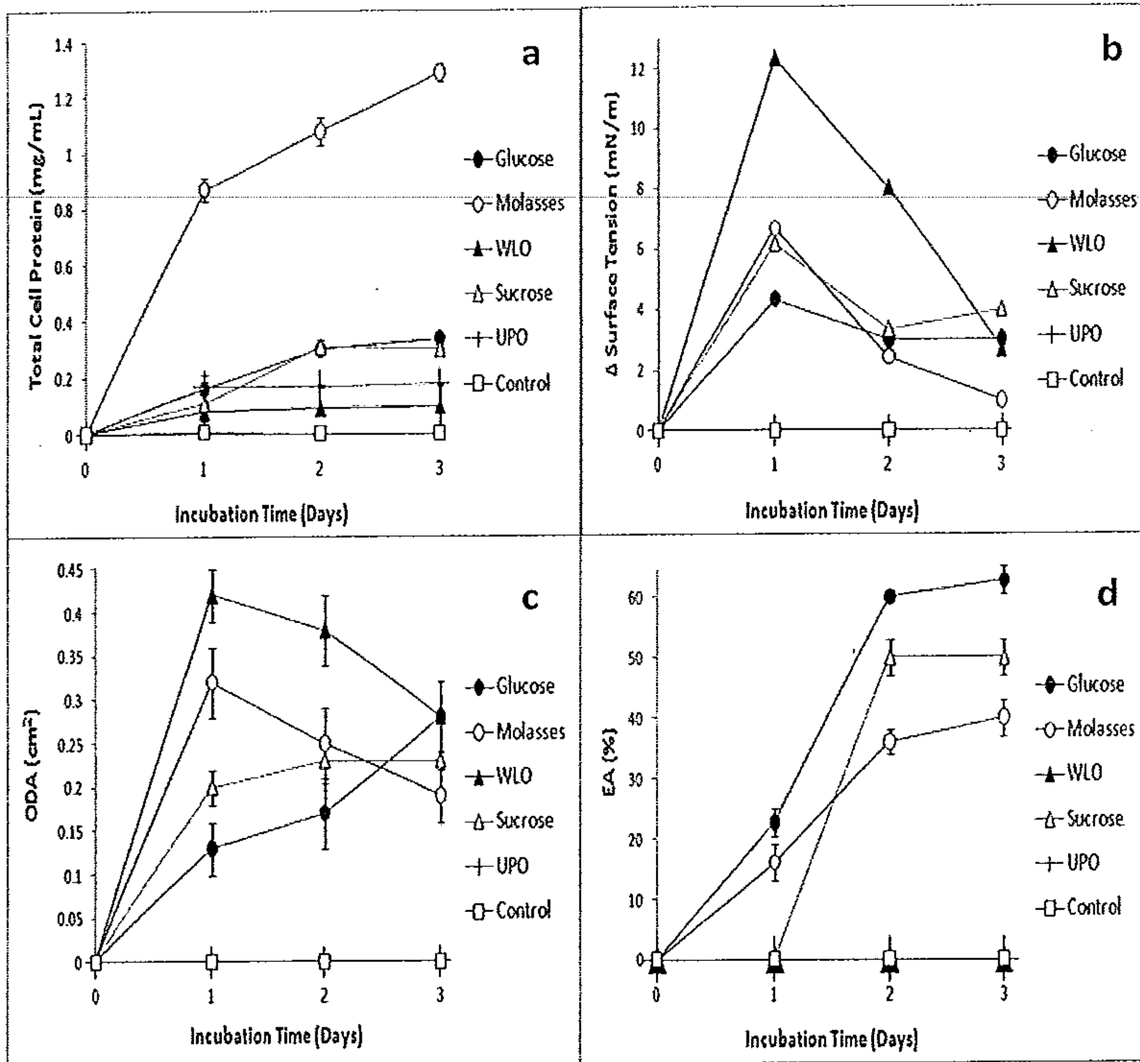

Figure 10. Effect of carbon sources (1%, w/v) on growth (a) and biosurfactant activity (b: Δ surface tension; c: ODA; d: EA) of *Achromobacter xylosoxidans* 207-4B in minimal salt medium, initial pH 7.0, at room temperature.

4.2 Effect of carbon source concentration on biosurfactant production

The result showed that increasing the concentration of WLO could accelerate the cell growth of *A. xylosoxidans* 207-4B, as evident from the total cell protein (Fig. 11b). The biosurfactant production from *A. xylosoxidans* 207-4B increased significantly as the WLO concentration increased up to 1%. The surface tension of the medium was reduced from 65 mN/m to 53.8 mN/m (Fig. 11a). When the concentration of WLO increased higher than 1%, biosurfactant activity decreased significantly, it may assume that *A. xylosoxidans* 207-4B produced another compound

that inhibited biosurfactant activity since the reduction of surface tension was decreased.

From the results, 1% of WLO was selected as a carbon source concentration for further study.

4.3 Effect of nitrogen source on biosurfactant production

The effect of nitrogen sources on biosurfactant production and growth by *A. xylosoxidans* 207-4B in the 1% (w/v) MSM containing WLO is shown in Figure 12. Growth and biosurfactant production of *A. xylosoxidans* 207-4B in the MSM with inorganic nitrogen sources such as NH_4HCO_3 and $NaNO_3$ at 0.1% (w/v) was compared with control (without nitrogen source) and the original MSM which added 0.1% (w/v) of $(NH_4)_2SO_4$. The amount of nitrogen in each nitrogen source was 1.604, 2.240 and 1.939 mmol for $(NH_4)_2SO_4$, NH_4HCO_3 and $NaNO_3$, respectively.

It was found that the highest cell growth and biosurfactant activity was obtained in the medium with $(NH_4)_2SO_4$ as a nitrogen source. Meanwhile, when cultivated in MSM without nitrogen source, *A. xylosoxidans* 207-4B exhibited the lowest cell growth and biosurfactant activity was not detected.

Biosurfactant production is influenced by the type of nitrogen source that present in the medium (Haba *et al.*, 2000). Type of nitrogen source is distributed among a wide variety of genera. Sodium nitrate was the best nitrogen source for growth and biosurfactant production for *Pseudomonas fluorescens* (Abouseoud *et al.*, 2008). According to the results, $(NH_4)_2SO_4$ was selected as a nitrogen source for further study.

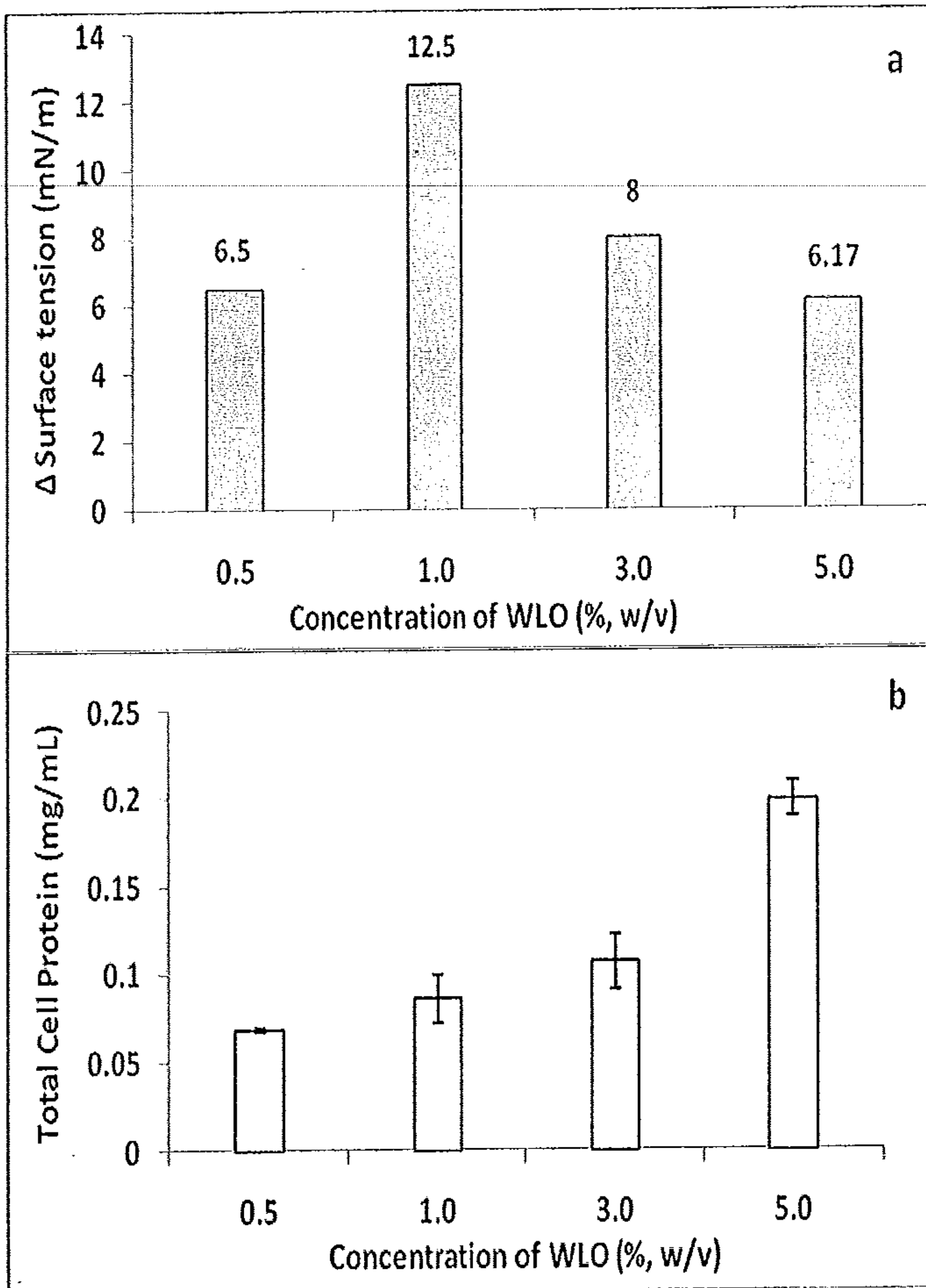

Figure 11. Effect of WLO concentration on biosurfactant activity (a) and growth (b) of *A. xylosoxidans* 207-4B in minimal salt medium, initial pH 7.0, at room temperature for 1 day.

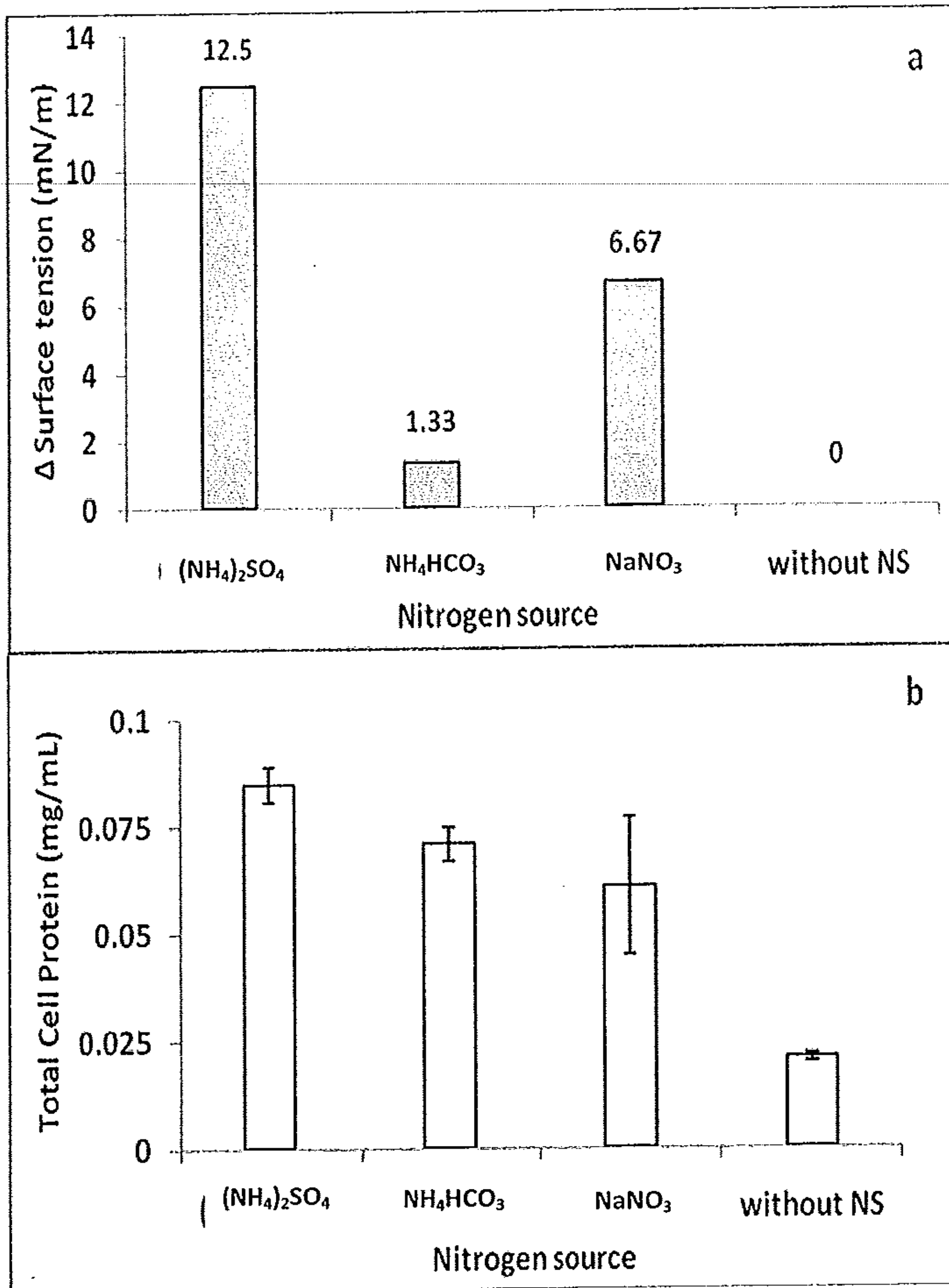

Figure 12. The effect of nitrogen sources (0.1%, w/v) on biosurfactant activity (a) and growth (b) of *A. xylosoxidans* 207-4B in minimal salt medium, initial pH about 7.0, at room temperature for 1 day.

4.4 Effect of nitrogen source concentration on biosurfactant production

The effect of $(NH_4)_2SO_4$ concentration on biosurfactant production by *A. xylosoxidans* 207-4B in the MSM containing 1% of WLO as carbon source is shown in Figure 13. $(NH_4)_2SO_4$ at the concentration of 0.1%, 0.2% and 0.3% (w/v) contained nitrogen 1.604, 3.208 and 4.812 mmol, respectively. It was found that growth of *A. xylosoxidans* 207-4B was decreased when increasing the concentration of $(NH_4)_2SO_4$. The highest cell growth (0.080 ± 0.004 mg/mL) and reduction of surface tension (65 mN/m to 52.83 mN/m) was obtained when MSM was supplemented with 0.1% of $(NH_4)_2SO_4$. The reduction of surface tension and cell growth were not significantly different when 0.2% and 0.3% of $(NH_4)_2SO_4$ were used. Thus, 0.1% of $(NH_4)_2SO_4$ was selected for further study. The result was in accordance with previous study that stimulation of rhamnolipid production by *Pseudomonas aeruginosa* take place under nitrogen-limited conditions (Haba *et al.*, 2000). In addition, production of rhamnolipid by *P. aeruginosa* 44T1 was started when nitrogen concentration reached low values (below 0.5 g/L) (Manresa *et al.*, 1991).

4.5 Effect of yeast extract on biosurfactant production

The highest biosurfactant production from *Nocardia amarae* was obtained when ammonium nitrate combination with yeast extract was applied (Moussa *et al.*, 2006). However, in the present study the addition of yeast extract did not give positive effect on biosurfactant activity and growth of *A. xylosoxidans* 207-4B (Fig. 14). Ammonium sulfate played an important role in the growth of cells as well as biosurfactant activity. The cells growth decreased significantly when $(NH_4)_2SO_4$ was reduced (Fig. 14b). The highest surface tension reduction was obtained at ammonium sulfate : yeast extract in the ratio 1 : 0.5 and followed by ammonium sulfate : yeast extract ratio 1 : 0 (Fig. 14a). Huszcza and Burczyk (2003) reported that yeast extract did not give significant influence on culture growth and surface tension reduction of culture broth for biosurfactant production by *Bacillus coagulans*. Guerra-Santos *et al.* (1984) reported that yeast extract, now ordinarily using for biosurfactant production, was not a favourable nitrogen source due to its

complexity.The cultivation medium without addition of yeast extract was preferable regarding to the economic point of view.

4.6 Effect of initial pH on biosurfactant production

The effect of initial pH of medium on growth and biosurfactant activity of *A. xylosoxidans* 207-4B was studied in the MSM containing 1% (w/v) of WLO and 0.1% (w/v) of ammonium sulfate with shaking speed at 200 rpm and incubated in room temperature. Initial pH of the medium was adjusted to 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0 (Fig. 15).

The highest biosurfactant activity was obtained when initial pH was adjusted to 7.0. It was shown by reduction of surface tension which decreased from 65 mN/m to 52 mN/m (Fig. 15a). However, the maximum growth of *A. xylosoxidans* 207-4B was obtained when initial pH of medium was adjusted to 6.5 (Fig. 15b). pH plays a role in determining the ability of bacteria to grow or thrive in particular environments because it majorly affected biological activities of prokaryotes (Cameotra and Makkar, 1998). The nature of some bacteria renders them more effective at acidic medium and its action also influenced by others factors (Swaminathan, 1997), however most of bacteria grow optimally within a narrow range of pH between 6.7 and 7.5.

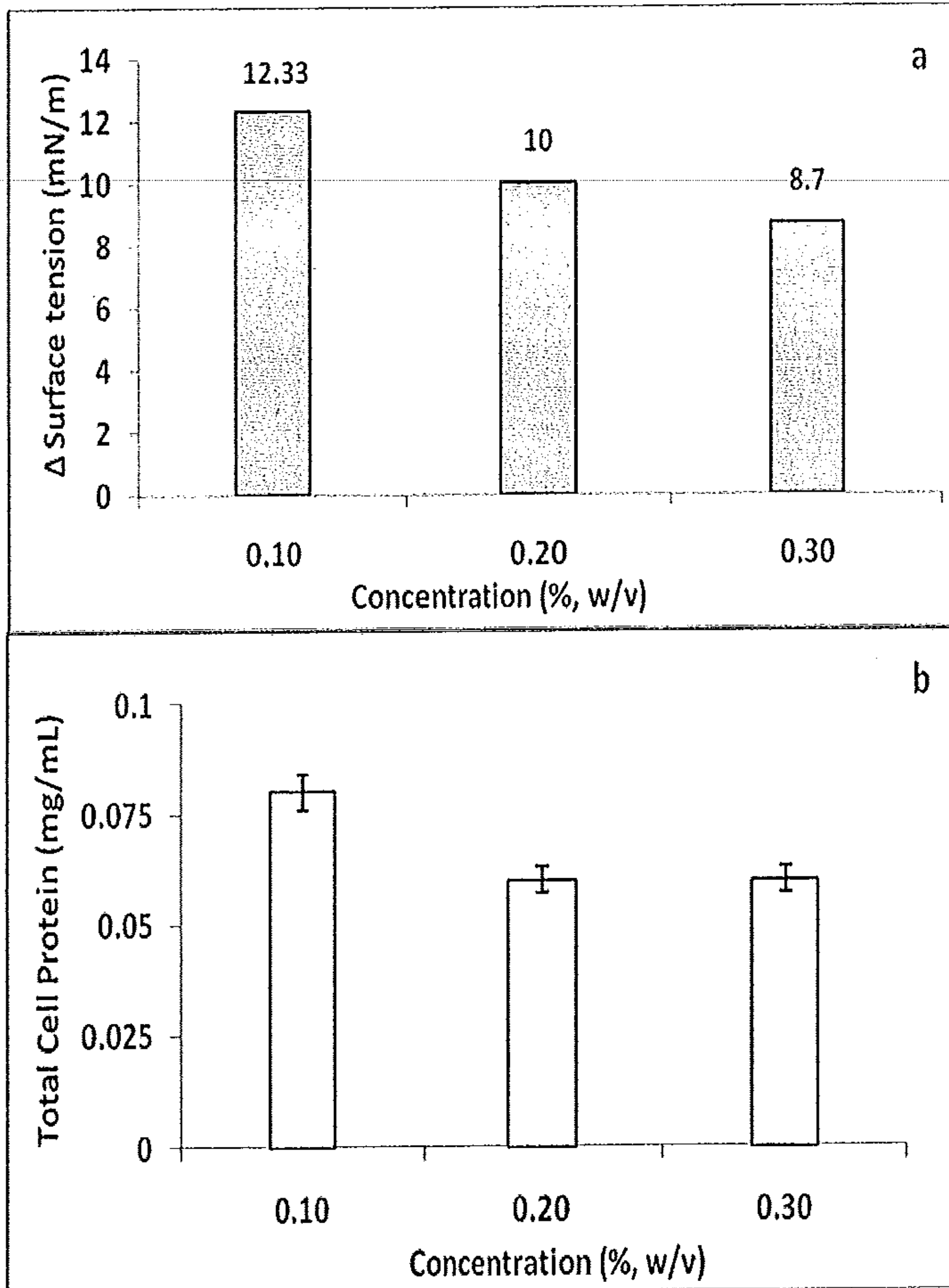

Figure 13. The effect of ammonium sulfate concentration on biosurfactant activity (a) and growth (b) of *A. xylosoxidans* 207-4B in minimal salt medium, initial pH 7.0, at room temperature for 1 day.

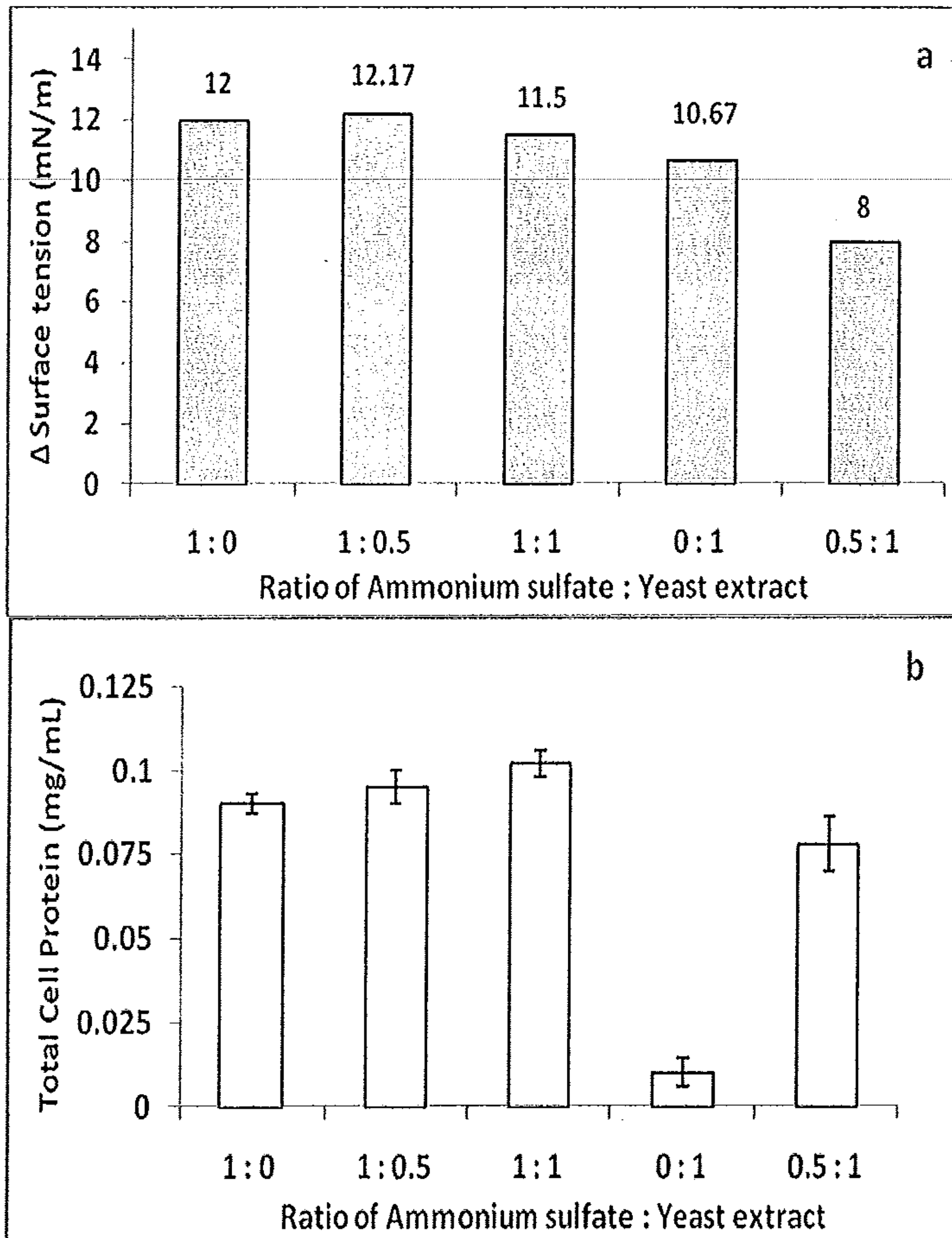

Figure 14. The effect of yeast extract addition on biosurfactant activity (a) and growth (b) of *A. xylosoxidans* 207-4B in minimal salt medium, initial pH 7.0, at room temperature for 1 day.

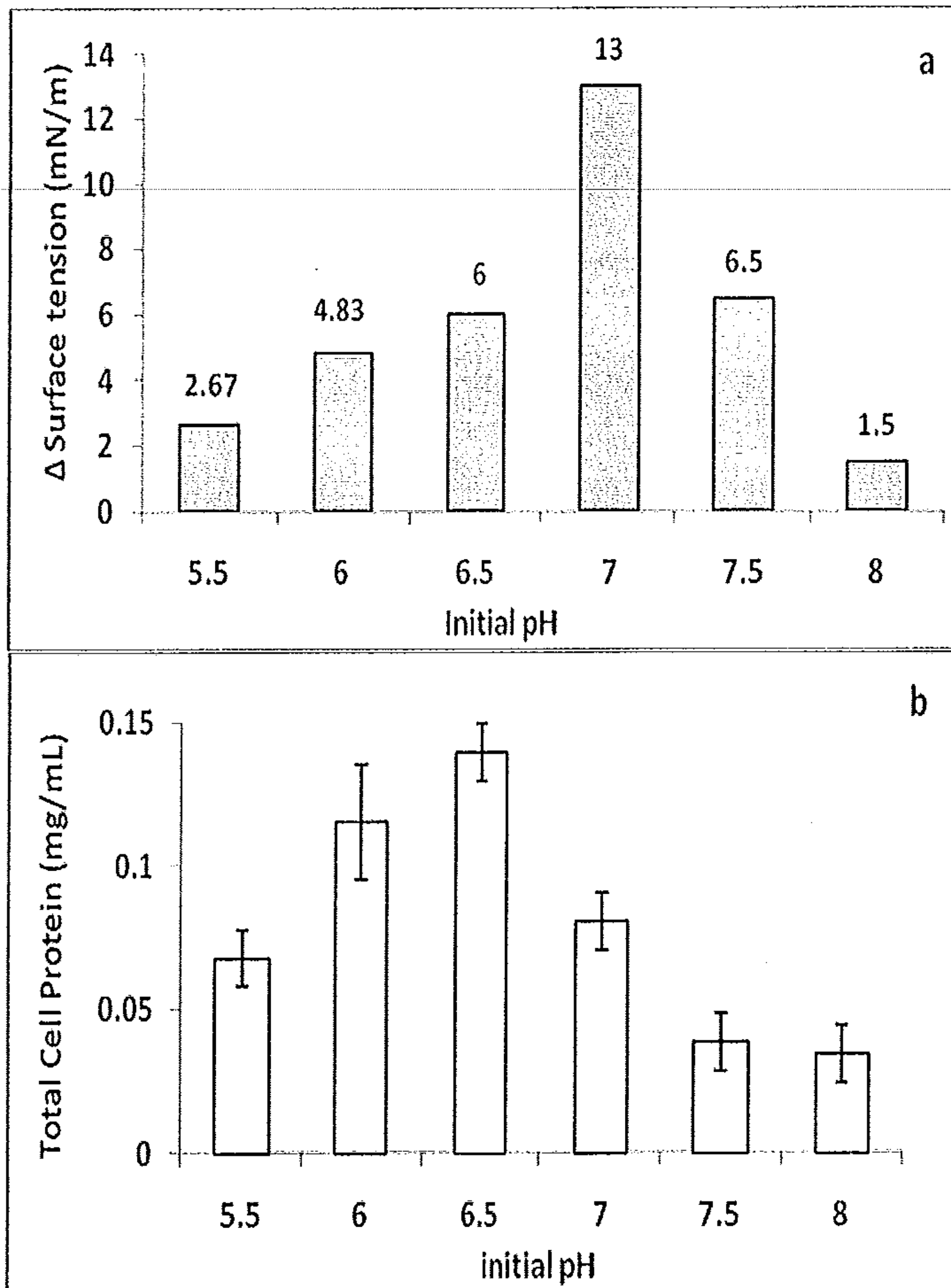

Figure 15. The effect of initial pH of medium on biosurfactant activity (a) and growth (b) of *A. xylosoxidans* 207-4B in minimal salt medium, at room temperature for 1 day.

4.7 Effect of shaking speed on biosurfactant production

Shaking speed affected to biosurfactant production and growth of *A. xylosoxidans* 207-4B (Fig. 16). The cell growth increased when shaking speed increased. The maximum cell growth was obtained when the shaking speed was at 250 rpm (Fig. 16b). However, the highest biosurfactant production was obtained when the shaking speed was at 200 rpm (Fig. 16a). Shaking speed affected the mass transfer efficiency of both oxygen and medium components and considered to the cell growth and biosurfactant formation of the aerobic bacterium, especially when it was grown in a shake flask. The effect of biotechnological parameter such as shaking speed can influence biosurfactant synthesis (Lotfabad *et al.*, 2009). Increasing shaking speed when cultivation performed in the shake flasks with the same headspace, may result in better dissolved oxygen (DO) (Yeh *et al.*, 2005).

The similar result also reported by Wei *et al.* (2005), they was studied the effect of shaking speed on rhamnolipid production by indigenous *Pseudomonas aeruginosa* J4 originating from petrochemical wastewater in the range of 50 – 250 rpm. They found that even the maximum growth was observed with shaking speed 250 rpm, but the optimum of biosurfactant production was reached when shaking speed on 200 rpm. Yeh *et al.* (2005) also reported that *Bacillus subtilis* obtained the maximum surfactin production when agitation speed was 200 rpm and then decreased with increasing agitation speed.

4.8 Effect of inoculum concentration on biosurfactant production

The effect of the inoculum concentrations (1 to 10 %) on the growth and biosurfactant production of *A. xylosoxidans* 207-4B are shown in Figure 17. The highest total cell protein was obtained with 10% (v/v) of inoculum (0.18 mg/mL). Meanwhile, the highest surface tension reduction was obtained with 5% of inoculum. Das and Mukhrejee (2007) reported that with increasing inoculum size did not have a significant impact in enhancing the biosurfactant production by *Bacillus subtilis* DM-03.

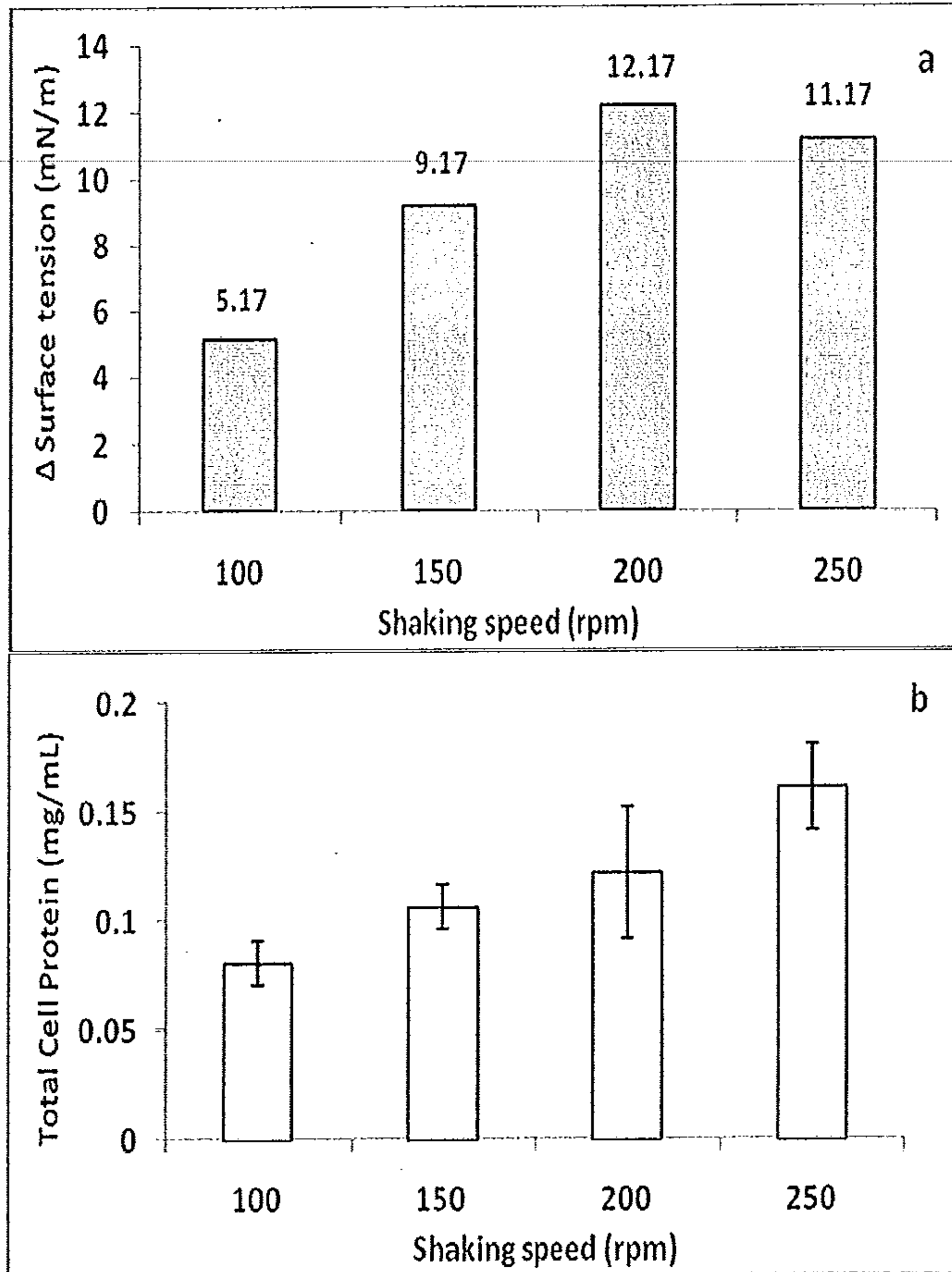

Figure 16. The effect of shaking speed on biosurfactant activity (a) and growth (b) of *A. xylosoxidans* 207-4B and in minimal salt medium, initial pH 7.0, at room temperature for 1 day.

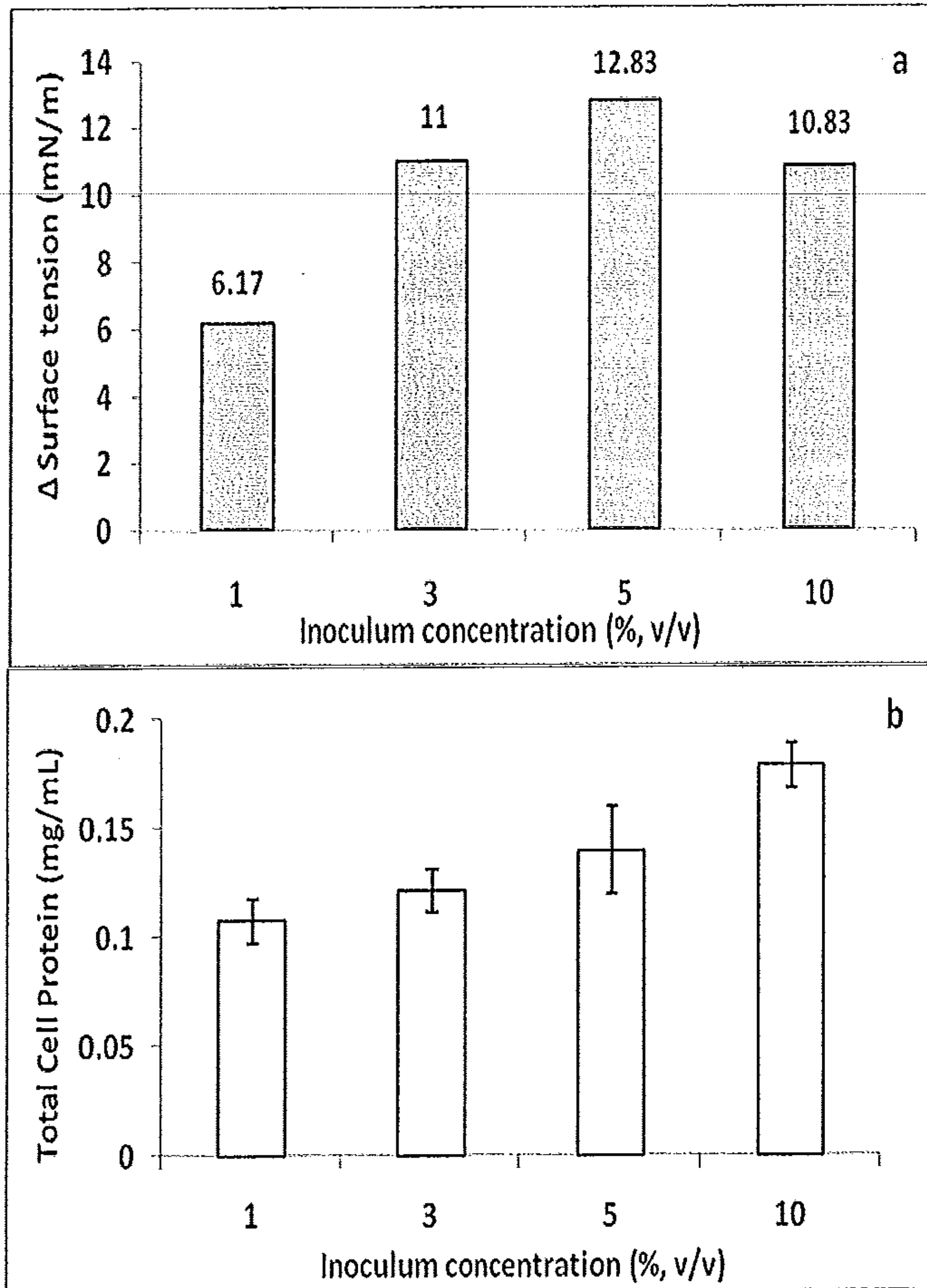

Figure 17. The effect of inoculum concentration on biosurfactant activity (a) and growth (b) of *A. xylosoxidans* 207-4B in minimal salt medium, initial pH 7.0, at room temperature for 1 day.

5. Time course of growth and biosurfactant production

Cultivation of *A. xylosoxidans* 207-4B in the 50 mL of minimal salt medium (contained 1% WLO, 0.1% of ammonium sulfate) with an initial pH 7.0 in 250 mL flask at room temperature with 200 rpm shaking speed is shown at Figure 18. *A. xylosoxidans* 207-4B gave highest reduction of surface tension at day 1. After day 1, the reduction of surface tension value was decreased. Meanwhile, the growth was increasing slowly from day 1 to day 5.

Nitschke *et al.* (2004) also reported biosurfactant production that produced by *Bacillus subtilis* 21332 with using cassava effluent medium was not directly associated with cell growth, the bulk of surfactant was synthesized during stationary phase. While *Bacillus subtilis* MTCC 1427 required 96 h of cultivation time in the minimal salt medium supplemented with molasses as the carbon source to produce biosurfactant was not growth associated (Makkar and Cameotra, 1997). Sadouk *et al.* (2008) also reported that biosurfactant produced by *Rhodococcus erythropolis*16 LM.USTHB was found not growth associated with the cultivation time up to 672 h in minimal salt medium supplemented with diesel oil as the carbon source.

6. Recovery of biosurfactant produced by *Achromobacter xylosoxidans* 207-4B

Crude extract of the biosurfactant was recovered from the culture supernatant of *A. xylosoxidans* 207-4B by precipitation with acid and extracted with solvents. Among three methods, chloroform/methanol extraction was the most efficient in biosurfactant recovery from culture supernatant (Table 8). Recovery yields of 23.40 mg/mL and 20.55 mg/mL were obtained when ethyl acetate and chloroform/methanol extraction were used, respectively. In addition, critical micelle concentrations were 0.055 mg/mL and 0.025 mg/mL when extracted by ethyl acetate and chloroform/methanol extraction, respectively. Precipitation with acid could not recover biosurfactant from culture supernatant. Most of biosurfactants recovered by acid precipitation tend to exhibit emulsification activity than reduce surface tension. In addition, it is considered as high molecular weight biosurfactant which consist of protein (Makkar and Cameotra, 1998; Huszcza and Burczyk, 2003; Abushady *et al.*, 2005).

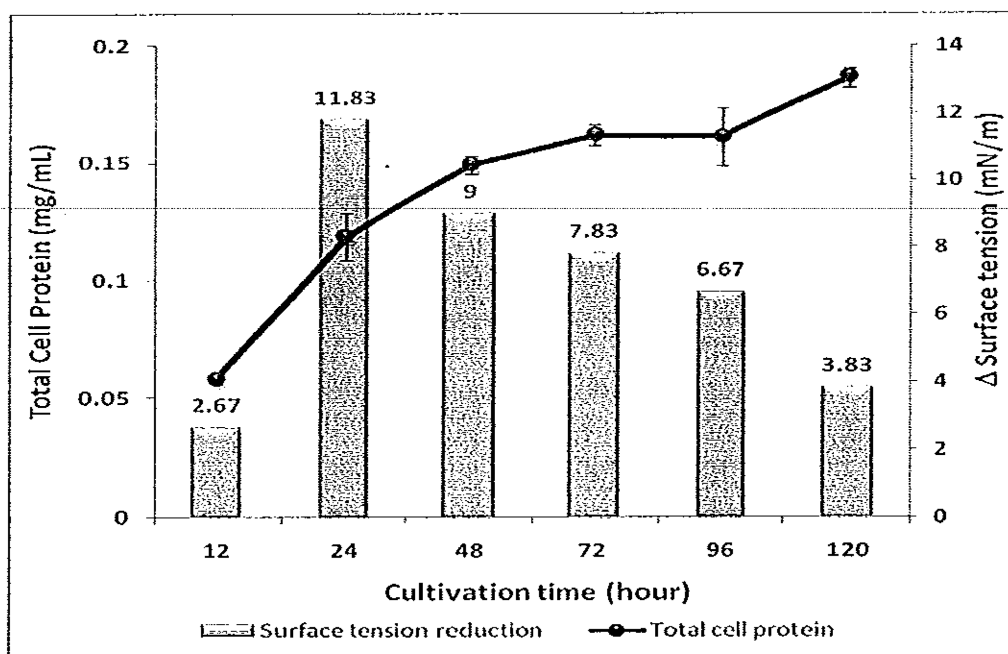

Figure 18. Time course of biosurfactant production and growth by *Achromobacter xylosoxidans* 207-4B in minimal salt medium with optimal condition (1% of WLO, 0.1% of ammonium sulfate, shaking speed 200 rpm, 5% of inoculum concentration, initial pH about 7.0, at room temperature for 1 day).

The crude biosurfactant extracted by the mixture of chloroform/methanol could reduce surface tension of water from 72 to 42.75 mN/m at the 0.5 mg/mL concentration. In addition, the CMC value of obtained biosurfactant was 0.025 mg/mL. This result was better than biosurfactants produced by *Flavobacterium* sp. strain MTN11 that exhibited CMC of 0.3 mg/mL (Bodour *et al.*, 2004) and biosurfactant produced by *Pseudomonas aeruginosa* exhibited CMC of 19 mg/mL (Rashedi *et al.*, 2005).

Mixtures of solvents were commonly used to facilitate adjustment of the polarity between the solvent as the extraction agent and the biosurfactant to be extracted (Desai and Banat, 1997; Kuyukina *et al.*, 2001). The structure of biosurfactant which was composed by hydrophilic and hydrophobic moieties, could easily extracted by this solvent system because the solvent system contained both non polar (chloroform, Log P = 1.97) and quite polar (methanol, Log P = -0.74) solvent.

Thus, it is better than single solvent (ethyl acetate, Log P = 0.73) (Sangster, 1989). The mixture of chloroform/methanol (2:1) system was selected for further study.

The CMC value of crude biosurfactant produced by *A. xylosoxidans* 207-4B was lower than two synthetic surfactant, SDS (Table 8). Important characteristic properties of a potent surface-active agent are its ability to lower the surface tension of an aqueous solution, and its low CMC (Kim *et al.*, 2000). The value of CMC affected the cost of application because with low value of CMC it means need less amount of biosurfactant for application step. The results suggested that the crude biosurfactant provided excellent properties in terms of the reduction of surface tension and a low value of CMC.

Table 7. Yield and critical micelle concentration (CMC) of crude biosurfactant produced by *Achromobacter xylosoxidans* 207-4B

Surfactant	Yield (mg/mL)	CMC (mg/mL)
Crude biosurfactant :		
- Extracted from ethyl acetate	23.40	0.055
- Extracted from $CHCl_3$.MeOH	20.55	0.025
- Acid precipitation	ND	ND
Synthetic Surfactant :		
- Sodium Dodecyl Sulfate (SDS)	-	2.200

ND: Not Determined

7. Characterization of crude biosurfactant

7.1 Effect of pH on biosurfactant activity

The effect of pH on activity of crude biosurfactant produced by *A. xylosoxidans* 207-4B is shown in Figure 19. Surface tension increased obviously with decreasing pH below 6. However, no changes in activity were noticeable in the pH range of pH 6-12.

Similar result had been reported for biosurfactant produced by *Bacillus subtilis* (Makkar and Cameotra, 2002) which stable in the range of pH 4.0 to 12.0. The crude biosurfactant produced by *Pseudomonas fluorescens* was also stable in a wide range of pH (2-12) (Abouseoud *et al.*, 2008) and the crude biosurfactant

produced by *Pseudomonas aeruginosa* SP4 that was stable at a pH range 5-11 (Pornsunthorntawee *et al.*, 2008).

7.2 The effect of temperature on biosurfactant stability

After the crude biosurfactant was incubated at 36-100°C for 1 h and at 110 and 121°C for 15 min, the residual activity was determined (Fig. 20). Temperatures ranging from 30-121°C did not show any influence on biosurfactant activity.

The similar result have been reported for biosurfactant produced by *Bacillus subtilis* MTCC 2423 (Makkar and Cameotra, 2002), *Pseudomonas fluorescens* (Abouseoud *et al.*, 2008) and *Pseudomonas aeruginosa* MR01 (Lotfabad *et al.*, 2009). Heat treatment of some biosurfactant caused no appreciable change in surfactant properties (lowering of surface tension and inter facial tension) (Fiechter, 1992).

7.3 The effect of salts on biosurfactant activity

The application of biosurfactant in environmental protection and enhanced oil recovery need a biosurfactant that have stability on high salinity condition. Thus, the effect of salts on biosurfactant activity was studied.

The effect of NaCl, $MgCl_2$ and $CaCl_2$ on the crude biosurfactant activity of *Achromobacter xylosoxidans* 207-4B is illustrated in Figure 21A and Figure 21B, respectively.

At very high NaCl concentration, biosurfactant activity of crude biosurfactant tended to decrease (Fig. 21a). Crude biosurfactant solutions exhibited no significant differences in surface tension as NaCl increased up to 12.5% (w/v). The concentration of salts in aquatic environments ranges from less than 0.05% (w/v dissolved salts) to saturated salt up to 30% and above. NaCl is a major component of seawater (Cameotra and Makkar, 1998). NaCl activated biosurfactant activity of many strains, which were isolated from seawater or petroleum reservoirs (Yakimov *et al.*, 1995).

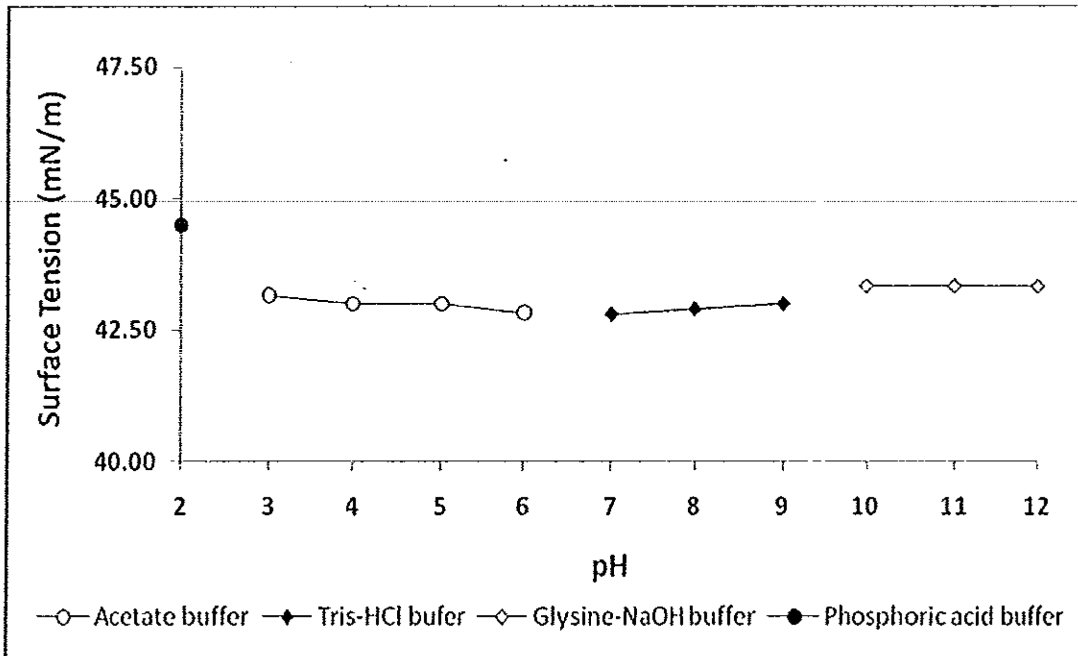

Figure 19. Effect of pH on activity of crude biosurfactant from *Achromobacter xylosoxidans* 207-4B (the crude biosurfactant was dissolved in the series of different pH buffer and kept for 24 h at 4°C before measuring surface tension).

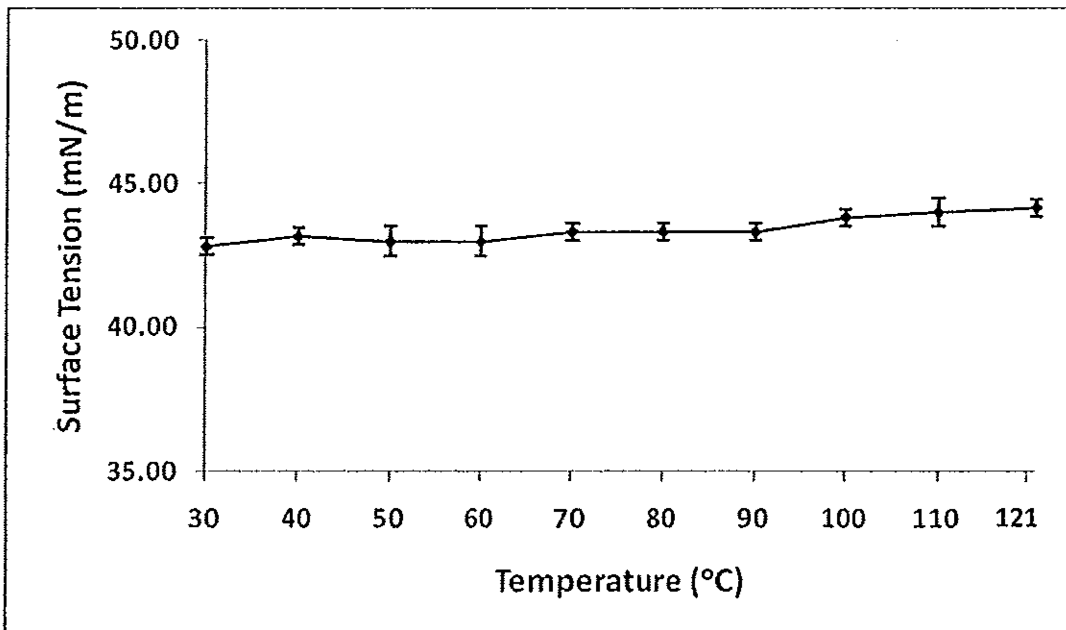

Figure 20. Effect of temperatures on stability of crude biosurfactant produced by *Achromobacter xylosoxidans* 207-4B.

The activity of crude biosurfactant produced by *Pseudomonas fluorescens* in the presence of salts with concentration of NaCl up to 20% was also reported by Abouseoud *et al.* (2008).

The effect of $MgCl_2$ and $CaCl_2$ on biosurfactant activity of *A. xylosoxidans* 207-4B is presented in Figure 21b. Calcium and magnesium salt are also present in seawater. Those divalent ions frequently break the oil/water emulsion (Kim *et al.*, 1997). However, $MgCl_2$ and $CaCl_2$ ranging from 0 to 0.2% (w/v) had no effect on biosurfactant activity of crude biosurfactant from *A. xylosoxidans* 207-4B.

The effect of seawater (pH 8.0) on biosurfactant activity of crude biosurfactant of *A. xylosoxidans* 207-4B was also investigated. Crude biosurfactant could reduce the surface tension of seawater from 77 mN/m to 43 mN/m. It indicated that combination of salts (NaCl, $MgCl_2$, $CaCl_2$, etc.) in seawater did not affect biosurfactant activity of crude biosurfactant from *A. xylosoxidans* 207-4B.

8. Identification of crude biosurfactant

The TLC analysis of crude biosurfactant from *A. xylosoxidans* 207-4B showed negative result with ninhydrin reagent, suggesting the absence of peptide. However, the results were positive with anisaldehyde and rhodamine B for sugar and lipid detection, respectively at R_f value of 0.54 (Fig. 22A and 22C). It indicated that biosurfactant produced by *A. xylosoxidans* 207-4B is glycolipid biosurfactant.

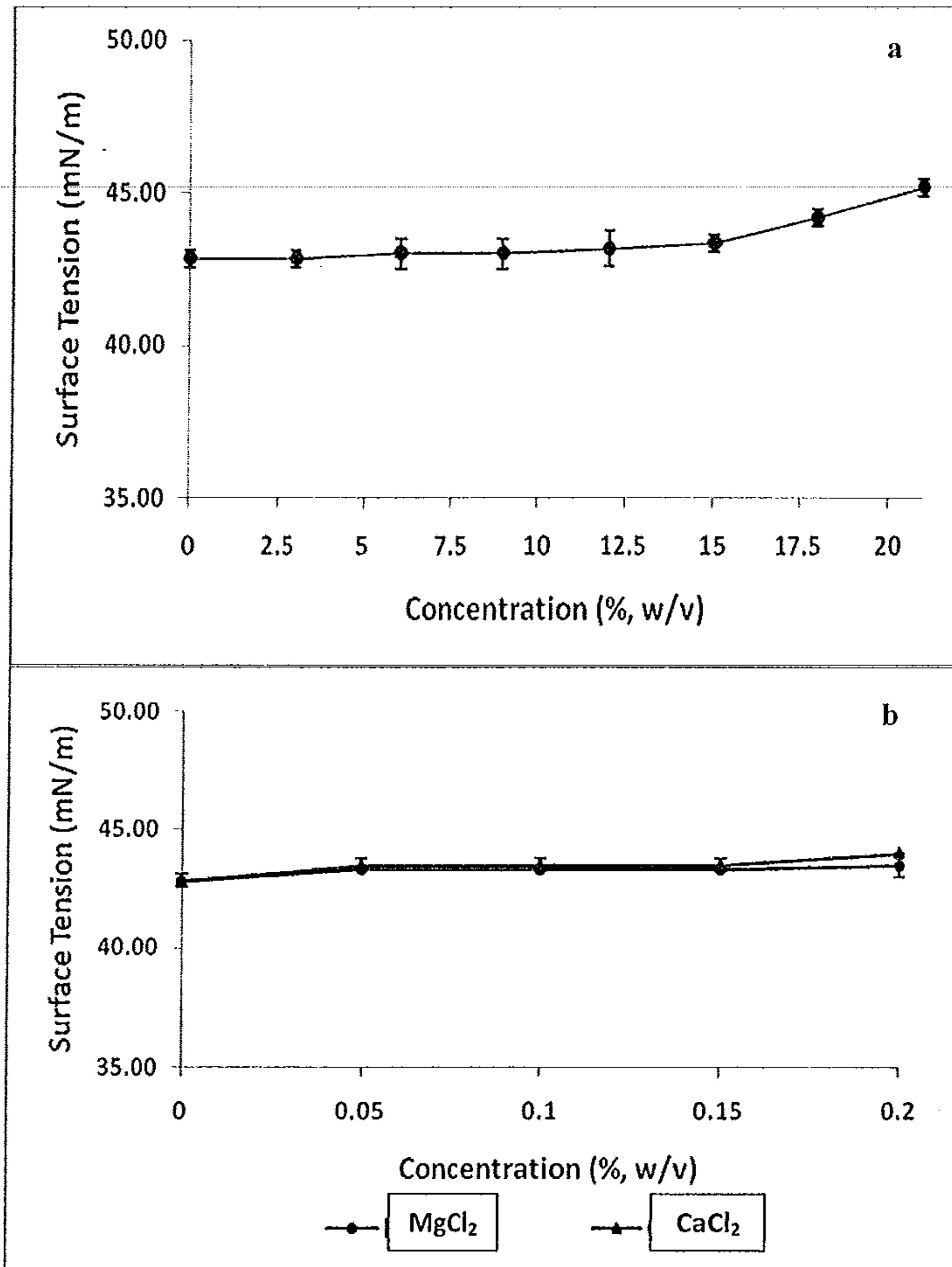

Figure 21. Effect of NaCl (a); $MgCl_2$ and $CaCl_2$ (b) on crude biosurfactant activity produced by *Achromobacter xylosoxidans* 207-4B.

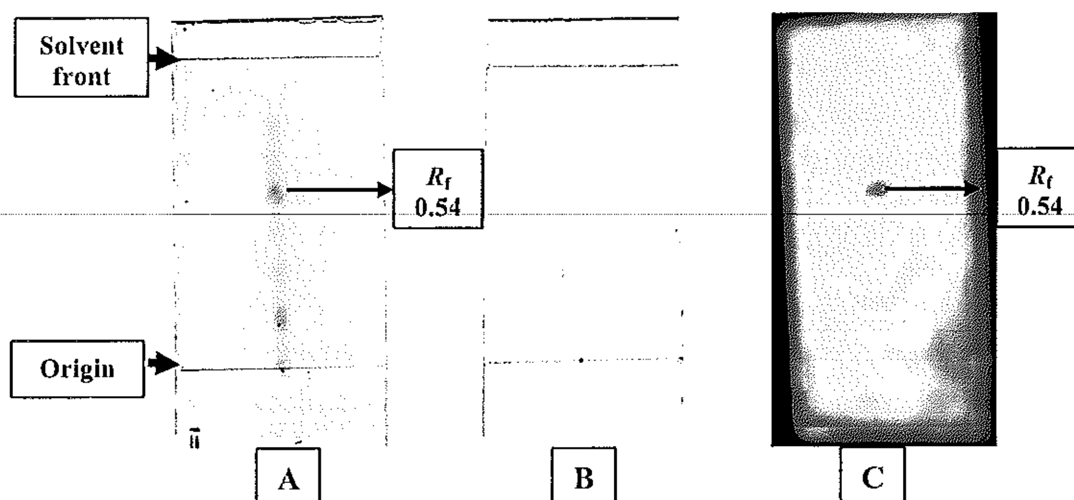

Figure 22. Thin layer chromatography analysis of the crude biosurfactant using hexane/ethyl acetate (7:3) (v/v) as a mobile phase, sprayed with *p*-anisaldehyde reagent (A); ninhydrin (B) and rhodamine B (C).

CHAPTER 4

CONCLUSION

Twenty bacterial consortia were isolated from mangrove sediment in Thungwa District, Satun Province, Thailand as waste lubricating oil degrader. Eighty bacterial isolates were isolated from those bacterial consortia when spread-plated on minimal salt medium supplemented with waste lubricating oil as a sole carbon source. Twenty-four isolates produced biosurfactant activity which exhibited emulsification activity and oil displacement activity when used waste lubricating oil and glucose as a carbon source. Strain 207-4B showed the highest biosurfactant production. It showed oil displacement activity about 0.356 cm^2 and exhibited emulsification activity about 50% when cultivated in a medium with waste lubricating oil and glucose as a carbon source, respectively. The strain 207-4B was identified as *Achromobacter xylosoxidans* 207-4B based on 16s rDNA sequence analysis. The optimum medium for the production of biosurfactant by *A. xylosoxidans* 207-4B contained 1% (w/v) of waste lubricating oil as a carbon source, 0.1% (w/v) of ammonium sulfate as a nitrogen source, initial pH of the medium at 7.0 and shaking speed at 200 rpm with 5% (v/v) of inoculum. It showed the maximum biosurfactant activity at 24 h of cultivation and could reduce surface tension of culture medium from 65 mN/m to 53.17 mN/m. The obtained culture supernatant was extracted twice with an equal volume of mixture of chloroform/methanol (2:1). The crude biosurfactant with yield 20.55 mg/mL was obtained and had critical micelle concentration of 0.025 mg/mL. The crude biosurfactant was stable at pH 6-12, temperature ranging from 30-12°C. The crude biosurfactant was stable in 0-0.2% (w/v) $MgCl_2$ and $CaCl_2$, however was not stable in NaCl higher than 15% (w/v). The preliminary identification of crude biosurfactant by TLC revealed that it consisted of sugar and lipid.

Suggestions

The results of this work lead to the following suggestions:

1. Purification and structure elucidation of biosurfactant produced by *Achromobacter xylosoxidans* 207-4B.
2. Application of biosurfactant produced by *Achromobacter xylosoxidans* 207-4B.

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Appendix

1. Culture media and preparation

1.1 Modified Minimal Salt Medium

waste lubricating oil	10.0	g
K_2HPO4	0.8	g
KH_2PO_4	0.2	g
$CaCl_2$	0.05	g
$MgCl_2$	0.5	g
$FeCl_3$	0.01	g
$(NH_4)_2SO_4$	1.0	g
NaCl	5.0	g

per liter of distilled water, pH 5.0. Autoclave at 121°C for 15 min.

1.2 Minimal Salt Medium Agar

waste lubricating oil	10.0	g
K_2HPO4	0.8	g
KH_2PO_4	0.2	g
$CaCl_2$	0.05	g
$MgCl_2$	0.5	g
$FeCl_3$	0.01	g
$(NH_4)_2SO_4$	1.0	g
NaCl	5.0	g
Agar	15	g

per liter of distilled water, pH 5.0. Autoclave at 121°C for 15 min.

2. Nucleotide sequence of 16s rDNA gene of *Achromobacter xylosoxidans* 207-4B

Sample name: 207-4B

Identify: *Achromobacter xylosoxidans*

16s rDNA Sequence

GGGGGAACCCTGATCCAGCCATCCCGCGTGTGCGATGAAGGCCTTCGGGT
TGTAAAGCACTTTTGGCAGGAAAGAAACGTCGTGGGTTAATACCCCGCGA
AACTGACGGTACCTGCAGAATAAGCACCGGCTAACTACGTGCCAGCAGCC
GCGGTAATACGTAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGC
GTGCGCAGGCGGTTCGGAAAGAAAGATGTGAAATCCCAGAGCTTAACTTT
GGAACTGCATTTTTAACTACCGGGCTAGAGTGTGTCAGAGGGAGGTGGAA
TTCCGCGTGTAGCAGTGAAATGCGTAGATATGCGGAGGAACACCGATGGC
GAAGGCAGCCTCCTGGGATAACACTGACGCTCATGCACGAAAGCGTGGG
GAGCAAACAGGATTAAATACCTGGTAGTCCCGCCCTAAACGATGTCAACT
AGCTGTTGGGGTCTTCGGACCTTGGTAGCGCAGCTAACGCGTGAAGTTGA
CCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGG
GACCCGCACAAGCGGTGGATGATGTGGATTAATTCGATGCAACGCGAAAA
ACCTTACCTACCCTTGACATGTCTGGAATGCCGAAGAGATTTGGCAGTGCT
CGCAAGAGAACCGGAACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTC
GTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCATTAGTT
GCTACGAAAGGGCACTTCTAATGAGACTGCCGGTGACAAACCGGAGGAA
GGTGGGGATGACGTCAAGTCCTCATGGCCCTTATGGGTAGGGCTTCACAC
GTCATACAATGGTCGGGACAGAGGGTCGCCAACCCGCGAGGGGGAGCCA
ATCCCAGAAACCCGATCGTAGTCCGGATCGCAGTCTGCAACTCGACTGCG
TGAAGTCGGAATCGCTAGTAATCGCGGATCAGCATGTCGCGGTGAATACG
TTCCCGGGTCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTTTAC
CAGAAGTAGTTAGCCTAACCGCAAGGGGGGCGATTACCACGGTAGGATTC
ATGACTGGGGTGAAGTCGTAACC

Blast Result

RID: AWE0A4BU011

Database: All GenBank+EMBL+DDBJ+PDB sequences (but no EST, STS, GSS,environmental samples or phase 0, 1 or 2 HTGS sequences) 7,335,116 sequences; 24,732,212,558 total letters

VITAE

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