CHAPTER IV
STRUCTURAL AND PHYSICOCHEMICAL CHARACTERIZATION OF CRUDE BIOSURFACTANT PRODUCED BY *Pseudomonas aeruginosa* SP4 ISOLATED FROM PETROLEUM-CONTAMINATED SOIL

4.1 Abstract

Pseudomonas aeruginosa strain SP4, isolated from petroleum-contaminated soil in Thailand, was used to produce a biosurfactant from a nutrient broth with palm oil as the carbon source. The key components of the crude biosurfactant were fractionated using HPLC-ELSD technique. With the use of ATR-FTIR spectroscopy, in combination with ^{1}H NMR and MS analyses, chemical structures of the fractionated components of the crude biosurfactant were identified as rhamnolipid species. When compared to synthetic surfactants, including Pluronic F-68, which is a triblock nonionic surfactant containing poly(ethylene oxide) and poly(propylene oxide), and sodium dodecyl sulfate, the crude biosurfactant showed comparable physicochemical properties, in terms of the surface activities. The crude biosurfactant reduced the surface tension of pure water to 29.0 mN/m with a critical micelle concentration of approximately 200 mg/l, and it exhibited good thermal and pH stability. The crude biosurfactant also formed stable water-in-oil microemulsions with crude oil and various types of vegetable oils, but not with short-chain hydrocarbons.

Keywords: Biosurfactants; Glycolipid; Rhamnolipid; *Pseudomonas aeruginosa*

4.2 Introduction

Surfactants, or surface active agents, can be classified into two main groups: synthetic surfactants and biosurfactants. Synthetic surfactants are produced by organic chemical reactions, while biosurfactants are produced by biological processes, being

excreted extracellularly by microorganisms such as bacteria, fungi, and yeast. When compared to synthetic surfactants, biosurfactants have several advantages, including high biodegradability, low toxicity, low irritancy, and compatibility with human skin (Banat et al., 2000; Cameotra and Makkar, 2004). Because of these superior properties, biosurfactants have a potential use in food, pharmaceutical, and cosmetic industries (Desai and Banat, 1997). Nowadays, the development of cost-effective bioprocesses for the production of biosurfactants is of great interest (Mercadé et al., 1993; Fox and Bala, 2000; Abalos et al., 2001; Wei et al., 2005; Nitschke and Pastore, 2006).

Based on the types of biosurfactant-producing microbial species and the nature of their chemical structures, biosurfactants are categorized into four main groups: lipopeptides and lipoproteins, phospholipids, polymeric surfactants, and glycolipids (Healy et al., 1996). Among these four groups, the most common biosurfactants that have been isolated and studied are the glycolipids. This type of biosurfactant is composed of carbohydrates in a combination with long-chain aliphatic acids or hydroxyl aliphatic acids. From the point of view of surfactant properties, the most widely studied glycolipids are rhamnolipids produced by *Pseudomonas aeruginosa* strains (Cameotra and Makkar, 2004).

Although there are several types of rhamnolipid species, all of them possess similar chemical structures (Torrens et al., 1998). In general, the two major types of rhamnolipid species are L-rhamnosyl-3-hydroxydecanoyl-3-hydroxydecanoate or monorhamnolipid (Rha-C_{10}-C_{10}) and L-rhamnosyl-L-rhamnosyl-3-hydroxydecanoyl-3-hydroxydecanoate or dirhamnolipid (Rha-Rha-C_{10}-C_{10}) (Déziel et al., 1999; Nitschke et al., 2005). The crude biosurfactant extracted from the liquid culture of *P. aeruginosa* strains were found to reduce the surface tension of water from 72 to 30 mN/m with a critical micelle concentration (CMC) in the range of 5 to 200 mg/l, depending on the components of the mixture (Finnerty, 1994; Nitschke et al., 2005).

With the use of a number of analytical methods such as high performance liquid chromatography (HPLC) equipped with a photodiode array detector or UV detector (Rendell et al., 1990; Schenk et al., 1995; Mata-Sandoval et al., 1999), and gas

chromatography/mass spectrometry (GC/MS) (Mata-Sandoval et al., 1999; Thanomsub et al., 2006), rhamnolipids, which are rhamnose-containing glycolipids, were identified as a mixture of several species. Although the above-mentioned methods are able to analyze the chemical structures of the rhamnolipid species, they are time-consuming techniques and do not provide a reliable quantification analysis. Therefore, liquid chromatography/mass spectrometry (LC/MS) (Déziel et al., 1999, 2000; Abalos et al., 2001; Chayabutra and Ju, 2001; Benincasa et al., 2004) and HPLC equipped with an evaporative light scattering detector (ELSD) (Noordman et al., 2000; Trummler et al., 2003) have been developed as efficient techniques for the analysis of the rhamnolipid species.

In this study, *P. aeruginosa* strain SP4, isolated from petroleum contaminated soil in Thailand, was used to produce a biosurfactant. The biosurfactant production was induced by the addition of palm oil. The key components of the crude biosurfactant were fractionated using HPLC-ELSD technique. The chemical structures of the fractionated components were elucidated, and their physicochemical properties were investigated in a comparison with those of the synthetic surfactants, including Pluronic F-68 and SDS.

4.3 Experimental

4.3.1 Materials

Pluronic F-68 was purchased from Sigma-Aldrich (USA) while SDS (96% purity) was supplied from Ajax Finechem (Australia). Acetonitrile (HPLC grade) was provided by Labscan Asia Co., Ltd. (Thailand). All chemicals were used as received without further purification.

4.3.2 Bacterial Strain and Culture Growth Conditions

P. aeruginosa SP4 was isolated from petroleum contaminated soil in Thailand. The isolated strain was maintained on nutrient agar slants at 37°C and was sub-cultured every 2 weeks.

4.3.3 Production and Extraction of Crude Biosurfactant

To produce crude biosurfactant, an inoculum was prepared by transferring the bacterial colonies into a nutrient broth, and the culture was incubated at 37°C in a shaking incubator at 200 rpm for 22 h. Then a nutrient broth containing 2% inoculum and 2% palm oil was incubated at 37°C under aerobic condition in a shaking incubator at 200 rpm for 48 h to obtain the highest microbial and surfactant concentration (Paisanjit, 2006). After that, the solution was centrifuged at 4°C and 8500 rpm for 20 min to remove the bacterial cells. The obtained supernatant was further treated by acidification to pH 2.0 using 6 M hydrochloric acid solution, and the acidified supernatant was left overnight at 4°C for the complete precipitation of the biosurfactants (Yakimov et al., 1995). After centrifugation, the precipitate was then dissolved in a 0.1 M sodium bicarbonate solution, followed by the biosurfactant extraction step with a solvent having a 2:1 chloroform-to-ethanol ratio at room temperature (Zhang and Miller, 1992). The organic phase was transferred to a round bottom flask connected to a rotary evaporator to remove the solvent, yielding a viscous honey-colored biosurfactant product. About 5.20 g of the crude biosurfactant was extracted per liter of culture medium.

4.3.4 Fractionation of Crude Biosurfactant

The components of the crude biosurfactant were fractionated using a high performance liquid chromatograph (HPLC) (an Alltech 580 autosampler, an Alltech HPLC pump, model 626, and an Inertsil® ODS-3 column) equipped with an evaporative light scattering detector (ELSD) (Alltech, 2000ES). The mobile phase solutions were an aqueous solution of 10% acetonitrile (A) and pure acetonitrile (B). Both eluents contained 0.1% trifluoroacetic acid. The gradient system was used, starting with B from 30% to 70% in 5 min and then from 70% to 90% in 15 min. After that, the gradient of B was raised again to 100% at the end of the process. The flow rate of the mobile phase was set constant at 0.5 ml/min and the sample injection volume was 50 µl. The ELSD drift tube temperature was maintained at 100°C while the nebulizer flow rate was kept constant at 1.5 l/min (Noordman et al., 2000). The gradual change in the affinity of the

mobile phase resulted in the fractionation of the components in the crude biosurfactant. All fractions eluted from the HPLC column at different retention times were collected. Each fraction was then evaporated to remove all of the eluents to obtain a high-purity biosurfactant-containing material.

4.3.5 Structural Characterization

The chemical structures of the components in the crude biosurfactant sample were preliminarily determined by using Fourier transform infrared (FT-IR) spectroscopy. Nuclear magnetic resonance (NMR) analysis and mass spectrometry were employed in order to identify the chemical structures of the components in the crude biosurfactant.

4.3.5.1 Fourier Transform Infrared Spectroscopy

FT-IR spectroscopy can be used to elucidate the chemical structures of some components in an unknown mixture by identifying the types of chemical bonds or the functional groups present in their chemical structures (Rodrigues, et al., 2006). In this present study, a Thermo Nicolet Nexus 670 FT-IR spectrometer equipped with an attenuated total reflectance (ATR) crystal accessory was used to provide a chemical analysis of the components in the crude biosurfactant. The spectra were collected at a resolution of 4 cm^{-1} and 32 scans with correction for atmospheric carbon dioxide.

4.3.5.2 Nuclear Magnetic Resonance Analysis

The ^{1}H NMR spectra of each fraction from the fractionation step were achieved from an FT-NMR 500 mHz spectrometer (JEOL, JNM-A500) using deuterated chloroform as a solvent for all samples.

4.3.5.3 Mass Spectrometry

The isolated fractions eluted from the HPLC column were directly subjected to analysis with mass spectrometry. The experiments were performed with a Waters mass spectrometer using electrospray in the positive mode and a scanning mass range of 300–900 Da.

4.3.6 Physicochemical Characterization

The physicochemical properties of the crude biosurfactant and its isolated fractions were investigated in comparisons with those of two commercial surfactants, Pluronic F-68 and SDS. Pluronic F-68 is the commercial trade name of a triblock nonionic surfactant composed of ethylene oxide (PEO) and propylene oxide (PPO) blocks in the form of PEO-PPO-PEO, while SDS is a widely used anionic surfactant.

4.3.6.1 Oil Displacement Test

The oil displacement test is a method used to measure the diameter of the clear zone, which occurs after dropping a surfactant-containing solution on an oil-water interface. The binomial diameter allows an evaluation of the surface tension reduction efficiency of a given biosurfactant. The oil displacement test was done by adding 50 ml of distilled water to a petri dish with a diameter of 15 cm. After that, 20 µl of crude oil was dropped onto the surface of the water, followed by the addition of 10 µl of an aqueous solution containing a surfactant concentration of 20 mg/ml, onto the surface of the oil. The diameters of the clear zones of triplicate experiments from the same surfactant sample were determined for an averaged value of the clear zone diameter (Rodrigues, et al., 2006).

4.3.6.2 Surface Tension Measurement

The surface tension of the aqueous solution at different surfactant concentrations was measured by using a du Nöuy ring-type tensiometer (Krüss, K10T). The surface tension measurement was carried out at 25±1°C after dipping the platinum ring in the solution for a while to attain equilibrium conditions. The measurement was repeated three times and an average value was obtained. The critical micelle concentration (CMC) was then determined from the break point of the surface tension versus its log of bulk concentration curve. For the calibration of the instrument, the surface tension of the pure water was measured before each set of experiments.

4.3.6.3 Measurement of Emulsification Activity

A mixture of 6 ml of the studied hydrocarbon or oil and 4 ml of 1 mg/ml of the crude biosurfactant or Pluronic F-68 or SDS was vortexed at a high speed

for 2 min. The emulsion activity was investigated after 24 h and the emulsification index (E_{24}) was calculated by dividing the measured height of the emulsion layer by the total height of the mixture and multiplying by 100 (Cooper and Goldenberg, 1987). The higher the emulsification index, the higher the emulsification activity of a tested surfactant.

4.3.6.4 Stability Testing

The crude biosurfactant sample and the two synthetic surfactants were prepared at the CMCs for the thermal stability test. The prepared surfactant solutions were incubated in a water bath at different temperatures and different time intervals before cooling to room temperature. The pH stability was investigated by adjusting the solutions to different pH values. For both thermal and pH stability testing, the surface tension was measured and used to indicate the stability.

4.3.6.5 Statistical Analysis

The experimental data are presented in terms of arithmetic averages of at least three replicates and the standard deviations are indicated by the error bars. The analyses were done using SigmaPlot software, version 8.02 (SPSS Inc., UK).

4.4 Results and Discussion

4.4.1 Fractions of Crude Biosurfactant

The crude biosurfactant obtained from the liquid culture of *P. aeruginosa* SP4 was first analyzed by using HPLC-ELSD technique. The key components of the crude biosurfactant were successfully separated into six main fractions eluted from the HPLC column at different elution times. As shown in Table 4.1, the crude biosurfactant contains those six fractions, named A, B, C, D, E, and F, about 0.68%, 1.54%, 73.48%, 9.55%, 13.35%, and 1.39%, respectively. Among the fractionated components, fraction C is the predominant component in the crude biosurfactant.

4.4.2 Chemical Structures of Biosurfactants

The chemical composition of each component fractionated from the crude biosurfactant was preliminarily investigated using ATR-FTIR technique. The ATR-FTIR spectra of the six components are quite similar, suggesting that the six fractions should have similar chemical structures. The important adsorption bands located at 3468, 2922, 2853, 1743, and 1300–1100 cm^{-1} indicate that all of them have chemical structures identical to those of rhamnolipids, which are composed of rhamnose rings and long hydrocarbon chains. The broad band appearing at 3468 cm^{-1} should be assigned to the O-H stretching vibrations of hydroxyl groups in the chemical structures of the biosurfactants. The strong adsorption peaks present at 2922 and 2853 cm^{-1} are expected to be the C-H stretching vibrations of the hydrocarbon chain positions. The characteristic peak displayed at 1743 cm^{-1} relates to the C=O stretching vibrations of the carbonyl groups while the C-O stretching bands at 1300–1000 cm^{-1} confirm the presence of the bonds formed between carbon atoms and hydroxyl groups in the chemical structures of the rhamnose rings.

With the use of ^{1}H NMR and MS analyses, the chemical structures of all the isolated fractions were revealed. The characteristic chemical shifts present in the ^{1}H NMR spectra confirm that all of the isolated fractions have the molecular structures of the rhamnolipid species. The presence of long hydrocarbon chains and rhamnose rings is indicated by the appearance of the characteristic chemical shifts in the region of 0.8–1.4 and 3.3–5.5 ppm, respectively (Wei et al., 2005).

In this study, the MS analysis was done in the positive electrospray ionization mode, so positively charged molecules were observed. Under these conditions, the molecules having an organic function like rhamnolipids tend to form sodium or potassium adducts; thus the signals appearing in the mass spectra are assigned to the sodiated or potassiated ions (Rendell et al., 1999; Déziel et al., 1999). From the mass spectra, the possible molecular weights were identified as 622, 476, 504, 530, 532, and 704, for fractions A, B, C, D, E, and F, respectively.

The mass spectrum of fraction C, which is the predominant component in the crude biosurfactant produced by *P. aeruginosa* SP4, shows the intense sodiated and disodiated molecular ions at *m/z* 527 and 549, respectively. The sodiated and disodiated fragment ions formed by the loss of the terminal lipid also appear at *m/z* 357 and 379, respectively. These data are consistent with the structure of monorhamnolipid (Rha-C_{10}-C_{10}). Most of the biosurfactants produced from various *P. aeruginosa* strains were reported to be dirhamnolipid (Déziel et al., 1999; Mata-Sandoval et al., 2001; Rahman et al., 2002). Only a few reports showed that monorhamnolipid was the predominant component (Arino et al, 1996; Sim et al., 1997; Costa et al., 2006). The difference in the predominant rhamnolipid species might result from the age of the culture, *P. aeruginosa* strains (Déziel et al., 1999), substrate composition, and culture conditions (Costa et al., 2006).

In the case of fraction B, the presence of signals at *m/z* 499 and 521 in the mass spectrum corresponds to the sodiated and disodiated molecular ions of C_{18} rhamnolipid, respectively. The loss of the terminal C_{10} generates fragment ions having signals at *m/z* 329 and 351, but the signals at *m/z* 357 and 379 also confirm the loss of the terminal C_8 of the rhamnolipid molecules. From the MS results, it can be concluded that fraction B is a mixture of Rha-C_8-C_{10} and Rha-C_{10}-C_8, indicating that the isomers of rhamnolipid molecules were not chromatographically resolved. By comparing the signal intensity, Rha-C_8-C_{10} was relatively more abundant than Rha-C_{10}-C_8. As previously described for rhamnolipid molecules containing two different 3-hydroxy fatty acid side chains, rhamnolipid molecules with the shorter 3-hydroxy fatty acid side chain are found to be more abundant than those with the longer chain connected to the rhamnose molecule at the same position (Déziel et al., 2000). In the case of the remaining fractions (A, D, E, and F), each of them was also identified as a mixture of the two structural isomers of rhamnolipid molecules, and their chemical structures are given in Table 1. For fractions D and F, unsaturated $C_{12:1}$ and $C_{14:1}$ rhamnolipid species were observed in the chemical structures of both fractions. Although it was previously described that the unsaturated fatty acid side chain was always found at the terminal end of rhamnolipid

molecules (Rendell et al.1999), our results indicate that the unsaturated 3-hydroxy fatty acid side chains locate at two positions; that is, the position connected to the rhamnose molecule and to the terminal end of the rhamnolipid species, which is consistent with the work of Déziel et al. (Déziel et al., 1999).

4.4.3 Physicochemical Properties

The oil displacement test is an indirect measurement of the surface activity of a surfactant sample tested against oil; a larger diameter represents a higher surface activity of the testing solution (Rodrigues et al., 2006). In this present study, the surface activities of the crude biosurfactant and its isolated fractions were investigated in comparisons with those of Pluronic F-68 and SDS. As shown in Figure 4.1, fraction C has the highest surface activity, while Pluronic F-68 exhibits the lowest among the tested samples. When compared to the synthetic surfactants, the surface activity of the crude biosurfactant was much higher than that of Pluronic F-68, but was slightly lower than that of SDS. Although the crude biosurfactant had a lower surface activity than SDS, the isolated fractions C, D, and F showed higher surface activities than this synthetic surfactant, SDS. In addition, fraction B also displayed a comparable surface activity to SDS. In a comparison between the isolated fractions and the crude biosurfactant, fractions B, C, D, and F exhibited higher surface activities than the crude biosurfactant, while fractions A, and E showed comparable surface activities.

The surface tension and emulsification index of the crude biosurfactant were measured in comparison to those of Pluronic F-68 and SDS. Figure 4.2 shows the plot of surface tension versus initial surfactant concentration of the crude biosurfactant, Pluronic F-68, and SDS. In the case of the crude biosurfactant, the surface tension rapidly decreased as the concentration of biosurfactants increased, and a minimum surface tension of 29.0 mN/m was found at an initial surfactant concentration greater than 200 mg/l. From the break point of surface tension *versus* its log of concentration curve, the CMC of the crude biosurfactant is approximately 200 mg/l. The obtained values of the minimum surface tension and the CMC of the crude biosurfactant are

consistent with the previously reported values (Mulligan, 2004). As shown in Figure 4.2, Pluronic F-68 and SDS are able to reduce the surface tensions of pure water to 42.8 and 28.6 mN/m, corresponding to the CMC values of 350 and 1280 mg/l, respectively. The results suggested that the crude biosurfactant provided excellent properties in terms of the reduction of surface tension and a low value of the CMC.

Regarding previous works, *Pseudomonas* rhamnolipids can effectively emulsify and stabilize emulsions with various types of hydrocarbons and oils such as linseed oil, almond oil, mineral oil (Benincasa et al., 2004), diesel (Haba et al., 2003; Wei et al., 2005), crude oil, kerosene, *n*-alkanes, aromatic compounds, coconut oil, and olive oil (Patel and Desai, 1997); however, the emulsification of rhamnolipids also depends on carbon sources used to produce rhamnolipids (Patel and Desai, 1997; Costa et al., 2006). Figure 4.3 shows the emulsification activity (E_{24}) of the crude biosurfactant produced by *P. aeruginosa* strain SP4 in a comparison with Pluronic F-68 and SDS using different hydrocarbons and oils. The crude biosurfactant was found to be able to form stable water-in-oil emulsions with crude oil and vegetable oils (palm oil, soybean oil, coconut oil, and olive oil), but they failed to emulsify short-chain hydrocarbons (pentane, hexane, heptane, toluene, and 1-chlorobutane). In contrast, both synthetic surfactants, Pluronic F-68 and SDS, were able to provide high emulsification activities of all oils, including short-chain hydrocarbons. The ability of the crude biosurfactant to form stable emulsions with vegetable oils indicated the potential use of these surface-active compounds as emulsifying agents in the food industry. Interestingly, the crude biosurfactant gave the highest emulsification activity on palm oil, probably because it was produced by using palm oil as a carbon source.

Figure 4.4a shows the effect of temperature on the surface tension of the crude biosurfactant produced from *P. aeruginosa* strain SP4 as compared to the two commercial surfactants, Pluronic F-68 and SDS. The results show that all tested surfactants were able to withstand a high temperature of 90°C up to 2 h without a reduction in surface tension. In addition, the heat tolerance of the tested surfactants was studied at a higher temperature of 120°C for 15 min by using an autoclave. For both the

crude biosurfactant and Pluronic F-68, the surface tension remained unchanged, indicating that both surfactants possess excellent heat tolerance. In the case of SDS, the surface tension increased remarkably from 28.72 mN/m to 34.95 mN/m after the heat treatment. The result suggested that deterioration of SDS occurred at a temperature of 120°C.

Figure 4.4b shows the effect of pH on surface tension in the presence of the crude biosurfactant, as compared to the two synthetic surfactants. The surface tension of SDS remained constant throughout the studied range of pH. In contrast, the surface tension increased slightly for the crude biosurfactant, but decreased slightly for Pluronic-F-68 when the pH of the solution was increased.

When compared to the synthetic surfactants, the crude biosurfactant exhibited superior properties in both heat and pH stabilities. Although SDS was able to retain its surface activity over a pH range from 3 to 11, it showed lower thermal stability than the crude biosurfactant. The deterioration of its surface activity might result from the hydrolysis at the hydrocarbon chain of the SDS molecule; however, this reaction occurs under severe conditions like autoclaving. These findings suggest that the robust characteristics of the crude biosurfactant are very beneficial for applications under extreme conditions of temperature and pH, such as in oil recovery and in the bioremediation of a polluted environment. Moreover, these biosurfactants have great potential for biomedical applications due to their robust heat tolerance even after being submitted to autoclave sterilization.

4.5 Conclusions

In this study, the crude biosurfactant produced from *P. aeruginosa* strain SP4 was chemically characterized as a mixture of eleven rhamnolipid species. Among them, the most relatively abundant component was identified as monorhamnolipid (Rha-C_{10}-C_{10}). When compared to synthetic surfactants, including Pluronic F-68 and SDS, the crude biosurfactant showed comparable physicochemical properties in terms of the

surface activities. The crude biosurfactant was able to reduce the surface tension of pure water to 29.0 mN/m and the CMC was found to be 200 mg/l, while Pluronic F-68 and SDS reduced the surface tension of water to 42.8 and 28.6 mN/m with CMC values of approximately 350 and 1280 mg/l, respectively. The crude biosurfactant also exhibited a robust tolerance for both heat and pH, and was able to form stable water-in-oil microemulsions with crude oil and various types of vegetable oils.

4.6 Acknowledgements

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4.7 References

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Table 4.1 Chemical structures of the isolated fractions of the crude biosurfactant produced by *P. aeruginosa* SP4

Fraction	Retention Time (min)	Peak Area (%)	Chemical Structures	Molecular Weight
A	4.68	0.68	Rha-Rha-C_8-C_{10} Rha-Rha-C_{10}-C_8	622
B	14.58	1.54	Rha-C_8-C_{10} Rha-C_{10}-C_8	476
C	19.38	73.48	Rha-C_{10}-C_{10}	504
D	22.25	9.55	Rha-C_{10}-$C_{12:1}$ Rha-$C_{12:1}$-C_{10}	530
E	25.12	13.35	Rha-C_{10}-C_{12} Rha-C_{12}-C_{10}	532
F	29.62	1.39	Rha-Rha-C_{10}-$C_{14:1}$ Rha-Rha-$C_{12:1}$-C_{12}	704

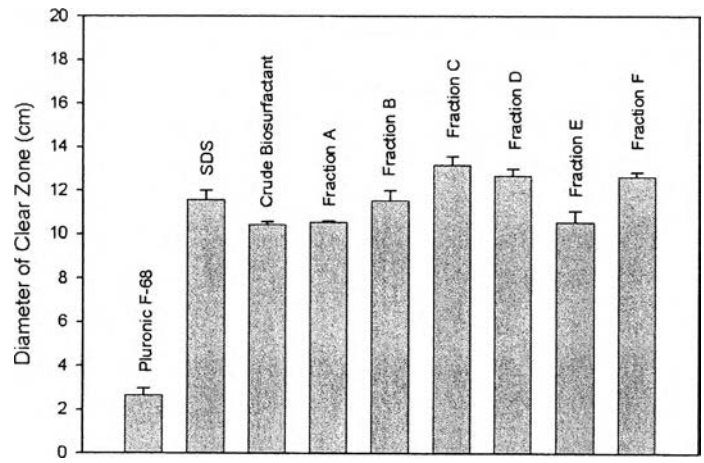

Figure 4.1 Diameter of the clear zones on the oil surface obtained from oil displacement testing with the crude biosurfactant produced by *P. aeruginosa* SP4 and its fractions compared with Pluronic F-68 and SDS at a surfactant concentration of 20 mg/ml.

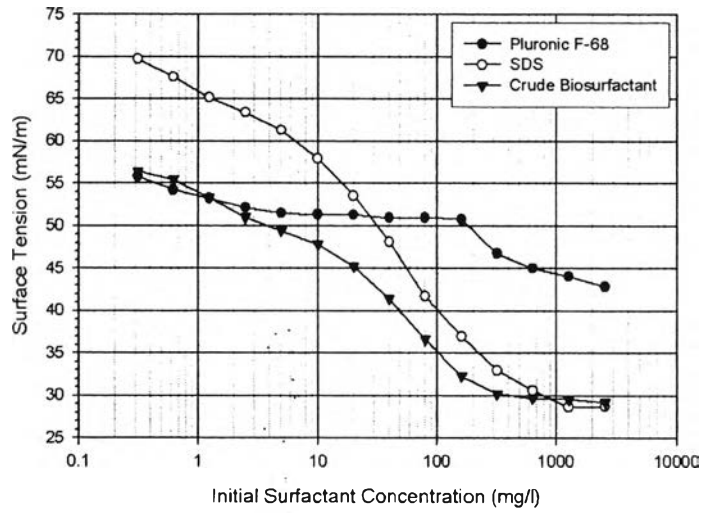

Figure 4.2 Surface tension versus concentrations of the crude biosurfactant produced by *P. aeruginosa* SP4 compared with Pluronic F-68 and SDS.

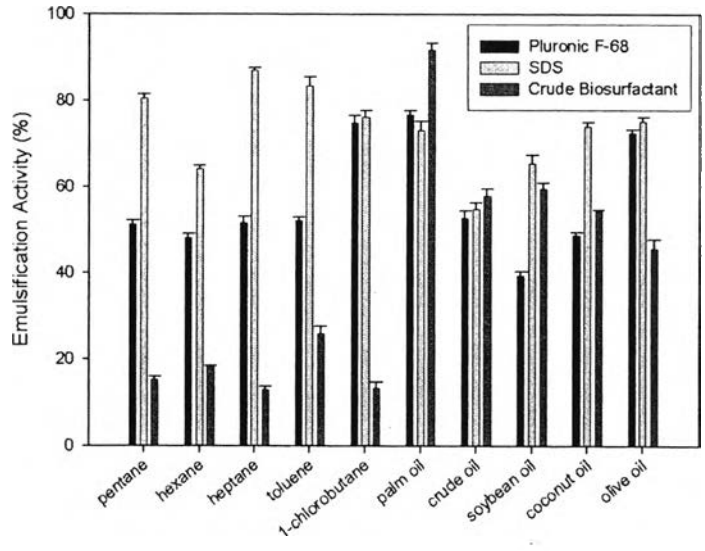

Figure 4.3 Emulsification activity (E_{24}) of the crude biosurfactant produced by *P. aeruginosa* SP4 compared with Pluronic F-68 and SDS.

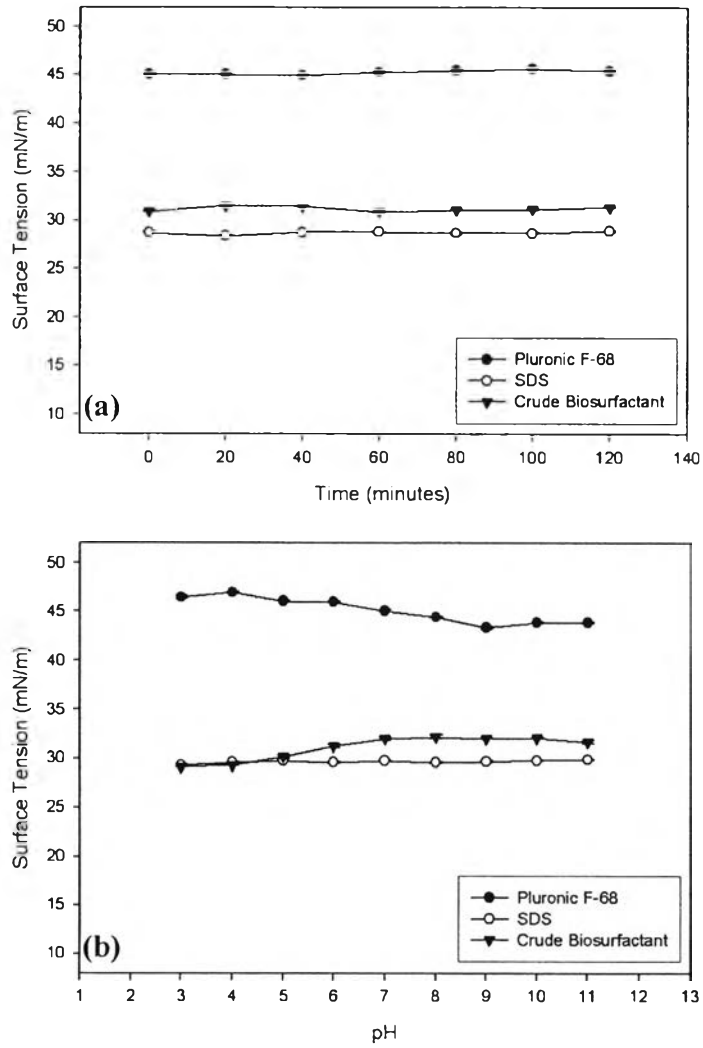

Figure 4.4 Stability of the crude biosurfactant as compared with Pluronis F-68 and SDS. (a) Surface tension of surfactants after heat treatment at 90°C with different heating times. (b) Effect of pH on surface activity of surfactants.